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Transformation of a Conditionally Peptidoglycan-Deficient Mutant of *Staphylococcus aureus* with Plasmid DNA

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A simple and reliable method for polyethylene glycol-induced plasmid transformation of a temperature-sensitive peptidoglycan-deficient mutant of *Staphylococcus aureus* is described. The procedure uses strains carrying the *tofA372* mutation grown under conditions that yield osmotically fragile cells capable of efficient wall regeneration. The peptidoglycan-deficient cells were transformed with plasmids pE194 and pI258 at frequencies comparable with those obtained with protoplasts prepared with lysostaphin treatment. A readily portable *tofA372* mutation was constructed by isolating an insertion of the erythromycin resistance transposon Tn*551* adjacent to *tofA372*. *tofA372* was shown by protoplast fusion and transformation analyses to be in the gene order *hly-421*–Ω[Chr::Tn*551*]1059–*tofA372*–*uraB232*–Ω[Chr::Tn*916*]1101–*thrB106* on the chromosome of *S. aureus* NCTC 8325.

Temperature-sensitive mutants of *Staphylococcus aureus* that become fragile and die when grown at the restrictive temperature (43°C) were initially described by Good and Pattee (6). Lysis of these mutants, termed TOF (for temperature-sensitive osmotically fragile), at the restrictive temperature was prevented by the addition of osmotic stabilizers such as NaCl and sucrose to the growth medium. Good and Tipper (5) subsequently showed that TOF mutants were defective in peptidoglycan precursor synthesis. They identified specific enzymatic defects in three of these mutants (in L-alanine-adding enzyme, D-glutamate-adding enzyme, and L-lysine-adding enzyme) that interfered with the addition of amino acids to the pentapeptide moiety of the peptidoglycan monomer (20).

This study was undertaken to investigate the usefulness of TOF mutants as transformation recipients for plasmid DNA. Effective transformation of *S. aureus* with plasmid DNA conventionally has used protoplasts prepared with lysostaphin. Unfortunately, the poor regeneration efficiency of enzymatically prepared protoplasts has hindered the recovery of genetically useful recombinants. The use of peptidoglycan-deficient TOF mutants as recipients for plasmid DNA was expected to have several advantages over the use of protoplasts prepared with lysostaphin. TOF mutants grown at the restrictive temperature accumulate peptidoglycan precursors in the cytoplasm and retain a substantial amount of cell wall. Because the cells are primed for subsequent wall synthesis and are free of any exogenously added wall-digesting enzyme, the regeneration efficiency should be higher than with true protoplasts. Several of the TOF mutants require osmotic stabilization for survival at 37°C and would therefore offer containment advantages for recombinant DNA. Finally, the procedure would be more cost-effective because lysostaphin is somewhat expensive.

Of the several TOF mutants available to our laboratory, ISP1224 (previously designated TOF-123 [6]), which contains a mutation (*tofA372*) responsible for a defective D-glutamate-adding enzyme (5), appeared to be the most useful for this application. Unlike the other TOF mutants, inhibition of growth was evident as low as 35°C and was complete above 41°C, suggesting that the degree of wall weakening at elevated temperatures was the greatest with this mutant.

The results presented in this report confirmed the above expectations and demonstrated that plasmid DNA can be efficiently introduced into peptidoglycan-deficient (PG^-) cells resulting from the *tofA372* mutation. The chromosomal locus occupied by *tofA372* was mapped by protoplast fusion and transformation, and an insert of the erythromycin resistance transposon Tn*551* was isolated adjacent to the *tofA372* locus, thereby providing the means of transferring the mutation to different strains of *S. aureus* by positive selection.

MATERIALS AND METHODS

Bacteria and bacteriophage. The strains of *S. aureus* used in this study are listed in Table 1 and were maintained as described previously (22). Phage 80α (14) was maintained by propagation on ISP8.

Media and reagents. All commercially available dehydrated culture media were supplemented with 20 μg of thymine and 5 μg each of adenine, guanine, cytosine, and uracil per ml. The composition of complete defined synthetic agar was modified by omitting the appropriate amino acids or uracil as needed to score nutritional markers (15, 18). Antibiotic resistance phenotypes were selected and scored on brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) agar containing the appropriate concentration of antibiotic (16, 22). The Tof^- phenotype was scored on Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.). Regeneration (R) agar, used for the regeneration of PG^- cells, was 30 g of Trypticase soy broth (TSB; BBL), 273 g of sucrose, 25 g of agar, 0.5 g of sodium citrate, 2.1 g of soluble starch, and deionized water to yield a total volume of 1 liter (22). Polyethylene glycol (PEG; molecular weight, 1,000 or 6,000; Sigma Chemical Co., St. Louis, Mo.) was prepared as a 35% (wt/vol) solution in TSCM buffer (100 mM Tris hydrochloride [pH 8.0], 500 mM sodium succinate, 40 mM $CaCl_2$, 10 mM $MgCl_2$) (27). Where indicated, osmotically fragile cells were stabilized in SMTB (sucrose-magnesium-Tris buffer) (22) or SMMP (sucrose-maleate-Penassy medium) (3).

Chromosomal and plasmid DNA isolations. *S. aureus* chromosomal DNA used for transformations was prepared as

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TABLE 1. Designation, genotype and origins of plasmids and *S. aureus* strains used

S. aureus strain or plasmid	Genotype or phenotype	Origin and/or reference
Strains		
ISP5	8325 *thy-101 thrB106 ilv-129 pig-131*	18
ISP41	8325 *nov-142 pig-131 fus-149*	22
ISP200	655(pPQ2)(pPQ3) Chr::Tn*554*	9
ISP479	8325-4(pPQ9) *pig-131*	15
ISP798	8325 *uraB232*::Tn*551 pig-131*	ISP565 (15) × ISP794 (22)[a]
ISP1224	655(pPQ2)(pPQ3) Chr::Tn*554 hisG15 trp-363 tofA372*	4
ISP1390	8325-4(pE194) *pig-131*	E. Lederberg[b] (7)
ISP1551	8325-4 Ω[Chr::Tn*4001*]1207 *pig-131*	12
ISP1766	8325 *pig-131 hly-421*::Em^r	16
ISP1855	8325 r1$^-$ r2$^-$ m31$^+$ m32$^+$ *pig-131* Ω[Chr::Tn*4001*]1212 *lys-115 trp-103 ilv-129 thrB106 ala-126 tmn-3106 hisG15 nov-142*	12
ISP1941	655(pPQ2)(pPQ3) *tofA372 hisG15 trp-363* Ω[Chr::Tn*4001*]1207	φ80α/ISP1551 × ISP1224[c]
ISP1944	655(pPQ3) *tofA372 hisG15 trp-363* Ω[Chr::Tn*4001*]1207	ISP1941 spontaneously cured of pPQ2
ISP1946	655(pPQ3) *hisG15 trp-363* Ω[Chr::Tn*551*]1059	φ80α/pooled ISP479 × ISP1944[d]
ISP1947	655(pPQ3) *tofA372 hisG15 trp-363* Ω[Chr::Tn*4001*]1207 Ω[Chr::Tn*551*]1059	φ80α/ISP1046 × ISP1944
ISP1949	8325 *nov-142 pig-131 fus-149* Ω[Chr::Tn*551*]1059	φ80α/ISP1947 × ISP41
ISP1950	8325-4 r$^-$ *pig-131 tofA372* Ω[Chr::Tn*551*]1059	φ80α/ISP1947 × RN4220
ISP2038	8325-4 r$^-$ *pig-131 tofA372* Ω[Chr::Tn*551*]1059 *ermB496*	Pc × ISP1950[e]
ISP2044	8325 Ω[Chr::Tn*916*]1101 Ω[Chr::Tn*551*]*1059 nov-142 fus-149 pig-131*	ISP1252 DNA × ISP1948
ISP2045	8325 Ω[Chr::Tn*916*]1101 Ω[Chr::Tn*551*]*1059 nov-142 fus-149 pig-131 thrB106 tofA372*	ISP1252 DNA × ISP1948
ISP2047	8325 Ω[Chr::Tn*916*]1101 *nov-142 fus-149 pig-131 tofA372*	ISP2045 DNA × ISP41
ISP2126	8325 Ω[Chr::Tn*916*]1101 *nov-142 fus-149 pig-131 tofA372 uraB232*::Tn*551*	ISP798 DNA × ISP2047
RN4220	8325 r$^-$ *pig-131*	J. J. Iandolo[f] (10)
Plasmids		
pE194	Em^r	See ISP1390
pPQ9	pI258 *blaI401 mer-14 repA36* Ω[Tn*551*]1	See ISP479

[a] For example, ISP585 × ISP794 means that ISP585 DNA was used to transform ISP794.
[b] Received from Esther Lederberg, Stanford University, Stanford, California.
[c] For example, phage 80α/ISP1551 × ISP1224 means phage 80α propagated on ISP1551 was used to transduce ISP1224.
[d] Phage 80α propagated on a population of ISP479 cells presumed to contain all possible Tn*551* chromosomal insertions was used to transduce ISP1944.
[e] Erythromycin-sensitive mutation in Tn*551* obtained by penicillin counterselection with ISP1950.
[f] Received from John J. Iandolo, Biology Division, Kansas State University, Manhattan, Ks.

described previously (18). Plasmids pE194 and pI258 were isolated from ISP1390 and ISP479, respectively, by a modified (8) alkaline lysis procedure (2). The concentration of purified (on CsCl-ethidium bromide gradients) plasmid DNA was estimated by UV absorbance (260 nm) determined by using a Uvikon spectrophotometer (model 810; Kontron Instruments, Zurich, Switzerland).

Genetic techniques. Phage 80α transducing lysates were propagated on donor strains as described previously (26) except as indicated below. Transductions were performed at a multiplicity of infection between 0.5 and 1.0 (21). Transductants were selected at the appropriate temperature (30 or 43°C) on BHI agar containing 5 μg of sodium citrate and 1 μg of erythromycin per ml.

A phage lysate containing random Tn*551* insertions was prepared by propagating phage 80α on a population of cells containing a random distribution of chromosomal insertions of Tn*551*. The cells from an overnight BHI agar slant culture of ISP479 grown at 30°C were uniformly suspended in saline, and appropriate dilutions were spread onto BHI agar plates containing 10 μg of erythromycin per ml and then incubated at 43°C for ca. 30 h. An estimated 2,500 colonies uniformly suspended in TSB were used to inoculate two 100-ml volumes of BHI containing 10 μg of erythromycin per ml in 300-ml nephelometer flasks to an initial optical density at 540 nm (OD_{540}) of 0.10. (Optical densities were measured throughout this study at 540 nm by using a Spectronic 20 spectrophotometer.) The flasks were gently shaken at 43°C until the OD_{540} was equal to 0.65. The cells were then harvested by centrifugation and used to prepare a phage 80α lysate.

Protoplast fusions and transformations were performed as described previously (22, 23). Recombinants were selected at 30°C on BHI agar containing 10 μg of the appropriate antibiotic(s) per ml. The cotransformation frequencies of two markers, A and B, are expressed as 1 - the cotransfer frequency (c), where c = AB/A (18).

Growth studies. Growth curve experiments were performed to analyze the development of osmotic fragility of Tof$^-$ strains under various broth culture conditions. The strains used in these experiments were harvested in saline from BHI agar slant cultures grown at 30°C for ca. 18 h. The cell suspensions were used to inoculate TSB-based media contained in 300-ml nephelometer flasks to an initial OD_{540} of 0.05. Except where indicated otherwise, the flasks were incubated with shaking (100 cycles/min) in a water bath at 30°C until the OD_{540} reached 0.45. Either the flasks were then directly transferred to the desired nonpermissive temperature, or the cells were then harvested (10,000 × g, 20 min) and added to fresh TSB-based media at the desired temperature. Incubation was continued for 6 h with shaking. Osmotic fragility was monitored over the course of incubation by the change in OD_{540} resulting from the addition of 0.2 ml of 0.5 M sodium dodecyl sulfate (SDS; final concentration, 0.02 M) to a 4.8-ml sample of the culture. OD_{540} readings were recorded before SDS addition and again 15 min after SDS addition at the temperature of the culture. Osmotic fragility was also monitored by spreading appropri-

ately diluted (in SMMP) samples of the culture onto R agar (for osmotically stabilized cells) or TSA (for osmotically shocked cells). All plates were incubated overnight at 30°C and then transferred to a humidified incubator at 30°C for an additional 4 days. The percentage of non-osmotically fragile cells in the population then was determined from the difference in CFU on the two media.

Plasmid transformations. Plasmid transformations were performed with lysostaphin-generated protoplasts (22), PG^- *tofA372*-carrying cells, and untreated walled cells as recipients. PG^- Tof^- recipient cells were prepared by suspending the cells from an overnight BHI agar slant culture incubated at 30°C in saline and inoculating a 100-ml volume of TSB contained in a 300-ml nephelometer flask to an initial OD_{540} of 0.05. This culture was incubated at 30°C with shaking (ca. 110 cycles/min) until the OD_{540} reached 0.45. The culture was then transferred either to 37°C and shaker incubated for 4 h (ISP1944) or to 39°C and shaker incubated for 3 h (ISP2038). The cells were then harvested (6,000 × *g*, 30 min, 4°C), and the pellet was washed in 10 ml of SMTB (2,500 × *g*, 7 min), and gently suspended in 2 ml of SMMP. Walled cell recipients were prepared as described above except that they were harvested immediately after attaining an OD_{540} of 0.45 at 30°C.

Once suspended in SMMP, all recipients were treated the same. The incidence of nonprotoplasted cells in the suspension was determined by spreading 0.1-ml samples of appropriately diluted (in SMMP) cells onto TSA. Total viable cells were determined by spreading 0.1-ml samples of appropriately diluted cells onto R agar. Direct counts of cells were determined by using a Petroff-Hausser counting chamber and phase-contrast microscopy.

In a plastic centrifuge tube, 200 μl of plasmid DNA in 10 mM Tris–1 mM disodium EDTA, pH 7.5 was mixed with 200 μl of 2× SMM buffer (3). To this mixture, 0.5 ml of the cell suspension was added and immediately followed by 1.5 ml of 35% PEG 1000 or PEG 6000. The tube was inverted on a rocker platform at 15 cycles/min for 30 min at room temperature. SMMP (5 ml) was added, the tube was inverted a few times, and the cells were pelleted (2,500 × *g*, 7 min). The cell pellet was gently suspended in 1 ml of SMMP and incubated for 4 h at 30°C to allow for expression. Samples, diluted and undiluted in SMMP, then were spread onto R agar plates containing 1 μg of erythromycin per ml to select for transformants. Total CFU/ml were determined by spreading 0.1-ml samples of appropriately diluted (in SMMP) material onto nonselective R agar. All plates were incubated overnight at 30°C before being transferred to a 30°C humidified incubator for an additional 4 days of incubation before all plates were scored.

RESULTS

Isolation of Tn*551* insertions near *tofA372*. Since *tofA372* confers a nonselectable phenotype, a selectable marker linked to this locus was needed to allow efficient strain constructions and to facilitate the mapping of this mutation on the chromosome. Therefore, insertions of Tn*551* near *tofA372* were obtained by using a modification of the procedure described previously (11) for isolating insertions of Tn*551* near markers of interest in *S. aureus*. Phage 80α propagated on an erythromycin-resistant (Em^r) population of ISP479 containing an essentially random distribution of Tn*551* chromosomal insertions was used to transduce ISP1944 (Em^s Tof^-), with selection for Em^r at 43°C. Four Em^r Tof^+ transductants were recovered. The ratio of Em^r Tof^+ transductants to the total number of Em^r transductants, determined by selection for Em^r at 30°C in the same experiment, was 1:7,500.

Phage 80α propagated on one of the Em^r Tof^+ transductants (designated ISP1946; Table 1) was used to transduce ISP1944 with selection for Em^r at 30°C. An Em^r Tof^- transductant was recovered (ISP1947) that carried a Tn*551* insertion (Ω[Chr::Tn*551*]1059) that was 74.7% cotransducible with *tofA372* by phage 80α. ISP1947 served as a source of a portable *tofA372* mutation in subsequent experiments to introduce *tofA372* into different genetic backgrounds by positive selection for Ω1059, including the restrictionless 8325-4 strain RN4220. It was expected that the RN4220 Tof^- construction, designated ISP1950, would provide a nonrestricting recipient capable of being transformed with plasmid DNA at high efficiency. Because many plasmids of interest in *S. aureus* have a selectable Em^r phenotype, ISP1950 was subjected to penicillin counterselection to obtain an Em^s derivative of the Tn*551* insert (19); this strain was designated ISP2038.

To investigate the possibility that more than one lesion was responsible for the Tof^- phenotype attributed to *tofA372*, ISP1944 and ISP2038 were transduced with phage 80α propagated on ISP2044, which carries Ω1059 and is Tof^+. Both strains yielded Em^r Tof^+ transductants at similar frequencies (74.7% for ISP1944 and 60.9% for ISP2038). These results are consistent with a single mutation being responsible for the Tof^- phenotype of each strain. Although the possibility of multiple mutations that are closely linked is not ruled out by these experiments, the reversion frequency of each strain to Tof^+ (about 10^{-8}) is typical of a single point mutation and suggests that a single lesion is involved.

Chromosomal map location of *tofA372*. The approximate chromosomal location of *tofA372* was estimated by mapping Ω1059. Protoplasts of ISP1949 (Em^r Fus^r Tc^s prototroph) and ISP1855 (Em^s Fus^s Tc^r multiple auxotroph) were fused, and the resulting Em^r Tc^r and Fus^r Tc^r recombinants were scored for the distribution of other markers. In both sets of recombinants, Ω1059 exhibited high (99 and 93%) coinheritance with Ω[Chr::Tn*4001*]1212, indicating close linkage between Ω1059 and Ω1212. Ω1212 is known to be closely linked to Ω[Chr::Tn*551*]40 and Ω[Chr::Tn*916*]1101 (12) (Fig. 1).

Several multifactorial transformations summarized in Table 2 were performed to define the chromosomal loci occupied by Ω1059 and *tofA372* relative to surrounding markers occupying known loci; the results are summarized in Fig. 2.

Growth studies. Consistent with previous reports (5, 6), 1 M NaCl or 2 M sucrose in TSA and 1 but not 2 M sucrose in TSB facilitated growth at 43°C of *S. aureus* 655 derivatives carrying the *tofA372* mutation. In this study, 1.2 M NaCl was shown to be required for confluent growth at 43°C, and 1.2 M KCl could replace NaCl. Glycerol (2 M) in TSA or TSB supported limited growth at 43°C. This limited growth is attributed to the slower growth rate at 30°C observed for ISP1944 in TSB plus 2 M glycerol compared with TSB alone.

It was initially assumed that Tof^- cells grown at 43°C with osmotic stabilization (1.2 M NaCl) would be sufficiently deficient in peptidoglycan to allow efficient uptake of plasmid DNA. However, transformation frequencies for ISP1944 and ISP2038 grown under these conditions were only slightly higher than the frequencies obtained with untreated walled cells and were substantially lower than those obtained with protoplasts prepared by lysostaphin treatment. These low frequencies were subsequently explained by the lack of osmotic fragility of cells grown under

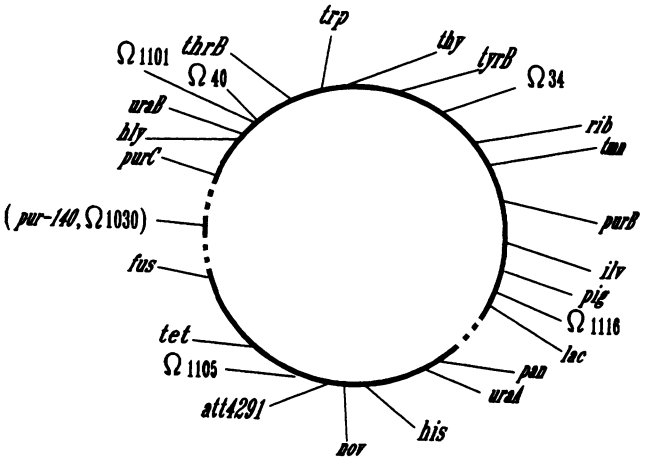

FIG. 1. Abbreviated chromosome map of *S. aureus* NCTC 8325 showing the markers used to map the *tofA372* mutation and other markers for reference purposes. Markers: *thy*, thymine requirement; *tyrB*, tyrosine requirement; Ω34, Ω1030, and Ω40, silent insertions of Tn*551*; *rib*, riboflavin requirement; *tmn*, constitutive tetracycline and minocycline resistance; *purB*, adenine plus guanine requirement; *ilv*, isoleucine-valine requirement; *pig*, absence of golden-yellow pigment; Ω1116, Ω1105, and Ω1101, silent insertions of Tn*916*; *lac*, lactose fermentation; *pan*, pantothenate requirement; *uraA* and *uraB*, uracil requirements; *his*, histidine requirement; *nov*, novobiocin resistance; *att4291*, attachment site for Tn*4291*; *tet*, inducible tetracycline resistance; *fus*, fusidic acid resistance; *pur-140* and *purC*, purine requirement; *hly*, α-toxin structural gene; *thrB*, threonine requirement; *trp*, tryptophan requirement. The map is a continuous genetic linkage map by transformation except where the circle is dashed. (Redrawn from reference 17.)

these conditions. Growth of ISP1944 in TSB containing 1.2 M NaCl at 43°C was monitored for osmotic fragility over the course of incubation (Fig. 3A) by exposing culture samples to SDS. Since bacterial cells with a damaged peptidoglycan layer are lysed preferentially by SDS (13), a decrease in OD_{540} can be taken as evidence of cell wall fragility. Cell wall fragility was also monitored by spreading diluted samples of the culture onto TSA (osmotically shocked cells) or R agar (osmotically protected cells) with incubation at 30°C with supportive humidity for 5 days. The difference in CFU per milliliter on the two media was taken as a measure of osmotic fragility. Significant osmotic fragility was not indicated by either method (Fig. 3A). Essentially the same results were obtained when the cells were stabilized with 1.2 M KCl (data not shown).

To compare the development of cell fragility at 43°C in osmotically stabilized and unstabilized cultures, logarithmically growing cells of ISP1944 were harvested from a TSB

TABLE 2. Summary of transformations to identify the linkage relationships of *hly-421*::Em^r, Ω[Chr::Tn*551*]1059, *tofA372*, *uraB232*::Tn*551*, Ω[Chr::Tn*916*]1101, and *thrB106*

Donor strain	Recipient strain	Marker selected (no. scored)	Unselected markers scored
ISP2045	ISP41	Ω1101 (952)	Ω1059, *tofA372*, and *thrB106*
ISP2045	ISP5	Ω1059 (277)	*tofA372* and Ω1101
ISP2126	ISP5	*uraB232*::Tn*551* (475)	*tofA372* and Ω1101
ISP2047	ISP1766	Ω1101 (1,006)	*tofA372* and *hly-421*::Em^r

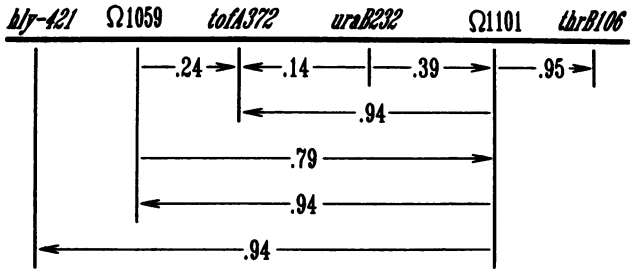

FIG. 2. Linkage relationship of the *hly-421*, Ω[Chr::Tn*551*]1059, *tofA372*, *uraB232*, Ω[Chr::Tn*916*]1101, and *thrB106* markers as determined from the data shown in Table 2. Arrows point from the selected to the unselected markers.

culture grown at 30°C and used to inoculate fresh TSB with or without 1.2 M NaCl to an OD_{540} of about 0.4. Cell fragility was monitored over a 6-h incubation period as described above (Fig. 3B and C). Growth was inhibited in the unstabilized culture, and the development of osmotic fragility and cell lysis occurred rapidly (Fig. 3C). In contrast, cells grown in the osmotically stabilized medium were substantially less fragile (Fig. 3B). This experiment was repeated with various concentrations of NaCl (0.2, 0.4, and 0.8 M) and sucrose (0.4, 0.6, 0.8, and 1.2 M) (data not shown). In each case, osmotic fragility decreased with increasing solute concentration.

From these results it was apparent that the remedial effect of high solute concentration was not merely the osmotic stabilization of fragile cells but phenotypic reversion of the Tof⁻ phenotype. If the mutant phenotype was still expressed in the presence of high solute concentrations, then increasing the solute concentration would yield a more osmotically fragile population of viable cells because PG⁻ cells would be protected against lysis. It was interesting to find that NaCl had essentially the same effect on wild-type cells exposed to D-cycloserine or glycine. Log-phase cells of RN4220 (Tof$^+$) were harvested from a 30°C TSB culture and transferred to TSB containing 200 μg of D-cycloserine per ml with or without 1 M NaCl. Because D-cycloserine is a known inhibitor of peptidoglycan biosynthesis (25), stabilized protoplasts were anticipated in the culture containing 1 M NaCl. However, NaCl had essentially the same effect on cells treated with D-cycloserine as it did on the Tof⁻ cells (data not shown). This experiment was repeated with 0.5 M glycine substituted for D-cycloserine. High concentrations of glycine were previously shown to induce protoplast formation in *S. aureus* and other bacteria (24). As with D-cycloserine, 1 M NaCl reversed the inhibitory effect of glycine (data not shown).

While the highest degree of osmotic fragility was obtained in cultures that were not osmotically stabilized, cell viability quickly declined during incubation. Inhibition of growth of ISP1944 was complete at 37°C or higher (Fig. 4A). To investigate the effect of growth rate on cell viability and the development of osmotic fragility, log-phase cells of ISP1944 grown at 30°C were harvested and then transferred to fresh TSB with continued incubation at 37°C. Although the acquisition of osmotic fragility was appreciably delayed, higher cell viability was maintained at 37 than at 43°C (data not shown). It subsequently was found that by directly transferring an early-log-phase culture (OD_{540}, 0.45) grown at 30 to 37°C, the cells became osmotically fragile sooner while maintaining the same level of viability (Fig. 5A). Under these conditions, the lowest percentage of nonosmotically fragile cells in the population was 0.049% at hour 4 of incubation,

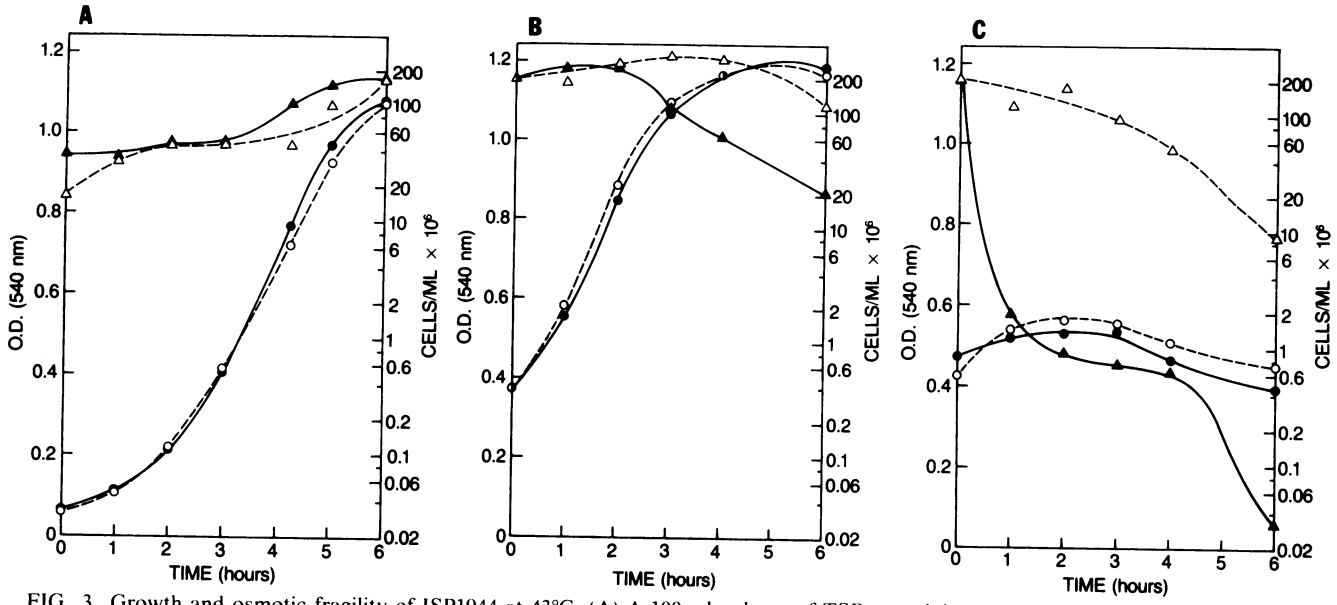

FIG. 3. Growth and osmotic fragility of ISP1944 at 43°C. (A) A 100-ml volume of TSB containing 1.2 M NaCl was inoculated from an overnight BHI agar slant culture grown at 30°C and incubated at 43°C with shaking. (B) Logarithmic-phase cells grown in TSB at 30°C were harvested by centrifugation, used to inoculate a 100-ml volume of TSB containing 1.2 M NaCl at 0 h, and then incubated at 43°C with shaking. (C) Logarithmic-phase cells grown in TSB at 30°C were harvested by centrifugation, used to inoculate a 100-ml volume of TSB at 0 h, and then incubated at 43°C with shaking. OD_{540}s of culture samples before (○) and after (●) the addition of 0.02 M SDS and cells per milliliter as indicated by CFU per milliliter on R agar (△) and TSA (▲) were used as measures of osmotic fragility.

while the percentage of CFU per milliliter at the time of transfer to 37°C that remained viable after 4 h was 54%. Among the growth conditions examined for ISP1944, this was taken as the optimal procedure for obtaining viable cells deficient in peptidoglycan and was used throughout the plasmid transformation studies with this strain.

The results from growth studies with the restrictionless Tof⁻ strain ISP2038 were similar to those obtained with ISP1944, but as illustrated in Fig. 4B, the minimum inhibitory temperature was about 2°C higher for ISP2038. The results of directly transferring an early-log-phase TSB culture of ISP2038 grown at 30°C to 39°C are shown in Fig. 5B. Because cell viability began to decline rapidly after 3 h of incubation, cells used in the plasmid transformation studies were harvested at this hour. The percentage of non-osmotically fragile cells at this time was 0.67%, while 93% of the CFU per milliliter at the time of transfer to 39°C remained viable.

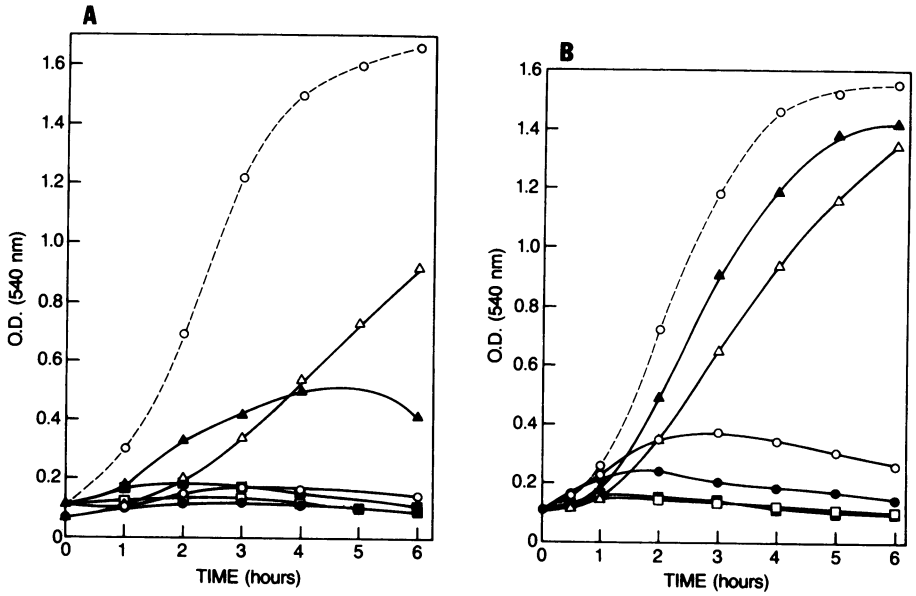

FIG. 4. Effect of temperature on the growth of ISP1944 (A) and ISP2038 (B). One-hundred-milliliter volumes of TSB were inoculated from an overnight 30°C BHI agar slant culture and incubated with shaking at either 30 (△), 35 (▲), 37 (○), 39 (●), 41 (□), or 43°C (■). Growth of the wild-type strains ISP200 (A) and RN4220 (B) at 37°C (– –○– –) are included for comparison.

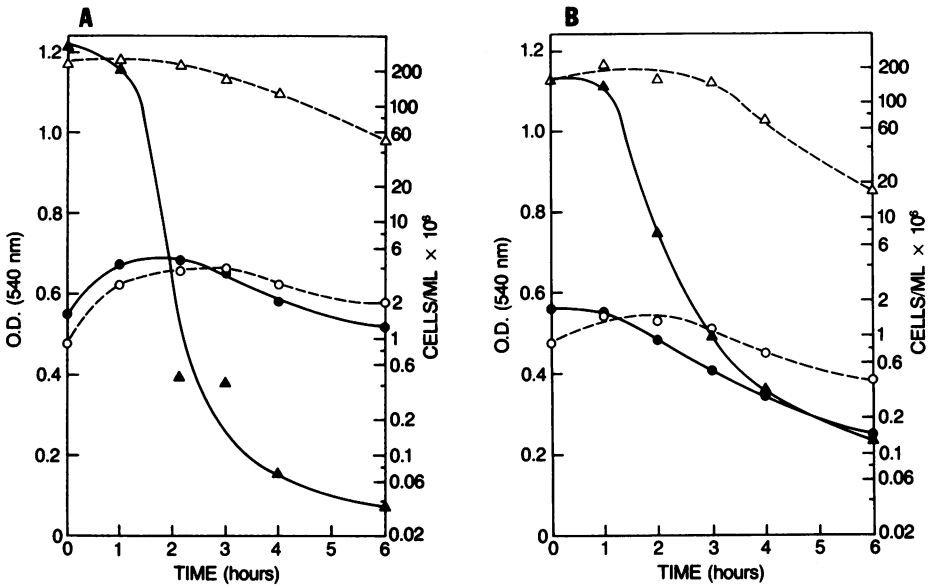

FIG. 5. Effect of a temperature shift from 30 to 37 (A) and 39°C (B) on the development of osmotic fragility of ISP1944 and ISP2038, respectively. For each strain, a flask containing logarithmically growing cells in 100 ml of TSB was shifted at 0 h to the nonpermissive temperature for continued incubation with shaking. OD_{540}s of the culture samples before (○) and after (●) the addition of 0.02 M SDS and cells per milliliter as indicated by CFU per milliliter on R agar (△) and TSA (▲) were used as measures of osmotic fragility.

Plasmid transformations. In the initial attempts to transform PG⁻ cells of ISP1944, the relationship between DNA concentration and the recovery of transformants was investigated by using the 2.3-megadalton Em^r plasmid pE194 (7). The PG⁻ recipient cells were prepared as described above, harvested, and suspended in SMMP buffer. Amounts of DNA from 0 to 15 μg were added to separate samples of this suspension, followed by 35% PEG 1000. The mixtures were gently inverted for 30 min before cells were harvested and suspended in SMMP buffer. After incubation at 30°C for 4 h, samples of each suspension were spread onto R agar with or without selection for Em^r. The results of this experiment (Table 3, experiment 1) demonstrated that plasmid DNA can be efficiently introduced into ISP1944 PG⁻ cells. Since a linear relationship between DNA concentration and transformation frequency was not observed, it would appear that a saturating concentration of DNA was obtained with 2 μg.

To determine the effect of plasmid size on transformation efficiency, ISP1944 cells prepared as in experiment 1 (Table 3) were transformed with either pE194 (2.3 megadaltons) or pI258 (18.4 megadaltons). Both equal amounts (2 μg each) and equal molar amounts (2 μg for pE194 and 16 μg for pI258) were used (Table 3, experiment 2). As expected, 2 μg of pE194 yielded many more transformants than 2 μg of pI258. However, the frequency of transformation with pI258 approached that of pE194 when equal molar amounts of DNA were used. In addition, when 35% PEG 6000 was used as the fusogen in place of PEG 1000, at least a twofold increase in transformation frequencies with both plasmids was observed (Table 3, experiment 3).

The results obtained when the restrictionless Tof⁻ strain ISP2038 was grown to osmotic fragility at 39°C and transformed with both plasmids (Table 3, experiment 4) show that PG⁻ cells of this strain are transformed at frequencies equivalent to those of ISP1944. The potential advantage of this strain in being able to be transformed with plasmids derived from donors in other restriction/modification groups was not assessed.

For comparison with PG⁻ recipients, protoplasts of RN4220 prepared with lysostaphin and walled cells (grown at 30°C) of ISP1944, ISP2038, and RN4220 were also transformed. The transformation frequencies were similar for PG⁻ cells and protoplasts (Table 3, experiment 5). However, because of the lower efficiency of regeneration, somewhat fewer transformants were recovered from the transformed protoplasts. Although walled cells were not efficiently transformed with pE194, a significant number of transformants were recovered. At least for relatively small plasmids, this demonstrates that cells with uncompromised walls are capable of taking up plasmid DNA. The fact that higher transformation frequencies were obtained with the walled Tof⁻ cells than with wild-type strain RN4220 suggests that the mutant cells have weakened walls when grown at 30°C.

DISCUSSION

The results of this study show that PG⁻ cells resulting from the *tofA372* mutation can be efficiently transformed with plasmid DNA. The transformation frequencies obtained with these cells with either pE194 or pI258 were comparable with those obtained with protoplasts prepared by lysostaphin treatment. More importantly, the regeneration frequency of PG⁻ Tof⁻ cells was consistently about 10 times higher than that of true protoplasts, resulting in the recovery of a greater number of transformants per microgram of DNA.

The osmotic fragility of the Tof⁻ strains ISP1944 and ISP2038 decreased with either a decrease in the incubation temperature or an increase in the concentration of NaCl or sucrose in the growth medium. The mechanism underlying the correction of the Tof⁻ phenotype by high solute concentrations remains unknown. Good and Tipper (5) reported that the *tofA372* mutation (in their study, the mutation in strain TOF-123) was responsible for a defective D-glutamate-adding enzyme and possibly for a defective L-alanine-adding enzyme as well. These soluble enzymes are required for the addition of D-glutamate and L-alanine to the pentapeptide moiety of peptidoglycan monomers (20). The results pre-

TABLE 3. Summary of plasmid DNA transformation experiments

Expt[a]	Recipient strain	Treatment[b]	Plasmid (μg of DNA)	Regeneration[c]		Transformation[d]	
				No.	Frequency	No.	Frequency
1	ISP1944	PG⁻ (37°C)	None	7.2×10^8	0.36	2.0×10^1	2.8×10^{-8}
			pE194 (2)	1.0×10^9	0.36	9.4×10^4	9.4×10^{-5}
			pE194 (4)	9.2×10^8	0.36	1.3×10^5	1.4×10^{-4}
			pE194 (6)	1.3×10^9	0.36	2.2×10^5	1.6×10^{-4}
			pE194 (9)	7.8×10^8	0.36	2.3×10^5	2.9×10^{-4}
			pE194 (12)	1.1×10^9	0.36	2.7×10^5	2.5×10^{-4}
			pE194 (15)	9.0×10^8	0.36	3.0×10^5	3.3×10^{-4}
2	ISP1944	PG⁻ (37°C)	None	1.3×10^9	0.35	1.5×10^1	1.2×10^{-8}
			pE194 (2)	1.4×10^9	0.35	4.6×10^4	3.3×10^{-5}
			pI258 (2)	1.4×10^4	0.35	2.0×10^3	1.4×10^{-6}
			pI258 (16)	1.4×10^9	0.35	1.4×10^4	1.0×10^{-5}
3	ISP1944	PG⁻ (37°C)	None	4.1×10^8	0.25	3.0×10^1	7.3×10^{-8}
			pE194 (2)	4.3×10^8	0.25	1.5×10^4	3.5×10^{-5}
			pI258 (2)	4.4×10^8	0.25	4.5×10^2	1.0×10^{-6}
			pE194[e] (2)	4.2×10^8	0.25	3.2×10^4	7.6×10^{-5}
			pI258[e] (2)	4.1×10^8	0.25	1.1×10^3	2.7×10^{-6}
4	ISP2038	PG⁻ (39°C)	None	5.8×10^8	0.20	2.0×10^1	3.4×10^{-8}
			pE194 (2)	6.0×10^8	0.20	4.2×10^4	7.0×10^{-5}
			pI258 (2)	5.9×10^8	0.20	5.0×10^2	8.5×10^{-7}
5	RN4220	Protoplasts	None	2.7×10^8	0.026	<1	$<3.7 \times 10^{-9}$
			pE194 (2)	3.1×10^8	0.026	1.6×10^4	5.2×10^{-5}
			pI258 (2)	1.3×10^8	0.026	1.3×10^2	1.0×10^{-6}
6	ISP1944	None	pE194 (2)	4.7×10^9	1.02	3.4×10^3	7.2×10^{-7}
	ISP2038	None	pE194 (2)	1.5×10^9	0.55	9.9×10^2	6.6×10^{-7}
	RN4220	None	pE194 (2)	6.2×10^9	0.48	1.2×10^3	1.9×10^{-7}

[a] For each experiment, the same initial cell suspension was used for all transformations of the indicated strain.
[b] PG⁻ cells were grown to osmotic fragility at the indicated temperature. Protoplasts were prepared with lysostaphin. "None" indicates that strains were grown at 30°C without additional treatment.
[c] Regeneration number, viable cells per milliliter (assayed on R agar); regeneration frequency, viable cells per milliliter before the addition of plasmid DNA divided by the total number of cells as determined by direct counts.
[d] Transformation number, Em^r transformants per milliliter (assayed on R agar containing 1 μg of erythromycin per ml); transformation frequency, Em^r transformants per milliliter divided by the total number of CFU (assayed on R agar).
[e] PEG 6000 (35%) was used with these two plasmids. All other experiments were performed with 35% PEG 1000.

sented by Good and Tipper (5) suggest that neither temperature nor direct interaction with the stabilizing solute affects the activities of the defective enzymes.

Since a variety of solutes can cause phenotypic reversion, it is likely that the remedial effect of high solute concentrations is simply an osmotic stabilization of the fragile cells, while the reversion of the Tof⁻ phenotype is secondary. This remedial effect must be general, since the bactericidal activities of D-cycloserine and high concentrations of glycine, both of which inhibit peptidoglycan biosynthesis (24, 25), were also reversed by 1 M NaCl. A clue to understanding this phenomenon may be taken from a recent study of penicillin-induced bacteriolysis in staphylococci (4). Giesbrecht et al. (4) determined that penicillin-induced lytic death results from an impairment in cross wall assembly during preparation for cell division. During nearly undisturbed continuation of other wall processes, this leads to cell separation in spite of an insufficiently assembled, unclosed cross wall. Because of the internal pressure of the PG⁻ cell, lytic cell death results from failure of the perforated wall along the division plane. This may be extrapolated to the effect of D-cycloserine, glycine, and the *tofA372* mutation, since, like penicillin, they all ultimately inhibit cross-linkage (transpeptidation) between the peptide subunits of peptidoglycan. Good and Tipper (5) presented scanning electron micrographs of TOF mutant cells grown at 43°C without osmotic stabilization that show cells which appeared to have fractured along nascent division planes. Osmotic stabilization would prevent cell lysis and thus allow more time for incorporation of stable peptidoglycan that escaped the inhibitory agent into the forming cross wall. In addition, time would be allowed for thickening of the peripheral wall by addition of new peptidoglycan monomers. Wall-thickening synthesis, separate from cross wall synthesis, has been reported to occur in *Staphylococcus* spp. (1). The decrease in growth rate resulting from lower incubation temperatures may have the same effect, assuming that the amino acid-adding enzyme activities do not concurrently decrease. The net result of transferring a logarithmically growing culture at 30 to 37°C for continued incubation without osmotic stabilization should then be a population of cells with thin layers of peptidoglycan in areas corresponding to past division planes. While there may not be enough cell wall in these areas to significantly interfere with plasmid DNA-cell membrane interaction, there is enough to prevent cell lysis in a hypotonic environment.

It is expected that *tofA372*-carrying cells grown to osmotic fragility will be applicable to other techniques traditionally requiring the use of *S. aureus* protoplasts, such as the isolation of plasmid and high-molecular-weight chromosomal DNAs and of cellular proteins susceptible to denaturation by the activity of lysostaphin or harsh lysis techniques. *tofA372*-carrying *S. aureus* may also prove to be advantageous for the introduction of plasmid DNAs by electroporation. The insertion of Tn*551* adjacent to *tofA372* will facilitate such applications by providing a means of transferring the *tofA372* mutation to different genetic backgrounds by positive selection.

Although the primary structure of *S. aureus* peptidoglycan and the mechanism of its biosynthesis are well known, little

is known of the genetic control of peptidoglycan synthesis. The chromosome mapping of *tofA372* is a small step towards an understanding of this process and towards determining the number and distribution of genes involved.

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