Isolation and Characterization of a Tn551-Autolysis Mutant of Staphylococcus aureus

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A Lyt− mutant with reduced autolytic activity was isolated after Tn551 mutagenesis of the methicillin-susceptible Staphylococcus aureus laboratory strain RN450. The Lyt− phenotype could be transferred back into the parent and into a variety of other S. aureus strains by transduction of the transposon marker. Southern analysis has located the Tn551 insert to a 3.2-kb HindIII DNA fragment on the Smal B fragment of the staphylococcal chromosome. The Lyt− phenotype included reduced rates of cell wall turnover and autolysis induced by detergent or methicillin treatment; however, the rate of methicillin-induced killing was not affected. Peptidoglycans prepared from the parental and mutant cells showed identical muropeptide compositions, as resolved by a high-resolution high-pressure liquid chromatography technique. On the other hand, LiCl extracts of the mutant cells contained reduced amounts of total protein and lower specific cell wall-degrading activity compared with those of extracts of parental cells. The profile of bacteriolytic enzymes as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed multiple band differences between mutant and parental cells; a major lytic band with properties characteristic of the staphylococcal endo-β-N-acetylmuramidase was completely absent from the Lyt− cells. The Lyt− phenotype transduced into a series of methicillin-resistant strains of both homogeneous and heterogeneous phenotypes caused only a modest decrease in the level of methicillin resistance, as determined by population analysis.

Autolytic enzymes, i.e., enzymes that break covalent bonds in the bacterial cell wall, are widespread among bacteria (7, 16). The activities of at least some of these enzymes are clearly involved with phenomena such as bacterial autolysis (induced by antibiotics or adverse physiological conditions) and cell wall turnover. Although speculations concerning the involvement of autolysins in cell wall enlargement, cell division, and/or morphogenetic processes have been repeatedly considered, the physiological function(s) of autolysins for the bacterium remains to be elucidated (21). Recent improvements in the techniques for detection of autolysins indicate that bacteria may contain multiple and, in some cases, a surprisingly large number of autolytic enzymes (11, 18), making the task of identification of physiological functions more complex.

Staphylococcus aureus contains at least three kinds of catalytically distinct autolytic enzymes: an amidase, an N-acetyl endoglucosaminidase, and an endopeptidase (8, 17, 19, 20). Molecular cloning of the glucosaminidase (I) and amidase (9) has been reported. Autolytic mutants of staphylococci have also been described (3, 10), and analyses of such mutants have yielded much interesting information about the control of these enzymes and their involvement with cell separation and wall turnover. Nevertheless, all of the mutants were generated by chemical mutagenesis, making a precise association of mutational site and phenotype difficult.

In this communication we describe the isolation and characterization of an autolytic mutant of S. aureus in which the lytic defect is clearly associated with the transposon (Tn551) insertional site. The easy selection for the Tn551 marker should allow the introduction of the lytic defect into various genetic backgrounds and thus help to elucidate the physiological function(s) of autolytic enzymes in this bacterium.

MATERIALS AND METHODS

Strains and growth conditions. The S. aureus strains used are listed in Table 1. Bacteria were grown in tryptic soy broth (TSB; Difco) with aeration at 37°C and plated on tryptic soy agar (TSA; Difco). Transposon mutants and transductants were grown in TSB containing 10 μg of erythromycin per ml in overnight cultures. For each experiment, the overnight culture was diluted 1:100-fold into prewarmed TSB without erythromycin to allow exponential growth. Growth was monitored by the increase in optical density at 620 nm (OD₆2₀) in a spectrophotometer (Pharmacia LKB, Piscataway, New Jersey) in cuvettes with a 1-cm path length.

Selection of Tn551 mutants. Tn551 was inserted into the chromosome as described previously (13). In short, RN2906, harboring the thermostresponsive plasmid pRN3208 carrying Tn551 with the erythromycin resistance determinant, was plated at different cell concentrations on TSA containing 100 μg of erythromycin per ml and incubated at 43°C for 48 h. The approximate frequency of insertion was 6 × 10⁻⁴. Colonies were replica plated onto TSA containing 0.25 mM Cd(NO₃)₂ and incubated at 43°C for 16 h to eliminate bacteria that have retained the whole plasmid (about 1% of all erythromycin-resistant colonies that grew at 43°C). Colonies that failed to grow in the presence of cadmium nitrate (indicating the loss of pRN3208) were selected. Each selected colony was grown in TSB containing 10 μg of erythromycin per ml at 37°C and used to screen for Lyt− mutants.

Screening for Lyt− mutants. Plates used for screening contained 10 ml of TSA bottom agar plus 3 ml of half-strength (0.5×) TSA containing heat-inactivated S. aureus RN450 cells (3 mg/ml) and about 200 CFU of Tn551-mutagenized bacteria to be screened. An additional 3 ml of 0.5×...
TSA was used to cover the surface of the agar plates. The plates were incubated at 37°C for 4 days. Uniform zones of clearing, approximately 5 mm in diameter, appeared around colonies of the parental strain, RN450. Colonies that exhibited smaller lysis zones were picked and examined. A single colony with a barely visible lysis zone around it was designated Lyt- mutant RUSAL1 and was used as the source of the Tn551-inactivated autolytic determinant(s) in all of the experiments.

**Transduction.** The method of Pattée (15) was used in a slightly modified form. A phase 80a lysate was prepared from donor strains and used to infect recipient cells. Transductants were selected in plates that contained erythromycin (30 μg/ml) in the 10-ml (total volume) bottom layer of agar; no drug was added to the middle (20-ml) layer or to the top (3-ml) layer; the top layer contained the bacteria.

**MIC determination and population analysis.** The MIC was determined by agar dilution as follows. Overnight cultures were diluted and spread on TSA plates containing different concentrations of methicillin. Colonies were counted after incubation at 37°C for 2 days. Population analysis was performed as previously described (22).

**Autolysis assay.** Triton X-100-stimulated autolysis was measured as described previously (6). Cells were grown exponentially to an OD$_{620}$ of 0.3. The culture was quickly chilled, and the cells were harvested by centrifugation (10,000 × g, 4°C, 5 min). Pellets were washed twice with cold distilled water and suspended to an OD$_{620}$ of 1.0 in 50 mM glycine–0.01% Triton X-100 buffer (pH 8.0). Autolysis was measured during incubation with aeration at 37°C as a decrease in OD$_{620}$ with a model 340 spectrophotometer (Sequoia-Turner Corp., Mountainview, Calif.).

**Cell wall turnover.** Cells were labeled for three to six generations (up to an OD$_{620}$ of 0.2 to 0.3) in 10 ml of TSB containing [3H]N-acetylglucosamine (18.5 KBq/ml, 3.7 GBq/mm) with aeration at 37°C. After labeling, the culture was centrifuged at room temperature for 5 min at 10,000 × g. The pellet was resuspended to an OD$_{620}$ of 0.02 in 50 ml of prewarmed fresh TSB containing cold 10 mM N-acetylglucosamine. Samples of 2 ml were withdrawn for the measurement of OD$_{620}$, and the radioactivity remaining in the cell wall was determined as previously described (6).

**Preparation of labeled cell wall and assay of autolytic activity.** [3H]N-acetylglucosamine (37 KBq/ml; specific activity, 214 GBq/mm) was added to exponentially growing cells. The cells were grown to an OD$_{620}$ of 0.5 for three generations and then harvested. Walls were prepared as described previously (4). To extract crude autolytic enzyme, cells were grown to an OD$_{620}$ of 1.0, chilled quickly, and centrifuged (10,000 × g, 4°C, 5 min). The cells were washed twice with cold distilled water and suspended in 3 M LiCl (0.6 g/ml of wet cells) for 30 min. The suspension was centrifuged, and the activity of autolytic enzyme in the extract (supernatant) was measured by its ability to solubilize radioactively labeled cell walls. The [3H]-labeled cell walls (0.2 mg/ml) were mixed with the extract and 70 mM sodium phosphate buffer (pH 7.0, 0.5-ml total volume) and incubated at 37°C. At intervals, 100-μl samples were withdrawn, mixed with 20 μl of formaldehyde (34%), and chilled on ice. After centrifugation at 4°C, radioactivity in 100 μl of

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a S, susceptible; R, resistant. Numbers within parentheses indicate MIC (micrograms per milliliter) for the cell majorly (22).
b Clinical MRSA isolate.
c Genetic outcross of RUSAL1 into the indicated recipient.
d Recipient in genetic outcross.
e Genetic backcross of RUSAL1 into the indicated recipient.
f Recipient in genetic backcross.
supernatant was counted in Ready Safe scintillation cocktail (Beckman). The activities of samples were calculated as percentages of the total radioactive label incorporated into the cell wall. Reference samples representing 100% of the incorporated radioactive label were digested completely with an excess amount of the extract from the staphylococcal strain RN450, and radioactivity was measured without centrifugation after formaldehyde was added. The protein concentrations of cell extracts were determined by the Coomassie blue assay (2).

**Bacteriolytic enzyme profiles on sodium dodecyl sulfate-polyacrylamide gels.** Bacteriolytic enzyme profiles were analyzed as described by LeClerc and Asselin (11). The gels contained heat-inactivated cells (1 mg of dry cells per ml) of either *S. aureus* RN450 or *Micrococcus luteus* ATCC 4698 (Sigma). After electrophoresis, the gels were washed for 1 h with several changes of distilled water and incubated in 0.1 M sodium phosphate buffer (pH 7.0) at 37°C for 24 h. The gels were stained in 0.1% methylene blue for 3 min and then destained extensively with distilled water overnight.

**Other methods.** Cell wall peptidoglycan was prepared and enzymatic cell wall hydrolases were analyzed by reversed-phase high-pressure liquid chromatography as described previously (4).

**RESULTS**

**Isolation of lytic mutant RUSAL1 and transduction of the Lyt" phenotype with the Tn551 marker.** After insertion of Tn551 into the chromosome of *S. aureus* RN450, 2,060 erythromycin-resistant colonies capable of growing at 43°C were obtained. Bacterial colonies were examined for autolytic activity in plates containing heat-inactivated *S. aureus* cells. Only 1 colony out of 2,060 examined exhibited a clearing zone that was substantially smaller than those of parental (RN450) colonies. This Lyt" colony was designated RUSAL1. Another property of RUSAL1 cultures was that they autolyzed more slowly than did cultures of RN450 during treatment with methicillin (10 μg/ml) (data not shown).

The Tn551 marker of RUSAL1 was backcrossed into the parent (RN450) by transduction. Backcrosses were obtained with a frequency of 3.3 × 10⁻⁹. Each of the three transductants examined showed the same Lyt" phenotype (small lysis zone around colonies and slow methicillin-induced autolysis) as RUSAL1. These findings indicated that the Lyt" phenotype of mutant RUSAL1 was caused by the Tn551 insertion into the chromosome. One of the Lyt" transductants, designated RUSAL2, was used in most of the physiological studies.

**Localization of the insertional site.** The chromosomal location of the Tn551 insert in RUSAL1 and its several transductants in a variety of *S. aureus* recipients was tested by pulsed-field electrophoresis of *SmaI* chromosomal fragments and probing with radioactively labeled Tn551 (Fig. 1). The location of Tn551 in RUSAL1 and RUSAL2 was also examined by conventional Southern analysis with Tn551 as a probe. The probe hybridized with DNA fragments of RUSAL1 and RUSAL2 in the same manner; no signal was detected with RN450. From the estimated lengths of the three Tn551-hybridizing fragments produced and from the known position of the two *Hind*III sites in *Tn917*, which has a sequence highly similar to that of Tn551, the insertion site of Tn551 could be located within a 3.2-kb *Hind*III fragment that was contained within a 17-kb *EcoR*I fragment (Fig. 2).

**Mechanism of lysis defect and cell wall composition.** The Tn551-interrupted genetic determinant may be involved with the synthesis of cell wall mureptides, causing the formation of an abnormal (autolysin-resistant) cell wall. Peptidoglycans prepared from the parental strain RN450 and from the isogenic Lyt" transductant RUSAL2 were purified, and the enzymatic hydrolysates containing disaccharide peptides were analyzed by a recently developed high-resolution tech-
nique (4). No differences in the muropeptide compositions of parental and mutant peptidoglycans were detected (Fig. 3).

Profile of bacteriolytic enzymes. Zymograms of sodium dodecyl sulfate extracts prepared from RN450 and RUSAL2 showed marked and multiple differences. With heat-inactivated S. aureus cells in the gel, several lytic bands were fainter in the RUSAL2 extracts and one particular band appeared to be more intense than the bands of corresponding mobility in the parental extracts. With heat-inactivated M. luteus cells as a substrate in the gels, a single intense lytic band of RN450 extracts was completely missing from the extracts of the lytic mutant RUSAL2 (Fig. 4). The electrophoretic mobility and the lack of lytic activity with S. aureus walls are consistent with this band representing the 51-kDa endo-β-N-acetylglucosaminidase described by Sugai et al. (18). Similar observations were made with LiCl extracts of the bacteria (data not shown).

Solubilization of radiolabeled cell wall by crude autolytic extracts. To compare the activities of autolytic enzymes of RUSAL2 with those of RN450, LiCl cell extracts and radiolabeled cell walls were prepared from both strains. The amount of protein in the LiCl extract from RUSAL2 (1.2 mg/g of dry cells) was much less than the amount of protein recovered in the extract of RN450 (3.4 mg/g of dry cells). This indicated that the amount of protein noncovalently

FIG. 2. Southern analysis of Tn551 insertion in S. aureus RUSAL1 and its backcross, RUSAL2. Total DNAs from RN450, RUSAL1, and RUSAL2 were digested with HindIII and/or EcoRI and probed with pRT1 (see Fig. 1 legend). The Xho1-HpaI fragment has two restriction sites for HindIII and no site for EcoRI. Lanes: HindIII digestion of RN450 (A), RUSAL1 (B), and RUSAL2 (C); EcoRI digestion of RN450 (D), RUSAL1 (E), and RUSAL2 (F); HindIII-EcoRI double digestion of RN450 (G), RUSAL1 (H), and RUSAL2 (I); molecular weight markers (lambda DNA digested with HindIII) (X).

FIG. 3. Separation of muropeptides by reversed-phase high-pressure liquid chromatography in strain RN450 (A) and the Lyt− Tn551 mutant RUSAL2 (B). Peptidoglycan was obtained and digested with a muramidase. Separation was done as described previously (4).

FIG. 4. Bacteriolytic enzyme profiles of S. aureus RN450 and RUSAL2. SDS-polyacrylamide gels containing heat-inactivated cells of S. aureus (A) or M. luteus (B) were prepared, and SDS cell extracts (13 μg of protein each) of RN450 (+) and RUSAL2 (−) were electrophoresed as described in Materials and Methods. After electrophoresis, the gels were washed with distilled water, incubated in 0.1 M phosphate buffer (pH 7.0) at 37°C for 24 h, and then assayed for enzyme activity.

FIG. 5. Solubilization of labeled cell wall by LiCl cell extracts of S. aureus RN450 or RUSAL2. Various amounts of crude bacteriolytic enzyme extracts from S. aureus RN450 or RUSAL2 were incubated with cell wall labeled with [3H]N-acetylglucosamine prepared from RN450. Symbols: 8 (■), 4 (△), 2 (●), 1 (□), 0.5 (▲), and 0.25 (○) μg of protein per ml. Samples were withdrawn at intervals, and the amount of radioactivity released into the supernatant was determined.
FIG. 6. Release of reducing sugars and free amino groups during incubation of the cell wall of S. aureus RN450 with LiCl cell extracts of strain RN450 or RUSAL2. Cell walls (1 mg/ml) were suspended in 20 mM sodium phosphate buffer (pH 7.0) and incubated with LiCl extracts (10 μg of protein per ml) from S. aureus RN450 (Δ, ○) or RUSAL2 (△, ▲). Samples were removed at intervals and boiled for 5 min to stop the reaction. The concentration of reducing sugars (○, ▲) and free amino groups (Δ, △) in the soluble fraction were determined as described previously (7, 14).

bound to the cell wall was three times less in RUSAL2 than in RN450. The extract of RN450 hydrolyzed the cell walls of RN450 and RUSAL2 with similar rates at all protein concentrations tested. The specific wall-hydrolyzing activity per milligram of protein in the LiCl extracts of RUSAL2 was about one-third of that of parental extracts (Fig. 5). Since the total amount of protein in the mutant extracts was already about three times less (per milligram of cell weight) than that in strain RN450, the autolytic activity of the mutant cells appears to be about nine times lower than that of the parental cells.

The amounts of reducing sugars and free amino groups released during hydrolysis of S. aureus cell walls in the RN450 and RUSAL2 LiCl cell extracts were compared (Fig. 6). The cell extract of RN450 released N-terminal amino groups about four times as fast as the RUSAL2 extract did. An even more pronounced difference was observed in the release of reducing sugars: glucosidase activity was virtually absent from RUSAL2 extracts.

Effect of the Lyt− mutation on some properties of the bacteria. Growing cultures of RN450 and RUSA12 were compared for their rates of growth and cell wall turnover, antibiotic (methicillin)-induced loss of viability, and rates of autolysis induced by treatments with detergent (Triton X-100) or methicillin.

RN450 and RUSAL2 each grew with a doubling time of 26 min in TSB at 37°C. We observed substantial differences in the cell wall turnover rates of various Lyt+ strains of S. aureus and their respective isogenic Lyt− derivatives generated via transduction with the Tn551-inactivated lyt gene(s) from RUSAL1; the degree of suppression of turnover rates clearly depended on the genetic background (Fig. 7).

Introduction of the Lyt− marker also suppressed methicillin-induced autolysis; however, the rates of loss of viability during methicillin treatment of the isogenic pairs of strains were similar (Fig. 8). The Lyt− transductants all showed greatly reduced rates of autolysis induced by Triton X-100 (Fig. 9).

The transposon-inactivated Lyt− determinant was transduced from the Tn551 mutant RUSAL1 into a number of methicillin-resistant S. aureus (MRSA) clinical isolates with different (homogeneous and heterogeneous) modes of expression of antibiotic resistance. The recipient strains showed widely different rates of methicillin-induced autolysis (the extremes being the very slow-lysing strain DU4916 and the fast-lysing strain COL), whereas all of the Lyt− transductant derivatives of these MRSA strains showed the reduced autolysis rate characteristic of RUSAL1 (data not shown).

Figure 10 compares the methicillin resistance levels (population analysis profiles) of the various staphylococcal strains used as recipients in the genetic crosses and their Lyt− derivatives. Introduction of the lytic defect had no detectable effect on the MIC values for MRSA strain DU4916 and the two methicillin-susceptible strains and only caused a modest shift in the direction of lower resistance in most of the other MRSA isolates tested. In only one of six Lyt− transductants of strain COL (RUSAL9) purified was there a more substantial (16-fold) decrease in the methicillin MIC. However, when RUSAL9 was used as a source of transducing lysate, which was then backcrossed into strain COL, most of the transductants again showed only a modest reduction of methicillin resistance (from the MIC for COL of 1,600 to 800 or 400 μg of methicillin per ml), and only 2 of the 10 transductants tested showed more substantial decreases in methicillin MICs (100 and 12.5 μg/ml, respectively).
FIG. 8. Suppression of methicillin-induced autolysis in the Lyt- strain of *S. aureus*. (A) Methicillin was added to exponentially growing cultures of the parental strain RN450 and its Lyt- transductant RUSAL2 at time zero, and the turbidity (OD620) of the cultures was monitored at intervals. (B) At the same intervals, samples were withdrawn, diluted, and plated on TSA for determination of viability. Symbols: control (○) and cultures incubated with 0.5 (△), 1.0 (□), 2.0 (●), 5.0 (▲), and 10 (■) μg of methicillin per ml.

DISCUSSION

The Lyt- mutant RUSAL1 appears to be the first Tn551-induced autolytic mutant of *S. aureus*. The successful transduction of the Lyt- phenotype via selection for the Tn551 marker into a variety of staphylococcal backgrounds indicates that the phenotype of this mutant is caused by the insertional inactivation of a chromosomal determinant. As to the nature of the inactivated gene, we can only speculate. Unlike the recently described auxiliary Tn551 mutants, in which autolysis and cell wall turnover were suppressed (6, 12), the Lyt- transposon mutant described here had no effect on the muropeptide composition of the cells. However, one should note that the analytical method used (4) would not resolve differences in other aspects of wall structure (e.g., degree of O acetylation, glycan chain length, etc.) that may influence autolysis. Nevertheless, the data together suggest that the autolytic phenotype may be related to some change(s) in the complement of autolytic enzymes that are detectable by the method of LeClerc and Asselin (11, 18). The most striking of the multiple autolytic band differences between the parental and isogenic Lyt- cells was the virtually complete absence from the mutant extract of a band that most likely represents the endo N-acetylglucosaminidase (19). The absence of this band also explains the lack of production of reducing groups from cell walls incubated with crude autolysin extracts from the mutant cells. However, the inactivated determinant is more likely to be a regulatory function than a structural gene for an autolysin, since the

FIG. 9. Suppression of detergent-induced autolysis in the Lyt- mutant of *S. aureus*. Bacterial strains were grown and labeled biosynthetically with [3H]-N-acetylglucosamine in their cell walls. Autolysis was induced by Triton X-100, and the rate of release of the radioactive label was measured. (A) Symbols: parent strain RN450 (○), Lyt- transductant RUSAL2 (●). (B) Symbols: Lyt- MRSA strain COL (○); Lyt- transductants RUSAL9 (●) and RUSAL10 (▲); RUSAL10; a Tn551-induced cell wall mutant of COL (△).
band differences between parental and mutant extracts were clearly multiple. The substantial (threelfold) decrease in the amounts of total cell wall-bound protein in RUSAL2 (as compared with those in the parental cells) suggests that the inactivated function is involved with the transport of autolysis to the outer surface of the bacterium.

A major advantage of the Tn551-linked Lyt− phenotype is in the relative ease with which the inactivated autolytic phenotype may be introduced into various genetic backgrounds for testing the physiological effects of the autolytic system. As expected, the Tn551-linked Lyt− marker resulted in the suppression of both detergent- and antibiotic-induced autolysis and cell wall turnover in all of the genetic backgrounds tested. On the other hand, inactivation of autolysis and suppression of cell wall turnover in MRSA isolates had only a minor effect on the level of methicillin resistance in most of the transductants. This finding is of importance, since other Tn551 mutants (MRSA auxiliary mutants [22]) were shown to cause dramatic parallel decrease both in the methicillin MIC and in the wall turnover rate of the highly and homogeneously resistant strain COL (6) and similar findings were reported for the so-called femA mutants of another MRSA strain (12). Furthermore, the reduced MIC and wall turnover cotransduced in the backcrosses (6). On the basis of this finding, it was proposed that cell wall turnover has a direct functional role in the expression of high-level antibiotic resistance in staphylococci, in a functional analogy with DNA excision repair, by providing a mechanism for the removal of structurally abnormal (and potentially lethal) pieces of cell wall material that may incorporate into the peptidoglycan of the cells during exposure to beta-lactam antibiotics (6).

A recent biochemical analysis of these auxiliary mutants showed that these bacteria produced peptidoglycan of drastically altered composition (5) and that the reduced rate of wall turnover in these mutants was most likely a conse-
quence of this change in the chemical structure of peptidoglycan, making these polymers poor substrates for the enzymes catalyzing cell wall turnover; this suggestion had been made earlier (12).

If the reduction of antibiotic resistance in the auxiliary mutants were, indeed, related to their gross slower rate of wall turnover, then a direct suppression of this process (e.g., in the autolysin-defective Tn551 mutant) would also be expected to reduce the level of antibiotic resistance in MRSA. The availability of the Tn551-linked autolytic mutant has allowed us to test this hypothesis. Transduction of the Lyt" phenotype caused only a minor decrease in the level of methicillin resistance, whereas cell wall turnover rates and autolytic rates were drastically reduced. Southern analysis of RUSAL9 (the Lyt" transductant of MRSA strain COL) and its backcrosses into COL (constructs RUSAL9-6 through 9-10; Fig. 10) showed that Tn551 was retained in its original position in all of these transductants, irrespective of whether they had lower methicillin MICs. These findings indicate that even a massive reduction in cell wall turnover need not necessarily bring about reduction in methicillin resistance. It is conceivable that the particular murine hydrolase needed to remove the fraudulent (antibiotic-induced) pieces of cell wall remains intact in the Lyt" Tn551 mutant. Alternatively, the findings may imply that the drastic reduction in methicillin resistance level observed in the auxiliary mutants was caused not by the slowdown in wall turnover but rather by some other biosynthetic misfunction related to the abnormal muropeptide composition of the mutant cell walls.

The relative ease with which the Tn551-linked L Y t" phenotype can be introduced into various staphylococcal backgrounds should make the RUSAL1 mutation a useful tool for testing the role of the staphylococcal autolytic system in other phenomena.

ACKNOWLEDGMENT

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REFERENCES