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Isolation and Characterization of a Mutant of *Staphylococcus aureus* Deficient in Autolytic Activity

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A mutant of *Staphylococcus aureus* H (RUS3) was isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The rate of autolysis of whole cells and isolated cell walls of RUS3 was less than 10% of the parent strain. In addition, the ability of the crude soluble enzyme isolated from RUS3 to degrade cell walls was negligible compared with the parent strain. The cell wall composition and the generation time of RUS3 were comparable to the parent strain. Unlike *S. aureus* H, RUS3 grew in clumps and did not undergo cell wall turnover. Both strains exhibited identical kinetics of killing by penicillin G. This may indicate that autolytic enzymes play a role in cell wall turnover and cell separation, but in *S. aureus* most of the autolytic activity is unrelated to the lethal effect of cell wall antibiotics.

Autolytic enzymes that degrade bacterial cell walls have been the subject of intensive study over the past decade and their mechanism of action has been well documented (9, 13). In marked contrast, the metabolic role of these ubiquitous enzymes is not clear. In one approach to this problem, investigators have studied mutant strains with altered autolytic activity. Unfortunately in the majority of such studies the observed differences in the lytic behavior were associated with changes in cell wall composition (7, 8, 16, 22), and not primarily due to a modification of the autolytic enzyme(s). Since *Staphylococcus aureus* H has a well-characterized autolytic enzyme system in which the *N*-acyl-muramyl-L-alanine amidase (amidase) activity predominates (20), we have chosen this model system for our investigation. In the present article we describe the isolation and properties of a Lyt$^-$ mutant derived from *S. aureus* H. Evidence is presented that the observed phenotypic differences are due to its defective autolytic enzyme and not related to any alteration of its substrate.

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MATERIALS AND METHODS

Strains and growth conditions. General conditions for maintenance, isolation, and characterization of the parental strain *S. aureus* H (Str) have been described (1). All cultures were grown in phytone-yeast extract-K_2HPO_4 (PYK) broth containing 0.2% glucose on a gyratory shaker at 37 C (12).

Selection procedure for Lyt$^-$ mutants. Nutrient agar plates containing either commercially available (Calbiochem) *Micrococcus lysodeikticus* or heat-killed *S. aureus* cells (1 mg/ml) (lyophilized) were spread with 100 to 200 colony-forming units (CFU) and incubated at 37 C for 24 h. They were then placed at 4 C and inspected every 24 h. With *S. aureus* H, a distinct zone of clearing appeared around the colonies on *M. lysodeikticus* plates within 24 h at 4 C and increased with continued incubation in the cold. The zone of clearing was less pronounced on *S. aureus* plates but could be clearly seen after 48 h of incubation at 4 C. Colonies that failed to exhibit any zone of clearing after 4 days of incubation at 4 C (on both *M. lysodeikticus* and *S. aureus* plates) were selected for further study.

Preparation of labeled substrate. Peptidoglycan labeled with L-[^{3}H]lysine was prepared from cells incubated in a defined medium in the presence of penicillin G (5 μg/ml) as described previously (21). The peptidoglycan residue was repeatedly washed with water and lyophilized. When needed, the material was suspended in 10 mM phosphate buffer (pH 7.20), sonicated briefly to disperse clumps, and adjusted to a concentration of about 8 mg/ml (about 3.3 $\times$ 10^6 counts/min per ml).

Preparation and assay of soluble autolytic enzyme. The soluble autolytic enzyme present in the cytoplasm was isolated by differential centrifugation after mechanical disintegration of cells (21). The 100,000 $\times$ *g* supernatant was dialyzed overnight at 4 C against phosphate buffer and stored at -20 C. Enzyme preparations were thawed only once and used within a week of isolation.

Activity of the soluble autolytic enzyme was measured by its ability to solubilize radioactivity

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from labeled peptidoglycan (21). Twenty microliters of L-[^{3}H]lysine-labeled peptidoglycan substrate (8 mg/ml) was mixed with crude enzyme and 10 mM phosphate buffer (pH 7.2) (final concentration) and incubated at 37 C. Final volume was usually 200 μl. Reaction was stopped by adding 1 ml of distilled water followed by a transfer to a boiling-water bath for 2 min. The mixture was filtered (0.45-μm HAWP membrane filter, Millipore) and 0.5 ml of the filtrate was assayed for radioactivity. A control containing heat-inactivated enzyme was run with every experiment.

Assay of cell wall turnover. Cell wall turnover, as indicated by the decrease in the amount of radioactivity associated with the cell wall peptidoglycan and teichoic acid was determined as previously described (24). For turnover studies, cells were labeled with *N*-[^{14}C]acetylglucosamine (GlcNAc). Specificity of GlcNAc in labeling the two cell wall polymers of *S. aureus* has been demonstrated previously (24).

Other methods. Methods for the preparation and analysis of cell walls, isolation of native cell walls, and the methods (both turbidimetric and radioactive) for following the rate of lysis of whole cells and cell walls have been all described previously (3, 10, 11). All determinations of CFU were done on plates of nutrient agar. The mutant cell suspension was routinely sonicated for 1 min (Biosonik-111; power setting, 20) before dilution and plating. Effectiveness of this level of sonication in the selective disaggregation of clumped cells of *S. aureus* has been demonstrated (2). Phosphate was determined by the method of Chen et al. (4), and protein was measured by the method of Lowry et al. (14). All radioactivity was measured in a Beckman LS 230 scintillation counter using a toluene-based scintillation fluid containing Triton X-100 (15). Counting efficiency was 78% for ^{14}C and 28% for ^{3}H.

RESULTS

Isolation and autolytic characteristics of Lyt$^-$ mutant. *S. aureus* H (Str) cells suspended in buffer were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1). About 80% of the cells were killed by the mutagen. Survivors were diluted into fresh PYK broth, and after 2 h at 37 C aliquots were diluted and spread on *M. lysodeikticus* plates. Colonies that failed to show any zone of clearing (about 1 in 6×10^3) were further tested on *S. aureus* plates. Of the 16 colonies selected on the *M. lysodeikticus* plates, 7 failed to exhibit any clearing on the *S. aureus* plates. These 7 colonies were cloned and grown in PYK broth, and their autolytic properties were compared with the wild type. Of these seven isolates, one strain designated RUS3 appeared to be the most promising and was selected for further study.

We first compared the rate of whole-cell lysis of *S. aureus* H with RUS3. In a typical experiment, the turbidity of *S. aureus* H cells (at 585 nm) suspended at 37 C in 10 mM phosphate buffer (pH 7.20) decreased about 50% during a 6-h period. The decrease in turbidity of RUS3 was less than 5% under comparable conditions. When isolated cell walls of the two strains were allowed to lyse by endogenous autolytic activity, similar results were obtained (Fig. 1). The optical data were confirmed by incubating native cell walls labeled with GlcNAc at 37 C. Over a period of 24 h at 37 C, less than 6% of the radioactivity present in cell walls of RUS3 was solubilized, whereas with *S. aureus* H cell walls prepared and incubated under identical conditions the value was about 80%.

The autolytic rate of RUS3 (both whole cells and isolated cell walls) could not be stimulated by changes in pH or ionic strength of the incubation medium. Also, growth in the presence of 1.0 M NaCl, which has been shown in certain cases to stimulate the rate of autolysis of *S. aureus* (10), had no discernible effect on RUS3.

The generation time of RUS3 as judged by increase in optical density (OD) was comparable to *S. aureus* H: about 30 min at 37 C in PYK broth. Observation by phase contrast microscopy showed that RUS3 grew in large clumps in a fashion analogous to another autolytic mutant (10), whereas *S. aureus* H appeared as well-separated cocci with little or no clumping. We tried to disaggregate the clumps by addition of exogenous lytic enzymes (lysostaphin or the soluble autolytic enzyme from *S. aureus* H). Although the soluble enzyme had no observable effect on the cells of RUS3 (it should be noted that this enzyme also does not lyse whole cells of *S. aureus* H), addition of lysostaphin led to a gross lysis of cells. All attempts to disaggregate RUS3 with lysostaphin (e.g., low concentration, incubation at 20 C) were unsuccessful. The RUS3 cells could be disaggregated by mild sonication, and the number of CFU obtained from a suspension of RUS3 cells after

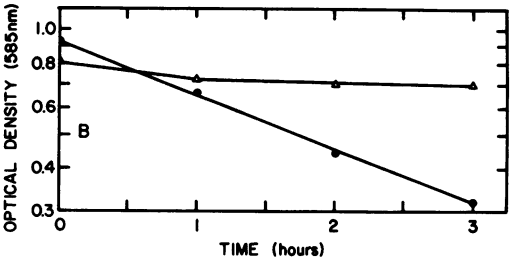

FIG. 1. *Autolysis of isolated cell walls of S. aureus H and RUS3. Native cell walls of S. aureus H and RUS3 suspended in 10 mM phosphate buffer (pH 7.20) were incubated at 37 C. At intervals, samples were withdrawn for determination of OD. S. aureus H walls (●), RUS3 walls (△).*

such a treatment was comparable to that of *S. aureus* H. The corresponding values for *S. aureus* H and RUS3 were 4×10^7 to 8×10^7 per ml and 3×10^7 to 6×10^7 per ml, respectively (per 0.1 OD unit at 585 nm).

One interesting property of RUS3 that might be related to its negligible autolytic activity is its marked ability to survive under nongrowing conditions. Thus, although the titer of *S. aureus* H cells suspended in distilled water at 37 C decreased from 7×10^{10} to 6×10^8 in 7 days, the corresponding decrease in the titer of RUS3 cells was only from 6×10^9 to 2×10^9. Similar results were obtained when the cells were suspended in phosphate buffer. Therefore, it might be possible to use this phenotype as an enrichment technique in the future isolation of Lyt$^-$ mutants.

Autolytic properties of the cytoplasmic enzyme. We next compared the activity of the soluble autolytic enzyme of the Lyt$^-$ mutant to that of *S. aureus* H. This was important since the bulk of the autolytic amidase of *S. aureus* H is known to be located in the cytoplasm (20). To increase the sensitivity of assay, the labeled substrate was isolated from cells incubated in the presence of penicillin (21). It may be seen that the ability of the crude enzyme from RUS3 to degrade cell wall peptidoglycan is negligible compared to the wild type (Fig. 2). Furthermore, in marked contrast to the parental enzyme, increasing additions of the mutant enzyme had very little effect on the release of radioactivity (Fig. 3). Identical results were obtained when the substrates were switched, e.g., when the labeled peptidoglycan was isolated from RUS3 instead of *S. aureus* H. The possibility of the presence of an inhibitor of lytic activity in RUS3 was ruled out by suitable mixing experiments.

Cell wall composition. The fact that both the peptidoglycan substrates (from *S. aureus* H and RUS3) could be degraded at the same rate by the parental enzyme suggested that there were no significant variations in these polymers. This was confirmed by the quantitative analysis of the cell walls (Table 1). It is evident that the cell wall composition of RUS3 (both peptidoglycan and teichoic acid) is very similar if not identical to the wild type. In addition, the ability of isolated cell walls of RUS3 to bind phage 52A was similar to that reported for *S. aureus* H (1). Since the phage receptor site in *S. aureus* involves both peptidoglycan and teichoic acid (1, 19), this further supports the contention that there are no significant variations in the cell wall composition of RUS3.

Cell wall turnover. We have previously demonstrated the role of amidase in the turnover of cell walls of *S. aureus* (24). It was of interest to examine if the cell walls of the Lyt$^-$ mutant could undergo turnover. The experimental de-

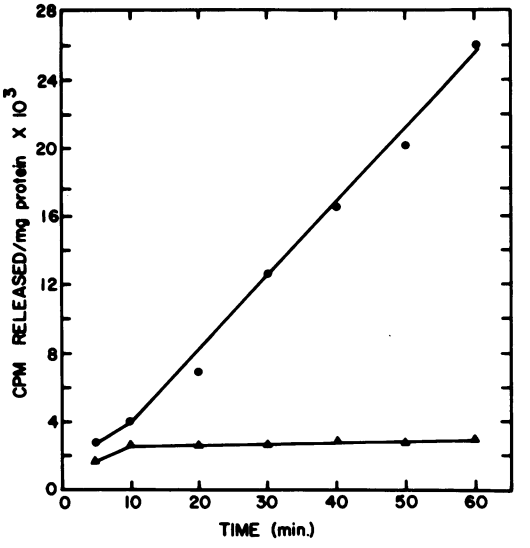

FIG. 2. *Kinetics of solubilization of labeled peptidoglycan by soluble autolytic enzyme from S. aureus H and RUS3. Peptidoglycan substrate labeled with L-[³H]lysine (from S. aureus H) was incubated with the 105,000 × g supernatant isolated from S. aureus H or RUS3 at 37 C. At intervals, reaction was terminated by boiling and the released radioactivity was determined. See text for details. S. aureus H enzyme (●), RUS3 enzyme (▲).*

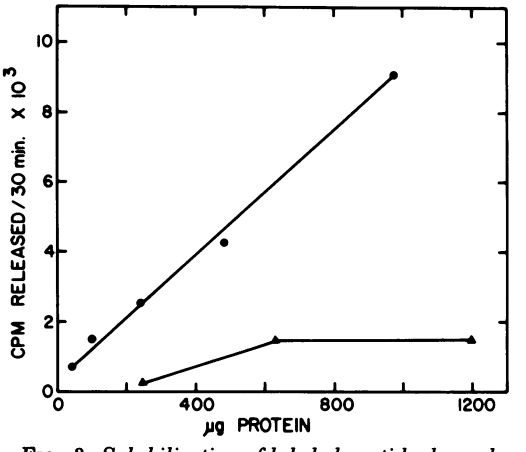

FIG. 3. *Solubilization of labeled peptidoglycan by increasing additions of soluble autolytic enzyme. Varying amounts of the crude autolytic enzyme from S. aureus H or RUS3 were incubated with peptidoglycan labeled with L-[³H]lysine (from S. aureus H) at 37 C for 30 min and the released radioactivity was assayed as described in the text. Symbols as in Fig. 2.*

sign for detection of cell wall turnover has been previously described (24). Briefly, the cells were labeled with [^{14}C]GlcNAc for four generations and then diluted into fresh prewarmed growth medium containing an excess of cold GlcNAc. Loss of radioactivity from cell wall polymers during growth in the nonlabeled medium is a direct measure of cell wall turnover. Figure 4 shows that the radioactivity associated with the cell wall peptidoglycan and teichoic acid of RUS3 did not change while the cells were growing, establishing the absence of cell wall turnover. This is in striking contrast to the behavior of *S. aureus* H where both peptidoglycan and teichoic acid were undergoing active turnover (Fig. 4).

Effect of cell wall antibiotics. It has been postulated that autolytic enzymes play a major role in the bactericidal effect of cell wall antibiotics (17, 18). In a Lyt$^-$ mutant of *Diplococcus pneumoniae*, marked resistance to cellular lysis and killing was observed with several antibiotics that inhibit the biosynthesis of cell wall (23). In the present study we compared the effects of penicillin G on the growth and viability of *S. aureus* H and RUS3. The minimum inhibitory concentration of penicillin G (by tube dilution assay) for both *S. aureus* H and RUS3 was determined to be 0.05 μg/ml. Figure 5 shows the effect of adding penicillin G to exponentially growing cultures of *S. aureus* H and RUS3. The growth rate of both cultures decreases in the presence of penicillin (especially at 5.0 μg/ml), but there is no evidence of cellular lysis even after 180 min of incubation with the antibiotic. The absence of gross cellular disintegration was further confirmed by examination of the cells from both cultures by phase contrast microscopy. Furthermore, penicillin-treated cells and control cells did not display

TABLE 1. *Cell wall composition of S. aureus H and RUS3*

S. aureus strain used	Wall component[a]						
	Muramic acid	Glucosamine	Glutamic acid	Glycine	Lysine	Alanine	Phosphorus
H	0.37	0.73	0.42	1.84	0.47	1.12	0.82
RUS3	0.41	0.78	0.42	1.98	0.48	1.15	0.84

[a] Expressed as micromoles per milligram of cell wall.

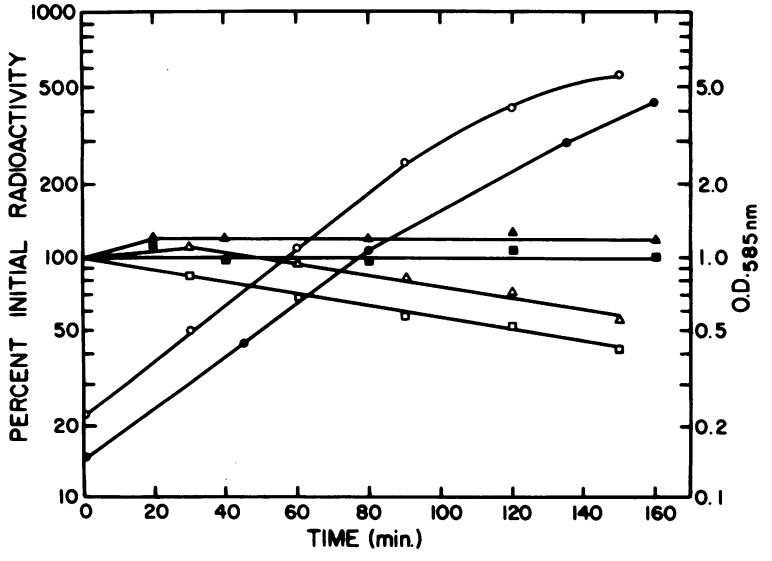

FIG. 4. *Turnover of cell wall polymers in S. aureus H and RUS3. S. aureus H and RUS3 cells growing in PYK broth were uniformly labeled with [^{14}C]GlcNAc (specific activity, 56 μCi/μmol; 0.2 μCi/ml). The cells were then harvested and diluted into fresh prewarmed PYK broth containing 100 mM cold GlcNAc. Samples were withdrawn at intervals and the radioactivity associated with the cell wall peptidoglycan and teichoic acid was determined after chemical fractionation as described in text. Growth of the cultures was monitored turbidimetrically. Symbols: ○, OD; △, teichoic acid; □, peptidoglycan. Open symbols, S. aureus H; closed symbols, S. aureus RUS3.*

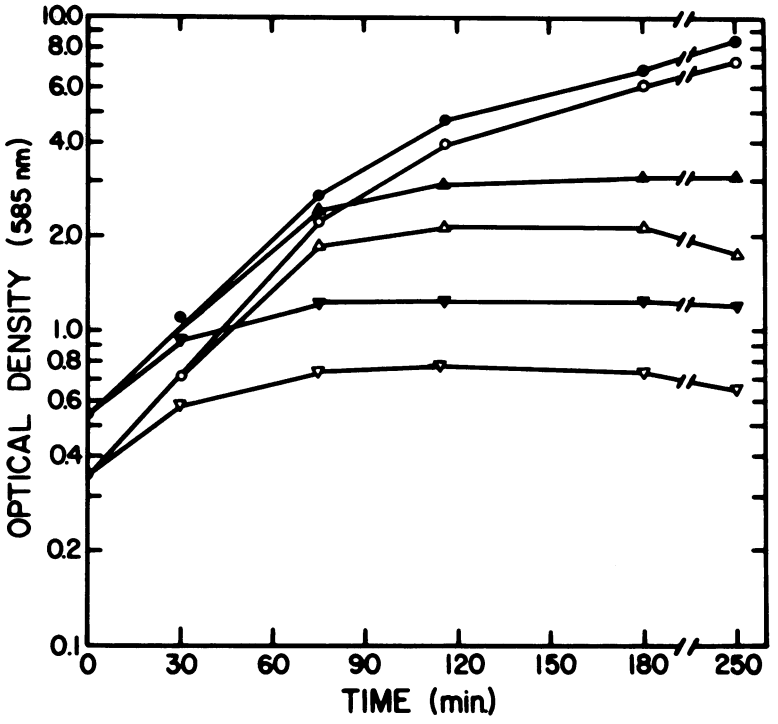

FIG. 5. *Effect of penicillin G on the growth of S. aureus H and RUS3. Penicillin G (at 0.05 and 5.0 μg/ml) was added to exponentially growing cultures of S. aureus H and RUS3 at zero time and the turbidity of the cultures was monitored at 585 nm. Symbols: ○, control; △ and ▽, penicillin at 0.05 and 5.0 μg/ml, respectively. Open symbols, S. aureus H; closed symbols, S. aureus RUS3.*

differences in the extent of release of macromolecules that absorb at 260 nm or in the suceptibility to lysis in the presence of 1% sodium dodecyl sulfate. In marked contrast, penicillin was found to have a dramatic effect on viability. Figure 6 shows the exponential decrease in the number of CFU of *S. aureus* H growing in the presence of penicillin G. The rate of killing is significantly higher with the higher concentration of penicillin. To our surprise, the kinetics of killing of RUS3 cells by penicillin G was identical to that of *S. aureus* H (Fig. 6). The above data were confirmed in three separate experiments. Similar results were obtained with D-cycloserine (data not shown). These findings strongly suggest that at least in *S. aureus* the primary lethal effect of penicillin may not be directly coupled to the autolytic activity.

DISCUSSION

Data presented in this article support the hypothesis that the primary defect of RUS3 is in the amidase. This could be caused either be a decreased level of autolytic enzyme or by an alteration in the structure of the protein. The possibility that the Lyt$^-$ phenotype is related to a modification of the cell wall substrate is unlikely because cell walls of the parental and mutant strains have identical composition and can bind phage 52A at similar rates. Furthermore, the isolated cell walls of both strains are digested at the same rate by the amidase isolated from the parental strain. In a previous study with a Lyt$^-$ mutant in *Streptococcus faecalis*, Pooley et al. showed that the mutant cell wall peptidoglycan was less cross-linked than the wild type and as a result was degraded more rapidly by the wild-type autolytic enzyme (16). Though we have not directly analyzed the degree of cross-linking of RUS3 walls, the substrate characteristics of RUS3 wall suggest no significant variation in its cross-linking. It should be further noted that, in contrast to the behavior of the *S. faecalis* mutants, the autolytic rate of RUS3 could not be stimulated by changes in ionic strength. It is remarkable that the mass doubling time of RUS3 is comparable to that of *S. aureus* H. This implies that the bulk of the autolytic activity (over 90%) in *S. aureus* is not essential for growth.

A relationship between alteration of autolytic behavior and clumping of cells in liquid culture has been noted before in other systems,

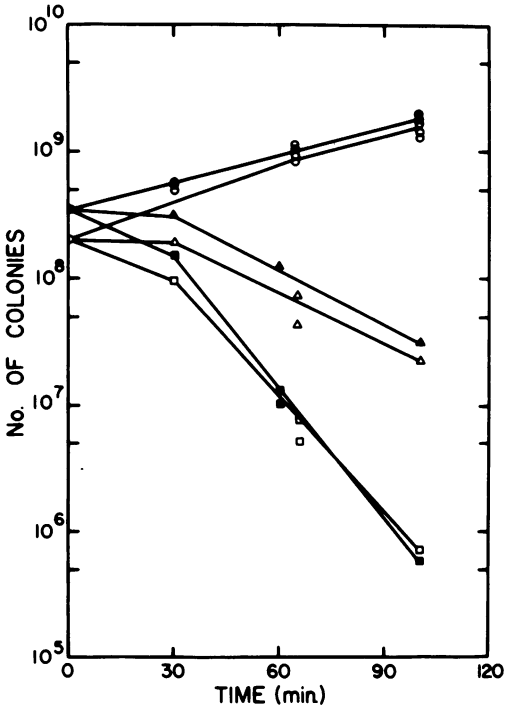

FIG. 6. *Effect of penicillin G on the viability of S. aureus H and RUS3. Penicillin G (at 0.05 and 5.0 μg/ml) was added to exponentially growing cultures of S. aureus H and RUS3 at zero time. At suitable intervals, samples were withdrawn, suitably diluted, and plated on nutrient agar. The RUS3 samples were sonicated before plating as described in text. Symbols: ○, control; △ and □, penicillin at 0.05 and 5.0 μg/ml, respectively. Open symbols, S. aureus H; closed symbols, S. aureus RUS3.*

suggesting a role of autolytic enzyme in cell separation (2, 7, 16, 22). To function in this role, the activity of these endogenous enzymes must be under exquisite control resulting in the nicking of selected bonds. Therefore, we were not surprised at the inability of exogenously added enzymes to disaggregate the clumps of RUS3. It is significant that viable cells can be recovered from the clumps (with an increase in CFU) after mild sonication. This suggests that the role of amidase in cell separation occurs at a late stage of the division cycle – after partitioning of the genome and growth of a functional cross wall.

The inability of the cell walls of RUS3 to undergo turnover is a nice illustration of the role of autolytic amidase in this process. To our knowledge, this is the first time such a correlation has been demonstrated in a Lyt⁻ mutant strain. This also reinforces the concept that the process of cell wall turnover is not essential for cell growth and division.

The observed effect of cell wall antibiotics on RUS3 is in striking contrast with the pneumococcal system where a Lyt⁻ strain was shown to have a marked resistance to similar antibiotics (23). Though we cannot explain the reason for this discrepancy, one major difference between the two systems is in the lytic effect of penicillin. Thus, unlike the pneumococcal system, addition of penicillin G to a growing culture of *S. aureus* does not result in any visible lysis or cellular disintegration – at least not within the first 2 to 3 h, during which time the viable titer declines exponentially (Fig. 5 and 6). This is true for both the wild-type strain, with its normal complement of autolytic enzyme, and the Lyt⁻ strain. This suggests that the role of autolytic enzyme in the killing of sensitive cells by antibiotics could vary widely and in *S. aureus* it probably plays a relatively minor role. This is reinforced by our observation that both with the wild-type and the mutant strains the rate of killing is proportional to the penicillin concentration (Fig. 6). It might be recalled that in *Escherichia coli*, which is lysed rapidly by penicillin, lower concentrations of penicillin lead to a more rapid loss of viability, a phenomenon referred to as the "zonal effect" (5, 6). This suggests that, unlike *S. aureus*, the lytic enzymes do play a significant role in *E. coli*; at low pencillin levels the biosynthetic machinery could stumble along making more uncrosslinked wall material that in turn would be digested more rapidly by the autolytic enzymes.

The three observations reported in this article: (i) absence of cell lysis, (ii) identical killing rates for both strains, and (iii) absence of zonal effect, suggest that autolytic enzymes are not primary killing agents when *S. aureus* is treated with penicillin. It might be that killing is related to the irreversible nature of damage caused by penicillin, i.e., once penicillin is fixed the cell loses its viability. Obviously, more work is needed to define the nature of the primary defect(s) responsible for penicillin-induced killing in *S. aureus*.

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