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Suppression of Autolysis and Cell Wall Turnover in Heterogeneous Tn*551* Mutants of a Methicillin-Resistant *Staphylococcus aureus* Strain

BOUDEWIJN L. M. DE JONGE, HERMINIA DE LENCASTRE,† AND ALEXANDER TOMASZ*

The Rockefeller University, 1230 York Avenue, New York, New York 10021

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Isogenic Tn*551* mutants of a highly and uniformly methicillin-resistant strain of *Staphylococcus aureus* were tested for their rates of autolysis and cell wall degradation in buffer and for cell wall turnover during growth. The normal (relatively fast) autolysis and turnover rates of the parent strain were retained in a Tn*551* mutant in which the insert was located within the *mec* gene and which produced undetectable levels of penicillin-binding protein 2A. On the other hand, autolysis and cell wall turnover rates were greatly reduced in auxiliary mutants, i.e., mutants in which the transposon caused conversion of the high-level and uniform resistance of the parent strain to a variety of distinct heterogeneous expression types and greatly decreased resistance levels. All of these mutants contained an intact *mec* gene and produced normal amounts of penicillin-binding protein 2A, and one of the mutations was located in the *femA* region of the staphylococcal chromosome (B. Berger-Bachi, L. Barberis-Maino, A. Strassle, and F. H. Kayser, Mol. Gen. Genet. 219:263–269, 1989). Autolysis rates were related to the degree of residual methicillin resistance and to the sites of Tn*551* insertion. Fast cell wall turnover may help expression of high-level methicillin resistance by providing a mechanism for the excision of abnormal (and potentially lethal) structural elements of the cell wall synthesized by the bacteria in the presence of methicillin.

While several genetic determinants have been identified as being essential for methicillin resistance, the biochemical (enzymatic) functions of these determinants remain unknown. All methicillin-resistant clinical strains of *Staphylococcus aureus* examined so far contain the *mec* gene (1, 10, 11, 13), a 1.2-kb DNA of foreign origin which codes for a 78-kDa protein capable of binding penicillin with very low affinity (PBP 2A) (7, 17, 23). It is also known that inactivation of *mec* by transposon insertion within the gene results in the cessation of the production of PBP 2A and a uniform drop of the MIC value from about 800 μg/ml to 4 μg/ml (12, 14). On the other hand, the biochemical function of PBP 2A remains unknown. It is generally assumed that this low-affinity PBP may act as a surrogate enzyme for cell wall synthesis under conditions in which the high concentration of a beta-lactam antibiotic in the environment inhibits the functioning of normal (high-affinity) staphylococcal PBPs. However, there is no direct experimental evidence for this.

In addition to *mec*, the phenotypic expression of high-level and uniform methicillin resistance also requires the presence of additional, auxiliary genes, the nature and gene product (factor X) (8) of which are also unknown (for a recent review, see reference 20). Evidence for the existence of these genes also comes from studies with transposon mutagenesis. Insertion of Tn*551* at several chromosomal locations outside the *mec* gene resulted in drastic alteration of the phenotypic expression of methicillin resistance (3, 9). Some of these inserts have been mapped in an area of the staphylococcal chromosome (*femA* [2]); others remain to be mapped. It has been suggested that these sites be referred to collectively as auxiliary genes (20) in reference to their operational definition as genetic elements that assist the *mec* gene in conveying a uniform, high-level antibiotic resistance to the bacteria. Several such auxiliary gene mutants have been described (9, 14). All of them carried intact *mec*, and most of them produced normal levels of PBP 2A. Most such mutants generate complex (heterogeneous) but reproducible antibiotic resistance phenotypes, with drastic reduction in the methicillin MIC for the majority of the bacteria (5).

It has been suggested that auxiliary genes involve the staphylococcal autolytic enzymes specifically, which would explain the homogeneous-versus-heterogeneous phenotypes of methicillin-resistant *S. aureus* (MRSA) (8, 19, 20). In addition, experimental evidence has been described indicating differences in the autolytic potential of various MRSA strains (15). Most recently, it has been reported that a heterogeneously resistant strain autolyzed more rapidly than a related, homogeneously resistant strain (6). However, in these previous studies the genetic relationships between the strains compared were not clear. Different isolates of *S. aureus* are known to differ in their intrinsic autolytic capacities (16), and therefore the differences detected in autolytic rates need not reflect differences in the methicillin resistance phenotype.

The purpose of the studies described here was to reexamine the question of autolytic rates in various phenotypic classes of MRSA by exploiting the availability in our laboratory of isogenic transposon mutants of a single highly and homogeneously resistant parental MRSA strain. These mutants have been well characterized for their resistance phenotypes, and they have been purified by genetic backcrosses into the common parental strain (7, 8, 14). By using these strains, we now show that mutations in auxiliary genes involved in methicillin resistance suppress the rates of autolysis and cell wall turnover. To our best knowledge, this observation provides the first direct experimental evidence linking an aspect of methicillin resistance with a specific biochemical function. The findings indicate that the auxiliary

* Corresponding author.
† Permanent address: Centro de Tecnologia Quimica e Biologica, Oeiras, and Universidade Nova de Lisboa, Lisbon, Portugal.

TABLE 1. Strains used in this study

Strain	Origin (reference)	MIC (μg/ml) of methicillin[a]	Resistance expression[b]	Intact *mec*[c]	PBP 2A[d]	Tn*551* insertion site	Autolysis[e] (rate)
COL	Parent (14)	800	Homogeneous	Yes	+	–	Fast (0.64 ± 0.02)
RUSA 4	Tn*551* mutant (12, 14)	4	Homogeneous	No	–	In *mec*	Fast (0.54 ± 0.04)
RUSA 4 BC1	Backcross into COL (this study)	4	Homogeneous	No	–	In *mec*	Fast (0.54 ± 0.02)
RUSA 12F	Tn*551* mutant (9)	12	Heterogeneous class 2	Yes	+	? (in auxiliary gene)	Slow (0.30 ± 0.05)
RUSA 12F BC 1	Backcross into COL (this study)	12	Heterogeneous class 2	Yes	+	? (in auxiliary gene)	Slow (0.28 ± 0.05)
RUSA 12F BC 2	Backcross into COL (this study)	12	Heterogeneous class 2	Yes	+	? (in auxiliary gene)	Slow (0.31 ± 0.04)
RUSA 12F BC 3	Backcross into COL (this study)	12	Heterogeneous class 2	Yes	+	? (in auxiliary gene)	Slow (0.30 ± 0.10)
RUSA 10	Tn*551* mutant (this laboratory)	3	Heterogeneous class 1	Yes	+	? (in auxiliary gene)	Slow (0.17 ± 0.05)
RUSA 10 BC 1	Backcross into COL (this study)	3	Heterogeneous class 1	Yes	+	? (in auxiliary gene)	Slow (0.18 ± 0.04)
RUSA III-3	Tn*551* mutant (2, 9)	3	Heterogeneous class 1	Yes	+	Ω2003 (*femA*)	Slow (0.18 ± 0.03)

[a] MICs were determined by agar dilution; the numbers refer to the MIC for most (≥99.9%) cells of the particular strain.
[b] Determined by population analysis.
[c] DNA isolated from the strains was tested for the specific activity of the *mec* marker by genetic transformation (10).
[d] PBP 2A was assayed by the fluorographic method (14).
[e] See Materials and Methods. Values are the averages of three experiments ± standard deviation.

genes are involved with the peptidoglycan metabolism of *S. aureus*. They also suggest that the expression of high-level resistance may require accelerated cell wall turnover.

(Parts of this work have bene presented at the 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, Ga., 21 to 24 October 1990 [4a].)

MATERIALS AND METHODS

Strains and growth conditions. Strains used are listed in Table 1. Tn*551* insertion mutants were isolated by methods described in reference 14. In the construction of backcrosses, chromosomal DNA isolated from the transposon mutants was introduced into the parent strain, COL, by genetic transformation, followed by the selection of erythromycin-resistant transformants (14). Chromosomal DNA and competent staphylococci were prepared by published procedures (18, 21). All strains were grown in tryptic soy broth (Difco, St. Louis, Mo.) with aeration at 37°C in a water bath (New Brunswick Scientific; 200 rpm). The transposon mutants and backcrosses were grown in the presence of 10 μg of erythromycin per ml. Growth was monitored by the increase in optical density at 620 nm (OD_{620}) in a spectrophotometer (Pharmacia LKB, Piscataway, N.J.) in cuvettes with a 1-cm path length.

Autolysis assay. Cells were grown exponentially for at least 16 generations to an OD_{620} of 0.3. The culture was then quickly chilled in an ice-ethanol bath until the temperature dropped below 10°C. After centrifugation (10,000 × *g*, 4°C, 5 min), the cells were washed once with ice-cold water. Pellets were suspended to an OD_{620} of 1.0 in a 50 mM glycine–Triton X-100 (0.01%) buffer, pH 8. Autolysis was measured during incubation with aeration at 37°C as a decrease in the OD_{620} with a model 340 spectrophotometer (Sequoia-Turner Corp., Mountain View, Calif.). Autolysis rates were defined as the reciprocal time (per hour) needed to achieve a 50% decrease in OD_{620}.

Cell wall turnover. Cells were labeled for 6 generations in tryptic soy broth containing 1 μCi and 5 μg of [^{3}H]*N*-acetylglucosamine (GlcNAc) per ml (Amersham, Arlington Heights, Ill.) by a published procedure (4). After being labeled, the cells were pelleted at room temperature for 5 min at 10,000 × *g*. The pellet was resuspended in tryptic soy broth prewarmed to 37°C. At regular time intervals, samples were taken, immediately frozen on dry ice, and stored at −20°C. Subsequently, the samples were extracted by boiling in 4% sodium dodecyl sulfate for 30 min and filtered onto 0.45-μm-pore-size membrane filters (Millipore). After being washed (3 times, each time with 5 ml of water), the filters were dried and radioactivity was counted in 5 ml of Ready Safe scintillation cocktail (Beckman).

Electron microscopy. The cells (100-μl samples) were fixed with 2.5% glutaraldehyde (1 ml) and put on ice. After 1 h, the cells were pelleted in an Eppendorf centrifuge and stored in 0.25% glutaraldehyde (4°C) until they were further processed for electron microscopy by methods previously described (22).

RESULTS

Autolysis rates of Tn*551* mutants. Relevant properties of the transposon mutants and genetic crosses used are shown in Table 1. The Tn*551* mutants fell into three distinct classes based on their effect on autolysis rates. The Tn*551* mutant RUSA 4, in which the insert is located within the *mec* gene (12), did not show any significant change in the fast autolysis rate of the parent cell (Fig. 1). The two Tn*551* mutants RUSA 10 and RUSA III-3 (both carrying intact *mec*) showed drastically reduced autolysis rates compared with the highly resistant parent strain (Fig. 1 and Table 1). And in another mutant, RUSA 12F, the reduction in the autolysis rate was less pronounced than in RUSA 10 or RUSA III-3. Furthermore, transformants of RUSA 10 and RUSA 12F, generated by backcrossing the mutant DNA into the parent strain (and selecting for transformants carrying the transposon marker), exhibited autolysis rates that were identical to those of the corresponding mutant.

The decrease in OD_{620} of the cell suspension incubating in

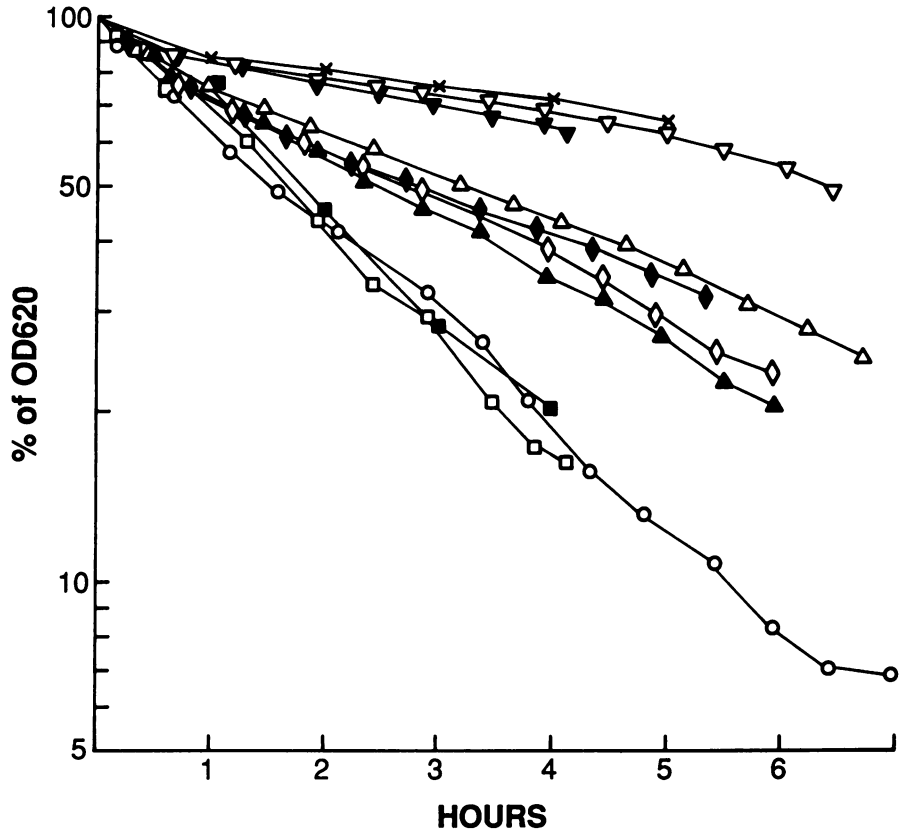

FIG. 1. Autolysis of a highly resistant MRSA strain and its isogenic Tn*551* mutants. Autolysis was measured as the decline in optical density, as described in Materials and Methods. Symbols: ○, quickly autolysing parental strain, COL; □ and ■, mutant RUSA 4 and its backcross RUSA 4 BC 1, respectively; △, ◇, ◆, and ▲, auxiliary mutant RUSA 12F and its three backcrosses RUSA 12F BC 1, BC 2, and BC 3, respectively; ▽, ▼, and ×, auxiliary mutant RUSA 10, its backcross RUSA 10 BC 1, and mutant RUSA III-3, respectively.

the autolysis buffer was accompanied by a parallel decrease in the titer of viable bacteria and by the release of UV-absorbing material (DNA and RNA) and protein into the supernatant (data not shown). For instance, in the experiment illustrated in Fig. 1, the initial titer of viable cells (determined immediately upon suspension of cells in the lysis buffer) of the quickly autolysing (parent) strain COL was 3×10^8 CFU/ml; the titer dropped to 3×10^6 CFU/ml (1% of the initial value) after 3 h in the autolysis buffer. In the case of the quickly autolysing mutant RUSA 4 (Tn*551* in the *mec* gene), the initial titer of viable cells fell from 2.6×10^8 CFU/ml to 1×10^6 CFU/ml. The titers of viable cells of the slowly autolysing strain RUSA 10 were 2.4×10^8 CFU/ml (initial titer) and 1.2×10^8 CFU/ml (50% of the initial titer) after 3 h.

When cells were used in which the peptidoglycan was prelabeled with [^{3}H]GlcNAc for four generations, a similar loss of radioactive cell wall material (from the sodium dodecyl sulfate-insoluble fraction of the cells) was also observed during autolysis (Fig. 2).

Cell wall turnover in Tn*551* MRSA mutants. The rates of cell wall turnover were compared in continuously labeled parental and RUSA 4 and RUSA 10 mutant cells by the procedure described in Materials and Methods. The rate of cell wall turnover was clearly slower in mutant RUSA 10 than in the parent strain, COL, and in RUSA 4 (Fig. 3).

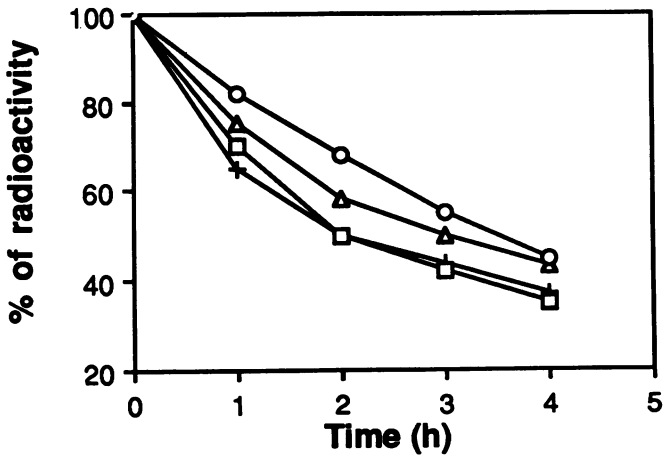

FIG. 2. Cell wall degradation during autolysis. Bacterial strains radiolabeled with [^{3}H]GlcNAc in their cell walls were allowed to autolyse, as described in the legend to Fig. 1, and the amounts of radioactivity retained in the cell wall fraction during autolysis were assayed, as described in Materials and Methods. Symbols: +, strain COL; □, △, and ○, mutants RUSA 4, RUSA 12F, and RUSA 10, respectively.

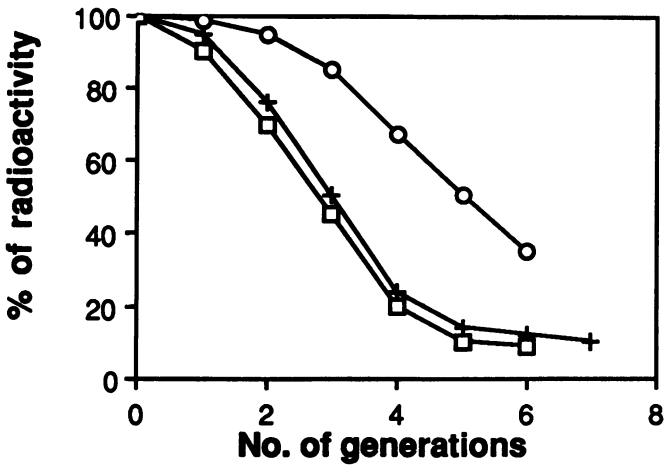

FIG. 3. Cell wall turnover of the highly resistant parent strain, COL (+), and the transposon mutants RUSA 10 (○) and RUSA 4 (□).

DISCUSSION

Until now, the only biochemical element associated with methicillin resistance was the 78-kDa PBP 2A, the product of the *mec* gene. However, the function of this protein is unknown. In this communication, we describe what appears to be a new biochemical correlate of methicillin resistance. Analysis of isogenic transposon mutants indicates that high-level (homogeneous) methicillin resistance is associated with high-level autolytic potential. Inactivation of several operationally defined auxiliary genes caused reduction in autolysis rates in parallel with the reduction of MICs. The link between reduced MICs and reduced autolysis was reproduced in genetic backcrosses, suggesting that they were caused by inactivation of the same, unique chromosomal site outside the *mec* gene.

Another process involving the activity of cell wall-degrading enzymes, cell wall turnover, was also shown to be suppressed in the auxiliary mutant (RUSA 10) examined. In addition to the clear difference in turnover rates, the parent strain and RUSA 10 also differed in that the mutant appeared to have a longer lag period before the net release of cell wall material began (Fig. 3). The reason for this is unclear at present.

We measured autolysis as a decrease in the optical density of a cell suspension in a buffer containing 0.01% Triton X-100, an agent known for its capacity to trigger autolysis in several bacteria. Since a decrease in OD_{620} alone does not necessarily reflect autolysis, additional (parallel) tests were also performed. These tests indicated that the decline in optical density was always accompanied by a decrease in the viable count and by the release of nucleic acids and proteins into the buffer with rates that were similar to the rates of

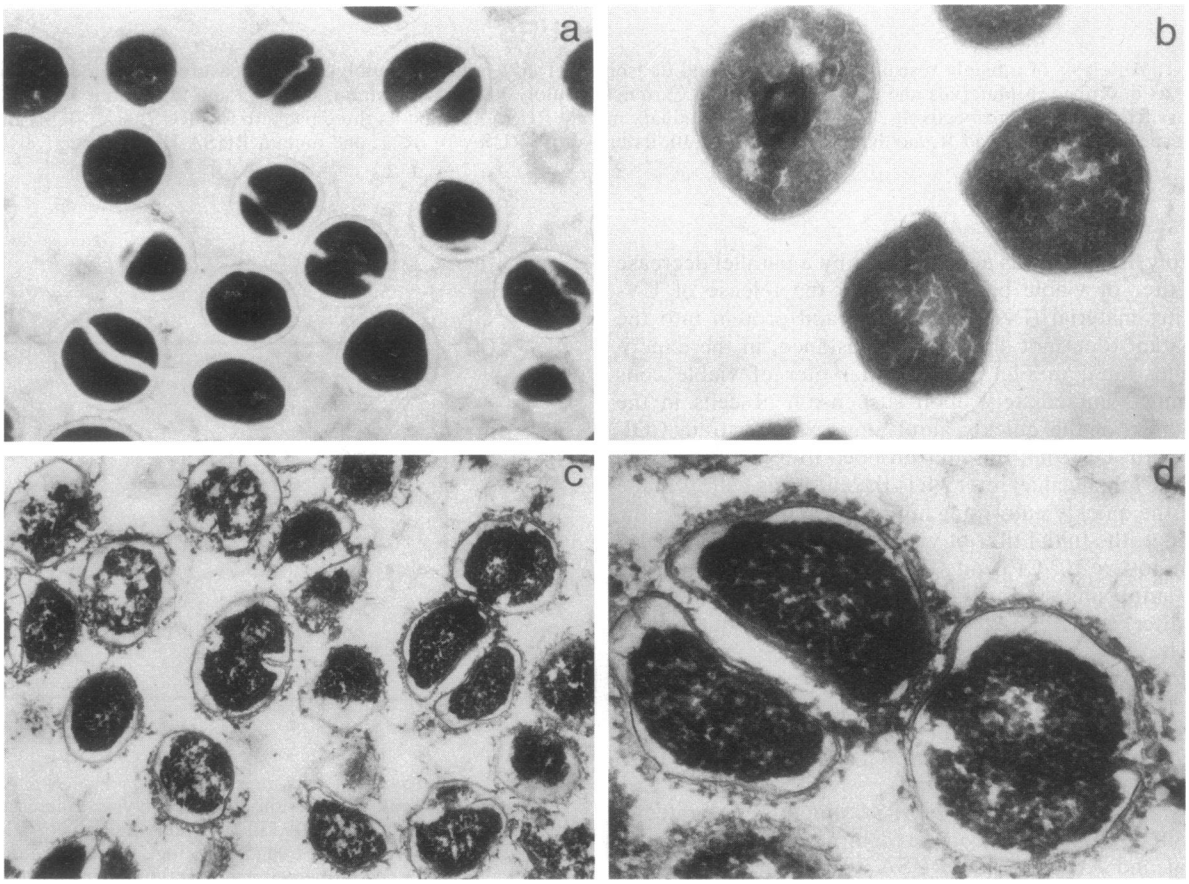

FIG. 4. Electron micrographic appearance of strain COL after autolysis. Bacteria were processed for transmission electron microscopy, as described in Materials and Methods. (a and b) Strain COL before autolysis. (c and d) Strain COL after 5 h of autolysis. Magnification, ×20,000 (a and c) and ×50,000 (b and d).

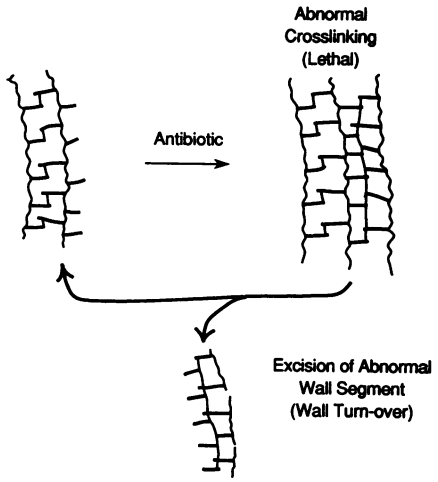

FIG. 5. Model for the proposed role of cell wall turnover as a wall repair mechanism assisting expression of high-level methicillin resistance. In the model, a differential rate of acylation of individual PBPs by the beta-lactam antibiotic (cell wall synthetic enzymes) generates a situation in which the proportion of free (unacylated) PBPs becomes abnormal in the cell; this abnormal assembly of PBPs then produces structurally abnormal segments in the cell wall (e.g., extra crosslinks or autolysin attachment sites). Unless removed, through cell wall turnover, this structural abnormality is lethal for the cell.

decline in optical density. Furthermore, the use of [^{3}H]GlcNAc-labeled cells showed loss of radioactive cell wall material from the sodium dodecyl sulfate-insoluble fraction during autolysis, indicating degradation of cell wall polymers. This was also confirmed by electron microscopy (Fig. 4).

Our findings together indicate that the auxiliary mutations, including a mutation in the *femA* locus (2), are directly or indirectly involved with the regulation or functioning of staphylococcal autolytic enzymes.

Our results are exactly the opposite to the data reported by Gustafson and Wilkinson, who showed a faster autolysis rate for a low resistant (heterogeneous) mutant compared with the highly resistant (homogeneous) parent strain (6). The reasons for this discrepancy are not clear. Autolysis rates are known to depend sensitively on the physiology of the cells. We found that for reproducible results, it was important to have the cultures growing in balanced exponential growth for at least 16 generations prior to the autolysis assay. In the study of Gustafson and Wilkinson, the strain with the lower (and heterogeneous) resistance level was isolated after 5-aminoacridine HCl mutagenesis and one wonders whether this treatment may not have also caused more-extensive genetic alterations in addition to lowering resistance to methicillin. Certainly, from the description, it appears that the mutant has also lost the penicillinase plasmid, which was present in the parent cell (6).

How the decreased autolytic potential and cell wall turnover contribute to the decreased methicillin susceptibility and complex (heterogeneous) phenotype of the MRSA mutants examined here is not known. We propose, as a working model, that an increased cell wall turnover rate may provide staphylococci with a mechanism to excise and eject cell wall material of abnormal composition made by an unbalanced assembly of synthetic enzymes during exposure to methicillin. A similar model was invoked to explain the inhibition of growth in penicillin-treated pneumococci (24). The rationale of this model is as follows. Because of their different intrinsic affinities for beta-lactam inhibitors, individual PBPs (cell wall synthetic enzymes) may be acylated to different degrees during the first minutes of contact with the antibiotic, resulting in a cell in which the proportion of free (unacylated) PBPs still capable of catalyzing wall assembly is abnormal. Such abnormal PBP assembly may then produce a structurally incorrect (e.g., abnormally crosslinked) cell wall, which would ultimately prevent cell division and growth. A high rate of wall turnover may act as a wall repair system by allowing excision and disposal of such anomalous segments of the cell wall (Fig. 5). This model has conceptual similarities to some models of DNA repair.

Our results show that autolysis was affected only when an auxiliary gene was inactivated; inactivation of the *mec* gene did not alter autolytic capacity. It remains to be seen whether auxiliary genes exert their effects on the autolytic properties of the bacteria as determinants of wall-degrading or wall-synthesizing enzymes or as regulators of autolytic activity.

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