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Opposing Roles of the *Staphylococcus aureus* Virulence Regulators, Agr and Sar, in Triton X-100- and Penicillin-Induced Autolysis

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The regulation of murein hydrolases is a critical aspect of peptidoglycan growth and metabolism. In the present study, we demonstrate that mutations within the *Staphylococcus aureus* virulence factor regulatory genes, *agr* and *sar*, affect autolysis, resulting in decreased and increased autolysis rates, respectively. Zymographic analyses of these mutant strains suggest that *agr* and *sar* exert their effects on autolysis, in part, by modulating murein hydrolase expression and/or activity.

Bacterial murein hydrolases have been shown to participate in a number of important biological processes occurring during cell growth and division, including cell wall synthesis, daughter cell separation, and peptidoglycan turnover and recycling (1, 6, 12, 17, 19). Because of their potential to destroy the cell wall, the expression and activity of these enzymes must be tightly controlled. Several studies indicate that murein hydrolase activity and autolysis are controlled at the transcriptional level (2, 3, 5–8, 16). In *Bordetella pertussis*, autolysis is regulated by the *bvg* virulence regulatory locus, resulting in the repression of autolysis during the phase shift from avirulence to virulence (6). Thus, in this system only the avirulent population of cells is susceptible to β-lactam antibiotics.

Autolysis assays. To examine the potential roles of *agr* and *sar* in the regulation of autolysis in *Staphylococcus aureus*, the Triton X-100- and penicillin-induced autolysis of *agr*, *sar*, and *agr sar* mutant strains was examined. To eliminate possible variations in genotype, strains used in this analysis were derived from the same parental strain, RN6390 (agr^+ sar^+). As shown in Fig. 1, the *sar* mutant ALC488 had a dramatically increased Triton X-100-induced autolysis rate (50% lysis in about 0.5 h) compared to the parent strain, RN6390 (50% lysis in 3.5 h). This autolysis rate was nearly identical to that exhibited by another *sar* mutant strain, ALC136 (data not shown). In contrast, the *agr* mutant, RN6911, exhibited a lower autolysis rate (50% lysis in approximately 5 h). The *agr sar* mutant, ALC135, exhibited a Triton X-100-induced autolysis rate that was intermediate (50% lysis in about 3.5 h) between those observed for the single-mutant strains. As shown in Fig. 2, the relative penicillin-induced autolysis rates for the respective mutants were similar to those induced by Triton X-100. The *sar* mutant ALC488 also exhibited a dramatic increase in penicillin-induced autolysis compared to the parental strain, RN6390. As with Triton X-100-induced autolysis, the *agr* mutant, RN6911, had a lower penicillin-induced autolysis rate than that of the parental strain and the rate for ALC135 was intermediate between those observed for the single-mutant strains.

Penicillin-induced killing. Recently, Piriz-Duran et al. (13) reported that *agr* and *sar* mutant strains exhibited reduced resistance to oxacillin, cefoxitin, and imipenem. The demonstration that these mutants also produced altered levels of penicillin-binding proteins led them to speculate that these changes may be responsible for the altered susceptibilities of these strains to β-lactam antibiotics. Initially, the results presented here in Fig. 2, which demonstrate that the *agr* mutant strain exhibited diminished penicillin-induced lysis compared to the parental strain, appeared to conflict with their data. However, a determination of the number of viable cells present revealed that the measurements of lysis did not accurately reflect cell viability and did not correlate with the observed changes in culture turbidity. In fact, the regulatory mutant strains were all more sensitive than the parental strain to penicillin-induced killing (Fig. 3), in agreement with the findings of Piriz-Duran et al. (13). The RN6911 culture viability 8 h after penicillin treatment was 6.5-fold lower than that of the RN6390 culture (0.2 and 1.3% viability, respectively). The ALC488 (*sar* mutant) and ALC135 (*agr sar* mutant) strains both exhibited dramatically increased sensitivity to the killing effects of penicillin (0.001 and 0.04% viability 8 h after penicillin treatment, respectively) compared to RN6390. These data indicate that *agr* and *sar* also affect the expression of factors, unrelated to murein hydrolases, that are involved in penicillin-induced killing. These factors could be analogous to the products of the hypothetical *cid* genes of *Streptococcus pneumoniae* which are required for the lysis-independent killing effects of penicillin (9).

Zymographic analysis. To determine the effects of the different regulatory mutations on the cell wall-associated murein hydrolase activity, the relative amounts and diversity of murein hydrolases produced by the different mutant strains were examined by zymography (Fig. 4). This analysis revealed dramatic differences in the cell wall-associated murein hydrolase profiles produced. Strains ALC488 (*sar* mutant) and ALC135 (*agr sar* mutant) produced high levels of a 32-kDa murein hydrolase (Fig. 4, lanes 4 and 5, respectively), unlike the parental strain, which produced barely detectable levels of this murein hydrolase (lane 2). In contrast, strain RN6911 (*agr* mutant) produced undetectable amounts of the 32-kDa murein hydrolase but produced increased levels of several high-molecular-weight (MW) (>75,000) murein hydrolases. These results suggest that the observed differences in autolysis of these strains might be attributable to changes in the expression of different murein hydrolases.

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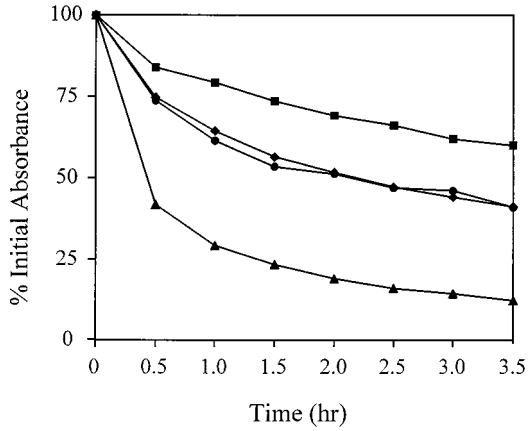

FIG. 1. Effect of *agr* and *sar* on Triton X-100-induced autolysis. *S. aureus* RN6390 (wild type) (diamonds), RN6911 (*agr* mutant) (squares), ALC488 (*sar* mutant) (triangles), and ALC135 (*agr sar* mutant) (circles) were grown to an optical density at 580 nm of 0.6 to 0.8. Triton X-100-induced autolysis assays were performed as described by Mani et al. (8) except that the cells were grown in NZY broth (3% NZ amine, 1% yeast extract). Triton X-100-induced autolysis was measured as the decline of optical density versus time and is expressed as the percent of the initial optical density. The data presented are representative of three independent experiments.

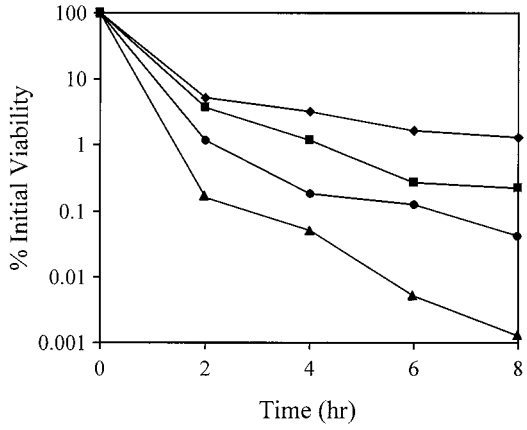

FIG. 3. Effect of *agr* and *sar* on penicillin-induced killing. Penicillin-induced killing of *S. aureus* RN6390 (wild type) (diamonds), RN6911 (*agr* mutant) (squares), ALC488 (*sar* mutant) (triangles), and ALC135 (*agr sar* mutant) (circles) was examined by performing viable cell counts every 2 h on the penicillin-treated cultures for which data are presented in Fig. 2. The viability of the cultures was assessed immediately prior to and every 2 h (for 8 h) after the addition of penicillin G by performing serial dilutions and spreading the cells on tryptic soy broth agar medium. The data presented are representative of three independent experiments.

A gene that likely encodes the 32-kDa murein hydrolase has recently been identified by Ramadurai and Jayaswal (15). This gene, designated *lytM*, encodes a 34.4-kDa protein that shares features with a secreted protein. The processed protein was predicted to have a molecular mass of 32 kDa, suggesting that this protein is the same as that which we have observed in our zymographic analysis (Fig. 4). Thus, the observation that the *sar* mutant exhibited increased levels of the 32-kDa murein hydrolase suggests that the expression of the *lytM* gene, or a factor that affects the activity of this murein hydrolase, is negatively regulated by Sar. In contrast, the *agr* mutant strain was observed to have increased levels of high-MW murein hydrolase activities, proteins that have been proposed to be the precursors of the *atl*-encoded AM and GL murein hydrolases (4, 11). Oshida et al. (11) have demonstrated that the processing of the high-MW murein hydrolases to the mature AM and GL forms can be inhibited with protease inhibitors. Thus, one explanation for the observed increase in the levels of high-MW murein hydrolases in the *agr* mutant strain is that Agr is required for the expression of a protease(s) involved in the proteolytic processing of these *atl*-encoded murein hydrolases.

It should be noted that a previous study of factors affecting autolysis and murein hydrolase activity in *S. aureus* indicated that the *agr* virulence factor regulatory locus had no effect on these processes (18). Although it is not clear why different results were obtained by our laboratories, it is possible that the wild-type strain that was used in that study had acquired a spontaneous *agr* mutation, similar to that previously described

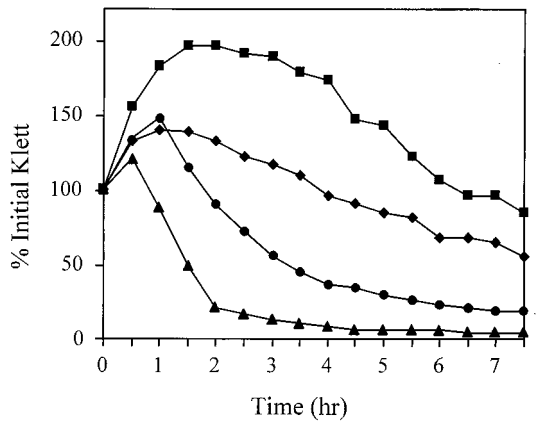

FIG. 2. Effect of *agr* and *sar* on penicillin-induced autolysis. Penicillin-induced autolysis of *S. aureus* RN6390 (wild type) (diamonds), RN6911 (*agr* mutant) (squares), ALC488 (*sar* mutant) (triangles), and ALC135 (*agr sar* mutant) (circles) was measured with a Klett-Summerson colorimeter (filter no. 60; 1 Klett unit = 5×10^6 CFU/ml). Penicillin G (final concentration of 0.4 μg/ml) was added to early-exponential-phase cells (20 Klett units) in tryptic soy broth at 37°C, and the changes in culture turbidity were monitored over an 8-h period. Autolysis was measured as the decline in culture turbidity versus time and is expressed as the percent of the initial Klett reading when penicillin was added. The data presented are representative of three independent experiments.

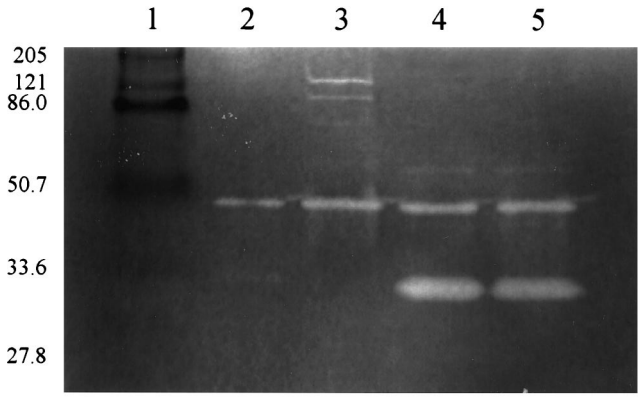

FIG. 4. Zymographic analysis of *S. aureus* murein hydrolases. Cell wall-associated proteins (1.5 μg) were extracted from *S. aureus* cells grown to early exponential phase (optical density at 580 nm of 0.6 to 0.8) in NZY broth and analyzed as described by Qoronfleh and Wilkinson (14). Murein hydrolase activities were visualized as zones of hydrolysis by staining the gel with methylene blue. Lane 2, RN6390 (wild type); lane 3, RN6911 (*agr* mutant); lane 4, ALC488 (*sar* mutant); lane 5, ALC135 (*agr sar* mutant). The sizes of the prestained broad-range MW standards (Bio-Rad Laboratories, Hercules, Calif.) are given in kilodaltons (lane 1).

by Novick et al. (10). The phenotypes of the strains used in our study include the exoprotein and cell wall-associated protein expression profiles that are known to be associated with the *agr* and *sar* mutations. The results of our studies, along with those of a study by Piriz-Duran et al. (13), indicate that the *agr* and *sar* regulatory loci have a significant effect on the susceptibility of *S. aureus* to β-lactam antibiotics. Thus, the bacteria may have evolved a regulatory strategy that functions to maximize their ability to evade host immune responses, while at the same time minimizing their intrinsic susceptibility to β-lactam antibiotics. Continued studies of the regulatory mechanisms that are involved in resistance to β-lactam antibiotics could lead to improved methods for the treatment of staphylococcal disease.

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