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Localized Perforation of the Cell Wall by a Major Autolysin: *atl* Gene Products and the Onset of Penicillin-Induced Lysis of *Staphylococcus aureus*

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We investigated the cell surface localization of the *atl* gene products of *Staphylococcus aureus* exposed to a lytic concentration (4 MIC) of penicillin G (PCG) by means of immunoelectron microscopy using anti-62-kDa *N*-acetylmuramyl-L-alanine amidase or anti-51-kDa endo-β-*N*-acetylglucosaminidase immunoglobulin G. Protein A-gold conjugates reacting with antigen-antibody complex localized at sites of defects of the cell wall at the nascent cross wall. Anti-62-kDa *N*-acetylmuramyl-L-alanine amidase or anti-51-kDa endo-β-*N*-acetylglucosaminidase immunoglobulin G inhibited the decreased turbidity caused by PCG-induced lysis and the formation of defects in the wall. The autolysis-defective mutant, *S. aureus* RUSAL2 (*atl*::Tn*551*), exposed to 4 MIC of PCG resisted autolysis and formation of the wall defect. These results suggest that activation or deregulation of the *atl* gene products at localized sites where formation of new cross wall was disturbed by PCG causes small defects in the cell wall in situ, eventually leading to general autolysis.

Staphylococcus aureus, which is sensitive to β-lactam antibiotics, undergoes autolysis after exposure to penicillin G (PCG). The morphology of PCG-treated *S. aureus* has been described in detail by Giesbrecht and coworkers (1–4, 6, 7). Their studies demonstrated that the PCG-induced autolysis of *S. aureus* starts with localized perforation of the cell wall at initiated nascent cross walls, followed by general lysis (4). They suggested that wall hydrolases contained in specific components of the cell wall, called murosomes, are responsible for this phenomenon. In normal cells, murosomes are thought to form minute pores through the peripheral cell wall above the completed cross wall to facilitate cell separation. Thus, the authors attributed wall defects to a morphogenetic defect of murosome-related wall hydrolases of PCG-treated cells during the initiation of cell separation. However, the identity of the wall hydrolase(s) and biochemical basis for the localized perforation of the cell wall remain unknown.

Autolysis-defective mutants of *S. aureus* have been isolated by transposon mutagenesis (8, 10). Cloning of one of the transposon insertion sites in the parent strain identified the unique bacteriolytic enzyme gene *atl* (9). The deduced amino acid sequence of *atl* suggested that the gene product is synthesized as 138-kDa bifunctional protein (ATL) with amidase and glucosaminidase domains. ATL is suggested to undergo proteolytic processing to mature 51-kDa endo-β-*N*-acetylglucosaminidase (51-kDa GL) and 62-kDa *N*-acetylmuramyl-L-alanine amidase (62-kDa AM) on the cell wall. The 51-kDa GL and 62-kDa AM from culture supernatant and cellular extract have been identified as cell cluster-dispersing enzymes (5, 13). Anti-immunoglobulin G (IgG) generated against purified 51-kDa GL or 62-kDa AM (anti-AM IgG or anti-GL IgG) inhibits cell separation of *S. aureus* and induces cells to form giant clusters. Mutants with little or no 51-kDa GL and 62-kDa AM activity grow in clusters (13, 14). Furthermore, immunoelectron microscopic studies have demonstrated that *atl* gene products are localized on the cell surface at the presumptive sites of cell separation (17). These data suggested that *atl* gene products are involved in the cell separation of *S. aureus*. On the other hand, they are also implicated in the β-lactam antibiotic-induced autolysis of *S. aureus* (10). However, their exact role in autolysis is ambiguous.

In this study, we investigated the localization of *atl* gene products in PCG-treated *S. aureus* by immunoelectron microscopy and found that they were localized at the site of wall defects. Our results suggest that the *atl* gene product(s) is involved in the initial perforation of the cell wall during the autolytic process of PCG-treated *S. aureus.*

MATERIALS AND METHODS

Bacterial strains and growth condition. *S. aureus* FDA209P (ATCC 6538), *S. aureus* RN450, Lyt$^-$ mutant RUSAL2 (RN450 *atl*::Tn*551*) (10), and *S. aureus* 2PF-18 (FDA209P Δprotein A) (17) were examined. Cells cultured in Trypto-soya broth (Nissui Pharmaceutical Co., Tokyo, Japan) were inoculated into fresh medium with an adjusted initial optical density at 660 nm at 0.05 and incubated with continuous agitation at 37°C. After shaking for 0.5 h, PCG potassium salt (Meiji Seika Kaisha) was added to the culture, which was continuously agitated in a rotary shaker. Cell growth was monitored at intervals by measuring the optical density or by counting CFU. Prior to monitoring, specimens were mildly sonicated to disperse clusters.

Antibodies. Anti-AM IgG and anti-GL IgG were prepared as described previously (12, 13).

Electron microscopy. Bacterial cells were harvested, washed twice with Dulbecco's phosphate-buffered saline (PBS), and suspended in PBS. The *atl* gene products on the cell surface were localized by protein A-gold labeling as described previously (11, 17). Briefly, cells were mixed with either anti-AM IgG or anti-GL IgG and incubated at 37°C for 1 h. After several washings in PBS, the bacteria were collected into a pellet and doubly fixed with 2.5% glutaraldehyde and 1% OsO_4. The samples were dehydrated in an ethanol series and then embedded in Spurr's Epon. Ultrathin sections were cut with an ultramicrotome with a diamond knife and examined in a JOEL JEM-2000 EXII electron microscope at 80 or 100 kV.

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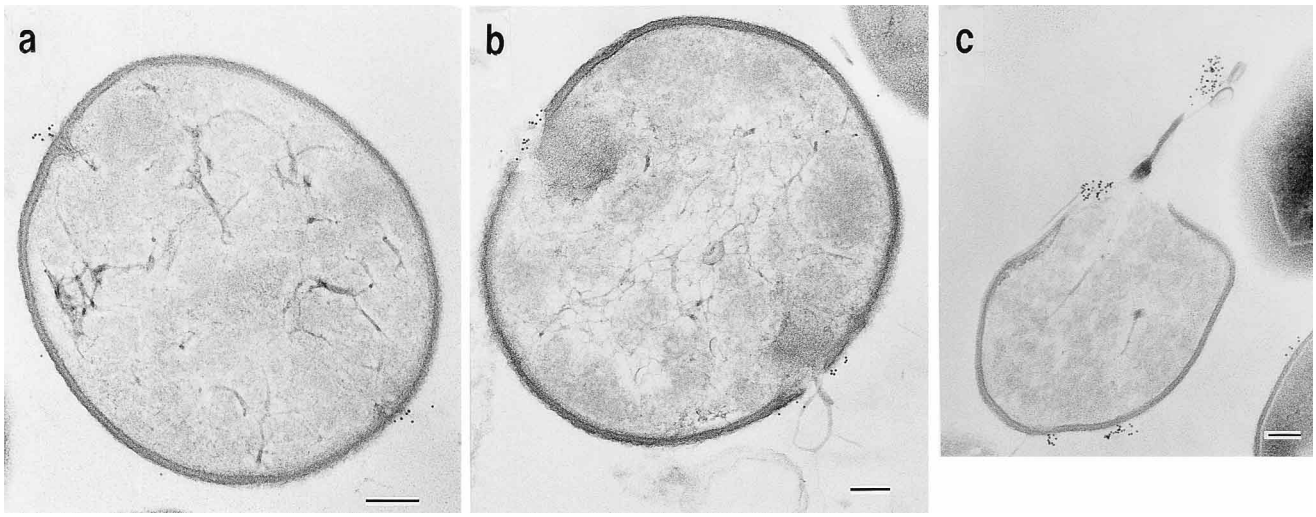

FIG. 1. Immunogold labeling of *atl* gene products on the cell surface of PCG-treated *S. aureus* by transmission electron microscopy. A lytic concentration (4 MIC) of PCG was added to exponentially growing cultures of *S. aureus* 2PF-18 at 0.5 h and incubated for 3 h. The cells were then immunolabeled with anti-AM IgG. The cell surface is labeled at presumptive sites for cross wall formation (a) and at cell wall defects (b and c). Bars indicate 100 nm.

RESULTS

We studied the localization of *atl* gene products on the cell surface of PCG-treated *S. aureus* by immunoelectron microscopy. Exponentially growing *S. aureus* 2PF-18 cells were incubated with a lytic dose of PCG (4 MIC, 0.02 μg/ml) for 3 h. The cells were then incubated with anti-AM IgG and colloidal gold-labeled protein A and processed for electron microscopy. Figure 1 shows cross sections of PCG-treated *S. aureus*. Cross wall formation was impaired by PCG, but the labeling particles were localized at incomplete cross walls (Fig. 1a). Figure 1b shows PCG-treated cells with defects in the presumptive cross wall. The labeled particles were localized at sites of wall defects. Most cytosolic materials were still retained in the cells, but fibrillar material had started extending from the wall defect (below right). The sites of the cell wall where labeled particles were found were not simple breaks but rather regions of substantial loss. Some labeled particles were also associated with fragments of wall material released from the cells (Fig. 1c). Similar results were obtained in assays using an anti-GL IgG, and control experiments using preimmune IgG and gold particles showed no immunolabeling (not shown). These results demonstrated that *atl* gene products are localized at the sites of initial perforation of the cell wall in *S. aureus* exposed to PCG. To determine whether the wall defect was produced by the activity of localized *atl* gene products, we investigated the effect of an antibody against *atl* gene products on autolysis induced by PCG. Exponentially growing *S. aureus* 2PF-18 was incubated with 4 MIC of PCG, followed by 10 μl of anti-AM IgG, 10 μl of anti-GL IgG, or 5 μl each of anti-AM IgG and anti-GL IgG, and turbidity was monitored. As shown in Fig. 2, the antibody inhibited the decreasing turbidity of the culture. Anti-AM IgG and anti-GL IgG synergistically inhibited the decrease in turbidity. We studied the inhibitory effects of antibodies at various concentrations and found that a mixture of 5 μl of each of the antibodies was maximally inhibitory (not shown). Addition of this mixture (anti-ATL) to *S. aureus* after PCG addition also inhibited the decrease in turbidity, although the inhibition was less the later the anti-ATL was added (data not shown). As predicted from previous studies (15, 16), anti-ATL did not inhibit the reduction in the viable count caused by PCG (not shown).

We examined the effect of anti-ATL on the formation of wall defects in PCG-treated *S. aureus* 2PF-18 by electron microscopy. Cells exposed to 4 MIC of PCG and anti-ATL for 0.5 h were incubated for a further 4 h and then harvested and processed for electron microscopic study. Figure 3a and b shows cells with a thickened cell wall, but there were no apparent complete wall defects caused by PCG. Figure 3a shows impaired cell separation resulting in the formation of a cell cluster due to inhibition of the cell-separating activity of *atl* gene products by anti-ATL (13). The cell wall was sometimes partially digested inside the nascent septum; however, peripheral portions appeared intact (Fig. 3b). The partial defect of cell wall may be due to steric hindrance of antibody interaction with *atl* gene products residing in the inner space of the cell wall. This could also explain the incomplete inhibition of the decrease in turbidity of PCG-treated *S. aureus* incubated with anti-ATL antibody.

S. aureus RUSAL2 was isolated as an autolysis-defective mutant constructed by Tn*551* insertion mutagenesis of *S. aureus* RN450 (10). This strain produces virtually no *atl* gene

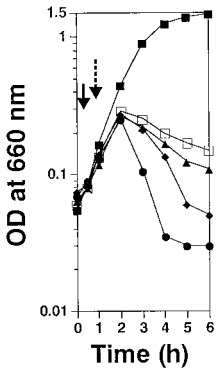

FIG. 2. Effect of anti-ATL antibody on PCG-induced *S. aureus* lysis. Four MIC of PCG was added to exponentially growing cultures of *S. aureus* 2PF-18 at 0.5 h (arrow), and antibodies were added 1 h later (arrow with dotted line). Turbidity of the cultures was monitored at intervals. Symbols: ■, control; ●, PCG alone; ▲, PCG and 10 μl of anti-GL IgG; ◆, PCG and 10 μl of anti-AM IgG; □, PCG and 5 μl each of anti-GL IgG and anti-AM IgG. OD, optical density.

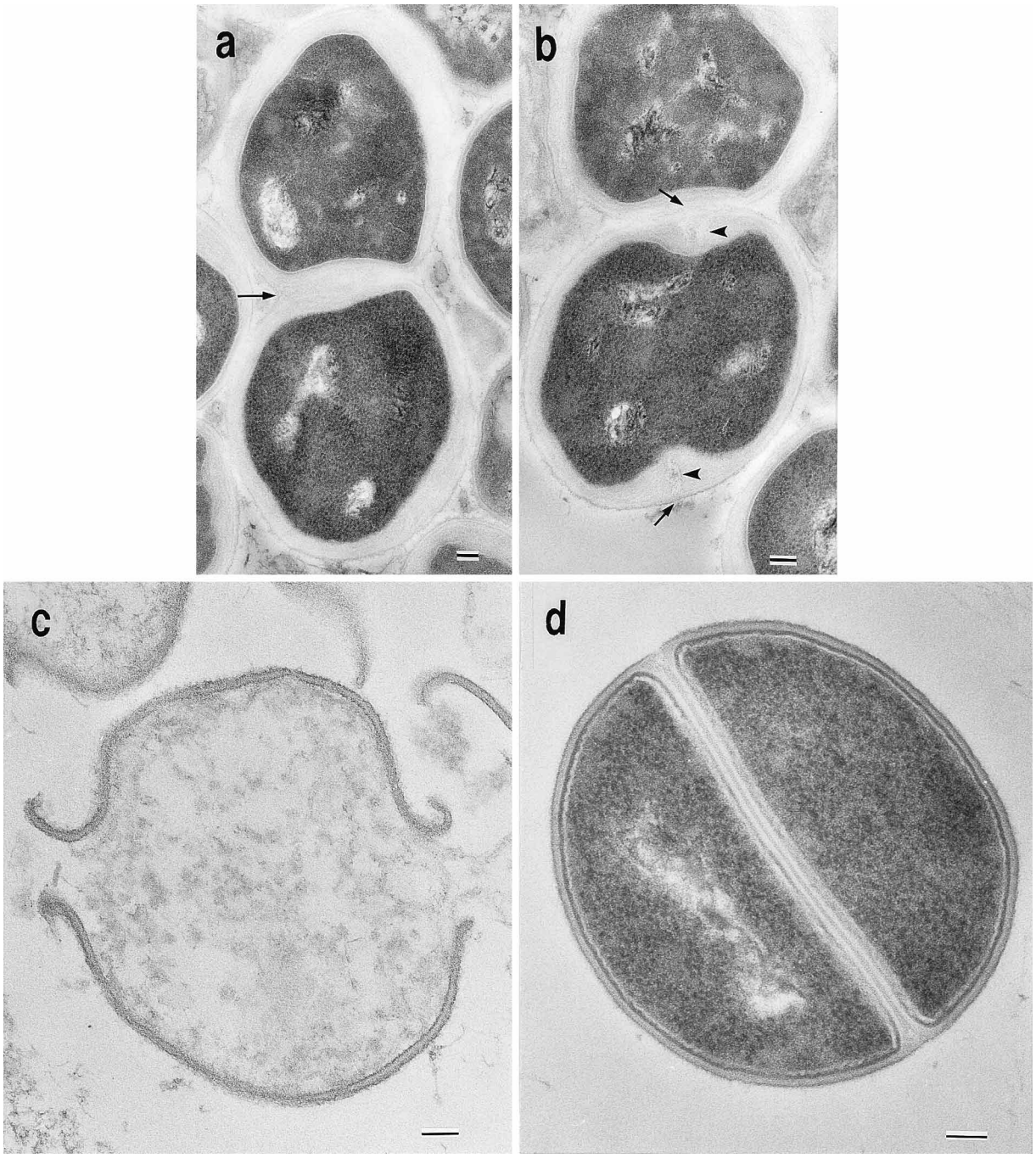

FIG. 3. Thin sections of *S. aureus* 2PF-18 incubated with 4 MIC of PCG and anti-ATL antibodies. A lytic concentration of PCG (4 MIC) was added to an exponentially growing culture of *S. aureus* 2PF-18 with (a and b) or without (c) 10 μl of anti-ATL antibodies at 0.5 h and incubated for a further 3.5 h. (d) Cells treated with neither PCG nor antibodies. The cells were processed for transmission electron microscopy. Bars indicate 100 nm.

products, and it does not undergo autolysis in the presence of an otherwise lytic dose of methicillin (10, 13). The fact that the *atl* mutants are viable indicates that there are undoubtedly multiple peptidoglycan hydrolases in *S. aureus*. We also examined cross sections of PCG-treated *S. aureus* RUSAL2 for small wall defects. Exponentially growing *S. aureus* RUSAL2 was incubated with 4 MIC of PCG (0.2 μg/ml) for 4 h, and then the cells were harvested and processed for electron microscopic study. No wall defects were found in the cells, although abnormal septum formation was observed (Fig. 4).

DISCUSSION

Since *atl* gene products with various molecular sizes are present on the cell surface, it is not yet clear which is the major autolysin(s) in vivo (5, 9). The synergistic inhibitory effect of

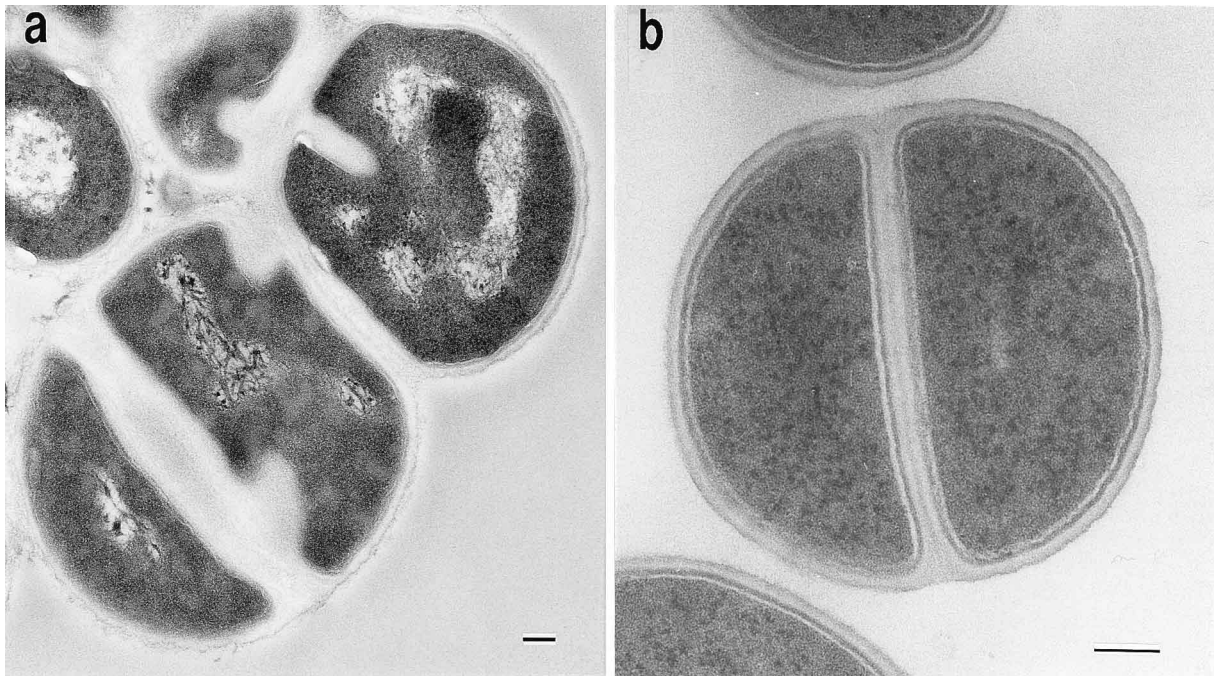

FIG. 4. Thin section of *S. aureus* RUSAL2 incubated with (a) or without (b) 4 MIC of PCG. Four MIC of PCG was added to exponentially growing cultures of *S. aureus* RUSAL2 at 0.5 h, and incubation proceeded for a further 3.5 h. The cells were then processed for transmission electron microscopy. Bars indicate 100 nm.

anti-AM IgG and anti-GL IgG on PCG-induced autolysis suggests that both glucosaminidase and amidase are involved. Our studies suggest that *atl* gene products are the wall hydrolases, predicted by Giesbrecht and coworkers (4), that are involved in formation of wall defects in PCG-treated *S. aureus*. The suggested roles of *atl* gene products in normal cells and in those exposed to PCG are shown in Fig. 5. Normally, the activation or deregulation of localized *atl* gene products at the peripheral cell wall above the completed septum starts digestion of old cell wall, which eventually separates daughter cells (17). When exposed to PCG, the cell wall localized at the presumptive site of cross wall formation is digested by *atl* gene products without forming a new cross wall, which causes a wall defect to form at the site. Whether the mode of activation or deregulation of *atl* gene products at localized sites in PCG-treated cells is similar to that in normal cells remains to be studied. Giesbrecht and coworkers suggested that there are about 20 sites at the periphery of the division plane where murosomes erupt (3, 4). During PCG-induced lysis, one or two eruptions of murosomes at nascent cross wall may cause initial perforation of the cell wall (4). The *atl* gene products localize at the periphery of the division plane as a ring structure, although it is not known whether they form a ring continuously around the cell (17). The wall defects created by *atl* gene products immediately release internal pressure, which may eventually push out membranous material at these sites. This material could be the PCG-induced murosomes described by Giesbrecht and coworkers (4).

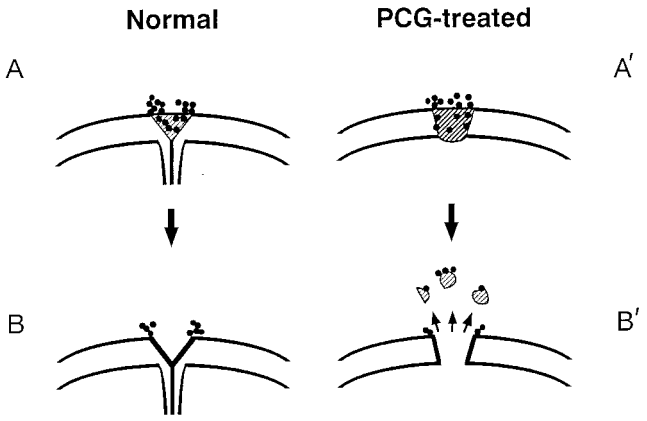

FIG. 5. A hypothetical model of the function of *atl* gene products in cell separation and in PCG-induced lysis of *S. aureus*. (A) Normal cells start to accumulate *atl* gene products at the presumptive site of cross wall formation. (B) After completing the cross wall, peripheral peptidoglycan connecting daughter cells (triangle with dotted line) is digested by *atl* gene products. (A′) PCG-treated cells cannot form new cross walls for the next division cycle, but *atl* gene products continue accumulating at the presumptive site of cross wall formation. (B′) Eventually, *atl* gene products are activated or deregulated in situ, which creates punched holes in the localized sites. The internal pressure releases cytoplasmic material from the holes, which triggers the activation or deregulation of cell-associated autolysin, including *atl* gene products, to induce general lysis of the cells.

Thin sections of autolytic, PCG-treated *S. aureus* 2PF-18 incubated with anti-ATL and PCG-treated *S. aureus* RUSAL2 had no apparent wall defects. However, the rate of methicillin-induced killing was affected by neither a Tn*551* insertion into *atl* (10) nor incubation with anti-ATL antibody. These results further support the notion that the formation of wall defect is not always prerequisite for the PCG-induced death of *S. aureus* (1, 2, 4).

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