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The Autolytic Enzyme LytA of *Streptococcus pneumoniae* Is Not Responsible for Releasing Pneumolysin

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It was previously proposed that autolysin's primary role in the virulence of pneumococci was to release pneumolysin to an extracellular location. This interpretation came into question when pneumolysin was observed to be released in significant amounts from some pneumococci during log-phase growth, because autolysis was not believed to occur at this time. We have reexamined this phenomenon in detail for one such strain, WU2. This study found that the extracellular release of pneumolysin from WU2 was not dependent on autolysin action. A mutant lacking autolysin showed the same pattern of pneumolysin release as the wild-type strain. Addition of mitomycin C to a growing WU2 culture did not induce lysis, indicating the absence of resident bacteriophages that could potentially harbor *lytA*-like genes. Furthermore, release of pneumolysin was unaltered by growth in 2% choline, a condition which is reported to inactivate autolysin, as well as most known pneumococcal phage lysins. Profiles of total proteins in the cytoplasm and in the supernatant media supported the hypothesis that release of pneumolysin is independent of pneumococcal lysis. Finally, under some infection conditions, mutations in pneumolysin and autolysin had different effects on virulence.

Pneumococci elaborate a variety of factors that contribute to virulence. These include the capsule, surface proteins like PspA, PspC, and PsaA, and proteins like pneumolysin (Ply), which must be released into the extracellular environment in order to affect host cells (10, 11, 28, 32). In addition, cell wall degradation products released as a result of the action of the enzyme autolysin (LytA) are known to mediate inflammation and toxicity in several animal models. LytA, the major pneumococcal lysin, is an *N*-acetylmuramoyl-L-alanine amidase (27). It can be activated to cause lysis of the bacteria in the stationary phase or upon penicillin treatment (25). Autolysin mutants have attenuated virulence in animal models compared to that of isogenic wild-type pneumococci. This suggests a role for autolysin and possibly for the inflammation that follows autolysis in pneumococcal virulence and pathogenesis (4, 5, 12). Previous studies demonstrated that immunity to pneumolysin and autolysin provide similar degrees of protection against intraperitoneal (i.p.) challenge (5, 12). Immunity to both factors did not result in greater protection than immunity to either one alone (22). It has been suggested that autolysin plays its role in pathogenesis in two ways: (i) by generating inflammatory cell wall degradation products and (ii) by releasing the pneumococcal cytoplasmic contents, including virulence factors such as pneumolysin (25).

Pneumolysin is a multifunctional cytolysin. It belongs to a family of related thiol-activated toxins synthesized by gram-positive bacteria of different genera. Pneumolysin is capable of a variety of detrimental effects on the components of the host immune system, and its role in pathogenesis has been established in several ways. Antibody titers to pneumolysin rise in humans following pneumococcal infection, indicating that the protein is synthesized by the bacteria while they are growing in the host (20). This conclusion is also supported by the fact that mutants lacking the ability to make pneumolysin are less virulent (6). Antibodies to pneumolysin confer partial protection against infection following intranasal or i.p. inoculation in mice (22). Pneumolysin has been implicated as playing a particularly important role in pneumococcal pneumonia (12). Immunofluorescence data on tissue sections show the presence of pneumolysin in the lungs of infected mice. Pneumolysin is produced by virtually all known strains of *Streptococcus pneumoniae*, although there appears to be some differential hemolytic activity among pneumolysins from different strains (23).

Earlier reports have shown that pneumolysin is located in the cytoplasm of pneumococci (21). The pneumolysin protein, unlike the other thiol-activated toxins, lacks an N-terminal signal sequence for transport out of the cytoplasm (36). This observation led to the hypothesis that pneumolysin is released upon autolysin-dependent autolysis of pneumococci (28). We have previously observed two patterns of extracytoplasmic release of pneumolysin (2). In a majority of the strains, including capsule type 2 strain D39, extracellular pneumolysin is not evident until late log phase. However, for strains WU2, A66.1, GB05 (capsular type 3), EF3296, EF5668 (capsular type 4), and DBL6A (capsular type 6A), the presence of extracellular pneumolysin could be detected in early log phase, much before generalized cell lysis. This early-log-phase release pattern may occur in one of two ways: (i) a specific autolysin-dependent nonlytic release of pneumolysin may occur prior to stationary phase and generalized cell lysis or (ii) pneumolysin may be released in an autolysin-independent manner.

In the present study, we evaluated the potential role of autolysin in pneumolysin release in two ways. One approach used autolysin-negative mutants of the virulent type 3 pneumococcus WU2 in which the autolysin gene was interrupted by

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TABLE 1. Strains used in this study

Strain	Capsule type	Phenotype	Relevant characteristic(s)	Reference or source
D39	2	D39	Mouse-passaged clinical isolate	24
AL2	2	D39 LytA$^-$	Defined autolysin-deficient *lytA* mutant of D39; Em^r	4
PLN	2	D30 Ply$^-$	Defined pneumolysin-deficient *ply* mutant of D39; Em^r	6
WU2	3	WU2	Mouse-passaged clinical isolate	9
SM121.10	3	WU2 LytA$^-$	Defined autolysin-deficient *lytA* mutant of WU2; Em^r	This study
SM321.1	3	WU2 LytA$^-$	Defined autolysin-deficient *lytA* mutant of WU2; Em^r	This study
SM122.2	3	WU2 LytA$^-$	Defined autolysin-deficient *lytA* mutant of WU2; Em^r	This study
UB2271	3	WU2 Ply$^-$	Defined pneumolysin-deficient *ply* mutant of WU2; Em^r	This study
UB1122	3	WU2 Ply$^-$	Defined pneumolysin-deficient *ply* mutant of WU2; Em^r	This study
UB1129	3	WU2 Ply$^-$	Defined pneumolysin-deficient *ply* mutant of WU2; Em^r	This study

insertion duplication mutagenesis (30). In the second approach, WU2 was grown in broth containing 2% choline chloride to suppress autolysin activity. Neither test supported a requirement for autolysis in pneumolysin release.

MATERIALS AND METHODS

Pneumococcal strains and growth conditions. Pneumococcal strains used in this study are listed in Table 1. The bacteria were routinely grown in Todd-Hewitt broth containing 0.5% yeast extract (THY) or on blood agar base (Difco Laboratories, Detroit, Mich.) supplemented with sterile 5% defibrinated sheep blood (Colorado Serum Company, Denver, Colo.). THY and blood agar plates were supplemented with erythromycin (0.3 μg/ml) for strains carrying an erythromycin resistance marker. When required, 2% choline chloride was added to THY.

Transformation. Competent WU2 cells were transformed with chromosomal lysates from AL-2 and PLN-A as previously described (38). The transformants were selected on blood agar plates supplemented with erythromycin (0.3 μg/ml). Each transformant was backcrossed three times into the wild-type WU2 background.

Southern blot analyses. Chromosomal DNAs were extracted from the indicated pneumococcal strains and digested with *Hin*dIII or *Cla*I under conditions described by the manufacturer (Promega, Madison, Wis.). The digests were loaded on a 1.0% agarose gel and separated by electrophoresis in Tris-borate-EDTA buffer. The DNAs were transferred to a nylon membrane (Micron Separations Inc., Westborough, Mass.) and then detected by hybridization with a PCR-generated autolysin-specific (primers 5′ TTTCTCGCACATTGTTGGG AAC 3′ and 5′ CGCTGACTGGATAAAGGCATTTG 3′; 709-bp PCR product) or pneumolysin-specific (primers 5′ TCTGTAACAGCTACCAAC 3′ and 5′ CAGAAATTCCTCTCTGTT 3′; 508-bp PCR product) DNA probe labeled with digoxigenin (Roche Molecular Biochemicals, Indianapolis, Ind.).

In vitro growth curves. Colonies from overnight growth on blood agar plates were inoculated into 5 ml of THY. The cultures were allowed to grow under static conditions to early log phase and then, based on their optical densities at 600 nm (OD_{600}), were diluted into 250 ml of fresh THY such that the density of the cells in the new medium was about 10^5 CFU/ml. The *lytA* mutant strains were grown without added antibiotics. The stability of the *lytA* mutation under these conditions was confirmed by plating on blood agar plates containing 0.3 μg of erythromycin per ml.

Samples were withdrawn at various time points between 0 and 10 h. At each time point, 5-ml aliquots were taken. One milliliter was used to determine the OD_{600}, and 50 μl of this sample was used to determine the viable counts. The remaining 4 ml was centrifuged at 7,000 rpm for 10 min in a Sorvall RC-5B superspeed centrifuge. The top 1 ml of the supernatant was taken to determine the extracellular pneumolysin hemolytic titer. The pellet was lysed with lysis buffer (0.01% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 0.015 M sodium citrate) and incubated at 37°C for 30 min. The final volume of the lysed pellet was 400 μl (1/10 of that of the original sample of 4 ml). The cytoplasmic hemolytic titers, described below, were divided by 10 so as to normalize the titers for the pellets and the supernatants so that both represented pneumolysin from the same number of cells.

When the samples were collected for Western blotting, 15-ml aliquots were taken at each time point. After centrifugation, the top 7 ml of the supernatant was recovered for total protein analysis. The supernatant proteins were concentrated by precipitation with 10% trichloroactetic acid (TCA) overnight at 4°C. The precipitated proteins were collected by centrifugation. The pellets were washed twice with acetone and resuspended in distilled water. The pellet was lysed as described above (final volume, 1.5 ml). For Western blotting, the samples were concentrated with 10% TCA as described above for supernatant proteins. The lysed pellets and the culture supernatants were frozen at −70°C until the hemolytic assay was performed.

Autolysin-deficient mutants are resistant to detergent lysis. Therefore, these strains were lysed using the FastPrep system (Bio 101, Vista, Calif.). Bacteria in the lysis buffer were shaken with silica and ceramic spheres for 20 s at 6,000 vibrations/min. This lysate was stored at −70°C until the assay was performed. The same procedure was followed for cells grown in choline-containing media.

Pneumolysin hemolytic assay. The hemolytic assay was performed as previously described (2). This semiquantitative endpoint assay measures the hemolytic activity of pneumolysin. The values reported are reciprocal titers (hemolytic units) and represent the highest threefold dilutions at which all the sheep red blood cells lysed. The maximum dilution of the lysed pneumococcal pellet that caused lysis of sheep red blood cells was designated the cytoplasmic pneumolysin titer. The hemolytic titer of the supernatant was designated the extracellular titer. The negative controls were a *ply* mutant strain (UB1122/UB2271), lysis buffer alone, THY alone, or phosphate-buffered saline alone. None of these controls caused lysis of sheep red blood cells under the assay conditions.

Western blot analysis. The amounts of total protein in the TCA-precipitated pellets and supernatant were determined by the Lowry method. Volumes containing 10 μg of total protein were mixed with 5× sample buffer, boiled for 5 min, and loaded on a Tris-HCl–10% polyacrylamide gel. The proteins in the gel were transferred to a nitrocellulose membrane, and the blot was probed with rabbit antisera to pneumolysin. The secondary antibody, goat anti-rabbit (H+L)-biotin, was used in conjunction with streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham, Ala.). The blots were developed using 1 M Tris, pH 8.8, containing Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Fisher Scientific, Springfield, N.J.).

Coomassie blue staining of total proteins. WU2 was grown at 37°C in THY. Samples (50 ml) were withdrawn at different times during growth. The bacteria were pelleted. The pelleted proteins were released by incubating them in lysis buffer as described above. The supernatant and pellet proteins were precipitated with 10% TCA overnight at 4°C, separated on a 10% polyacrylamide gel, and visualized by staining with Coomassie brilliant blue R-250.

Effect of mitomycin C on growing pneumococcal cultures. WU2 was grown as three sets of cultures at 37°C up to an OD_{600} of approximately 0.3. To one set, mitomycin C was then added to a final concentration of 0.1 μg/ml to induce possible lysogenic bacteriophages (31). Penicillin was added at 0.1 μg/ml to another set of culture as a positive control for lysis. The third set received nothing and served as a negative control for lysis. Incubation of all three cultures was continued, and growth was monitored by determinations of OD over the next 5 h.

Infections of mice. Female CBA/CaHN-XID/J and BALB/cByJ mice, 6 to 8 weeks old, were obtained from Jackson Laboratories (Bar Harbor, Maine). Pneumococci for infections were from frozen stock cultures containing a known concentration of viable bacteria. Aliquots were diluted with lactated Ringer's solution as required to achieve the desired challenge dose. The reported number of CFU injected into the mice was based on quantitation on blood agar plates. CBA/N mice were challenged i.p. with 300 CFU or intravenously (i.v.) with 200 CFU of pneumococci per mouse in 100 μl of Ringer's solution. The BALB/cByJ mice were challenged i.p. with 3×10^6 CFU per mouse in 200 μl of Ringer's solution or i.v. with 2.5×10^7 CFU per mouse in 200 μl of Ringer's solution.

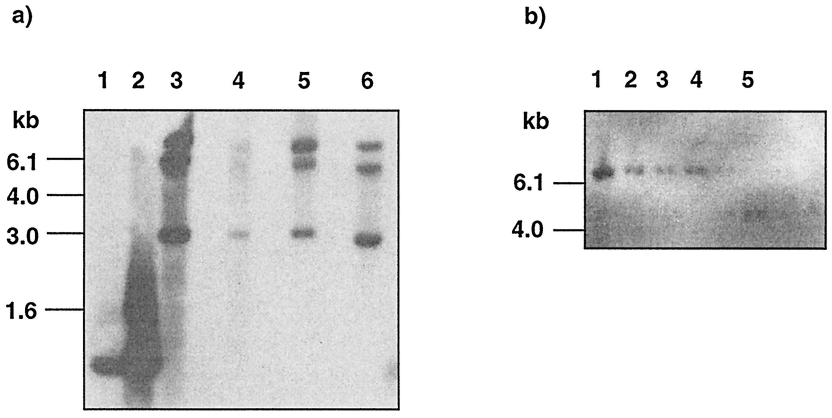

FIG. 1. Southern blot analysis of transformants. Chromosomal DNAs were extracted from the various pneumococcal strains and digested with *Hin*dIII (a) or *Cla*I (b). The samples were electrophoresed on a 1% agarose gel and subjected to Southern blot analyses, using a digoxigenin-labeled PCR-generated probe specific for the autolysin gene (a) or the pneumolysin gene (b). (a) Lane 1, D39 (wild type); lane 2, WU2 (wild type); lane 3, AL-2 (D39 *lytA* mutant); lane 4, SM321.3 (WU2 *lytA* mutant); lane 5, SM121.10 (WU2 *lytA* mutant); lane 6, SM122.2 (WU2 *lytA* mutant). (b) Lane 1, UB2271 (WU2 *ply* mutant); lane 2, UB1122 (WU2 *ply* mutant); lane 3, UB1129 (WU2 *ply* mutant); lane 4, PLN-A (D39 *ply* mutant); lane 5, WU2 (wild type).

RESULTS

Construction of autolysin- and pneumolysin-negative type 3 strains of pneumococci. Competent WU2 cells were transformed with chromosomal DNAs isolated from pneumolysin-negative (PLN-A) and autolysin-negative (AL-2) mutant strains of the D39 background (4–6, 38). Two independent transformants were obtained in each case. The transfer of the mutations was confirmed by Southern blot analysis of restriction endonuclease-digested chromosomal DNAs from the relevant strains (Fig. 1). The inactivation of *ply* and *lytA* was also confirmed by Western blot analyses using immune sera against autolysin and pneumolysin, respectively (data not shown). The mutation in the autolysin gene also resulted in a reduced ability of pneumococci to lyse when they were grown into stationary phase. As previously observed (8), the mutation in *lytA* also caused the pneumococci to grow in short chains with few or no free diplococci (data not shown). The cell lysates of the pneumolysin mutants were also confirmed to lack hemolytic activity (data not shown).

In vitro growth kinetics and pneumolysin production in THY. WU2 releases pneumolysin into the extracellular environment well before the cell lysis that occurs during stationary phase (2). To determine if the extracellular release of pneumolysin was dependent on the presence of autolysin, we compared the release of pneumolysin from WU2 with that of its isogenic autolysin mutant SM121.10 (Fig. 2). The data shown are representative of the results obtained with the two independent WU2 *lytA* mutants. Autolysin mutants show minimal autolysis and are resistant to lysis by deoxycholate-containing lysis buffer (4, 5, 33). A lysing matrix containing silica and ceramic spheres was used to efficiently lyse the autolysin-negative cells. The results with wild-type WU2 confirmed the release of pneumolysin to the medium, as reported previously (2). Like WU2, autolysin-negative SM121.10 also expressed detectable extracellular hemolytic activity within about 4 h of growth in vitro.

The isogenic autolysin-negative mutant released pneumolysin in a manner comparable to that of the corresponding wild-type strain. The cytoplasmic pneumolysin levels for both cultures rose steadily over the period of growth. The titers attained by the *lytA* mutant were a little lower than those achieved by wild-type WU2. The somewhat lower pneumolysin titers of the mutant, compared to those of WU2, may be explained in

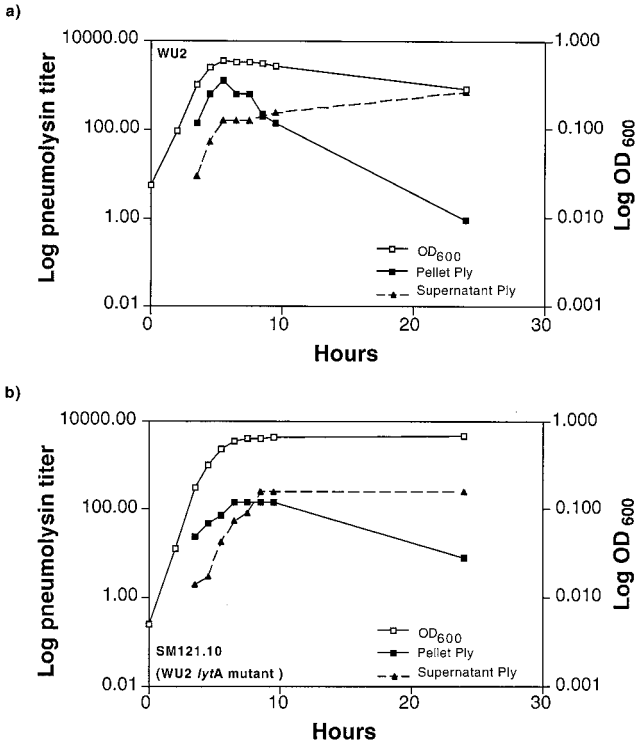

FIG. 2. Pneumolysin expression and release by wild-type and autolysin-deficient pneumococci. WU2 and SM121.10 (WU2 *lytA* mutant) were grown at 37°C in THY. Samples were withdrawn at different times, and the OD were determined (right *y* axis). The pneumolysin titers for cell pellets and supernatants were determined (left *y* axis). The pneumolysin titers for the first two points were below the detection limits of the assay (titer = 3) and were not plotted.

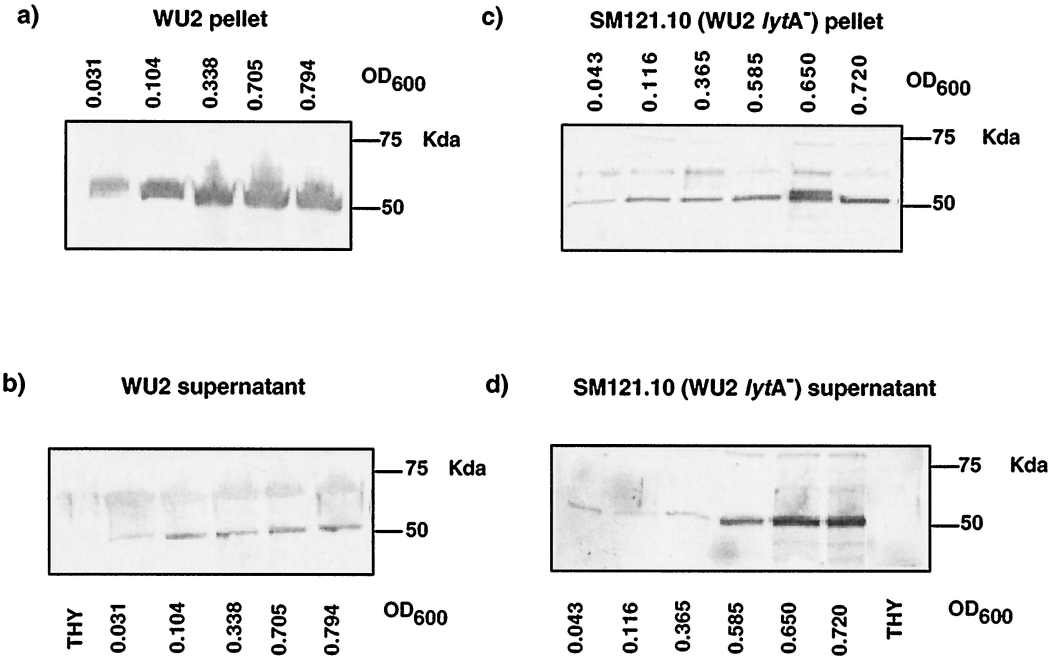

FIG. 3. Western blot analyses of pneumolysin expression during growth. Samples were withdrawn at different times during growth. Pellets were lysed to release the cytoplasmic proteins. Supernatant proteins were concentrated by precipitation with 10% TCA. Pneumolysin was detected using an antipneumolysin rabbit polyclonal antibody.

part by the slower growth of the mutant compared to that of the wild-type strain. It may also be attributed to the fact that, even with mechanical disruption, the autolysin-deficient strain released only half the amount of protein released from wild-type cells (data not shown). The procedure of mechanical disruption itself did not contribute to a reduced level of pneumolysin, as assays with cell lysates of the wild-type culture prepared with and without mechanical disruption yielded the same pneumolysin hemolytic titer (data not shown). The difference between the *lytA* mutant and wild-type strain in terms of the levels of pneumolysin was reduced, but not completely eliminated, when the pneumolysin titers were divided by the amount of total protein (data not shown).

With both cultures, the extracellular hemolytic activity of pneumolysin was first detectable within 4.5 h of growth (OD_{600} for WU2 = 0.316, OD_{600} for SM121.10 = 0.313) and continued to rise thereafter. With WU2, the highest titer achieved in the supernatant during the first 10 h of growth was 243, which was the same as that attained by the *lytA* mutant. After incubation for 24 h, the supernatant titer of WU2 rose to approximately 729, presumably because of the release of cytoplasmic contents due to lysis of the cells (culture OD_{600} = 0.2). On the other hand, after 24 h, the supernatant titer for the *lytA* mutant remained around 243 (culture OD_{600} = 0.67). The hemolytic assay was negative with UB2271 and UB1122, which are WU2 strains carrying a mutation in the *ply* gene (data not shown). We ruled out the possibility of the presence of extracellular pneumolysin due to incomplete centrifugation, because assays with D39 (which makes pneumolysin but does not secrete it during exponential growth) do not yield a similar extracellular pneumolysin profile (reference 2 and data not shown). These observations indicated that a deficiency in autolytic function does not impair the ability of strain WU2 to secrete pneumolysin.

A Western blot analysis of the pneumolysin (cell pellet and supernatant) obtained at different time points during the first 10 h of growth showed the presence of pneumolysin protein in the supernatants of both the WU2 and SM121.10 cultures prior to mid-log phase. This also indicated that pneumolysin was secreted into the external medium in the absence of autolysis (Fig. 3).

Effect of mitomycin C on growing pneumococcal cultures. It has been reported that many pneumococcal isolates contain prophages (31). The prophages of pneumococci encode lytic enzymes, a majority of which show a high homology to the host *lytA* (31, 37). Mitomycin C induces the lytic cycles of some bacterial prophages (31). It was important to determine if WU2 carried prophages which might be able to cause lysis of host cells and release pneumolysin. To test for prophage presence, mitomycin C was added to a growing culture of WU2. The culture was then monitored over the next 5 h (Fig. 4). Positive control tubes containing penicillin showed lysis of the cultures over the incubation period, with the OD_{600} dropping from 0.4 to 0.069. The test culture containing mitomycin C, however, did not show this drop in OD_{600} and was always comparable in OD to the culture without mitomycin C. We also failed to observe any effect of mitomycin C on the amounts of pneumolysin in the supernatants and pellets of the growing cultures (data not shown). These results failed to reveal any functional prophage in WU2 that could have led to lysis and pneumolysin release during growth.

Growth in the presence of choline. High concentrations of choline in the broth inhibit the action of LytA (8, 19) as well as the action of all but one bacteriophage-encoded lytic enzymes

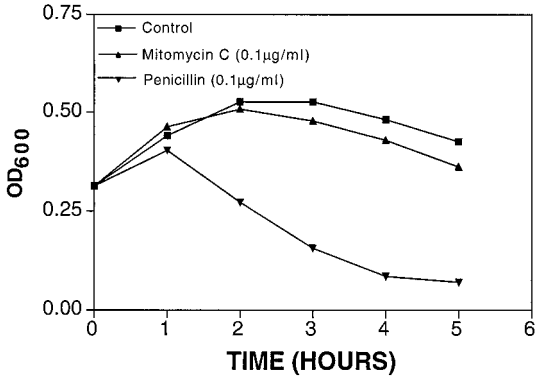

FIG. 4. Effect of mitomycin C on growth of WU2. WU2 was grown in THY at 37°C until the OD_{600} was approximately 0.3. Then 0.1 μg of mitomycin C or penicillin or nothing was added. The OD of the cultures were monitored over the next 5 h.

responsible for phage-mediated lysis. Like autolysin, the phage-encoded enzymes must be able to bind choline of the host pneumococcal cell wall teichoic acid for activity (13, 15, 31). WU2 was grown in THY containing 2% choline chloride, and samples were collected at various times to determine cytoplasmic and supernatant pneumolysin activity. WU2 grown in THY without added choline served as a control (Fig. 5). As expected, the choline-grown cells grew in short chains (data not shown) and displayed a loss of the autolytic property during the entire 24-h period of growth. Under both growth conditions, the cell-associated hemolytic activity due to pneumolysin was first detected by 4 h of growth (THY OD_{600} = 0.3; THY plus choline OD_{600} = 0.1), with a subsequent rise over the period of logarithmic growth (Fig. 5). At the 10- and 24-h time points, low cytoplasmic activity was recorded in the choline-treated culture, presumably due to degradation following cell death. The fact that lower cytoplasmic pneumolysin levels were observed at 24 h in the absence than in the presence of choline is consistent with the cell lysis that occurs without added choline. Hemolytic titers in the supernatants indicated that pneumolysin was released in both the THY- and the choline-grown cultures as early as 4 h into the growth cycle, at which time the cultures were in early-to-mid-log phase. The supernatant hemolytic titers of the choline-grown culture were remarkably similar to those of the culture grown in the absence of choline. As previously observed in Fig. 2, the levels in the supernatant increased over time under both conditions. The highest titer attained under both growth conditions was 243. Also, as previously observed, the extracellular pneumolysin titer rose between 10 and 24 h in the THY-grown culture (titer, 729), presumably due to cell lysis. The choline-grown culture behaved like the *lytA* mutant SM121.10, with the 24-h extracellular titer of pneumolysin (titer, 243) being similar to that attained at the end of the first 10 h of growth. Unlike with the *lytA* mutant, which showed a slight reduction in overall pneumolysin levels compared to those of the wild-type strain, growth in a high concentration of choline did not affect pneumolysin levels. This might be explained by the fact that *ply* is 7 kb downstream from *lytA*. Thus, the insertion into *lytA* may have had a polar effect on *ply* expression.

Analyses of total protein synthesis and secretion during growth. In order to further test the possibility that limited lysis associated with early and mid-exponential phase might explain the presence of extracellular pneumolysin, we examined the total protein profiles of whole-cell lysates and supernatants at different times during growth. The proteins were separated on a polyacrylamide gel and visualized by Coomassie blue staining (Fig. 6). The comparison of cytoplasmic and supernatant protein profiles did not provide any evidence for autolysis occurring during log-phase growth. The protein profiles of the whole-cell lysates were distinct from the corresponding profiles of the supernatant proteins. Major bands present in the whole-cell lysates were not represented among those proteins in the concentrated supernates, as would have occurred if cytoplasmic proteins had entered the supernatant through cell lysis. The supernatant profile also differed from that of samples from an identically concentrated sterile medium, indicating that it depicts a group of transported proteins. A semiquantitative dye reduction assay was also performed to check for the presence of lactate dehydrogenase, a cytoplasmic marker, in the supernatant samples. The levels of lactate dehydrogenase in the pellet samples were approximately sixfold more than the levels in the supernatants (which were equal to that of the negative control in medium alone). The assay was also performed with the autolysin mutant SM121.10, as well as with WU2 grown in THY containing choline. The results in both these cases were essentially the same as that obtained with

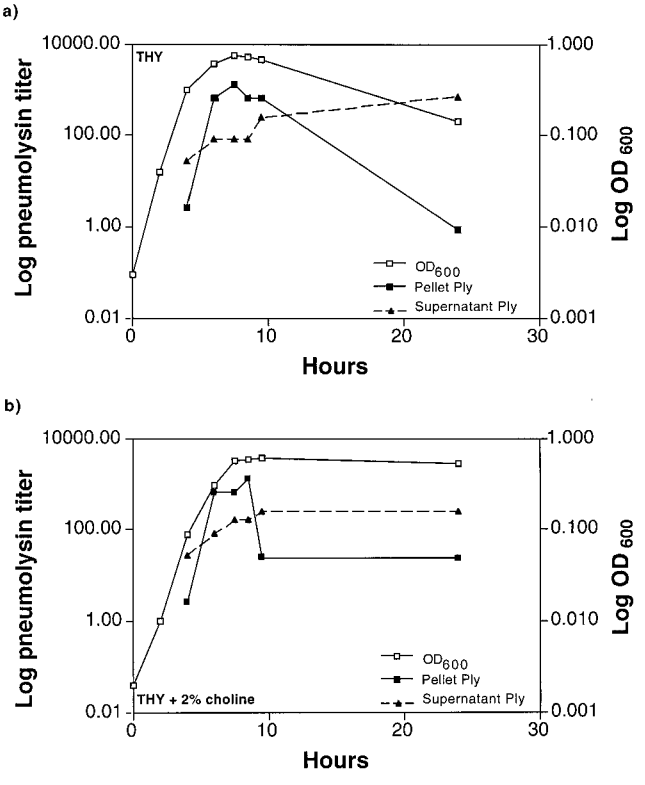

FIG. 5. Pneumolysin expression and release by WU2 in the presence and absence of choline. WU2 was grown in THY (a) or THY plus 2% choline chloride (b). Samples were withdrawn at different times, and the OD were determined (right *y* axis). Pneumolysin titers of the pellets and the supernatants were estimated (left *y* axis). The pneumolysin titers for the first two points were below the detection limits of the assay (titer = 3) and were not plotted.

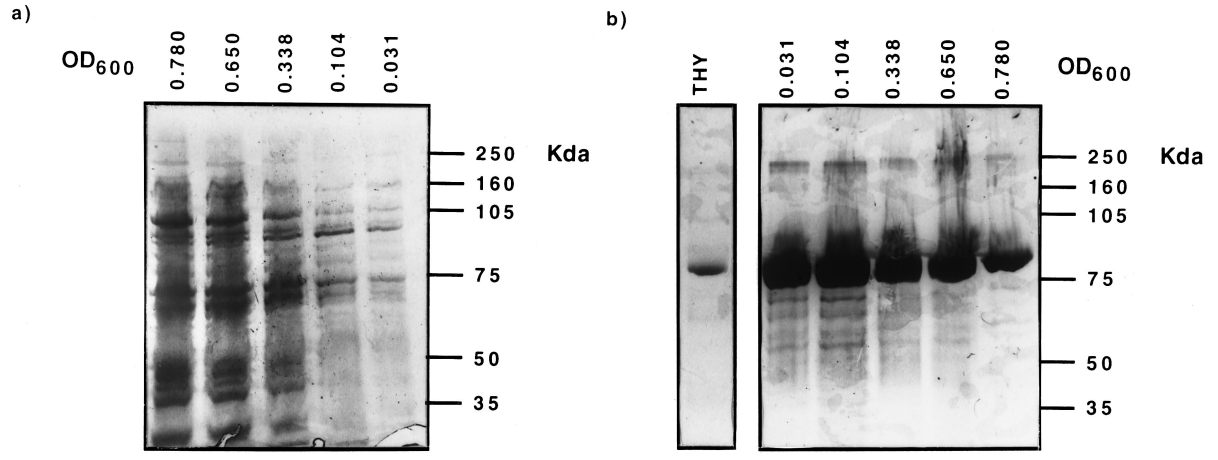

FIG. 6. Analyses of total proteins in the cytoplasm and supernatant during growth. WU2 was grown in THY at 37°C. Samples were withdrawn at various times. The cells were pelleted and lysed. The proteins present in the lysed pellets (cytoplasmic) and the culture supernatant (secreted) were precipitated with 10% TCA. The pellet (a) and supernatant (b) proteins were separated on a 10% polyacrylamide gel and stained with Coomassie brilliant blue R-250. THY alone was run as a control for the supernatant samples (b).

wild-type WU2, indicating no evidence of lysis during growth (data not shown).

These findings and the results with supernatant and cytoplasmic proteins support the idea that the extracellular release of pneumolysin is not dependent upon autolytic activity but that it might occur via active secretion of the pneumolysin protein by an unidentified mechanism.

Mouse virulence studies. Since the autolysin mutants behaved similarly to wild-type WU2 with respect to pneumolysin release, we examined the relative effects of the autolysin and pneumolysin mutations on virulence in this strain. Some of these experiments were conducted with CBA/N mice, which are highly susceptible to infection with pneumococci and lack the partially protective levels of antibodies to phosphocholine (26). When CBA/N mice were challenged i.v. with 200 CFU of bacteria, it was observed that wild-type WU2 and both of the autolysin-deficient mutants, SM121.10 and SM321.3, exhibited similar high levels of virulence (the median time to death being 48 h in all three cases). In contrast, the two pneumolysin null mutants, UB1122 and UB2271, which were used as controls, showed highly significant attenuations of virulence. In previous studies where immunologically normal mice were challenged by the i.p. route, it was observed that autolysin and pneumolysin-negative mutants had similar effects on virulence. Thus, we also challenged BALB/cByJ mice i.v. and i.p. with wild-type and mutant strains. By both routes of infection (Fig. 7b and d), a loss of LytA or pneumolysin caused an attenuation in virulence. This result was consistent with published results for immunologically normal mice (4–6, 12). To determine if the difference in the effect of a *lytA* null mutation in CBA/N mice compared to that in BALB/c mice was specific to the route of infection (i.v.), we challenged CBA/N mice with 300 CFU by the i.p. route. (Fig. 7c). In this case, we observed a partial attenuation of both mutants. However, the mice challenged with the *lytA* mutant strains died sooner (median time to death = 48 h) than the mice challenged with the *ply* mutant strains (median time to death = 96 h). This difference was significant ($P = 0.0032$; Rank test) and confirms the i.v. results with CBA/N mice which indicated that pneumolysin and autolysin have different effects on virulence.

These results indicated that at lower challenged doses, in CBA/N mice, the loss of LytA function did not greatly impair the ability of the pneumococci to cause an infection.

DISCUSSION

Unlike the thiol-activated toxins produced by other gram-positive pathogens, pneumolysin lacks an N-terminal export signal sequence for transport to the extracellular environment (36). However, two findings make it clear that pneumolysin acts extracellularly (1, 12). Antibody to pneumolysin is able to inhibit pneumolysin-dependent virulence in vivo (22). Pneumolysin-deficient strains can be complemented during coinfection with pneumolysin-sufficient strains, again indicating the importance of extracellular pneumolysin (1). It was thus assumed that the extracellular presence of pneumolysin was due to autolysin-mediated lysis of the pneumococci in the stationary phase of bacterial growth (4). Several lines of experimental evidence supported this idea. Autolysin-negative mutants of D39 created by insertion duplication mutagenesis did not release intracellular pneumolysin unless purified autolysin was supplied exogenously (4). Moreover, mutagenesis of *lytA* did not result in an additive attenuation of virulence in the *ply* deletion background, consistent with a common pathway (3, 5, 12). However, unlike strain D39, strain WU2 and a few other strains express extracellular pneumolysin prior to stationary phase (2). This finding raised the possibility that pneumolysin might be able to be released by a nonlytic mechanism. In the present study we observed that *lytA* mutants of WU2 were capable of secreting pneumolysin in a manner comparable to that of the wild-type WU2 parent. Although the LytA amidase is the major pneumococcal autolysin, *S. pneumoniae* also encodes other lytic enzymes. These include a glucosaminidase, a murein hydrolase, LytB, and an autolytic lysozyme, LytC (14, 17, 18). Although the exact roles of these enzymes are as yet unknown, it has been hypothesized that they may function in

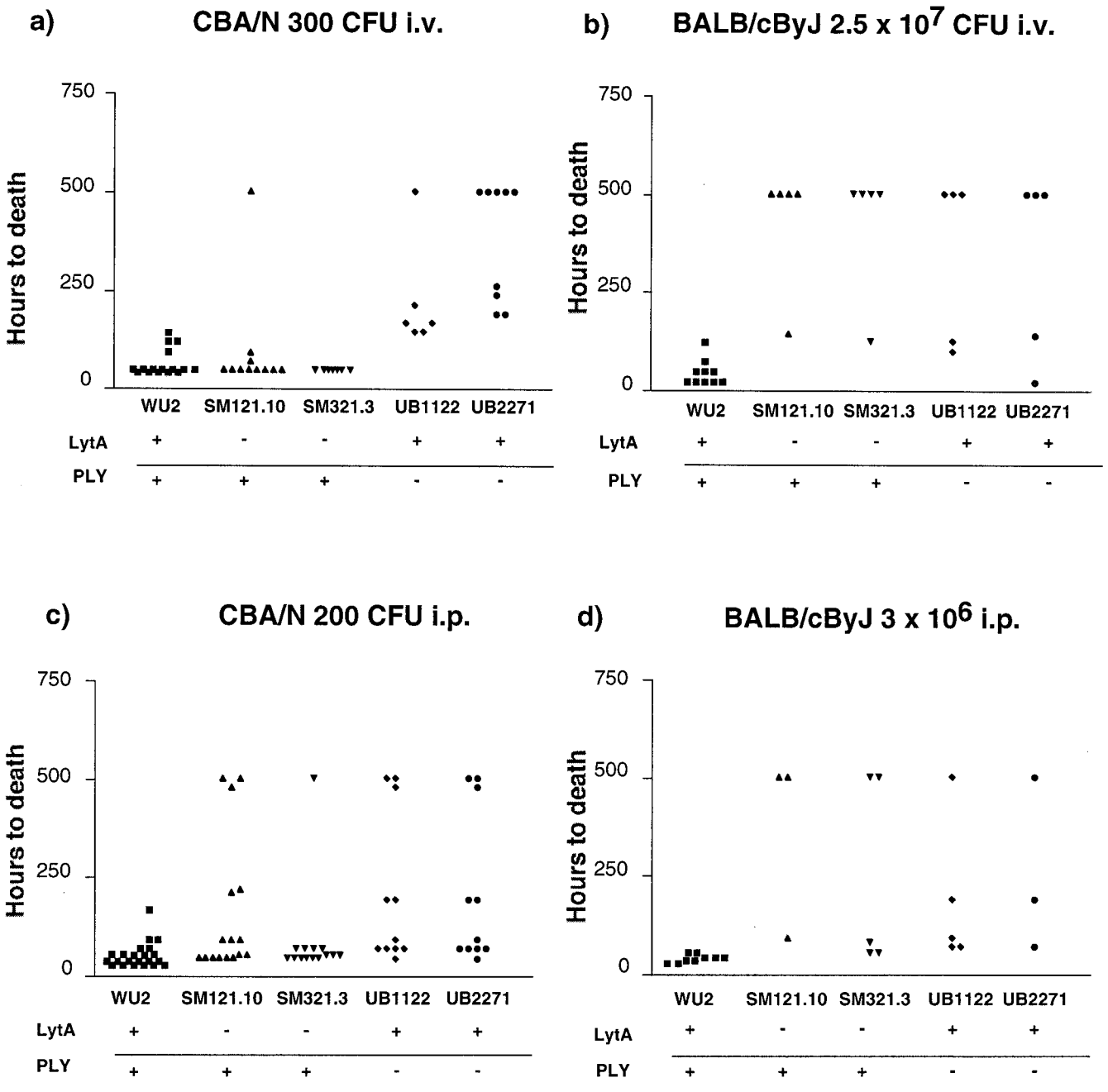

FIG. 7. Effect of inactivation of *lytA* on the virulence of WU2. CBA/N mice (a and c) or BALB/cByJ mice (b and d) were challenged i.v. (a and b) or i.p. (c and d). The hours to death were determined in each case. Control mice were given Ringer's solution. Test mice were given 300 CFU (a), 2.5×10^7 CFU (b), 200 CFU (c), 3×10^6 CFU (d).

cell division and release of DNA during competence (14, 17, 18). All of these enzymes are purified on the basis of their ability to bind to choline. In this regard, they function like LytA in requiring an attachment to choline-containing cell wall teichoic acid for their activity and in being inhibited by high levels of choline present in the growth medium. Our results indicated that pneumolysin activity was detected at remarkably similar levels for strains grown in the presence or absence of 2% choline chloride, at which concentration LytA and other known autolytic enzymes are completely inhibited (8, 17). Thus, the release of pneumolysin into the extracellular environment did not require the activity of any known pneumococcal autolysin.

The log-phase release of pneumolysin from WU2 also did not appear to be dependent on a lysogenic bacteriophage. Pneumococcal phages contain genes that encode lysins required for the liberation of phage progeny. All but one of the known pneumococcal phage lysins display an absolute requirement for choline on the cell wall teichoic acid for their activity, as does LytA. Mitomycin C, which often induces phage lytic cycles, did not cause lysis of the cells in a growing WU2 culture, indicating that this strain was not host to functional phages.

While this study did not rule out the possibility that WU2 was carrying a defective phage particle, lysis by the lysin of such a particle should still have been inhibited by the choline-containing medium described above.

It has been reported that the Cp17 lysin from the pneumococcal bacteriophage Cp-7 functions independently of choline (16). As a result, such an activity will not be inhibited by growth in the presence of choline. However, if in an exponentially growing culture a significant number of bacteria were undergoing autolysis, then one would expect to find cytoplasmic proteins in the supernatant. Our results showed that the cytoplasmic and the extracellular protein profiles were very different from each other.

Previous in vivo data demonstrated that the absence of functional pneumolysin could alter the net growth kinetics of pneumococci in a mouse model of bacteremia (1). The growth of wild-type strain D39 increases exponentially from 10^5 to $\sim 10^{10}$ CFU in the blood at the time of death of the mice. In contrast, the net growth of the pneumolysin-negative strain PLN-A increases like that of wild-type D39 until it reaches 10^6 to 10^7 CFU/ml of blood and then results in a chronic infection at $\sim 10^6$ CFU/ml. In a mixed infection with D39, PLN-A follows the same growth kinetics as D39 (1). This result and the observation that antibody to pneumolysin can protect against sepsis (29) demonstrate that the effect of pneumolysin on pathogenesis must be mediated extracellularly and that pneumolysin is secreted by some mechanism at levels in blood as low as 10^6 CFU/ml (1). This bacterial density of PLN-A is comparable to that attained by in vitro-grown WU2 at about the time that the extracellular pneumolysin is first detected.

BALB/cByJ mice are relatively resistant to pneumococcal sepsis, and fatal infection requires a high challenge dose. In these mice mutations in *lytA* and *ply* had similar effects on virulence. In the highly susceptible CBA/N mice, however, the situation was different. At the low challenge doses used in these mice, the loss of pneumolysin had a large effect on virulence. In contrast, the loss of autolysin had no effect in i.v. infection and had significantly less effect than the loss of pneumolysin on the virulence of an i.p. infection. This observation makes it clear that autolysin and pneumolysin have different effects on virulence and that autolysin is not a requirement for pneumolysin's effect on virulence in WU2. The ability of actively growing pneumococci to release pneumolysin may be necessary for optimal infection. Indeed, it has been observed that pneumolysin facilitates the multiplication of pneumococci and the spread of the bacterium from the lungs to the blood. Pneumolysin is thought to play a role in enhancing the survival of the bacteria in vivo (12). It has been demonstrated that live D39 cells can induce concentration- and time-dependent nitric oxide (NO) production by murine macrophages (7). This role has been attributed to pneumolysin, since the pneumolysin-negative mutant of D39, PLN-A, was unable to induce the production of NO under similar conditions (7). In those experiments, care was taken to demonstrate that the stimulation of NO production did not require bacterial lysis and so could not be due to the cell wall, which is also known to be a mediator of NO production (7). From those results, one could also conclude that bacterial autolysis is not required for the release of pneumolysin in vivo. If this is the case, then the attenuated virulence of the AL-2 strain (the autolysin-negative mutant of D39) must be attributable to a pneumolysin-independent mechanism. However, it is also possible that different strains of pneumococci have evolved distinct mechanisms for the release of pneumolysin.

In *Helicobacter pylori* it had been shown that the urease subunits UreA and UreB, as well as HspA and HspB, are released into the extracytoplasmic space via a process of specific secretion and not via autolysis, as previously thought. These proteins, like pneumolysin, also lack any known secretory signals (35).

Since the extracellular secretion of pneumolysin by D39 is not observed in vitro, it appears that the in vitro expression kinetics of pneumolysin by D39 might be quite different from that seen in vivo. One could speculate that some factor(s) in the blood acts as an environmental cue for the release of pneumolysin. In the presence of such a factor strains like D39 might release pneumolysin at low cell densities. As a part of this scenario, it could be assumed that WU2 has lost its ability to regulate the release of pneumolysin and thus that it releases it extracellularly even in an in vitro culture in the absence of an in vivo stimulus. Alternatively, it is possible that regulation of pneumolysin release occurs by different mechanisms in D39 and WU2. Although we have not compared genomic sequences of D39 and WU2 *ply* genes, the high structural and antigenic conservation at this locus has been well established (23).

Since the pneumococcal genome does not harbor any known sequences indicative of a type III secretory pathway (34), it would then appear that pneumolysin is released by a non-type II, non-type III mechanism.

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