INFECTION AND IMMUNITY, June 1996, p. 2240–2245 Vol. 64, No. 6
0019-9567/96/$04.00+0

Relationship between Phase Variation in Colony Morphology, Intrastrain Variation in Cell Wall Physiology, and Nasopharyngeal Colonization by *Streptococcus pneumoniae*

JEFFREY N. WEISER,[1,2]* ZDISLAW MARKIEWICZ,[3] ELAINE I. TUOMANEN,[3] AND JAVAID H. WANI[1]

Department of Pediatrics, Children's Hospital of Philadelphia,[1] and Department of Microbiology, University of Pennsylvania School of Medicine,[2] Philadelphia, Pennsylvania, and Laboratory of Molecular Infectious Disease, Rockefeller University, New York, New York[3]

Received 22 February 1996/Accepted 3 April 1996

Streptococcus pneumoniae **undergoes phase variation in colony morphology, which has been implicated as a factor in the pathogenesis of pneumococcal disease. Phenotypic differences between opaque and transparent colony forms correlate with differences in rates of autolysis. This study examined whether differences in autolysis are caused by differences in expression of the major amidase, LytA, or the structure of its peptidoglycan substrate. No significant difference was detected by high-pressure liquid chromatography analysis of stem peptides released after treatment of purified peptidoglycan with amidase. Differences in the rate of digestion of purified cell walls, furthermore, did not correlate with susceptibility to autolysis. Lower levels of autolysis in opaque variants, however, was associated with decreased levels of immunodetectable LytA on colony immunoblots and Western blots (immunoblots). Diminished cell-surface-associated LytA in opaque variants was also demonstrated by whole-cell inhibition enzyme-linked immunosorbent assay. Since transparent variants have been shown both to colonize the nasopharynx more efficiently in an animal model and to express more surface-exposed LytA, it was determined whether LytA contributes to colonization in a neonatal rat model of pneumococcal carriage. Defined mutants in the *lytA* gene were used to show that there was no significant contribution by LytA to nasopharyngeal colonization in this model. Although the expression of LytA was shown to undergo phase variation in association with colony morphology, *lytA* mutants are still capable of phenotypic variation in colony morphology, which suggests that other factors are responsible for intrastrain differences which affect colonization.**

Streptococcus pneumoniae, the pneumococcus, undergoes spontaneous, reversible variation (phase variation) which is apparent as differences in colony opacity on transparent agar surfaces (22). These differences in colony morphology appear to be relevant to the pathogenesis of pneumococcal disease. Organisms from transparent variants are able to colonize the nasopharynx efficiently in an animal model of pneumococcal carriage, whereas isogenic opaque variants are deficient in colonization (22). In addition, transparent variants have been shown to be more adherent to human endothelial cells and type II lung cells in culture (7). Cytokine stimulation of lung cells accentuated differences in adhesion between opaque and transparent variants through a mechanism involving the platelet-activating factor receptor (6). It has been proposed that pneumococci bind the platelet-activating factor receptor via the phosphorylcholine on the cell wall teichoic acid. In addition, there is direct evidence that pneumococcal cell wall components mediate adherence (14). However, the specific cell surface constituents of the pneumococcus that are associated with the transparent phenotype and contribute to colonization or the ability of the organism to attach to host cells in vitro have not been identified. The role of the opaque phenotype in the pathogenesis of pneumococcal disease has not yet been defined (22).

* Corresponding author. Mailing address: 802A Abramson Building, Children's Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104. Phone: (215) 573-3510. Fax: (215) 573-9068. Electronic mail address: Weiser@A1.mscf.upenn.edu.

Although the biochemical basis of the variation in colony opacity remains unknown, several observations have indicated that the cell wall and its degradation in stationary phase may differ in association with colony opacity. When electron micrographs of opaque and transparent pneumococci at an equivalent phase in their growth were compared, transparent, but not opaque, organisms appeared to be in the process of breaking down their cell walls (autolysis) (22). Further evidence linking cell wall breakdown and colony morphology was provided by a recently identified chromosomal locus which affects the frequency of phase variation (17). Mutagenesis of a putative regulatory region in this locus generated an unstable, rapidly autolyzing mutant. These findings suggested that the opaque variant may be deficient in enzymatic degradation of its peptidoglycan.

The major murein hydrolase of the pneumococcus is an *N*-acetylmuramoyl-L-alanine amidase referred to as autolysin (LytA), which requires binding to choline on teichoic acid for its activity (5, 13). Although this enzyme is conserved among pneumococcal isolates, it is unclear what advantage autolysis confers on the organism. LytA has been implicated as a virulence determinant of the pneumococcus, although the specific contribution of the autolysin to pathogenesis remains unclear (2, 3, 20). In one report, the relative virulence of *lytA* mutants constructed in an encapsulated background was reduced compared with that of their parent strain after intranasal challenge of mice (2). In a separate study, however, the relative levels of virulence of *lytA* mutant and *lytA*$^+$ pneumococci rendered encapsulated by transformation were equivalent after intraperi-

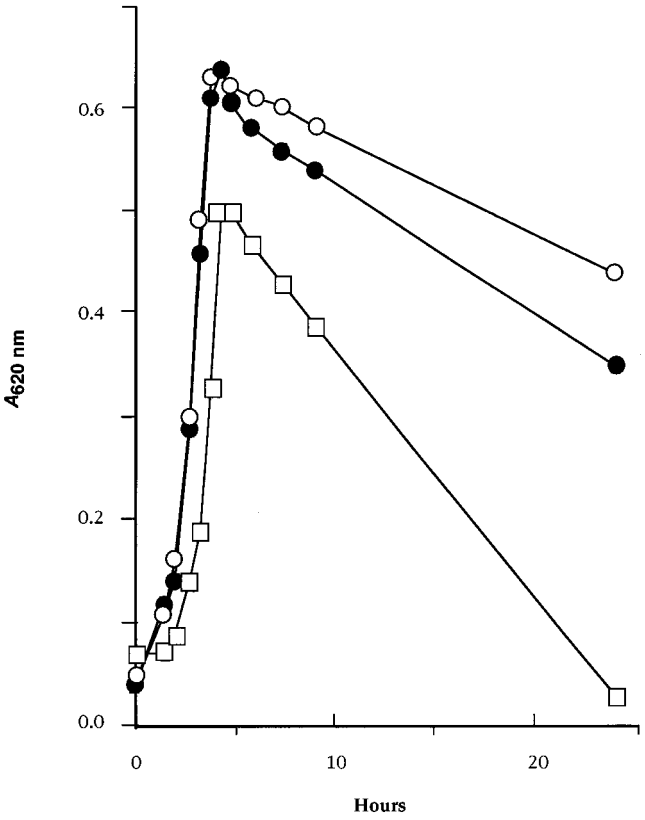

FIG. 1. Growth curves of pneumococcal phenotypic variants and mutants of strain R6. Strains were grown at 37°C in a semisynthetic medium, and the A_{620} was measured at the times indicated. Solid circles, R6 opaque variant; open squares, R6 transparent variant; open circles, *lytA* mutant RUP24.

toneal challenge (20). These studies suggest that if LytA contributes to pathogenesis, it may be during steps involved in nasopharyngeal colonization.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Spontaneous opaque and transparent variants of laboratory strain R6 and a type 18C clinical isolate, P45, were used in this study (1). R6 variants were grown in semisynthetic medium (C+Y medium [pH 8.0]) at 37°C without shaking (15). P45 variants were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). Broth cultures were plated on tryptic soy plates containing 1% agar onto which 100 μl of catalase (5,000 U) (Worthington Biochemical Co., Freehold, N.J.) was added. Cultures were grown at 37°C overnight in a candle extinction jar, which provided the atmosphere of increased CO_2 necessary for optimal growth on this medium. Colony morphology was assessed with a stereo zoom microscope with oblique transmitted illumination as previously described (23). All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., unless stated otherwise.

Cell wall preparation. The walls of pneumococci from exponentially growing cultures were purified as previously described (13). To obtain pure peptidoglycan, the walls were additionally treated with 48% hydrofluoric acid for 24 h at 4°C. The peptidoglycan was labelled by growing bacteria in C_{DEN}, a chemically defined medium, containing L-[4,5-^{3}H]lysine (Amersham) at final concentrations of 2.5 μCi and 10 μg/ml (19).

Amidase digestion of cell walls and peptidoglycan. Pneumococcal amidase was isolated from *Escherichia coli* CM21 carrying the cloned *lytA* gene on plasmid pGL80 (11). The enzyme was purified and converted to an active form in a single affinity chromatography step by using a Hi TrapQ column (Pharmacia). After the loaded column was washed with 1.5 M NaCl in 50 mM Tris-HCl buffer, pH 6.9, elution was done with 2% choline chloride in the same buffer. The pure enzyme was used to remove stem peptides from peptidoglycan as previously described (12). The digestion of peptidoglycan was confirmed by adding a trace amount of labelled wall (20,000 cpm) and monitoring the release of radioactivity in the soluble phase. In some experiments, the removal of stem peptides by amidase was assessed by observing the decrease in turbidity of the peptidoglycan suspension at A_{620} over time.

HPLC analysis of stem peptide composition. Peptides removed from peptidoglycan with amidase were separated by high-pressure liquid chromatography (HPLC) (Beckman Gold System) using a Vydac 218TP54 column (The Separations Group, Hesperia, Calif.) (12). Elution was carried out with a 129-ml linear gradient from 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Fractions were monitored at A_{210}.

Western blotting (immunoblotting) and colony immunoblotting. Bacterial cultures at equivalent cell densities, based on the optical density at 620 nm, were divided into cell and supernatant fractions by centrifugation. In other samples, phosphate-buffered saline (PBS)-washed cells were resuspended in 1/10 of the original culture volume and washed in PBS with or without 2% choline chloride for 15 min at 4°C. Samples were stored at −20°C, and the volume loaded was adjusted on the basis of colony counts. Samples from equivalent numbers of cells in loading buffer were heated at 100°C for 5 min prior to separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% acrylamide separating gels. The electrotransfer of proteins onto Immobilon-P (Millipore Co., Bedford, Mass.) and Western blotting were carried out as described previously, except that the membrane was immersed in methanol prior to wetting (21). For colony immunoblots, colonies were lifted onto nitrocellulose membranes and allowed to dry. Plates were reincubated for 8 h for regrowth of colonies to determine their phenotypes. The immunoblotting of membranes was carried out as follows. Membranes were first washed twice with Tris-saline blocking buffer (TSBB) (10 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 0.5% Tween 20, and 0.02% sodium azide) and incubated for 16 h with polyclonal antiserum raised against the C-terminal domain of LytA (diluted 1 in 5,000 in TSBB). After five washes in TSBB, goat anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, Calif.) was added, and the membrane was incubated for another 2 h. Reactivity was visualized by using the nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate substrate system (18) after five washes with TSBB and a subsequent wash with alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 5 mM $MgCl_2$).

Whole-cell inhibition ELISA. R6 bacterial lysate, the source of LytA antigen, was prepared by growing cells to an optical density at 620 nm of 0.5 and subsequent cell lysis by vortexing with glass beads (20 to 120 mesh). The lysate was collected after centrifugation at 1,000 × *g* for 10 min, and 100 μl of a 1-in-50 dilution of the lysate was fixed in 0.1 M sodium carbonate on a 96-well polystyrene microtiter plate by incubation for 16 h at 37°C. The plate was washed five times with buffer consisting of PBS (pH 7.4), sodium azide (0.02%), and Brij 35 (0.05%). Bacterial cells were washed with PBS and adjusted to the desired cell density on the basis of optical density. Cells were incubated with 200 μl of antibody to LytA diluted in PBS to a concentration to generate twice the half-maximal ELISA reading. Cells were incubated at 37°C with shaking for 2 h. Cells were pelleted, and 100 μl of the supernatant was added to the microtiter plate to which the R6 lysate had been fixed and incubated at 37°C for 16 h with shaking. After incubation for 2 h with goat anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase, the A_{405} was determined as previously described (4).

Nasopharyngeal colonization. The phenotypes of mutants to be compared in the animal model were confirmed by Western blot analysis using the antibody to LytA. Nasopharyngeal colonization of neonatal rats by pneumococci was carried out as described previously (22). For each experiment, 10 pups were each given an intranasal inoculum of 10^6 CFU. Colonization was assessed at 1, 3, and 8 days postinoculation. To allow for accurate quantification of colonization by type 3 strains, which are mucoid on agar surfaces, the fluids obtained from nasopharyngeal washes were first mixed with 20 ml of tryptic soy agar at 42°C and allowed to solidify.

RESULTS

Growth characteristics of opaque and transparent variants. The in vitro growth characteristics of phenotypic variants of strain R6 with opaque and transparent colony morphologies were compared (Fig. 1). In contrast to the transparent variant, the opaque variant did not undergo autolysis at an appreciable rate at 37°C upon entry into the stationary phase of growth. The growth characteristics of the opaque variant resembled those of strain RUP24, which contains a defined mutation in the major amidase gene, *lytA* (20). On the basis of this observation, the possibilities that differences in autolysis were caused either by altered expression of the amidase or by heterogeneity in its peptidoglycan substrate were considered separately.

Comparison of stem peptides from the peptidoglycan of opaque and transparent variants. The cell walls of isogenic opaque and transparent variants were initially compared by analyzing stem peptides removed by amidase digestion of pu-

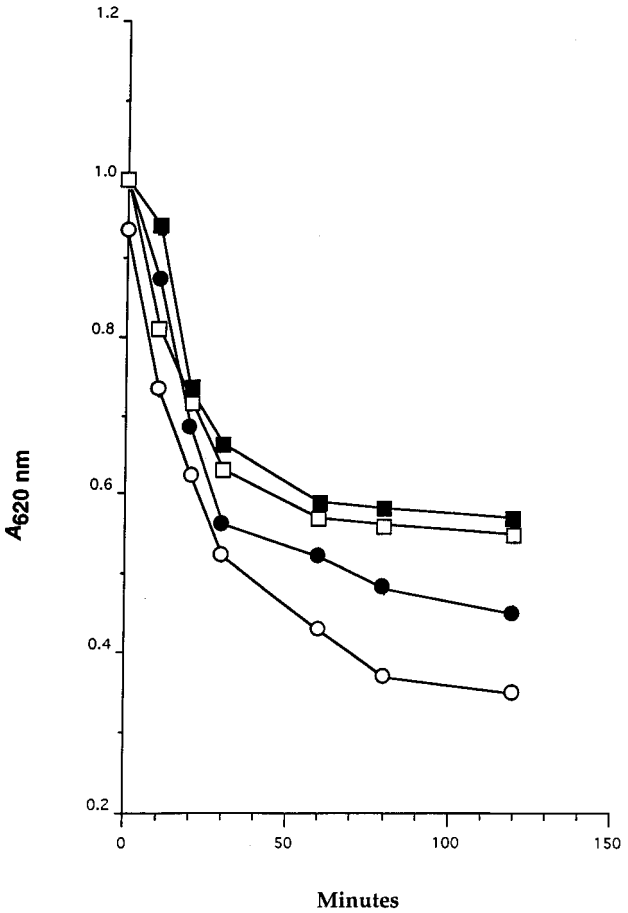

FIG. 2. Digestion of whole cell wall preparations and pure peptidoglycan by amidase. Digestion was measured as decreasing A_{620} over time. Shown are the results for the R6 opaque variant (open symbols) and the R6 transparent variant (solid symbols) with (squares) or after treatment with hydrofluoric acid to release teichoic acid, leaving peptidoglycan (circles). Data are the means of three independent determinations.

rified peptidoglycan. The patterns of HPLC fractionation were virtually identical on repeated runs of these materials (data not shown). The only difference between the two separations was a consistent twofold increase in the content of a minor stem muropeptide in the peptidoglycan of the opaque variant (6.0%) compared with that of the transparent variant (3.1%). The structure of this peptide, whose position on the chromatogram shows it to be a dimeric species, has not been identified (12). Additional studies showed that purified amidase was able to digest whole cell wall preparations from both opaque and transparent variants (Fig. 2). In fact, digestion of the whole cell wall preparation from the transparent variant was slightly slower than that from the opaque variant. These differences were more apparent after hydrofluoric acid treatment to remove teichoic acid (Fig. 2). This finding, however, does not provide an explanation for the diminished rates of autolysis associated with the opaque phenotype.

Phase variation in the expression of LytA. Since differences in the rates of autolysis could not be attributed to differences in the peptidoglycan substrate, the possibility that the levels of amidase expression vary in association with colony phenotype was examined. The technique of colony immunoblotting allowed direct comparison of the colony phenotype and the expression of the autolysin. Marked differences between opaque and transparent variants of strain R6 were detected in the amount of immunodetectable LytA. Transparent colonies, which show increased autolysis, were associated with far greater reactivity with an anti-LytA antibody than were opaque colonies. This finding is demonstrated in the case of strain P45 because unlike strain R6, it undergoes phase variation in colony morphology at high frequency, which allowed the identification of phenotypic revertants (17) (Fig. 3). Spontaneous reversion of the transparent variant to an opaque form was associated with decreased reactivity with the antibody to LytA. The results from colony immunoblotting suggested that there is phase variation in the amount of LytA accessible to antibody on the surface of the pneumococcus, which correlates with colony morphology.

Differences in the amount of immunodetectable LytA were also demonstrated by Western blot analysis of whole-cell lysates. When equal numbers of cells of opaque and transparent variants of strains R6 and P45 were compared on Western blots, the amount of LytA in the cell fraction of cultures was consistently higher in the transparent variant (Fig. 4, lanes 3 and 9). Similar samples from the *lytA* mutant RUP24 demonstrated that with this antiserum, the differences between variants were specific to the LytA protein. In addition to the increase in cell-associated LytA, the supernatants from cultures of the transparent variant, but not the opaque variant, contained small amounts of immunoreactive LytA (Fig. 4, lanes 4 and 10). Therefore, the increase in cell-associated LytA in the transparent phenotype could not be attributed to decreased release from cells with this phenotype, since significant amounts of immunodetectable LytA were present only in culture supernatants from transparent pneumococci. These results confirmed the correlation between the transparent phenotype and higher levels of cell-associated LytA.

To determine whether differences in the amount of LytA affected the quantity of the enzyme on the cell surface, variants of strain R6 and P45 were treated with choline and the amounts of immunodetectable LytA eluted from equivalent numbers of cells were compared by Western blot analysis. LytA is anchored to the cell by attaching to choline on teichoic acid and is released in the presence of high concentrations of choline (5). As expected, incubation in 2% choline led to the release of LytA from cells of both phenotypes. The larger amounts released by transparent variants indicated that the differences in LytA associated with this phenotype are attributable to increased levels of the protein on the cell surface (Fig. 4, lanes 13 and 17).

In order to confirm the differences seen on colony immunoblots and Western blots, the relative amounts of surface-bound LytA were compared by whole-cell inhibition ELISA. Cells at different densities were used to absorb anti-LytA antibody, and the amount of the free antibody was assayed by ELISA. Controls with the *lytA* mutant RUP24 had no effect on the amount of free antibody to LytA. The smaller amount of free antibody after absorption with equivalent numbers of cells of the transparent phenotype compared with that of the opaque phenotype indicated that transparent organisms bound and removed more antibody because of greater amounts of surface-exposed LytA (Table 1).

Contribution of LytA to nasopharyngeal colonization. The transparent phenotype, which expresses more cell-surface-associated LytA, has also been shown to colonize the nasopharynx in an animal model more efficiently than does the opaque phenotype (21). The hypothesis that LytA contributes to the ability of the pneumococcus to colonize the mucosal surface of the nasopharynx was examined by using previously constructed insertional mutants in the *lytA* gene. It was necessary to use

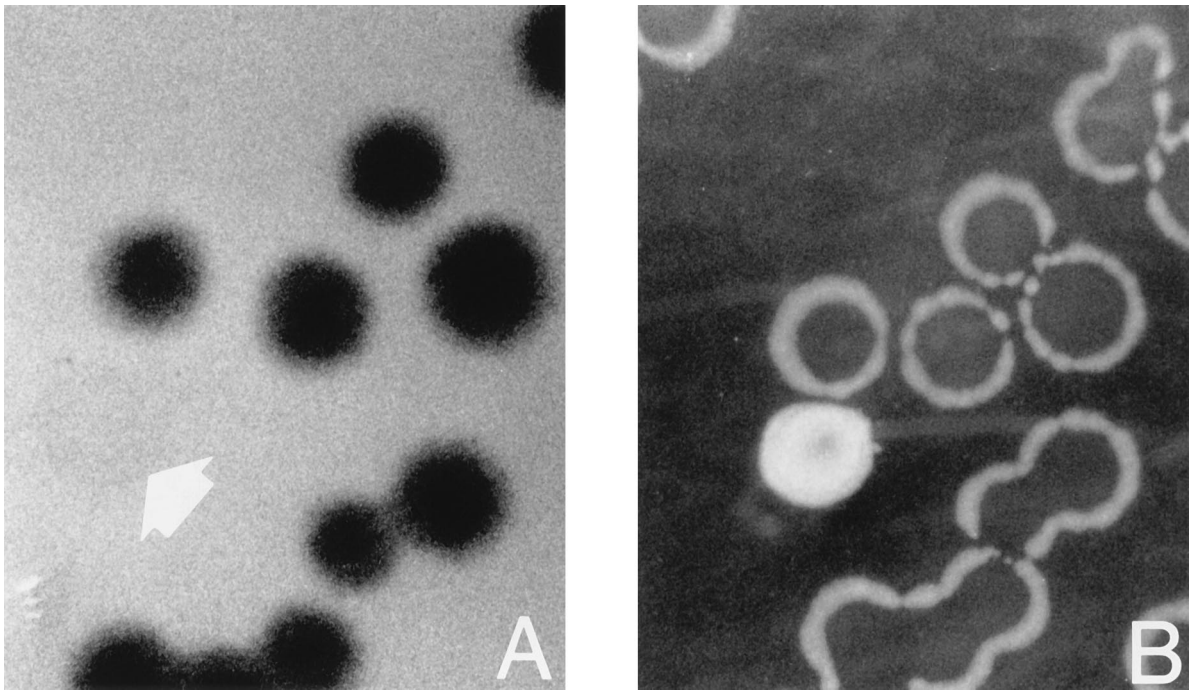

FIG. 3. (A) Colony immunoblot of strain P45 of transparent colony phenotype with an anti-LytA antibody. (B) After blotting, the same colonies were regrown and photographed with oblique transmitted light to show colony morphology. The position of a colony which is less reactive by immunoblotting corresponds to that of a spontaneously arising variant with an opaque phenotype and is shown by an arrow. Magnification, ×72.

mutants in an encapsulated background to achieve satisfactory levels of colonization. Two mutants in *lytA* (AL-2 and AL-6), a corrected mutant (AL-6R), and the serotype 2 parent strain, D39 (2), were compared in an infant rat model of pneumococcal colonization. Differences in colonization between strains based on the number of organisms in nasal washes in the days after inoculation did not correlate with the expression of LytA. This result was confirmed by comparing an unrelated set of defined mutants in *lytA* (A^-1 and A^-2) to their serotype 3 parent strain (WT) and a corrected mutant (A^+BT) (3) (Table 2). Again, quantitative differences in colonization between strains did not correlate with differences in the expression of LytA by Western blot analysis. This suggests that differences in the expression of LytA are not a significant factor in the greater ability of transparent pneumococci to colonize the mucosal surface of the nasopharynx.

DISCUSSION

Pneumococci of a clonal origin have been shown to be a heterogeneous population with respect to several important features, including growth characteristics. Differences within a population, which are readily observed as variations between opaque and transparent colony morphologies, correlate with susceptibility to autolysis after reaching stationary phase, a process which results from the enzymatic degradation of the cell wall. The diminished autolysis of the opaque variant was found to be associated with decreased surface expression of the

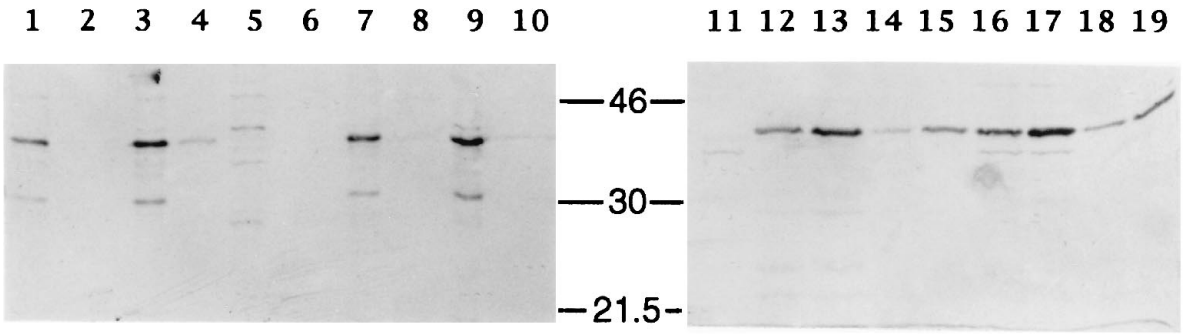

FIG. 4. Western blot analysis using antiserum against LytA. Cultures containing equivalent numbers of cells from opaque and transparent variants were divided into cell and supernatant fractions, and the amounts of LytA in these fractions were compared. In separate experiments, equivalent numbers of cells from opaque and transparent variants were washed in PBS with or without 2% choline chloride to compare the amounts of LytA eluted from cells. The band at approximately 29 kDa appears to be a degradation product of LytA since it is not present in the *lytA* mutant RUP24. Lanes 1, 3, 5, 7, and 9, whole-cell lysates; lanes 2, 4, 6, 8, and 10, culture supernatant fractions; lanes 11 to 13, 16, and 17, eluates with 2% choline chloride; lanes 14, 15, 18, and 19, controls without choline chloride. Samples were loaded as follows: lanes 1, 2, 12, and 14, R6 with opaque phenotype; lanes 3, 4, 13, and 15, R6 with transparent phenotype; lanes 5, 6, and 11, *lytA* mutant RUP24; lanes 7, 8, 16, and 18, P45 with opaque phenotype; and lanes 9, 10, 17, and 19, P45 with transparent phenotype. The positions of size markers (in kilodaltons) are indicated.

TABLE 1. Whole-cell inhibition ELISA comparing the abilities of the opaque and transparent phenotypes of strain R6 to absorb antibodies to LytA

Cell density (A_{620})	A_{405}[a] (% inhibition[b])		
	Opaque R6	Transparent R6	RUP24 (LytA$^-$)
0.5	0.289 (39.4)	0.179 (63.0)	0.484
0.4	0.304 (36.2)	0.198 (59.0)	0.477
0.3	0.410 (15.6)	0.269 (44.6)	0.486
0.2	0.424 (3.8)	0.371 (15.9)	0.441
0.1	0.481 (−4.1)	0.430 (6.9)	0.462
0.0	0.474 (−0.4)	0.457 (3.1)	0.472

[a] Data are the averages of two determinations.
[b] Calculated as the difference between the A_{405} readings for equivalent numbers of cells of the R6 phenotype indicated and strain RUP24.

major murein hydrolase of the pneumococcus. Although interstrain variations in the functions and sequences of pneumococcal autolysins have been described, this is the first report of intrastrain variation in the expression of this enzyme (9). A further implication of this finding is that LytA undergoes reversible, spontaneous variation in surface expression (phase variation).

Differences in the amount of extracellular LytA were most notable when colony morphology was compared with reactivity to an anti-LytA antibody by colony immunoblotting. To eliminate the possibility that this observation was an artifact of differential binding of opaque and transparent organisms to nitrocellulose filters, other techniques were used to confirm this finding. Differences in the amount of LytA were also noted when whole-cell lysates were compared by Western blotting. Western analysis of proteins eluted from cells by high concentrations of choline and a whole-cell inhibition ELISA was also used to show that the transparent phenotype expresses higher levels of autolysin on the cell surface. The presence of LytA in culture supernatants suggests that the release of LytA or the ability of LytA to associate with the surface of the pneumococcus may differ between transparent and opaque variants. The release of this protein into the culture supernatant has not previously been noted, and the mechanism for its secretion has not been established, as it lacks a typical prokaryotic signal sequence (8).

In contrast to results suggesting a correlation between the colony phenotype and the amount of LytA, no substantial differences in the structure of the population of stem peptides in the peptidoglycan were noted. Other structural differences may change the susceptibility of the cell wall substrate to autolytic degradation and thereby contribute to the resistance of opaque variants to lysis. It has been reported that in other species, differences in O acetylation, for example, may affect autolysis (10). Alternatively, modifications to the glycan strands of peptidoglycan may hinder absorption of this enzyme to the substrate (16). It remains possible that there are differences in the sugar backbone of peptidoglycan. In fact, small differences in the rates at which opaque and transparent cell walls were degraded by purified amidase were observed. These differences were even more pronounced after the release of teichoic acid from peptidoglycan. The significance of these differences is unclear since it was found that hydrolysis of the transparent cell wall was slower, even though autolysis of transparent organisms is more rapid than that of opaque variants. It was concluded, therefore, that the smaller amount of autolysin associated with opaque pneumococci is the major factor responsible for the diminished rate of autolysis of this phenotype.

It is difficult to speculate on why the pneumococcus might vary expression of this degradative enzyme on its surface, since it is unclear why this organism has evolved to be particularly efficient at autolysis. In this study, the question of whether LytA contributes to the ability of the pneumococcus to occupy its biological niche on the mucosal surface of the nasopharynx was addressed. A neonatal rat model, shown to be a sensitive assay to demonstrate quantitative differences in colonization, was used to compare defined mutants in *lytA* (22). Encapsulated strains containing defined mutations in *lytA* which had been shown to be impaired in the ability to cause sepsis after intraperitoneal or intranasal inoculation were compared with their parent strains. Although the transparent phenotype has been shown to colonize the nasopharynx more efficiently and was shown here to have higher levels of cell surface LytA, no correlation between the expression of LytA and the ability to colonize the nasopharynx could be demonstrated in this model. It was concluded that the more efficient colonization by transparent variants is not attributable to increased amounts of cell surface LytA.

It was previously reported that like *lytA* mutants, the opaque variant was resistant to the lytic effects of deoxycholate (17). The addition of LytA to cultures of opaque variants, however, was not sufficient to cause lysis in the presence of deoxycholate, indicating that differences in the expression of LytA may not fully account for differences in autolysis (17). Furthermore, although surface expression of LytA is an accurate marker of colony phenotype and a source of variation in colony morphology, LytA expression is not necessary to observe differences in colony opacity. Variation in colony morphology is found in *lytA* mutants, such as RUP24, that do not express immunodetectable autolysin (17). This suggests that although LytA expression varies in association with opacity, other factors which have not yet been defined also contribute to phenotypic variation detectable as altered colony morphology.

TABLE 2. Contribution of autolysin (LytA) to nasopharyngeal colonization of neonatal rats by *S. pneumoniae*

Strain[a]	Characteristics	10^3 CFU/ml of nasal wash on day postinoculation[b]		
		1	3	8
WT	Serotype 3, *lytA*$^+$	220 ± 97	59 ± 30	110 ± 73
A$^-$1	WT, *lytA*	150 ± 86	104 ± 53	74 ± 14
A$^-$2	WT, *lytA*	ND	66 ± 40	120 ± 58
A$^+$BT	A$^-$1, *lytA*$^+$	ND	80 ± 54	64 ± 36

[a] Strains have previously been described (2).
[b] Data are the means ± standard deviations for 20 pups from two separate experiments. ND, not determined.

ACKNOWLEDGMENTS

Antibody to LytA was generously provided by Rubens Lopez. We thank J. C. Paton and A. Tomasz for providing bacterial strains.

J. N. Weiser is a Lucille P. Markey Charitable Scholar. Z. Markiewicz is a J. W. Fulbright Visiting Scholar. This work was supported by the Lucille P. Markey Charitable Trust (J.N.W.) and NIH grant AI27913 (E.I.T.).

REFERENCES

1. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the nature of the chemical nature of the substance inducing transformation of pneumococcal types. J. Exp. Med. **79:**137–157.
2. **Berry, A. M., R. A. Lock, D. Hansman, and J. C. Paton.** 1989. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. Infect. Immun. **57:** 2324–2330.
3. **Berry, A. M., J. C. Paton, and D. Hansman.** 1992. Effect of insertional

inactivation of the genes coding pneumolysin and autolysin on the virulence of *Streptococcus pneumoniae* type 3. Microb. Pathog. **12:**87–93.
4. **Blake, M. S., C. M. Blake, M. A. Apicella, and R. E. Mandrell.** 1995. Gonococcal opacity: lectin-like interactions between Opa proteins and lipooligosaccharide. Infect. Immun. **63:**1434–1439.
5. **Briese, T., and R. Hakenbeck.** 1985. Interaction of the pneumococcal amidase with lipoteichoic acids and choline. Eur. J. Biochem. **146:**417–427.
6. **Cundell, D. R., N. P. Gerard, C. Gerard, I. Idanpaan-Heikkila, and E. I. Tuomanen.** 1995. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. Nature (London) **377:**435–438.
7. **Cundell, D. R., J. N. Weiser, J. Shen, A. Young, and E. I. Tuomanen.** 1995. Relationship between colonial morphology and adherence of *Streptococcus pneumoniae*. Infect. Immun. **63:**757–761.
8. **Diaz, E., E. Garcia, C. Ascaso, E. Mendez, R. Lopez, and J. L. Garcia.** 1989. Subcellular localization of the major pneumococcal autolysin: a peculiar mechanism of secretion in *Escherichia coli*. J. Biol. Chem. **264:**1238–1244.
9. **Díaz, E., R. López, and J. L. García.** 1992. Role of the major pneumococcal autolysin in the atypical response of a clinical isolate of *Streptococcus pneumoniae*. J. Bacteriol. **174:**5508–5515.
10. **Dougherty, T. J.** 1985. Involvement of a change in penicillin target and peptidoglycan structure in low-level resistance to β-lactam antibiotics in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **28:**90–95.
11. **Garcia, J. L., E. Garcia, and R. Lopez.** 1987. Overproduction and rapid purification of the amidase of *Streptococcus pneumoniae*. Arch. Microbiol. **149:**52–56.
12. **Garcia-Bustos, J. F., B. T. Chait, and A. Tomasz.** 1987. Structure of the peptide network of pneumococcal peptidoglycan. J. Biol. Chem. **262:**15400–15405.
13. **Garcia-Bustos, J. F., and A. Tomasz.** 1987. Teichoic acid-containing muropeptides from *Streptococcus pneumoniae* as substrates for the pneumococcal autolysin. J. Bacteriol. **169:**447–453.
14. **Geelen, S., C. Bhattacharyya, and E. Tuomanen.** 1993. The cell wall mediates pneumococcal attachment to and cytopathology in human endothelial cells. Infect. Immun. **61:**1538–1543.
15. **Lacks, S., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme activity in pneumococcus. Biochim. Biophys. Acta **39:**508–517.
16. **Markiewicz, Z., and A. Tomasz.** 1990. Protein-bound choline is released from the pneumococcal autolytic enzyme during absorption of the enzyme to cell wall particles. J. Bacteriol. **172:**2241–2244.
17. **Saluja, S. K., and J. N. Weiser.** 1995. The genetic basis of colony opacity in *Streptococcus pneumoniae*: evidence for the effect of box elements on the frequency of phenotypic variation. Mol. Microbiol. **16:**215–227.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Tomasz, A.** 1964. A chemically defined medium for *Streptococcus pneumoniae*. Bacteriol. Proc. **64:**29.
20. **Tomasz, A., P. Moreillon, and G. Pozzi.** 1988. Insertional inactivation of the major autolysin gene of *Streptococcus pneumoniae*. J. Bacteriol. **170:**5931–5934.
21. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
22. **Weiser, J. N., R. Austrian, P. K. Sreenivasan, and H. R. Masure.** 1994. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect. Immun. **62:**2582–2589.
23. **Weiser, J. N., E. I. Tuomanen, D. R. Cundell, P. K. Sreenivasan, R. Austrian, and H. R. Masure.** 1994. The effect of colony opacity variation on pneumococcal colonization and adhesion. J. Cell. Biochem. **18A:**S55.

Editor: A. O'Brien