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Additive Attenuation of Virulence of *Streptococcus pneumoniae* by Mutation of the Genes Encoding Pneumolysin and Other Putative Pneumococcal Virulence Proteins

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Although the polysaccharide capsule of *Streptococcus pneumoniae* has been recognized as a sine qua non of virulence, much recent attention has focused on the role of pneumococcal proteins in pathogenesis, particularly in view of their potential as vaccine antigens. The individual contributions of pneumolysin (Ply), the major neuraminidase (NanA), autolysin (LytA), hyaluronidase (Hyl), pneumococcal surface protein A (PspA), and choline-binding protein A (CbpA) have been examined by specifically mutagenizing the respective genes in the pneumococcal chromosome and comparing the impact on virulence in a mouse intraperitoneal challenge model. Mutagenesis of either the *ply*, *lytA*, or *pspA* gene in *S. pneumoniae* D39 significantly reduced virulence, relative to that of the wild-type strain, indicating that the respective gene products contribute to pathogenesis. On the other hand, mutations in *nanA*, *hyl*, or *cbpA* had no significant impact. The virulence of D39 derivatives carrying a *ply* deletion mutation as well as an insertion-duplication mutation in one of the other genes was also examined. Mutagenesis of either *nanA* or *lytA* did not result in an additional attenuation of virulence in the *ply* deletion background. However, significant additive attenuation in virulence was observed for the strains with *ply-hyl*, *ply-pspA*, and *ply-cbpA* double mutations.

Streptococcus pneumoniae is an important human pathogen, causing life-threatening invasive diseases such as pneumonia, meningitis and bacteremia, as well as less serious but highly prevalent infections such as otitis media and sinusitis. The high morbidity and mortality associated with pneumococcal disease are exacerbated by the rate at which this organism is acquiring resistance to multiple antibiotics (23). Polyvalent pneumococcal vaccines based on purified capsular polysaccharides have been available for nearly two decades, but their clinical efficacy has been limited by poor immunogenicity in high-risk groups (particularly young children) (16). Furthermore, antipolysaccharide antibodies confer a strictly serotype-specific protection, and only 23 of the 90 known serotypes are covered by existing formulations. The problem of poor vaccine immunogenicity in children is being addressed by conjugation of the polysaccharides to protein carriers. However, serotype coverage will be more limited, as it is unlikely that more than 11 serotypes will be included in such conjugate formulations. In view of this, much recent attention has focused on the possibility of developing vaccines based on pneumococcal protein antigens common to all serotypes (1, 12, 34).

Pneumococcal proteins which contribute to pathogenesis are obvious candidates for inclusion in such vaccines, and of those proteins studied to date, the thiol-activated toxin pneumolysin (Ply) and pneumococcal surface protein A (PspA) are the best characterized (12, 33, 35). Ply is a multifunctional protein having both cytotoxic and complement activation properties (11, 38). It is located in the cytoplasm but is released when pneumococci undergo autolysis (33, 35). PspA is a member of a family of structurally related choline-binding surface proteins (19, 20, 46, 47); its precise function is uncertain, although it has recently been shown to be capable of binding human lactoferrin (21). Both Ply and PspA are protective immunogens, and mutagenesis of the genes which encode them attenuates virulence of *S. pneumoniae* (1, 3, 7, 9, 10, 12, 13, 31, 45). The major pneumococcal autolysin (LytA) is also a choline-binding protein (19, 20) which contributes to virulence by mediating the release of Ply and possibly also inflammatory cell wall degradation products (4, 9, 26). A further choline-binding protein, CbpA (also referred to as SpsA), has recently been shown to bind the secretory component of secretory IgA (22) and also appears to be an adhesin for cytokine-activated epithelial and endothelial cell lines (39). Pneumococci also produce a hyaluronidase (Hyl) (6) and at least two neuraminidases (NanA and NanB) (5, 14, 27), but the contributions of these to pathogenesis are uncertain (28, 36).

Clearly, development of an effective protein-based vaccine depends on a thorough understanding of the roles of the various putative virulence proteins in pathogenesis, as well as their relative contributions to virulence. Cost considerations will place a limit on the number of different antigens which might be included, and so it is crucial that the most important virulence determinants be covered. In the present study we have compared the virulence of wild-type *S. pneumoniae* D39 with otherwise isogenic derivatives carrying mutations in the genes encoding Ply, NanA, LytA, Hyl, PspA, or CbpA. The virulence of D39 derivatives carrying a *ply* deletion mutation as well as an insertion-duplication mutation in one of the other genes was also examined.

MATERIALS AND METHODS

Bacterial strains. The virulent type 2 *S. pneumoniae* strain D39 (NCTC 7466) and its highly transformable, nonencapsulated derivative Rx1 have been described previously (2, 40). Derivatives of D39 with an insertion-duplication mutation in *lytA* (designated LytA$^-$) or with an in-frame deletion mutation in *ply* encoding a derivative of Ply lacking amino acids 55 to 437 (designated ΔPly) have also been described previously (4, 7). The pVA891-directed *pspA*-negative *S. pneumoniae* Rx1 derivative WG44-1 (31) was kindly provided by D. E. Briles. Pneumococci were routinely grown in Todd-Hewitt broth with 0.5% yeast extract (THY) or on blood agar. Where appropriate, erythromycin was added to media at a concentration of 0.2 μg/ml.

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Escherichia coli K-12 DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was grown in Luria-Bertani broth (30) with or without 1.5% Bacto-agar (Difco Laboratories, Detroit, Mich.). Where appropriate, chloramphenicol or erythromycin was added to the growth medium at a concentration of 25 or 125 μg/ml, respectively.

Transformation. Transformation of *E. coli* with plasmid DNA was carried out by standard methods with $CaCl_2$-treated cells. *S. pneumoniae* Rx1 and D39 were transformed with chromosomal or plasmid DNA as described previously (48). Pneumococcal transformants were selected on blood agar containing 0.2 μg of erythromycin per ml.

Southern hybridization analysis. Chromosomal DNA from the various *S. pneumoniae* D39 derivatives was restricted and electrophoresed on 1.0% agarose gels with a Tris-borate-EDTA buffer system, as described by Maniatis et al. (30). DNA was transferred to nylon membranes (Hybond N^+; Amersham, Little Chalfont, Buckinghamshire, England) as described by Southern (41), hybridized to probe DNA, and washed at high stringency, as described by Maniatis et al. (30). Probes specific for the various putative virulence genes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany), according to the method of Feinberg and Vogelstein (17). The templates used were a 1.2-kb *Hin*dIII fragment containing the complete *lytA* gene (20), a PCR product containing nucleotides (nt) 220 to 1986 of *pspA* (46), a *Cla*I/*Eco*RI fragment comprising nt 1377 to 2786 of *hyl* (6), an *Eco*RI/*Sph*I fragment comprising nt 615 to 1803 of *nanA* (14), and a PCR product comprising nt 481 to 680 of *cbpA* (22). Washed filters were developed with antidigoxigenin-alkaline phosphatase conjugate and a 4-nitroblue tetrazolium salt (NBT)–5-bromo-4-chloro-3-indolylphosphate (X-phosphate) substrate system (Boehringer Mannheim), according to the manufacturer's instructions.

Virulence factor assays. *S. pneumoniae* D39 derivatives were grown in THY at 37°C to an A_{600} of 0.3. Cells from 1 ml of culture were pelleted by centrifugation and lysed by resuspension in 100 μl of a mixture containing phosphate-buffered saline, pH 7.2, and 0.1% sodium deoxycholate. Pneumolysin activity in each lysate was quantitated by a hemolysis assay using human erythrocytes, as described previously (37). Neuraminidase and hyaluronidase were also assayed as previously described, using 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid and umbilical cord hyaluronic acid, respectively, as substrates (6, 27).

Western blot analysis. Proteins in *S. pneumoniae* lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (24) and electrophoretically transferred from SDS-PAGE gels onto nitrocellulose filters, as described by Towbin et al. (43). Filters were probed with mouse anti-PspA or mouse anti-LytA (used at a dilution of 1:1,000) followed by goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Bio-Rad Laboratories, Richmond, Calif.). Enzyme-labelled bands were visualized with an NBT–X-phosphate substrate system (Boehringer Mannheim).

Virulence studies. *S. pneumoniae* strains were grown overnight on blood agar (supplemented with erythromycin where appropriate), inoculated into serum broth (meat extract broth plus 10% horse serum), and incubated at 37°C for 3 h. Production of type 2 capsule was confirmed by the Quellung reaction, using antisera obtained from Statens Seruminstitut, Copenhagen, Denmark. Cultures were then diluted in serum broth to the appropriate density, and 0.1-ml volumes were injected intraperitoneally (i.p.) into groups of 12 or 13 BALB/c mice. Survival time was recorded.

Intranasal challenge studies were performed on QS mice which had been anesthetized by i.p. injection with 0.06 mg of sodium pentobarbitone (Nembutal; Boehringer Ingelheim, Sydney, Australia) per g of body weight. Aliquots (50 μl each) of 3-h serum broth cultures of the various *S. pneumoniae* strains, diluted when appropriate with serum broth to give a density of 10^8 CFU/ml, were then introduced into the nostrils. Mice regained consciousness after approximately 1 h, and survival time was recorded.

Differences in median survival time between groups were analyzed by the Mann-Whitney U test (two tailed). Differences in the overall survival rate between groups were analyzed by the Fisher exact test.

RESULTS

Construction and characterization of *S. pneumoniae* mutants. *S. pneumoniae* D39 derivatives with insertion-duplication mutations in various genes were constructed by using plasmid pVA891, which encodes chloramphenicol and erythromycin resistance and can replicate in *E. coli* but not in *S. pneumoniae* (29). The first step of the mutagenesis procedure involves cloning an internal fragment of the respective gene into pVA891. For *nanA*, a 637-bp *Hin*dIII-*Sph*I fragment corresponding to nt 1210 to 1847 of the *nanA* open reading frame (ORF) (14) was cloned into *Hin*dIII-*Sph*I-digested pVA891. For *hyl*, a 673-bp *Cla*I-*Nco*I fragment corresponding to nt 1286 to 1959 of the *hyl* ORF (6) was cloned into the *Cla*I site of pVA891. A 200-bp internal fragment of *cbpA*, corresponding to nt 481 to 680 of the *cbpA* ORF (22) was amplified by PCR with primers designed with reference to the *cbpA* sequence deposited in GenBank (accession no. Y10818), with *S. pneumoniae* D39 DNA as the template. This was blunt-end ligated into the *Eco*RV site of pVA891. Each of these constructs was transformed into *E. coli* DH5α.

In a previous study (10) we found that the efficiency of direct transformation of the encapsulated type 2 strain D39 to erythromycin resistance, using recombinant pVA891 derivatives, was very low, even in the presence of exogenous competence factor. Therefore we adopted a two-step approach, initially transforming the highly transformable *S. pneumoniae* Rx1 with plasmid DNA purified from the various *E. coli* DH5α clones. Chromosomal DNA from representative erythromycin-resistant transformants from each reaction was subjected to Southern hybridization analysis to confirm interruption of the respective gene with the pVA891 sequences, by using probes specific for pVA891 and either *nanA*, *hyl*, or *cbpA* (results not shown). DNA from these derivatives, as well as from the *psaA*-negative Rx1 derivative WG44-1, was then used to transform the encapsulated parental strain D39, and erythromycin-resistant transformants were isolated from two independent transformation experiments for each interrupted gene. Chromosomal DNA from each of these was subjected to Southern hybridization analysis using probes specific for the respective putative virulence gene or pVA891, to confirm interruption of the respective D39 gene with the vector sequences (Fig. 1). *S. pneumoniae* D39 transformants with confirmed insertion-duplication mutations in *nanA*, *hyl*, *pspA*, or *cbpA* were designated $NanA^-$, Hyl^-, $PspA^-$, and $CbpA^-$, respectively.

Pneumococci with mutations in *ply* as well as the other genes were constructed by transformation of *S. pneumoniae* D39 ΔPly with chromosomal DNA from the various Rx1 derivatives or from *S. pneumoniae* D39 $LytA^-$. Again, interruption of the respective gene in erythromycin-resistant transformants isolated from two independent transformation experiments was confirmed by Southern hybridization analysis (Fig. 1). Absence of the *ply* ORF in each of these double mutants was also confirmed by PCR, as previously described (7). *S. pneumoniae* D39 ΔPly transformants with confirmed insertion-duplication mutations in *nanA*, *hyl*, *pspA*, *lytA*, or *cbpA* were designated ΔPly-$NanA^-$, ΔPly-Hyl^-, ΔPly-$PspA^-$, ΔPly-$LytA^-$, and ΔPly-$CbpA^-$, respectively.

To confirm that the various single or double mutations did not affect the in vitro growth rate, the *S. pneumoniae* D39 derivatives were grown overnight on blood agar, inoculated into serum broth, and incubated at 37°C for 5 h. During this period, there was no significant difference in growth rate between any of the mutants and wild-type D39, as judged by viable count (result not shown). To confirm the phenotype of the various *S. pneumoniae* D39 derivatives, lysates of fresh THY cultures were tested with the hemolysis assay for Ply activity and direct enzyme assays for NanA and Hyl. The pneumolysin titer of the wild-type *S. pneumoniae* D39 lysate was 2,048 hemolytic units (HU) per ml of culture, but ΔPly lysates contained <0.2 HU of pneumolysin per ml (the sensitivity limit of the assay). Pneumolysin activity was also undetectable in any of the ΔPly double mutants. In contrast, all other *S. pneumoniae* D39 derivatives expressed the wild-type level of pneumolysin activity (2,048 HU/ml). Wild-type D39 and ΔPly lysates contained 48.7 and 48.9 mU of neuraminidase activity per ml, respectively, but no activity (that is, <0.15 mU/ml) could be detected in lysates of either $NanA^-$ or ΔPly-$NanA^-$. Similarly, D39 and ΔPly lysates contained 84.8 and 83.6 U of hyaluronidase activity per ml, respectively, but no activity could be detected in lysates of either Hyl^- or ΔPly-Hyl^-. Expression of PspA and LytA was assessed by Western blot analysis using

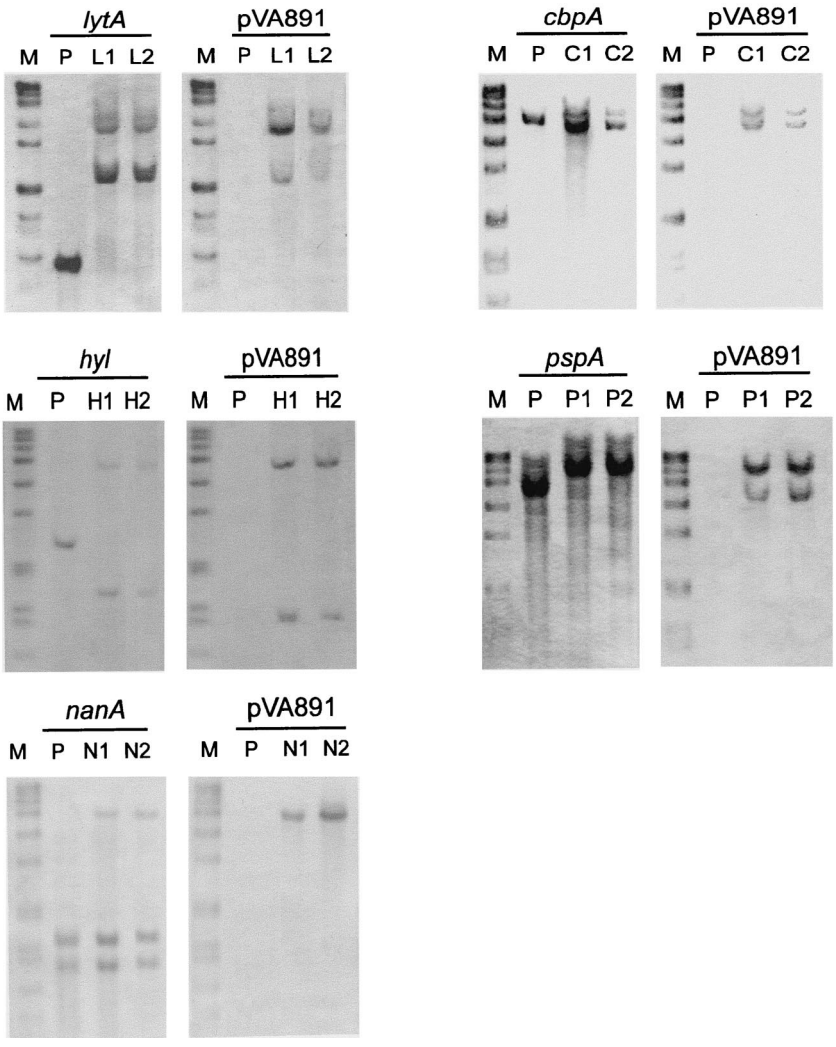

FIG. 1. Southern hybridization analysis of insertion-duplication mutants. Chromosomal DNA from the indicated *S. pneumoniae* derivatives was digested with *Hin*dIII (for *lytA* and *nanA* mutants), *Eco*RI (for *cbpA* and *hyl* mutants), or *Cla*I (for *pspA* mutants). Replicate digests were subjected to Southern hybridization analysis using probes specific for the respective virulence factor gene (*lytA*, *cbpA*, *hyl*, *pspA*, or *nanA*) and pVA891, as described in Materials and Methods. Lanes: M, prelabelled DNA size marker (bacteriophage SPP1 DNA restricted with *Eco*RI; sizes from top to bottom are 8.56, 7.43, 6.11, 4.90, 3.64, 2.80, 1.95, 1.88, 1.52, 1.41, and 1.16 kb); P, ΔPly; L1, LytA$^-$; L2, ΔPly-LytA$^-$; C1, CbpA$^-$; C2, ΔPly-CbpA$^-$; H1, Hyl$^-$; H2, ΔPly-Hyl$^-$; P1, PspA$^-$; P2, ΔPly-PspA$^-$; N1, NanA$^-$; N2, ΔPly-NanA$^-$.

polyclonal mouse antisera raised against purified LytA and PspA (anti-CbpA was not available) (Fig. 2). The anti-PspA serum labelled two species in both D39 and ΔPly lysates with approximate sizes of 75 and 155 kDa, but neither of these species was detectable in lysates of PspA$^-$ or ΔPly-PspA$^-$. The anti-PspA serum used was raised against a 43-kDa N-terminal fragment of PspA purified from recombinant *E. coli* expressing a truncated *pspA* gene from *S. pneumoniae* D39 (47). This fragment does not contain the choline-binding repeat domain common to several pneumococcal surface proteins, and so the presence of two immunoreactive bands is not a consequence of cross-reaction with another protein species. Talkington et al. (42) have previously reported an identical phenomenon with monoclonal anti-PspA for several *S. pneumoniae* strains including D39. They demonstrated that the low- and high-molecular-weight immunoreactive species corresponded to PspA monomers and noncovalently linked PspA dimers, respectively. The anti-LytA serum labelled a single species of the expected molecular size in both D39 and ΔPly lysates but not in lysates of LytA$^-$ or ΔPly-LytA$^-$ (Fig. 2). With both sera, all

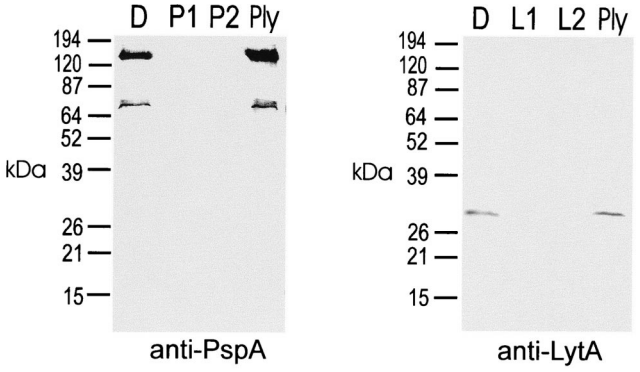

FIG. 2. Western immunoblot analysis. Lysates of the indicated *S. pneumoniae* D39 derivatives were separated by SDS-PAGE, electroblotted, and probed with mouse anti-PspA or mouse anti-LytA, as described in the Materials and Methods. Lanes: D, D39; P1, PspA$^-$; P2, ΔPly-PspA$^-$; Ply, ΔPly; L1, LytA$^-$; L2, ΔPly-LytA$^-$. The mobilities of protein size markers are also indicated.

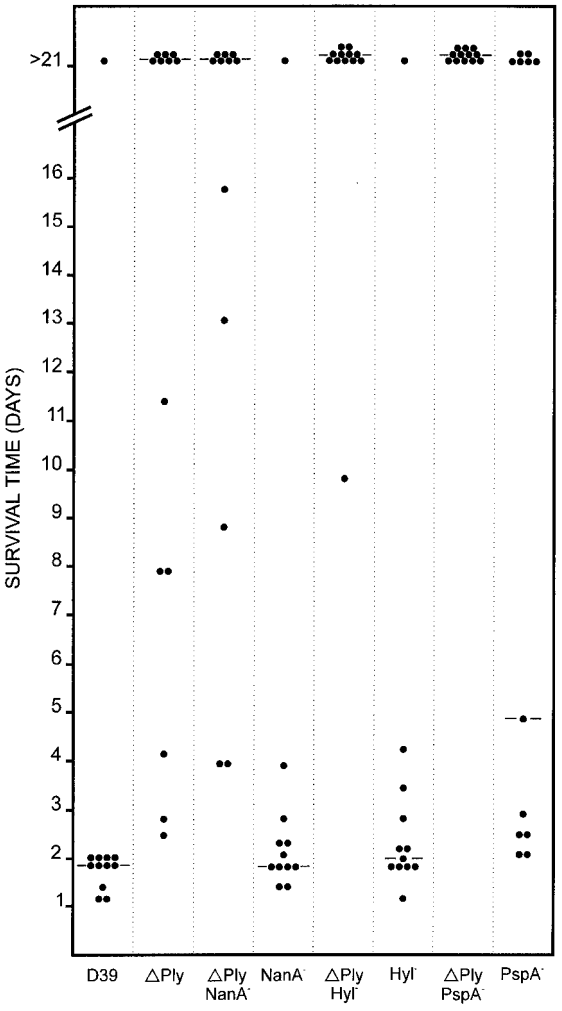

FIG. 3. Survival times of mice after i.p. challenge. Groups of 12 or 13 BALB/c mice were injected i.p. with approximately 10^3 CFU of the indicated strains. Each datum point represents one mouse. The horizontal lines denote the median survival time for each group.

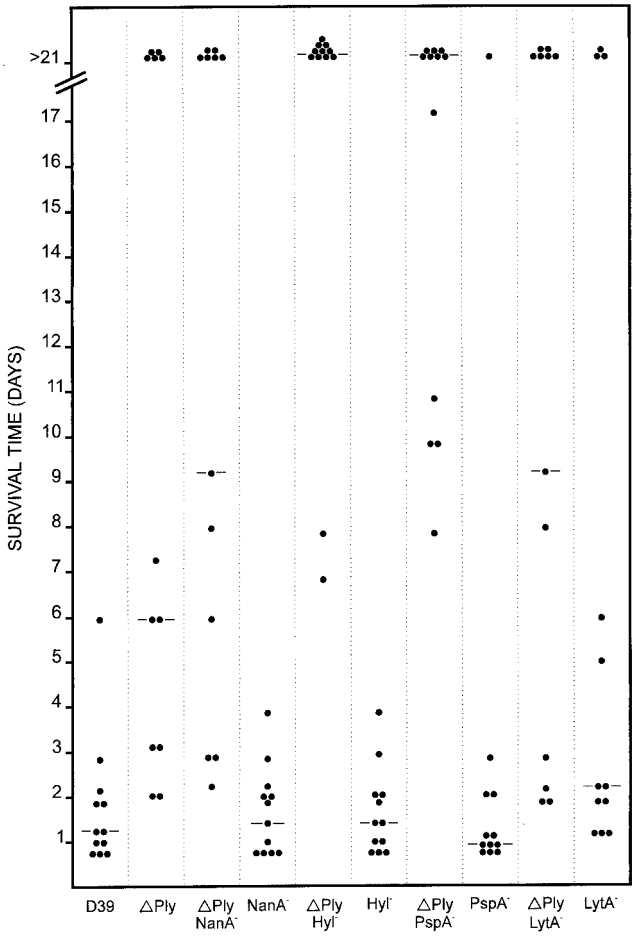

FIG. 4. Survival times of mice after i.p. challenge. Groups of 12 BALB/c mice were injected i.p. with approximately 10^5 CFU of the indicated strains. Each datum point represents one mouse. The horizontal lines denote the median survival time for each group.

the other *S. pneumoniae* D39 derivatives yielded immunoblot patterns similar to that seen for the wild type (result not shown).

Virulence studies. As an initial comparison of virulence, groups of 12 or 13 BALB/c mice were challenged i.p. with either D39, ΔPly, NanA$^-$, Hyl$^-$, PspA$^-$, ΔPly-NanA$^-$, ΔPly-Hyl$^-$, or ΔPly-PspA$^-$, at a dose of 10^3 CFU (Fig. 3). There was no significant difference in either median survival time or overall survival rate between groups challenged with D39, NanA$^-$, and Hyl$^-$. However, both the median survival time and the survival rate for the ΔPly group were significantly greater than those for the D39 group ($P < 0.002$ and $P < 0.025$, respectively). Similarly, both the median survival time and the survival rate for the PspA$^-$ group were significantly greater than those for the D39 group ($P < 0.002$ and $P < 0.05$, respectively), but they were not significantly different from those for the ΔPly group. Mice challenged with ΔPly-NanA$^-$ had a median survival time of >21 days and a survival rate of 7 of 12, which was indistinguishable from those for the ΔPly group (>21 days and 7 of 13). On the other hand, significant increases in survival rate relative to the ΔPly group were observed in the ΔPly-Hyl$^-$ and ΔPly-PspA$^-$ groups (survival rates were 11 of 12 and 12 of 12, respectively; $P < 0.05$ and $P < 0.025$, respectively). The survival rate for the ΔPly-PspA$^-$ group was also significantly higher than the rate of 6 of 12 obtained for the PspA$^-$ group ($P < 0.025$). The difference in survival time between the ΔPly-PspA$^-$ and PspA$^-$ groups also reached statistical significance ($P < 0.05$). Both the median survival time and survival rate for the ΔPly-Hyl$^-$ group (>21 days and 11 of 12) were markedly greater than that for the Hyl$^-$ group (1.9 days and 1 of 12) ($P < 0.002$ and $P < 0.001$, respectively). In a separate experiment, the i.p. virulence of ΔPly, LytA$^-$, and ΔPly-LytA$^-$ was compared at a dose of 10^3 CFU. However, there were no significant differences in either the median survival times or survival rates between any of these groups (result not presented).

When the comparative virulence of the various strains tested above was assessed at a higher i.p. dose (10^5 CFU), essentially similar results were obtained (Fig. 4). However, at this dose, a significant difference in virulence between D39 and PspA$^-$ was not detectable. Of the various D39 derivatives with mutations in a single gene, only ΔPly had a significantly greater survival time and higher survival rate than the wild-type strain ($P < 0.002$ and $P < 0.025$, respectively). LytA$^-$ was also significantly less virulent than D39 as judged by survival time ($P < 0.05$), but the survival rate was not significantly greater. Again, the double mutant ΔPly-Hyl$^-$ was less virulent than ΔPly, as judged by

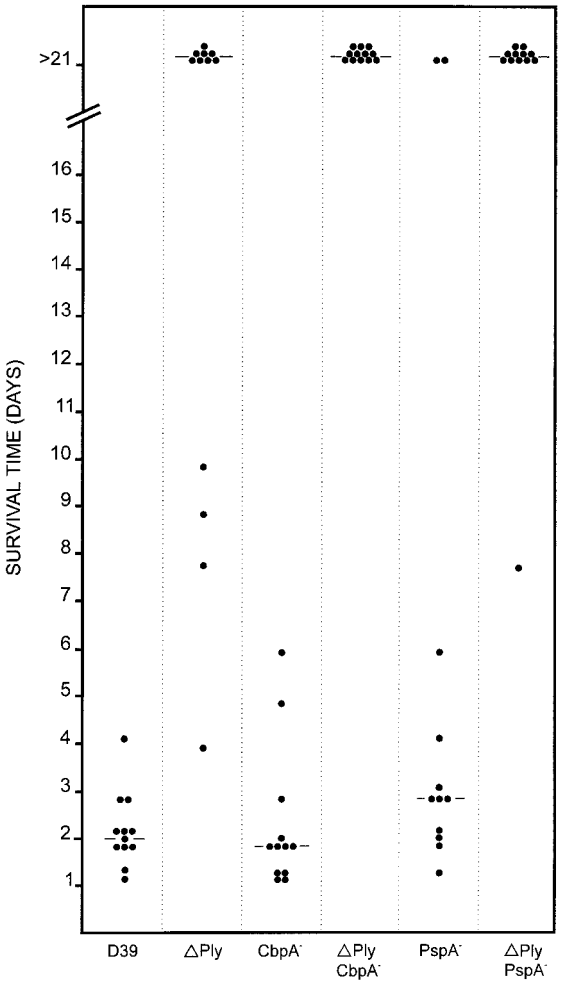

FIG. 5. Survival times of mice after i.p. challenge. Groups of 12 BALB/c mice were injected i.p. with approximately 5×10^3 CFU of the indicated strains. Each datum point represents one mouse. The horizontal lines denote the median survival time for each group.

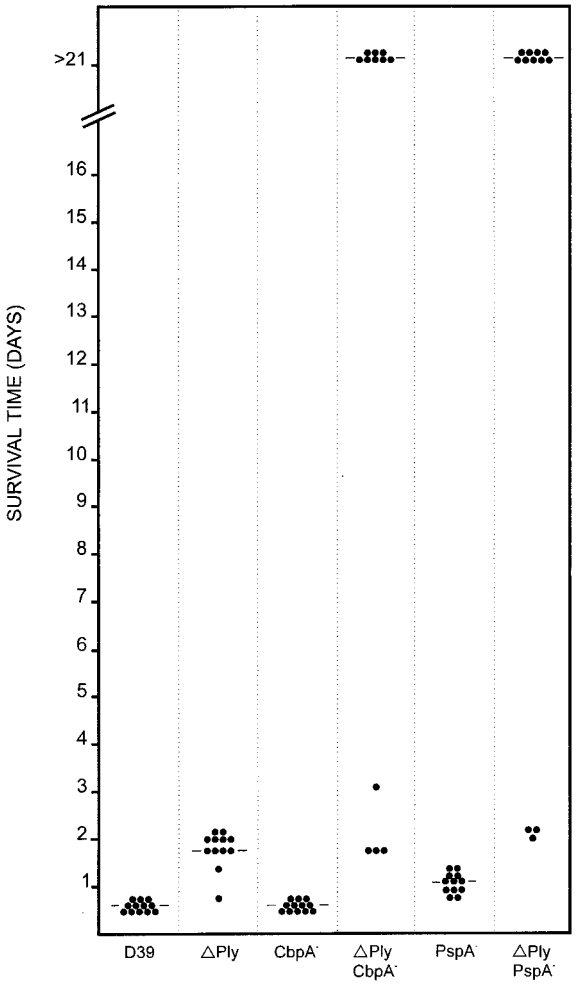

FIG. 6. Survival times of mice after i.p. challenge. Groups of 12 BALB/c mice were injected i.p. with approximately 8×10^6 CFU of the indicated strains. Each datum point represents one mouse. The horizontal lines denote the median survival time for each group.

both survival time and survival rate ($P < 0.05$ and $P < 0.05$, respectively). ΔPly-PspA$^-$ was also less virulent that PspA$^-$ as judged by both survival time and survival rate ($P < 0.002$ and $P < 0.025$, respectively). However, the difference in median survival time between the ΔPly-PspA$^-$ group (>21 days) and the ΔPly group (5.9 days) did not quite reach statistical significance ($0.05 < P < 0.1$). Furthermore, there was no significant difference in the virulence of ΔPly, LytA$^-$, and ΔPly-LytA$^-$.

In the second series of experiments, the virulence of D39, ΔPly, PspA$^-$, CbpA$^-$, ΔPly-PspA$^-$, and ΔPly-CbpA$^-$ was compared by challenging groups of 12 mice i.p., initially at a dose of 5×10^3 CFU (Fig. 5). Of the D39 derivatives with single mutations, ΔPly was the least virulent; both survival time and survival rate were significantly greater than those for either PspA$^-$ ($P < 0.002$ and $P < 0.025$, respectively), CbpA$^-$ ($P < 0.002$ and $P < 0.005$, respectively), and D39 ($P < 0.002$ and $P < 0.005$, respectively). The median survival time for the PspA group was significantly different from that for the D39 group ($P < 0.05$), but there was no significant difference in survival rate. However, there was no significant difference in the virulence of CbpA$^-$ and D39 as judged by either criterion. Although the overall survival rates for the groups challenged with ΔPly-PspA$^-$ or ΔPly-CbpA$^-$ (11 of 12 and 12 of 12, respectively) were numerically greater than that for the ΔPly group (8 of 12), this was not statistically significant. Accordingly, the i.p. challenge dose was increased to 8×10^6 CFU of each strain (Fig. 6). At this dose, none of the mice challenged with D39 or D39 derivatives with single mutations survived. However, the median survival times for the ΔPly and PspA$^-$ groups (1.75 and 1.12 days, respectively) were significantly different from that for the D39 group (<0.75 days) ($P < 0.002$ in both cases). The median survival time for the CbpA$^-$ group (<0.75 days) was indistinguishable from that for the D39 group. The differences in median survival time between the ΔPly group and either the PspA$^-$ or CbpA$^-$ group were also significant ($P < 0.002$ in both cases). The D39 derivatives with double mutations, ΔPly-PspA$^-$ and ΔPly-CbpA$^-$, were significantly less virulent than either D39 or any of the single mutants, as judged by both median survival time ($P < 0.002$, except for ΔPly-CbpA$^-$ versus ΔPly, for which P is <0.02), and survival rate ($P < 0.005$).

As confirmation of these findings, additional ΔPly-PspA$^-$, ΔPly-CbpA$^-$, and ΔPly-Hyl$^-$ mutants were isolated as described above, but from independent transformation experiments. The virulence of these independent mutants was then

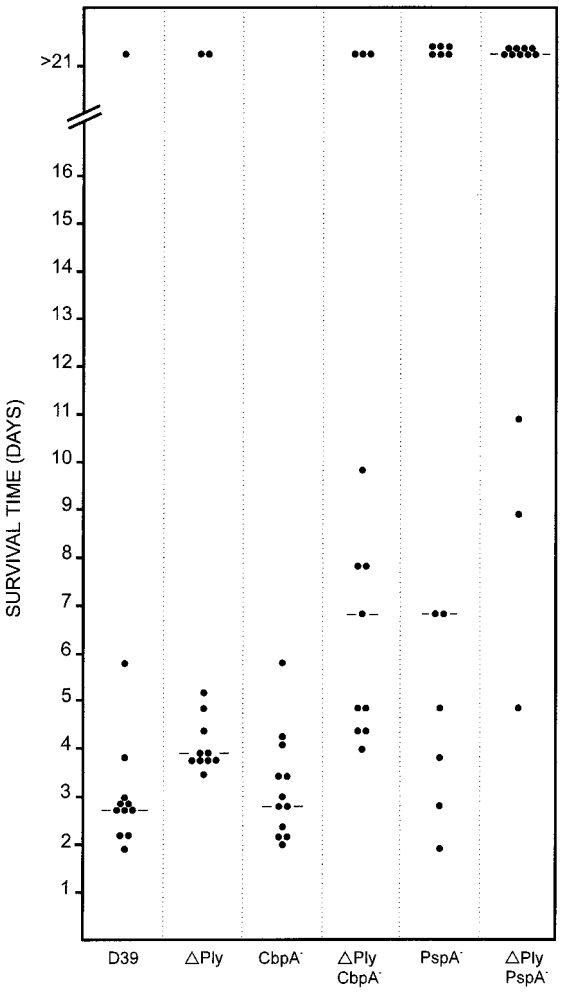

FIG. 7. Survival times of mice after intranasal challenge. Groups of 12 QS mice were anesthetized and challenged intranasally with approximately 5×10^6 CFU of the indicated strains. Each datum point represents one mouse. The horizontal lines denote the median survival time for each group.

compared with that of ΔPly, and with that of the original ΔPly-PspA$^-$, ΔPly-CbpA$^-$, and ΔPly-Hyl$^-$ mutants, by i.p. challenge at a dose of approximately 10^5 CFU. The virulence of these mutants relative to that of ΔPly was essentially as reported above, and there was no significant difference in either median survival time or overall survival rate between the respective pairs of independent mutants (results not presented).

In view of the previous report that CbpA may be an adhesin for cytokine-activated lung cells and that *cbpA* mutants have diminished capacity to colonize the nasopharynx of infant rats (39), virulence studies were also carried out with a mouse intranasal challenge model (Fig. 7). Both ΔPly and PspA$^-$ were less virulent than D39, as judged by median survival time ($P < 0.02$ in both cases). The survival rate of the PspA$^-$ group was also significantly greater than that of the D39 group ($P < 0.05$). However, the intranasal virulence of CbpA$^-$ was not significantly different from that of D39, as judged by either survival time or survival rate. Nevertheless, the ΔPly-CbpA$^-$ group survived significantly longer than the ΔPly group ($P < 0.05$) and the CbpA$^-$ group ($P < 0.002$). Both the median survival time and the survival rate of the ΔPly-PspA$^-$ group were significantly greater than those of the ΔPly group ($P < 0.002$ and $P < 0.01$, respectively). However, although both the survival time and survival rate of the ΔPly-PspA$^-$ group (>21 days and 9 of 12) were numerically greater than those of the PspA$^-$ group (6.8 days and 6 of 12), these differences did not reach statistical significance.

DISCUSSION

Although it has been known for a number of years that mutations in genes encoding Ply, PspA, and LytA reduce the virulence of *S. pneumoniae* (4, 10, 31), comparatively little is known of the impact of mutations in genes encoding other putative virulence factors. With the exception of a comparison of *lytA* and *ply* mutations in a type 3 pneumococcus (9), no previous studies have directly compared the virulence of strains with single mutations in the various virulence factor genes. Moreover, the impact of mutations in multiple virulence factor genes has not been examined before. In the present study, we have shown that *S. pneumoniae* D39 derivatives with either a deletion mutation in *ply* (ΔPly) or insertion-duplication mutations in *lytA* (LytA$^-$) or *pspA* (PspA$^-$) had significantly lower virulence for mice than did wild-type D39, as judged by both survival time and survival rate after i.p. challenge. In the i.p. model, the impact of the *ply* mutation was quantitatively greater than the *pspA* mutation, since when higher doses were tested, ΔPly was significantly less virulent than PspA$^-$. However, the virulence of ΔPly was not significantly different from that of LytA$^-$. In contrast, mutations in *nanA*, *hyl*, or *cbpA* did not result in detectable reduction in i.p. virulence. The effects on virulence observed for the various insertion-duplication mutants are not attributable to polar effects on downstream sequences, because in each case, strong transcription termination signals are located immediately 3′ to the interrupted gene.

When the impact of combinations of the *ply* and other mutations was examined, a D39 derivative deficient in production of both Ply and NanA was no less virulent than the strain carrying the *ply* mutation on its own. The single mutant NanA$^-$ was also fully virulent, suggesting that this neuraminidase plays a minimal role in the pathogenesis of pneumococcal sepsis. This is essentially in accordance with our previous finding that immunization with purified NanA confers only very weak protection against challenge with wild-type D39, and immunization with NanA and Ply provided no more protection than that achieved by immunization with Ply alone (28). The interpretation of both these findings is complicated to some extent by the fact that *S. pneumoniae* produces at least one other functional neuraminidase, NanB (5), which may have compensated for the absence or neutralization of NanA. Examination of the partial *S. pneumoniae* type 4 genome sequence (available at ftp://ftp.tigr.org/pub/data/s_pneumoniae/) also indicates the presence of an ORF on contig SP34 (designated *nanC*) which encodes a polypeptide with the structural features of a neuraminidase exhibiting approximately 50% deduced amino acid sequence identity to NanB. However, we have previously shown that the specific activity of NanA is much greater than NanB, and NanB also has a significantly lower pH optimum (5). When assayed at physiological pH with the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid, lysates of NanA$^-$ exhibited less than 0.3% of the neuraminidase activity of D39 (result not presented). Of course, it remains a possibility that the specific activity of NanB (and perhaps also NanC) may be higher with natural substrates, or that the expression of either *nanB* or *nanC* is specifically upregulated in vivo. We are currently attempting to construct

D39 derivatives with mutations in all three neuraminidase-encoding genes in order to resolve the remaining uncertainties concerning the role of these enzymes in pathogenesis of pneumococcal disease.

The D39 derivative deficient in production of both Ply and LytA was no less virulent than strains carrying either mutation on its own. We have previously demonstrated that although purified Ply and LytA were protective immunogens in mice against challenge with virulent pneumococci, no additive protection occurred when mice were immunized with both antigens (26). Furthermore, immunization with LytA provided no protection whatsoever against challenge with a Ply-negative pneumococcus. This suggested that the principal role of LytA in pathogenesis of invasive pneumococcal disease (at least in the i.p. challenge model) was to mediate release of Ply from the cells in vivo (26). This led us to predict that mutagenizing both *ply* and *lytA* would not result in additive attenuation of virulence; this prediction was upheld by the findings of the present study.

In contrast to the results above, the double mutants ΔPly-Hyl$^-$, ΔPly-CbpA$^-$, and ΔPly-PspA$^-$ were all significantly less virulent than any of the D39 derivatives with single mutations. This was unexpected for ΔPly-Hyl$^-$ and ΔPly-CbpA$^-$, because the single mutants Hyl$^-$ and CbpA$^-$ appeared to be as virulent as D39, even at the lowest dose tested. The additional attenuation of virulence achieved by mutagenizing two virulence factor genes was very considerable indeed. At the maximum i.p. dose tested (8×10^6 CFU), the survival rates for mice challenged with ΔPly-CbpA$^-$ and ΔPly-PspA$^-$ were 67 and 75%, respectively. The i.p. 50% lethal dose of wild-type D39 in this strain of mice is $<10^2$ CFU. Thus, mutagenesis of either of these pairs of virulence genes resulted in at least a 10^5-fold increase in 50% lethal dose. Such a massive impact on virulence has been observed previously only by transposon mutagenesis of *S. pneumoniae* genes essential for polysaccharide capsule production (44) or insertion-duplication mutagenesis of *psaA* (8), which encodes a permease with specificity for Mn^{2+} (15) and possibly also Zn^{2+} (25). However, mutagenesis of *psaA* has recently been reported to have pleiotropic effects, including reduced expression of CbpA and other potentially important choline-binding surface proteins (32).

In a previous study, Rosenow et al. (39) demonstrated that CbpA-deficient pneumococci exhibit a reduced capacity to colonize the nasopharynges of infant rats, but there was no apparent impact on virulence in a model of sepsis. While our findings for CbpA$^-$ are consistent with the latter result, the additional attenuation of virulence of ΔPly-CbpA$^-$ with respect to ΔPly clearly indicates that CbpA plays a measurable role in pathogenesis of systemic disease. This is consistent with the finding that this protein is an adhesin for cytokine-activated epithelial and endothelial cells (39). The apparent involvement of CbpA in nasopharyngeal colonization also prompted us to examine the virulence of the various mutants in an intranasal challenge model. One would predict that *cbpA* mutations would have a more significant impact on virulence in models such as this, which require the pneumococcus to penetrate the respiratory mucosa. However, these studies yielded findings analogous to those obtained with the i.p. challenge model; CbpA$^-$ had virulence similar to that of D39, but ΔPly-CbpA$^-$ was significantly less virulent than either ΔPly or CbpA$^-$.

The additive attenuation of virulence observed by mutagenizing *ply* as well as either *pspA*, *hyl*, or *cbpA* indicates that Ply and the other virulence proteins have independent functions in the pathogenesis of systemic pneumococcal disease. It follows from this that if the biological functions of these proteins can be blocked by antibody, then immunization with combinations of Ply and either Hyl, PspA, or CbpA might provide a higher degree of protection against *S. pneumoniae* than immunization with Ply alone. Ply has previously been shown to provide a significant degree of protection against multiple serotypes of *S. pneumoniae* (1). This protection is presumably due to neutralization of free toxin released from the pneumococcus by autolysis, and anti-Ply antibodies would not be expected to promote opsonophagocytic clearance. In contrast, antibodies directed against surface proteins might be expected to result in opsonization if they are not obscured by the polysaccharide capsule. In fresh *S. pneumoniae* cultures, most of the Hyl activity is cell associated (6), which is consistent with the presence of the gram-positive cell surface anchorage domain (LPXTGE) (18) near its C terminus. However, to date we have not been able to demonstrate any protection in a mouse model, using purified Hyl as the immunogen (36). The N-terminal portion of the choline-binding protein PspA has been predicted to have a coiled-coil structure reminiscent of the M proteins of group A streptococci (46), and this might be expected to protrude through the capsule. Although the N-terminal region is highly variable, PspA contains conserved epitopes which elicit antibodies protective against multiple *S. pneumoniae* serotypes (13, 45). CbpA is structurally similar to PspA; the C-terminal choline-binding domains have >90% amino acid sequence identity, and although there is no sequence similarity, the N-terminal portion of CbpA is also predicted to have a coiled-coil structure (22, 39). Like PspA, the N-terminal region of CbpA is highly variable, and it is not yet known whether this region contains common epitopes capable of eliciting protection against challenge with heterologous *S. pneumoniae* strains. Notwithstanding this uncertainty, examination of the protective efficacy of immunization with a combination of Ply and either PspA or CbpA is clearly warranted.

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