INFECTION AND IMMUNITY, Dec. 1996, p. 5255–5262
0019-9567/96/$04.00+0

Vol. 64, No. 12

Sequence Heterogeneity of PsaA, a 37-Kilodalton Putative Adhesin Essential for Virulence of *Streptococcus pneumoniae*

ANNE M. BERRY AND JAMES C. PATON*

Molecular Microbiology Unit, Women's and Children's Hospital, North Adelaide, S.A., 5006, Australia

Received 21 June 1996/Returned for modification 12 August 1996/Accepted 26 September 1996

***psaA* encodes a 37-kDa putative pneumococcal surface adhesin. Although its complete nucleotide sequence has been determined, its contribution to the pathogenicity of *Streptococcus pneumoniae* has not previously been assessed. In this study, we used a PCR-amplified internal fragment of the *psaA* gene from *S. pneumoniae* type 2 strain D39 cloned in pVA891, to direct the construction of D39 derivatives in which the *psaA* gene had been specifically interrupted, by insertion-duplication mutagenesis. Two independent D39 *psaA* mutants ($PsaA^-_1$ and $PsaA^-_2$) were significantly less virulent (as judged by intranasal or intraperitoneal challenge of mice) than either the wild-type D39 strain or a derivative of $PsaA^-_1$ in which the *psaA* gene had been reconstituted by back-transformation with an intact copy of the cloned gene. pVA891-directed mutagenesis of an open reading frame (designated ORF3) immediately 3′ to *psaA* or insertion of pVA891 between *psaA* and ORF3 had no impact on intranasal virulence. However, a small but significant difference in virulence was observed between these two derivatives and the parental D39 strain in a low-dose intraperitoneal challenge model, suggesting that the ORF3 product may also contribute to pathogenesis. Adherence of $PsaA^-_1$ to A549 cells (type II pneumocytes) was only 9% of that for D39, while the ORF3-negative strain exhibited intermediate adherence (23%). This is the first functional evidence that PsaA is an adhesin. Sequence analysis of the *psaA* gene from D39 indicated significant deviation from that previously published for the homolog from *S. pneumoniae* R36A. The deduced amino acid sequences of mature PsaA from the two strains had only 81% homology, with the bulk of the variation occurring in the amino-terminal portion.**

In spite of the availability of antimicrobial therapy, invasive disease due to *Streptococcus pneumoniae* continues to cause high morbidity and mortality throughout the world. The increasing problems caused by drug-resistant pneumococci and the suboptimal efficacy of polysaccharide vaccines have prompted a resurgence of research into the mechanisms of pathogenesis of this organism. Although the polysaccharide capsule has been recognized as a sine qua non of virulence (3), much recent attention has focused on the role of pneumococcal proteins in pathogenesis (26). The best-characterized protein virulence factors are the thiol-activated toxin pneumolysin and pneumococcal surface protein A (PspA). Immunization with either of these proteins has been shown to provide protection against challenge with virulent pneumococci (1, 27, 35). Moreover, specific inactivation of their respective genes in the pneumococcal chromosome by insertion-duplication mutagenesis also significantly reduces virulence for mice (5–7, 22).

The 37-kDa pneumococcal protein now referred to as PsaA was first detected by Russell et al. (29) with monoclonal antibodies, and Talkington et al. (34) have recently demonstrated that, like pneumolysin and PspA, it is a protective immunogen in mice. A clue as to its possible function was provided by sequence analysis of the cloned *psaA* gene, which demonstrated a significant degree of deduced amino acid sequence homology with putative fimbrial adhesins of *Streptococcus sanguis* and *Streptococcus parasanguis* (31). In the present study, we have examined the contribution of *psaA* to pneumococcal pathogenesis directly, by constructing a defined *psaA*-negative pneumococcus and comparing its virulence with that of the otherwise isogenic parental strain. We have also obtained evidence for potentially significant amino acid sequence variation between PsaAs from different strains of *S. pneumoniae.*

* Corresponding author. Mailing address: Molecular Microbiology Unit, Women's and Children's Hospital, North Adelaide, S.A., 5006, Australia. Phone: 61-8-8204 6302. Fax: 61-8-8204 6051. Electronic mail address: patonj@wch.sa.gov.au.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *S. pneumoniae* strains used were a virulent type 2 strain, D39 (4) (obtained from the National Collection of Type Cultures, London, United Kingdom; strain number NCTC7466), and its nonencapsulated, highly transformable derivative Rx1 (32). 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.

Escherichia coli K-12 DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was grown in Luria-Bertani broth (21) with or without 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). Where appropriate, chloramphenicol, ampicillin, or erythromycin was added to the growth medium at concentrations of 25, 50, and 125 μg/ml, respectively.

Plasmid pVA891, which encodes chloramphenicol and erythromycin resistance and can replicate in *E. coli*, but not in *S. pneumoniae*, has been described previously (20). pBluescript-SK was obtained from Stratagene, La Jolla, Calif., and plasmid pGEM7Zf(+) was obtained from Promega Corporation, Madison, Wis.

Bacterial transformation. Transformation of *E. coli* with plasmid DNA was carried out with $CaCl_2$-treated cells as described by Brown et al. (8). *S. pneumoniae* Rx1 and D39 were transformed, with chromosomal or plasmid DNA, essentially as described previously (38). Pneumococcal transformants were selected on blood agar containing 0.2 μg of erythromycin per ml.

DNA manipulations. *S. pneumoniae* chromosomal DNA used in Southern hybridization experiments was extracted and purified as previously described (25). Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (24). Analysis of recombinant plasmids was carried out by digestion of DNA with one or more restriction enzymes under the conditions recommended by the supplier. Restricted DNA was electrophoresed in 0.8 to 1.5% agarose gels with a Tris-borate-EDTA buffer system as described by Maniatis et al. (21).

Southern hybridization analysis. Chromosomal DNA (approximately 2.5 μg) was digested with appropriate restriction enzymes, and the digests were electrophoresed on agarose gels in Tris-borate-EDTA buffer. DNA was then transferred to a positively charged nylon membrane (Hybond N^+; Amersham, Little Chalfont, Buckinghamshire, England) as described by Southern (33), hybridized to digoxigenin (DIG)-labelled probe DNA, washed, and then developed with anti-DIG-alkaline phosphatase conjugate (Boehringer, Mannheim, Germany)

and 4-nitroblue tetrazolium–X-phosphate substrate according to the manufacturer's instructions. DIG-labelled lambda DNA, restricted with *Hin*dIII, was used as a DNA molecular size marker.

DNA sequencing and analysis. Nested deletions of pneumococcal DNA cloned into pBluescriptSK were constructed by the method of Henikoff (16) with an Erase-a-Base kit (Promega). This DNA was transformed into *E. coli* DH5α, and the resulting plasmid DNA was characterized by restriction analysis. Double-stranded template DNA for sequencing was prepared as recommended in the Applied Biosystems sequencing manual. The sequence of both strands was then determined with dye-labelled primers on an Applied Biosystems model 373A automated DNA sequencer. The sequence was analyzed with DNASIS and PROSIS version 7.0 software (Hitachi Software Engineering, San Bruno, Calif.). Amino acid sequence alignments were performed with the program CLUSTAL (17).

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 quellung reaction, using antisera obtained from Statens Seruminstitut, Copenhagen, Denmark. Cultures were then diluted to a density of 5×10^7 CFU/ml, and 0.1-ml volumes were injected intraperitoneally into groups of 12 to 13 BALB/c mice. Survival time was recorded.

Intranasal challenge studies were performed on QS mice which had been anesthetized by intraperitoneal injection with 0.06 mg of sodium pentobarbitone (Nembutal; Boehringer Ingelheim, Sydney, Australia) per g of body weight. Aliquots (50 μl) 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 χ^2 test (two-tailed).

Adherence of *S. pneumoniae* to A549 cells. A549, a human lung alveolar carcinoma (type II pneumocyte) cell line, was obtained from the Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and maintained in Dulbecco's modified Eagle's medium with 1% fetal calf serum. *S. pneumoniae* strains from fresh overnight blood agar cultures were inoculated into THY broth and grown at 37°C until they reached mid-logarithmic phase (A_{600} of 0.1 to 0.15; approximately 10^8 CFU/ml). Cultures were diluted to 5×10^6 CFU/ml in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (without antibiotics), and 1-ml aliquots were inoculated onto washed A549 monolayers in 24-well tissue culture trays. After incubation for 2 h at 37°C, the culture fluid was removed from each well and monolayers were washed five times with phosphate-buffered saline, pH 7.5 (PBS). The A549 monolayers were then detached from the plate by treatment with 100 μl of 0.25% trypsin–0.02% EDTA. Epithelial cells were then lysed by addition of 400 μl of 0.025% Triton X-100, and 100-μl aliquots (and serial 10-fold dilutions thereof) were plated on blood agar to determine the total number of adherent bacteria. Assays were performed in quadruplicate, and results presented are the mean ± standard error. The significance of differences between means was analyzed by the unpaired Student's *t* test (two-tailed).

Nucleotide sequence accession number. The nucleotide sequence of *psaA* from *S. pneumoniae* D39 has been deposited with GenBank under accession number U40786.

RESULTS

Construction of *psaA*-negative *S. pneumoniae*. The first step in construction of a *psaA*-negative pneumococcus involved cloning an internal portion of the *psaA* gene into pVA891. This vector is a deletion derivative of the *Escherichia-Streptococcus* shuttle plasmid pVA838, which has lost the capacity to replicate autonomously in streptococci but retains a streptococcal gene encoding erythromycin resistance. We have previously used pVA891 to direct the insertion-duplication mutagenesis of pneumococcal genes encoding pneumolysin, autolysin, and polysaccharide capsule biosynthesis (6, 7, 15). Published sequence data for *psaA* from *S. pneumoniae* R36A (31) were used to design PCR primers 5′-GACCCTCACGCTTGGCTC AATCTC-3′ and 5′-ATAGGCAGATGGGACGCCGTAGG C-3′) to amplify a 254-bp fragment, corresponding to nucleotides 411 to 664 of the *psaA* coding sequence, from *S. pneumoniae* D39. This fragment was blunt-ligated into the *Sma*I site of pGEM7Zf(+) and cloned into *E. coli* DH5α. The *psaA* fragment was then excised from this recombinant plasmid with *Xba*I and *Cla*I and cloned into similarly digested pVA891.

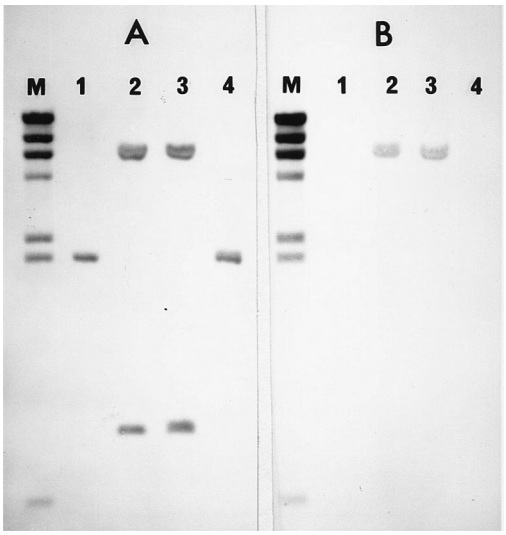

FIG. 1. Southern hybridization analysis. Chromosomal DNA from the indicated D39 derivatives was digested with *Cla*I, electrophoresed, and subjected to Southern hybridization analysis using DIG-labelled probes specific for *psaA* (A) or pVA891 (B). Lanes: M, DNA size markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb); 1, D39; 2, PsaA$^-{}_1$; 3, PsaA$^-{}_2$; 4, PsaA$^+$BT.

In a previous study (7), we found that the efficiency of direct transformation of the encapsulated type 2 strain D39 to erythromycin resistance, with recombinant pVA891 derivatives, was very low, even in the presence of competence factor derived from the highly transformable D39 derivative Rx1. Therefore, we adopted a two-step approach for the construction of D39*psaA*$^-$. Firstly, we transformed the nonencapsulated strain Rx1 with the pVA891 construct carrying the internal *psaA* fragment and isolated erythromycin-resistant transformants. Chromosomal DNA from one of the transformants was subjected to Southern hybridization analysis to confirm interruption of the *psaA* coding region with the pVA891 sequences (result not shown). DNA from this derivative was then used to transform the encapsulated parental strain D39, and erythromycin-resistant transformants were isolated from two independent transformation experiments. Chromosomal DNA from each of these was digested with *Cla*I and subjected to Southern hybridization analysis using *psaA*- and pVA891-specific probes (Fig. 1). The *psaA* probe reacted with a single 2.0-kb *Cla*I fragment in the wild-type D39 DNA, but in the two erythromycin-resistant transformants (designated PsaA$^-{}_1$ and PsaA$^-{}_2$), *psaA*-reactive sequences were located on 0.8- and 7.2-kb *Cla*I fragments. The larger fragment in each of these digests also reacted with the pVA891 probe. This hybridization pattern is consistent with insertion of the recombinant pVA891 derivative (which has a size of approximately 6.0 kb and has a single internal *Cla*I site) into the chromosomal *psaA* locus. Mutagenesis of the *psaA* gene did not affect in vitro growth of pneumococci; PsaA$^-{}_1$ and PsaA$^-{}_2$ grew at the same rate as D39 in THY medium over a 4-h period at 37°C, as judged by A_{600} and viable count (result not shown).

Isolation of *psaA* and generation of a *psaA*-positive back-transformant of PsaA$^-{}_1$. A *psaA*-positive back-transformant of PsaA$^-{}_1$ was required for use as a control in comparative virulence studies. Construction of such strains involves transformation of the erythromycin-resistant PsaA$^-{}_1$ with a linear fragment of DNA containing a complete copy of the *psaA* gene and selection for loss of erythromycin resistance. In order to isolate such a fragment, we subjected D39 DNA to PCR am-

plification using primers designed on the basis of the published sequence for *psaA* from strain R36A (31). The primers were 5′-AAGGAGAAACATATGAAAAAAATCGCTTCT-3′ and 5′-GAGTTTGAAGCTTTATTTGTTCAAACCTTC-3′, which would be expected to amplify a 957-bp fragment with *Nde*I and *Hin*dIII sites at each extremity (see underlined sections) to facilitate subsequent subcloning into expression vectors if required. However, no PCR product was obtained with these primer sequences with D39 or Rx1 DNA as template. This suggested the possibility of nucleotide sequence variations between the corresponding region of these strains and R36A. We therefore employed an alternative strategy for isolation of *psaA* based on rescue into *E. coli* of the pVA891 replicon along with the flanking 5′ and 3′ DNA sequences from $PsaA^-_1$. Chromosomal DNA from this strain was digested with *Cla*I (for rescue of 5′ *psaA* sequences) or *Kpn*I (for 3′ flanking sequences), recircularized, and transformed into *E. coli* DH5α with chloramphenicol selection. Plasmid DNA was extracted from representative transformants and digested with *Eco*RI (which cuts once within the duplicated central portion of *psaA*) and *Cla*I (which cuts 5′ and 3′ to the *psaA* coding region). An intact copy of *psaA* was then obtained by ligation of the separate 5′ and 3′ portions, and this was cloned into *Cla*I-digested pBluescript SK. This enabled isolation of large amounts of the intact *psaA* gene as a 2.0-kb *Cla*I fragment, which was used to back-transform $PsaA^-_1$. Back-transformants were isolated after enrichment in the presence of both erythromycin and ampicillin, as previously described (7). Chromosomal DNA from one of the erythromycin-sensitive back-transformants (designated $PsaA^+$BT) was subjected to Southern hybridization analysis (Fig. 1) which confirmed loss of the pVA891 sequences, as well as reconstitution of the *psaA* locus.

Virulence studies. In order to assess the contribution of *psaA* to pneumococcal virulence, groups of 14 to 15 QS mice were challenged by the intranasal route with approximately 5×10^6 CFU of either wild-type D39, $PsaA^-_1$, $PsaA^-_2$, or $PsaA^+$BT, as described in Materials and Methods. For comparison, a further group of mice was challenged with a derivative of D39 (designated Ply^-) in which the gene encoding pneumolysin (a proven pneumococcal virulence factor) had been inactivated by pVA891 insertion (6) (Fig. 2). Mice challenged with D39 had a median survival time of 2.75 days. Those challenged with Ply^- survived significantly longer (median survival time, 3.75 days; $P < 0.002$). However, all mice challenged with $PsaA^-_1$ or $PsaA^-_2$ survived; the difference in overall survival rate between mice challenged with these strains and those receiving either D39 or Ply^- was highly significant ($P < 0.001$). In contrast, mice challenged with $PsaA^+$BT had a median survival time indistinguishable from that with D39.

For intraperitoneal challenge studies, groups of 12 to 13 BALB/c mice were challenged intraperitoneally with approximately 5×10^6 CFU of the various strains, and survival time was recorded (Fig. 3). As was observed for the intranasal challenge model, the virulence of D39 and $PsaA^+$BT was indistinguishable (median survival time was 1.75 days for both groups). Although there was no significant difference in overall survival rate in this model system between the groups of mice challenged with $PsaA^-_1$ or $PsaA^-_2$ and those which received D39, the median survival times (2.8 and 3.0 days for $PsaA^-_1$ and $PsaA^-_2$, respectively) were significantly greater ($P < 0.02$). Ply^- appeared to be the least virulent strain in the intraperitoneal challenge model; the overall survival rate was significantly greater than that for all other strains ($P < 0.001$). However, pneumococci isolated from the heart blood of the mice

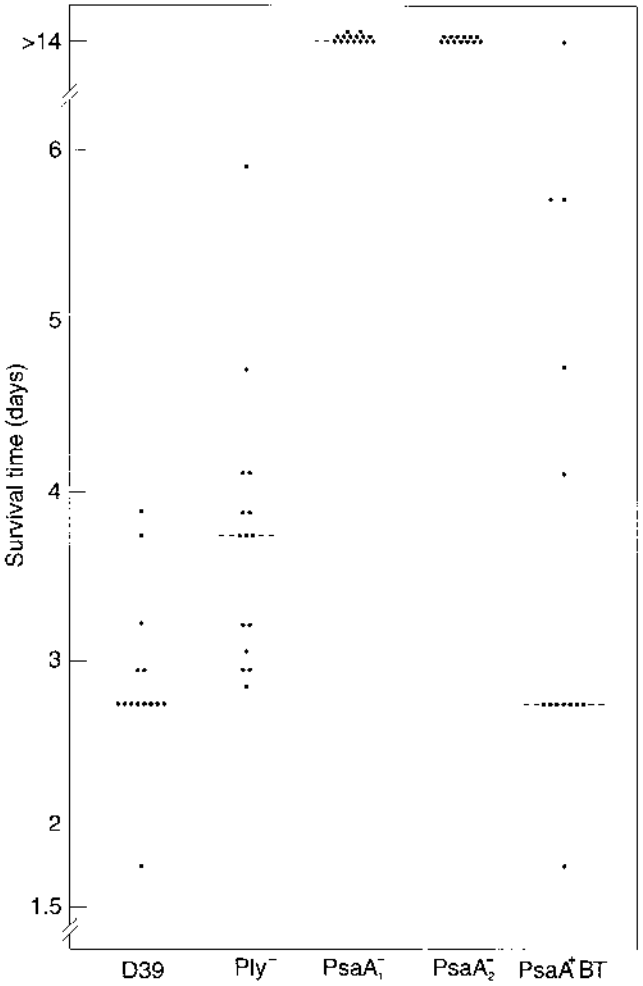

FIG. 2. Intranasal challenge. Groups of mice were challenged intranasally with approximately 5×10^6 CFU of the indicated strains. The survival time of each mouse is indicated. The broken lines denote the median survival time for each group.

which died as a result of challenge with $PsaA^-_1$ or $PsaA^-_2$ were all erythromycin sensitive. This suggested that the pVA891 sequences might have been eliminated in vivo. Chromosomal DNA extracted from these isolates was then subjected to Southern hybridization analysis, which showed that pVA891-reactive sequences were no longer present. Moreover, the *psaA*-specific probe reacted with a single *Cla*I fragment of 2.0 kb, the same size as seen in digests of D39 DNA (results not presented). Further evidence for reconstitution of the *psaA* gene was obtained by PCR amplification using primers (based on our own sequence data) specific for the 5′ and 3′ termini of the *psaA* open reading frame (ORF). This resulted in amplification of a PCR product of identical size (930 bp), when DNA from either D39 or any of the erythromycin-sensitive revertants was used as template (result not presented). These data suggest that, at some stage after injection into the mice (when erythromycin selection was no longer being applied), genetic recombination occurred, resulting in the elimination of the pVA891 sequences and reconstitution of an intact (uninterrupted) *psaA* operon. These recombinants were sufficiently virulent to overcome the mice. Attempts to prevent reversion in vivo by oral administration of erythromycin stearate to the mice were not successful.

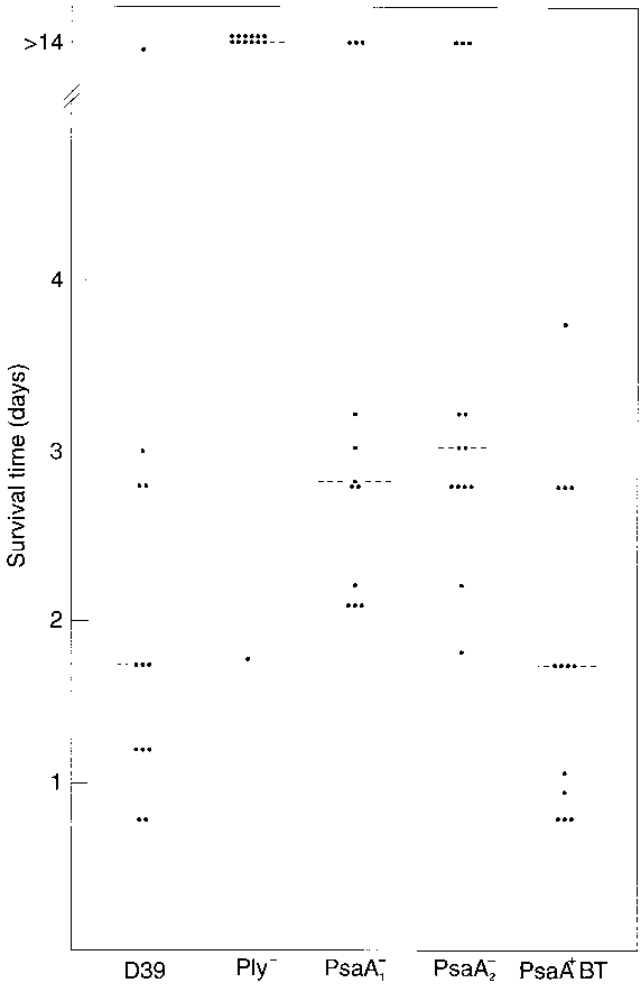

FIG. 3. Intraperitoneal challenge. Groups of mice were injected intraperitoneally with approximately 5×10^6 CFU of the indicated strains. The survival time of each mouse is indicated. The broken lines denote the median survival time for each group.

Nucleotide sequence analysis of *psaA* from *S. pneumoniae* D39. The failure of primers based on the published sequence of *psaA* from *S. pneumoniae* R36A to direct PCR amplification of the complete *psaA* gene from D39 suggested the possibility of nucleotide sequence variation between strains, at least at the N- or C-terminal coding regions. For this reason, we determined the complete nucleotide sequence for *psaA* from strain D39, which we had isolated by plasmid rescue from PsaA$^-_1$, as described above. The 1,900-bp sequence shown in Fig. 4 commences 233 nucleotides upstream of *psaA* and includes the 3′ portion of an open reading frame (designated ORF1) described by Sampson et al. (31). The sequence includes an additional 0.4 kb 3′ to that reported previously (31), which demonstrates the presence of a third ORF (designated ORF3) sufficient to encode an 18-kDa protein. There are no obvious stemmed loop structures between *psaA* and ORF3, suggesting the possibility that the two genes are cotranscribed. However, a very stable stemmed loop structure (Gibbs free energy, −26.2 kcal/mol [ca. −110 kJ/mol]) is located immediately 3′ to ORF3.

Additional mutagenesis and virulence studies. The presence of a potentially cotranscribed ORF downstream from *psaA* raised the possibility that the effects of insertion-duplication mutagenesis of *psaA* on virulence might be attributable to polar effects on ORF3. To examine this, two additional D39 derivatives were constructed. The 3′ portion of the *psaA* gene and an internal fragment of ORF3 (nucleotides 866 to 1218 and 1465 to 1586, respectively, in the sequence shown in Fig. 4) were each amplified by PCR and subcloned into pVA891. Transformation of *S. pneumoniae* with the former construct resulted in insertion of the pVA891 sequences immediately downstream from an intact (reconstituted) copy of *psaA*, followed by the duplicated 3′ portion of *psaA* and ORF3 (this D39 derivative was designated PsaA$^+$). The other construct resulted in generation of a D39 derivative with an insertion-duplication mutation in ORF3 (designated ORF3$^-$) (Fig. 5). The structures of the *psaA* operons in these erythromycin-resistant transformants were confirmed by Southern hybridization analysis of chromosomal DNA using *psaA*-, ORF3-, and pVA891-specific probes (results not shown).

Groups of QS mice were then challenged intranasally with 5×10^6 CFU of D39, PsaA$^-_1$, PsaA$^+$, or ORF3$^-$ (Fig. 6). There was no significant difference in survival time among the D39, PsaA$^+$, and ORF3$^-$ groups (respective median survival times were 3.1, 2.8, and 2.8 days). In contrast, all mice challenged with PsaA$^-_1$ survived. Groups of QS mice were also challenged intraperitoneally with 3×10^3 CFU of D39, PsaA$^-_1$, PsaA$^+$, or ORF3$^-$ (Fig. 7). This dose is much lower than that used in the previous intraperitoneal challenge experiment using BALB/c mice, and all mice challenged with PsaA$^-_1$ survived (i.e., there were no erythromycin-sensitive revertants). Both the median survival time and the overall survival rate of mice challenged with PsaA$^-_1$ were significantly greater than those for mice challenged with D39, PsaA$^+$, or ORF3$^-$ (P values ranged from <0.001 to <0.025). Interestingly, however, the median survival times for mice challenged with PsaA$^+$ or ORF3$^-$ were slightly but significantly elevated with respect to D39 (1.44 and 1.52 versus 1.06 days, respectively) ($P < 0.01$ and < 0.001, respectively). Also, the overall

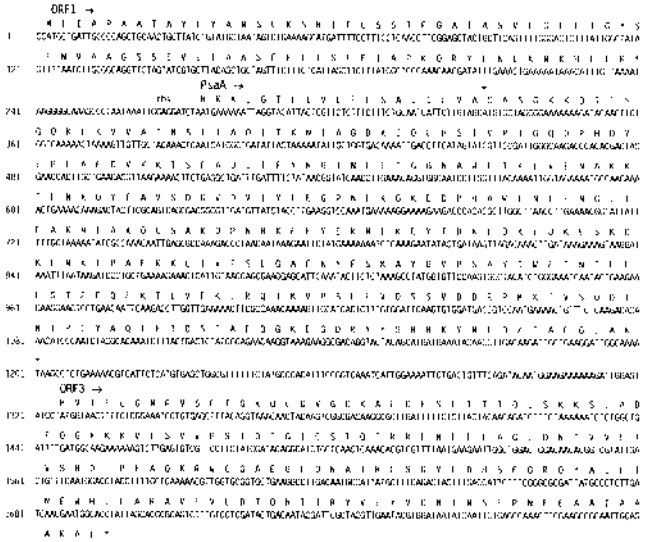

FIG. 4. Nucleotide sequence of *psaA* from *S. pneumoniae* D39. The sequence is numbered in accordance with that deposited with GenBank and shows the 3′ portion of ORF1, as well as the complete coding sequences for PsaA and ORF3. The deduced amino acid sequences are shown in single-letter code immediately above the first nucleotide of each codon. Ribosome binding sites (denoted by rbs) are double underlined, and the region encoding a putative transcription terminator (a stemmed loop structure with a Gibbs free energy of −26.2 kcal/mol) downstream of ORF3 is underlined. The putative signal peptidase cleavage site for PsaA is indicated with a vertical arrow.

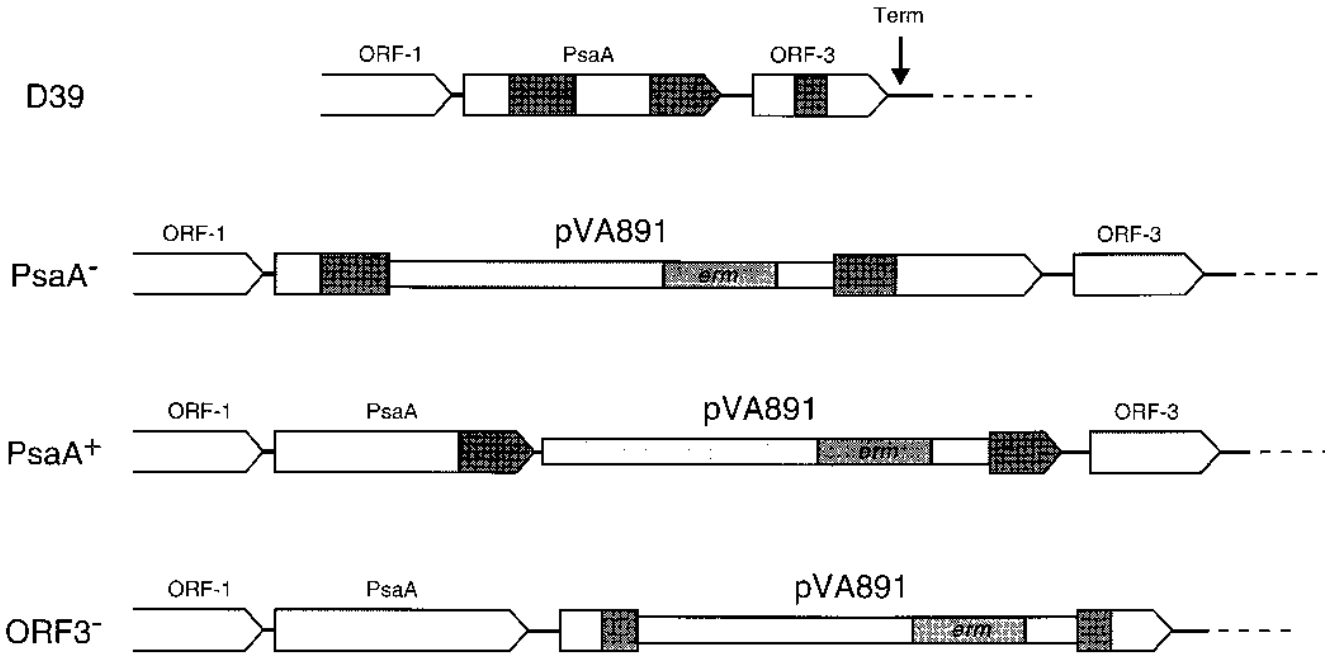

FIG. 5. Mutagenesis of the *psaA* operon of *S. pneumoniae* D39. The location of the strong transcription terminator downstream of ORF3 is shown on the map for D39, which also shows the regions subcloned into pVA891 for generation of mutants (shaded). For each of the D39 derivatives PsaA$^-$, PsaA$^+$, and ORF3$^-$, the duplicated region of DNA is shaded.

survival rate of mice challenged with ORF3$^-$ (6 of 12) was significantly different from both D39 (0 of 12) and PsaA$^-_1$ (12 of 12) ($P < 0.02$). Thus, mutagenesis of ORF3 impacts to some extent on the virulence of *S. pneumoniae* D39, but the effect is detectable only when mice are challenged with low doses by the intraperitoneal route and is not seen at all in the intranasal challenge model.

Effect of mutagenesis of *psaA* on adherence of pneumococci. Two different assays were used to examine whether the effects of mutagenesis of *psaA* on virulence could be correlated with reduced in vitro adherence of pneumococci. The first employed agglutination of neuraminidase-treated bovine erythrocytes as a model for adherence to receptors with exposed GlcNAcβ1-3Gal, the glycoconjugate implicated in attachment of pneumococci to oropharyngeal cells (2). Assays were conducted as described by Cundell et al. (11), but no difference was observed between the degrees of hemagglutination when either D39 or PsaA$^-_1$ was tested at a concentration of 5×10^8 CFU/ml (result not presented).

The capacity of pneumococci to adhere directly to A549 cells (a type II pneumocyte cell line) was also examined, as described in Materials and Methods. At a dose of 5×10^6 CFU,

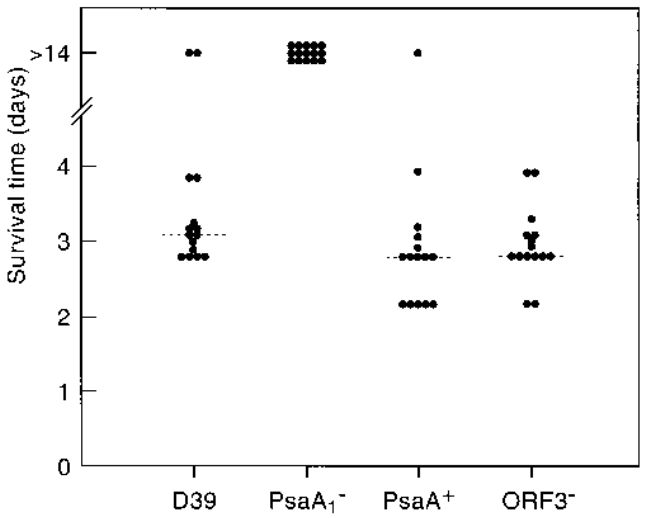

FIG. 6. Intranasal challenge. Groups of QS mice were challenged intranasally with approximately 5×10^6 CFU of the indicated strains. The survival time of each mouse is indicated. The broken lines denote the median survival time for each group.

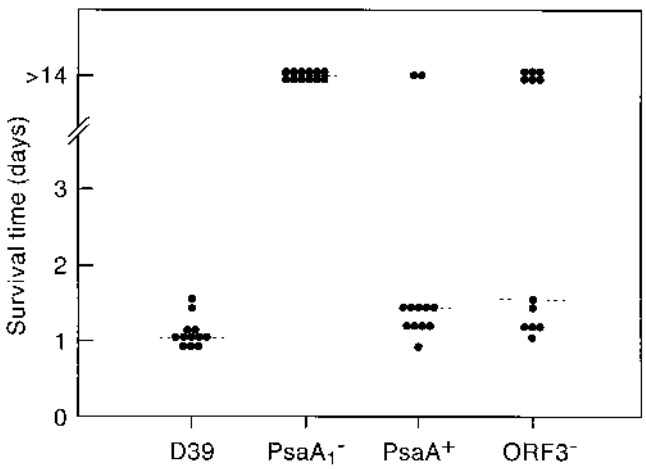

FIG. 7. Intraperitoneal challenge. Groups of QS mice were injected intraperitoneally with approximately 3×10^3 CFU of the indicated strains. The survival time of each mouse is indicated. The broken lines denote the median survival time for each group.

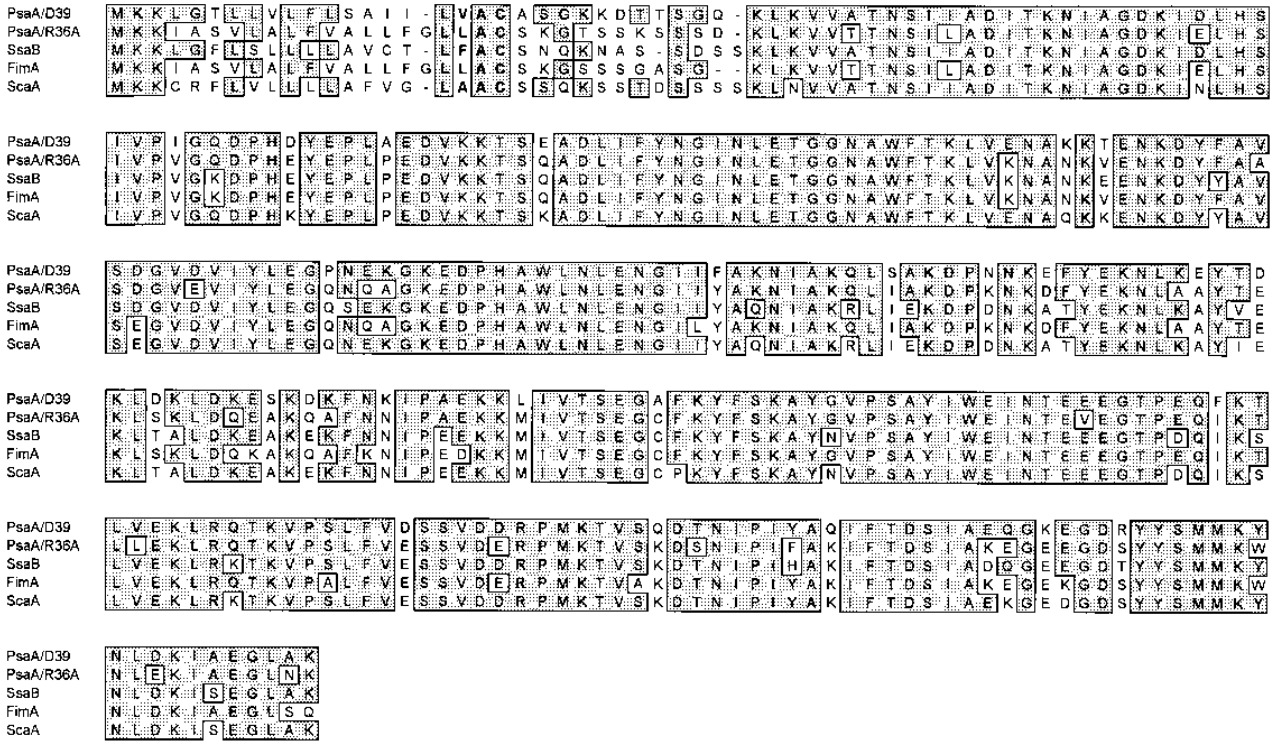

FIG. 8. Amino acid sequence alignment of PsaA/D39 and related proteins. The deduced amino acid sequence for PsaA/D39 was aligned with those published for PsaA from strain R36A (PsaA/R36A) (31) and the closely related putative fimbrial adhesins SsaB from *S. sanguis* (14), FimA from *S. parasanguis* (12), and ScaA from *S. gordonii* (18), by using the program CLUSTAL (17). Residues with identity to PsaA/D39 are boxed and shaded. The position of the consensus prolipoprotein cleavage site (L-X-X-C) (residues 17 to 20 for PsaA/D39) in each of the proteins is shown in boldface.

total adherence of *S. pneumoniae* D39 was 1,090 ± 331 CFU per well, compared with 98 ± 18 CFU per well for $PsaA^-_1$ ($P < 0.01$). Interestingly, the D39 derivative in which ORF3 had been interrupted exhibited intermediate adherence (255 ± 40 CFU per well), which was significantly different from that for both D39 ($P < 0.01$) and $PsaA^-_1$ ($P < 0.01$).

Amino acid sequence comparison. The deduced amino acid sequence for the product of the *psaA* gene from *S. pneumoniae* D39 (designated PsaA/D39) is shown in Fig. 8, aligned with those published for PsaA from strain R36A (PsaA/R36A) (31) and the closely related putative fimbrial adhesins SsaB from *S. sanguis* (14), FimA from *S. parasanguis* (12), and ScaA from *Streptococcus gordonii* (18). There was a similar degree of identity (77.4 to 79.9%) between PsaA/D39 and each of these proteins. Although substantial variation in amino acid sequence occurred in the signal peptide, the 12 residues at the N termini of the mature polypeptides also showed little similarity with each other. Indeed, PsaA/D39 and PsaA/R36A have identical amino acids at only 3 of the 12 positions. The overall identity between mature PsaA/D39 and mature PsaA/R36A is 81%. The deduced amino acid sequences of the C-terminal portion (79 residues) of ORF1 from D39 also differed from that reported for *S. pneumoniae* R36A (31), exhibiting only 72% identity. Similarly, the deduced amino acid sequence of ORF3 from D39 exhibited only 80% identity to the 50 N-terminal residues for ORF3 from R36A (31). The C-terminal portion of D39 ORF1 and the complete D39 ORF3 exhibited 71 and 76% identity with the respective homologs from the *fimA* operon of *S. parasanguis* (9).

DISCUSSION

The possible involvement of PsaA in the pathogenesis of pneumococcal disease has been suggested by the fact that the purified protein is a protective immunogen in mice (34). However, a role for PsaA in adhesion to mucosal or other cells has been hypothesized on the basis of a high degree of sequence homology between PsaA and fimbrial adhesins produced by other streptococci (31). In the present study, we have provided direct evidence for the involvement of the *S. pneumoniae psaA* locus in pathogenesis. We have shown that insertion-duplication mutagenesis of the *psaA* gene of the type 2 strain D39 significantly reduces its virulence for mice in both an intranasal and an intraperitoneal challenge model. The observed effects were not due to poorer growth of the mutated strains, and McDaniel et al. (22) have previously demonstrated that random insertion of pVA891 into the chromosome has no effect on the virulence of *S. pneumoniae* D39. Neither were the observed effects due to cotransformation of other pneumococcal DNA sequences during construction of the mutants, as $PsaA^-_1$ and $PsaA^-_2$ were derived from independent transformation reactions but had identically reduced virulence. Moreover, reconstitution of a wild-type *psaA* locus in $PsaA^-_1$ by back-transformation with a cloned copy of the *psaA* gene restored virulence to that of the parental D39 strain. In the intranasal challenge model, $PsaA^-_1$ and $PsaA^-_2$ were avirulent at the dose tested (5×10^6 CFU). Interestingly, mice receiving a similar dose of a pneumolysin-negative D39 derivative all succumbed to challenge, albeit with a significantly greater median survival time than mice receiving D39. However, comparative virulence data for the intraperitoneal chal-

lenge studies are more difficult to interpret, because of apparent instability of the *psaA* mutation in vivo, where it was not possible to maintain erythromycin selection. We have not observed such instability in any of the other pVA891-directed *S. pneumoniae* mutants that we have constructed (5–7, 15), a fact which serves to highlight the very potent in vivo selective advantage conferred by an intact *psaA* locus.

Sampson et al. (31) presented Northern (RNA) blot data which suggested that *psaA* is transcribed as part of a polycistronic mRNA of approximately 3 kb, and sequence analysis of flanking DNA indicated the presence of additional ORFs upstream and downstream of *psaA*. The additional 3′ sequence data presented in the present study indicate that, in *S. pneumoniae* D39, the downstream ORF3 is sufficient to encode a protein of 18 kDa. There are no apparent transcription termination sites in the pneumococcal chromosome between *psaA* and ORF3, but a very stable stemmed loop structure was found immediately 3′ to ORF3. A similar polycistronic structure has also been reported for the other closely related streptococcal adhesin operons (12, 14, 18). The two ORFs upstream of *fimA*, the *psaA* homolog of *S. parasanguis*, encode an ATP-binding protein and a hydrophobic membrane protein with homology to members of a superfamily of ATP-binding membrane transport systems (13). However, the function of the downstream ORF3 (which has 76% amino acid identity with D39 ORF3) is unknown. The fact that the *psaA* operon may encode a transport system is interesting given the recent report that mutagenesis of either of two peptide permease loci (*plpA* and *ami*) significantly reduced adherence of pneumococci to both A549 cells and endothelial cells (11). However, these peptide permeases are unrelated to PsaA.

Fenno et al. (13) found that in *S. parasanguis* transcription of ORF3 was not necessarily linked to *fimA*, but it was possible that in *S. pneumoniae*, insertion of pVA891 within the *psaA* ORF could have polar effects on ORF3. We have shown that insertion of pVA891 between the *psaA* ORF and ORF3 in D39 (generating strain PsaA$^+$) or direct insertion-duplication mutagenesis of ORF3 (ORF3$^-$) did not result in a detectable impact on intranasal virulence. However, a statistically significant (albeit modest) reduction in virulence of PsaA$^+$ and ORF3$^-$ with respect to D39 was observed in a low-dose intraperitoneal challenge model. Nevertheless, PsaA$^+$ and ORF3$^-$ were significantly more virulent than PsaA$^-_1$ (which was completely avirulent in this model), confirming that mutation of *psaA* itself is principally responsible for the massive reduction in virulence. Thus, we conclude that a functional *psaA* gene is essential for full virulence of *S. pneumoniae.*

At present, the precise in vivo function of PsaA is uncertain. It has been proposed as a pneumococcal adhesin on the basis of homology to FimA (31). Fenno et al. (13) have shown that FimA is not the fimbrial subunit protein of *S. parasanguis*, but it is located at the tips of these structures. FimA is directly involved in adherence of these bacteria to the salivary pellicle of dental surfaces, and recently *fimA*-negative mutants have been shown to be less virulent than wild-type *S. parasanguis* in a rat endocarditis model (9). In this system, FimA appears to mediate adherence of bacteria to fibrin associated with vegetations. The putative role of PsaA as a pneumococcal adhesin is supported by our finding that PsaA$^-_1$ had significantly less in vitro adherence to A549 cells than either D39 or ORF3$^-$. However, adherence of ORF3$^-$ was intermediate between D39 and PsaA$^-_1$, which is consistent with its intermediate virulence in the low-dose intraperitoneal challenge model.

The possibility of nucleotide sequence variation at one or both of the termini of the *psaA* gene was suggested by our failure to amplify the complete *psaA* gene from *S. pneumoniae* D39 with primers designed on the basis of the published sequence for the homolog from strain R36A. Moreover, restriction analysis of the 254-bp internal *psaA* fragment from D39 used to construct the pVA891-directed mutants indicated the presence of an *Eco*RI site not predicted from the R36A sequence. Sequence analysis of the *psaA* gene cloned by rescue of the pVA891 replicon along with flanking DNA from the chromosome of PsaA$^-_1$ confirmed this. The deduced amino acid sequence of PsaA/D39 has only 78.4% identity with PsaA/R36A (81% identity in the processed polypeptide). In fact, PsaA/D39 has a higher degree of homology with ScaA produced by *S. gordonii* (79.7%), and SsaB produced by *S. sanguis* (79.9%), than it has with PsaA/R36A. Conversely, PsaA/R36A is more closely related to FimA of *S. parasanguis* (92.3% identity) than it is to PsaA/D39.

The findings of this study may have implications for the development of vaccines based on pneumococcal protein antigens. The dramatic impact of mutagenesis of *psaA* on virulence implies that PsaA plays a very important role in the pathogenesis of pneumococcal disease. This is consistent with the recent report that it is a protective immunogen in mice (34). Nevertheless, the N terminus of mature PsaA may be subject to a significant degree of variability. Strain-to-strain variability within the putative signal peptide of PsaA may be of little consequence, but we found that the 12 residues at the N terminus of processed PsaA/D39 were identical to PsaA/R36A at only three positions. In contrast, there was 84.5% amino acid identity for the remainder of the proteins. Interestingly, Sampson et al. (30) have recently presented data based on restriction analysis of PCR products, suggesting that *psaA* is highly conserved among pneumococci belonging to different capsular serotypes. It is not known, however, whether sequences at the 5′ end of the gene were specifically examined. Although immunofluorescent staining of intact pneumococci suggests that at least some epitopes are exposed on the cell surface (29), at present there is insufficient information available to make predictions of the structure and localization of specific domains within PsaA. PsaA and the related streptococcal proteins all contain the prolipoprotein recognition sequence L-X-X-C at the carboxyl end of their signal peptides, which might suggest that the N terminus is closely associated with the cell membrane, anchored via an N-acyl glyceride cysteine. This prediction, however, is not supported by the immunoelectron micrographs of Fenno et al. (13) showing FimA localized at the tips of *S. parasanguis* fimbriae.

The possibility of antigenic variation between strains has important consequences for candidate vaccine antigens. This has been examined in detail for two other proteins under consideration for inclusion in subsequent generations of pneumococcal vaccines. The primary amino acid sequence of pneumolysin appears to be extremely stable (23), and immunization with a derivative of the protein genetically made into a toxoid has been shown to protect mice against at least nine different *S. pneumoniae* serotypes (1). Moreover, pneumolysin has been shown to be a very effective carrier for otherwise poorly immunogenic polysaccharide antigens in conjugate vaccine formulations (19, 28). PspA is also a proven protective antigen (35), but it is subject to antigenic variation (10), principally as a consequence of sequence variation in the N-terminal portion of the protein (35, 37). Notwithstanding this, PspA appears to contain cross-protective epitopes, as Tart et al. (36) have recently reported that a truncated PspA derivative elicited protection against at least seven challenge strains. Our finding that an intact *psaA* locus is essential for full pneumococcal virulence indicates that PsaA warrants further consideration as a candidate pneumococcal vaccine antigen. Sequence variability

appears to be largely confined to the N terminus, but the question of cross-serotype protective efficacy needs to be resolved.

ACKNOWLEDGMENTS

We are grateful to Ursula Talbot for assistance with the adherence assays.

This work was supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

1. **Alexander, J. E., R. A. Lock, C. C. A. M. Peeters, J. T. Poolman, P. W. Andrew, T. J. Mitchell, D. Hansman, and J. C. Paton.** 1994. Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. Infect. Immun. **62:**5683–5688.
2. **Andersson, B., T. Dahmen, T. Freijd, H. Leffler, G. Magnusson, G. Noori, and C. Svanborg-Eden.** 1983. Identification of an active disaccharide unit of a glycoconjugate receptor of pneumococci attaching to human pharyngeal epithelial cells. J. Exp. Med. **158:**559–570.
3. **Austrian, R.** 1981. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. Rev. Infect. Dis. **3**(Suppl.)**:**S1–S17.
4. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. **79:**137–158.
5. **Berry, A. M., J. E. Alexander, T. J. Mitchell, P. W. Andrew, D. Hansman, and J. C. Paton.** 1995. Effect of defined point mutations in the pneumolysin gene on the virulence of *Streptococcus pneumoniae*. Infect. Immun. **63:**1969–1974.
6. **Berry, A. M., J. C. Paton, and D. Hansman.** 1992. Effect of insertional inactivation of the genes encoding pneumolysin and autolysin on the virulence of *Streptococcus pneumoniae* type 3. Microb. Pathog. **12:**87–93.
7. **Berry, A. M., J. Yother, D. E. Briles, D. Hansman, and J. C. Paton.** 1989. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. Infect. Immun. **57:**2037–2042.
8. **Brown, M. C. M., A. Weston, J. R. Saunders, and G. O. Humphreys.** 1979. Transformation of *E. coli* C600 by plasmid DNA at different phases of growth. FEMS Microbiol. Lett. **5:**219–222.
9. **Burnette-Curley, D., V. Wells, H. Viscount, C. L. Munro, J. C. Fenno, P. Fives-Taylor, and F. L. Macrina.** 1995. FimA, a major virulence factor associated with *Streptococcus parasanguis* endocarditis. Infect. Immun. **63:**4669–4674.
10. **Crain, M. J., W. D. Waltman II, J. S. Turner, J. Yother, D. F. Talkington, L. S. McDaniel, B. M. Gray, and D. E. Briles.** 1990. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. Infect. Immun. **58:**3293–3299.
11. **Cundell, D. R., B. J. Pearce, J. Sandros, A. M. Naughton, and H. R. Masure.** 1995. Peptide permeases from *Streptococcus pneumoniae* affect adherence to eucaryotic cells. Infect. Immun. **63:**2493–2498.
12. **Fenno, J. C., D. J. LeBlanc, and P. Fives-Taylor.** 1989. Nucleotide sequence analysis of a type 1 fimbrial gene of *Streptococcus sanguis* FW213. Infect. Immun. **57:**3527–3533.
13. **Fenno, J. C., A. Shaikh, G. Spatafora, and P. Fives-Taylor.** 1995. The *fimA* locus of *Streptococcus parasanguis* encodes an ATP-binding membrane transport system. Mol. Microbiol. **15:**849–863.
14. **Ganeshkumar, N., P. M. Hannam, P. E. Kolenbrander, and B. C. McBride.** 1991. Nucleotide sequence of a gene coding for a saliva binding protein (SsaB) from *Streptococcus sanguis* 12 and possible role of the protein in coaggregation with actinomyces. Infect. Immun. **59:**1093–1099.
15. **Guidolin, A., J. K. Morona, R. Morona, D. Hansman, and J. C. Paton.** 1994. Nucleotide sequence of an operon essential for capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* type 19F. Infect. Immun. **62:**5384–5396.
16. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
17. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. Gene **73:**237–244.
18. **Kolenbrander, P. E., R. N. Andersen, and N. Ganeshkumar.** 1994. Nucleotide sequence of the *Streptococcus gordonii* PK488 coaggregation adhesin gene, *scaA*, and ATP-binding cassette. Infect. Immun. **62:**4469–4480.
19. **Lee, C.-J., R. A. Lock, T. J. Mitchell, P. W. Andrew, G. J. Boulnois, and J. C. Paton.** 1994. Protection of infant mice from challenge with *Streptococcus pneumoniae* type 19F by immunization with a type 19F polysaccharide-pneumolysoid conjugate. Vaccine **12:**875–878.
20. **Macrina, F. L., R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell, and K. R. Jones.** 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. Gene **25:**145–150.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **McDaniel, L. S., J. Yother, M. Vijayakamur, L. McGarry, W. R. Guild, and D. E. Briles.** 1987. Use of insertional inactivation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). J. Exp. Med. **165:**381–394.
23. **Mitchell, T. J., F. Mendez, J. C. Paton, P. W. Andrew, and G. J. Boulnois.** 1990. Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2. Nucleic Acids Res. **18:**4010.
24. **Morelle, G.** 1989. A plasmid extraction procedure on a miniprep scale. Focus **11.1:**7–8.
25. **Paton, J. C., A. M. Berry, R. A. Lock, D. Hansman, and P. A. Manning.** 1986. Cloning and expression in *Escherichia coli* of the *Streptococcus pneumoniae* gene encoding pneumolysin. Infect. Immun. **54:**50–55.
26. **Paton, J. C., P. W. Andrew, G. J. Boulnois, and T. J. Mitchell.** 1993. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. Annu. Rev. Microbiol. **47:**89–115.
27. **Paton, J. C., R. A. Lock, and D. J. Hansman.** 1983. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. Infect. Immun. **40:**548–552.
28. **Paton, J. C., R. A. Lock, C.-J. Lee, J. P. Li, A. M. Berry, T. J. Mitchell, P. W. Andrew, D. Hansman, and G. J. Boulnois.** 1991. Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to *Streptococcus pneumoniae* type 19F polysaccharide. Infect. Immun. **59:**2297–2304.
29. **Russell, H., J. A. Tharpe, D. E. Wells, E. H. White, and J. E. Johnson.** 1990. Monoclonal antibody recognizing a specific protein from *Streptococcus pneumoniae*. J. Clin. Microbiol. **28:**2191–2195.
30. **Sampson, J., Z. Furlow, and D. Williams.** 1995. Conservation of the 37-kDa protein gene among *Streptococcus pneumoniae* serotypes, abstr. G42. *In* Program and abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
31. **Sampson, J. S., S. P. O'Connor, A. R. Stinson, J. A. Tharpe, and H. Russell.** 1994. Cloning and nucleotide sequence analysis of *psaA*, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus* sp. adhesins. Infect. Immun. **62:**319–324.
32. **Shoemaker, N. B., and W. R. Guild.** 1974. Destruction of low efficacy markers is a slow process occurring at a heteroduplex stage of transformation. Mol. Gen. Genet. **128:**283–290.
33. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
34. **Talkington, D. F., B. G. Brown, J. A. Tharpe, A. Koenig, and H. Russell.** 1996. Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). Microb. Pathog. **21:**17–22.
35. **Talkington, D. F., D. L. Crimmins, D. C. Voellinger, J. Yother, and D. E. Briles.** 1991. A 43-kilodalton pneumococcal surface protein, PspA: isolation, protective abilities, and structural analysis of the amino terminal sequence. Infect. Immun. **59:**1285–1289.
36. **Tart, R. C., L. S. McDaniel, B. A. Ralph, and D. E. Briles.** 1995. Truncated *Streptococcus pneumoniae* PspA molecules elicit protective immunity against fatal pneumococcal challenge in mice, abstr. B65. *In* Program and abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
37. **Yother, J., G. L. Handsome, and D. E. Briles.** 1992. Truncated forms of PspA that are secreted from *Streptococcus pneumoniae* and their use in functional studies and cloning of the *pspA* gene. J. Bacteriol. **174:**610–618.
38. **Yother, J., L. S. McDaniel, and D. E. Briles.** 1986. Transformation of encapsulated *Streptococcus pneumoniae*. J. Bacteriol. **168:**1463–1465.

Editor: A. O'Brien