INFECTION AND IMMUNITY, July 1989, p. 2037–2042
0019-9567/89/072037-06$02.00/0

Vol. 57, No. 7

Reduced Virulence of a Defined Pneumolysin-Negative Mutant of *Streptococcus pneumoniae*

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Received 24 October 1988/Accepted 27 March 1989

Insertion-duplication mutagenesis was used to construct a pneumolysin-negative derivative of *Streptococcus pneumoniae*. This was achieved by first transforming the nonencapsulated strain Rx1 with a derivative of the vector pVA891 carrying a 690-base-pair DNA fragment from the middle of the pneumolysin structural gene. DNA was extracted from the resultant erythromycin-resistant, pneumolysin-negative rough pneumococcus and used to transform *S. pneumoniae* D39, a virulent type 2 strain. Several erythromycin-resistant transformants were obtained from two independent experiments, and none of these produced pneumolysin. Southern blot analysis confirmed that the pneumolysin gene in these transformants had been interrupted by the plasmid-derived sequences. The pneumolysin-negative mutants showed reduced virulence for mice compared with D39, as judged by survival time after intranasal challenge, intraperitoneal 50% lethal dose, and blood clearance studies. Pneumolysin production was reinstated in one of the mutants by transformation with the cloned pneumolysin gene, with the concomitant loss of erythromycin resistance; the virulence in mice of this isolate was indistinguishable from that of D39. These results confirm the involvement of pneumolysin in pneumococcal pathogenesis.

Streptococcus pneumoniae is an important human pathogen, which despite the availability of antimicrobial therapy, continues to cause considerable morbidity and mortality throughout the world. However, the precise molecular mechanisms whereby the organism invades and damages host tissues have yet to be elucidated.

Nevertheless, there is an increasing body of evidence that pneumolysin, a thiol-activated toxin produced by virtually all clinical isolates of *S. pneumoniae*, is directly involved in pathogenesis. In vitro studies showed that very low doses of purified pneumolysin inhibit the bactericidal properties of human polymorphonuclear leukocytes and macrophages (16, 18) as well as the proliferative response of human lymphocytes to mitogens (7). Higher toxin doses also cause activation of the classical complement pathway and depletion of serum opsonic activity (20). Thus, pneumolysin could function in pathogenesis by inhibiting phagocytic clearance of invading pneumococci as well as by interfering with the establishment of a humoral immune response. Immunization of mice with purified pneumolysin results in a significantly increased survival time after intranasal challenge with virulent *S. pneumoniae* (19).

To facilitate the molecular genetic assessment of the role of pneumolysin in pathogenesis, we have previously cloned the *S. pneumoniae* gene encoding the toxin in *Escherichia coli* (17). In the present paper, we describe the construction of a defined pneumolysin-negative derivative of an encapsulated pneumococcus by insertion-duplication mutagenesis and compare its virulence with that of its otherwise isogenic parental type.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *S. pneumoniae* strains used were a virulent type 2 strain D39 (1) (obtained from the National Collection of Type Cultures, London, United Kingdom; strain NCTC 7466) and its nonencapsulated, highly transformable derivative Rx1 (21). These organisms were routinely grown in Todd-Hewitt broth–0.5% yeast extract (THY) or on blood agar. *E. coli* K-12 DH1 (9) was grown in Luria-Bertani medium (14) with or without 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.). Plasmid pJCP20, a derivative of pBR322 carrying the complete *S. pneumoniae* pneumolysin gene, has been described previously (17). Plasmid pVA891 has also been described previously (13).

***S. pneumoniae* chromosomal DNA extraction.** *S. pneumoniae* chromosomal DNA for use in Southern blot hybridization experiments was extracted and purified as previously described (17). When DNA was to be used for transformation experiments, the above procedure was followed only as far as the deoxycholate-induced lysis step, after which the crude extracts were diluted 10-fold in 1× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate) and heated at 65°C for 15 min.

Transformation. Transformation of *E. coli* with plasmid DNA was carried out with $CaCl_2$-treated cells as described by Brown et al. (4). *S. pneumoniae* Rx1 and D39 were transformed as previously described (24). Briefly, for Rx1, cells were grown to the optimum culture density (approximately 10^8/ml) in competence medium (THY, 0.2% bovine serum albumin, 0.01% $CaCl_2$) and stored in aliquots at −70°C after the addition of 10% glycerol. A 1/10 volume of donor DNA was added to freshly thawed competent Rx1, and the mixture was incubated at 37°C for 2 h before being plated on blood agar containing 0.2 μg of erythromycin per ml. D39 was grown to a density of 3×10^8/ml in THY, diluted 100-fold in competence medium–10% glycerol, and stored at −70°C. A freshly thawed 0.5-ml aliquot was then incubated with an equal volume of filter-sterilized competent Rx1 culture supernatant for 20 min at 37°C to induce competence. Donor DNA was then added, and cells were incubated and plated out as for Rx1.

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Pneumolysin assay. Pneumolysin activity was determined by hemolysis assay as previously described (19).

Southern blot analysis. Chromosomal DNA extracted from the various pneumococcal strains was digested with the appropriate restriction enzymes under the conditions recommended by the supplier. Digests were electrophoresed on 0.8% agarose gels with a Tris-borate-EDTA buffer system as described by Maniatis et al. (14). DNA was transferred to nitrocellulose as described by Southern (22) and then hybridized to probe DNA, washed, and autoradiographed as described by Maniatis et al. (14). Probe DNA was labeled with ^{32}P by the method of Feinberg and Vogelstein (6).

Virulence studies. Intranasal challenge studies were performed on Prince Henry Hospital mice which had been anaesthetized by intraperitoneal (i.p.) injection with 2 μg of fentanyl citrate (David Bull Laboratories, Melbourne, Australia) and 2 mg of metomidate (Hypnodil; Janssen Pharmaceutica, Beerse, Belgium) in 0.2 ml of saline. Aliquots (50 μl) of 4-h serum broth cultures of the various *S. pneumoniae* strains (diluted when appropriate with serum broth) were then introduced into the nostrils. Mice regained consciousness after approximately 1 h. Survival time was recorded, and the results were analyzed by using the Mann-Whitney U test (one tailed).

BALB/c mice were used for i.p. 50% lethal dose (LD_{50}) studies. Serial 10-fold dilutions of fresh 4-h serum broth cultures of the various pneumococci were prepared in serum broth, and 0.1-ml aliquots were injected i.p. into groups of four mice.

Mice used in the blood clearance studies were CBA/N females. These mice are immune deficient and are unable to respond to most polysaccharide antigens, including the pneumococcal capsule, but respond well to protein antigens (3). Consequently, these mice are particularly susceptible to pneumococci and would therefore be expected to enable the detection even of small differences in virulence between strains. Bacteria were grown in THY to 1×10^8/ml and diluted to 5×10^6/ml in Ringer solution, and 0.2 ml was injected via the tail vein. Blood samples were collected at various times from the retro-orbital plexus, and appropriate dilutions were plated onto blood agar with or without erythromycin.

RESULTS

Construction of pneumolysin-negative *S. pneumoniae*. Pneumolysin-negative *S. pneumoniae* was constructed by insertion-duplication mutagenesis using the vector pVA891 (13). This is a deletion derivative of the *Escherichia-Streptococcus* shuttle plasmid pVA838 (12), which has lost the capacity to replicate autonomously in streptococci. However, pVA891 retains a streptococcal gene encoding erythromycin resistance. The first stage of the mutagenesis procedure involved cloning an internal fragment of the (previously cloned) pneumolysin-coding sequence (17) into pVA891. To achieve this, a 690-base-pair fragment was excised from pJCP20 by digestion with *Sau*3A1 and purified after electrophoresis on a low-melting-point agarose gel. This was ligated into pVA891 which had been linearized with *Bam*HI (Fig. 1) and was transformed into *E. coli* DH1. The recombinant plasmid was purified from its *E. coli* host and used to transform *S. pneumoniae*. Homologous recombination between the 690 base pairs of pneumococcal DNA in the plasmid and the *S. pneumoniae* chromosomal pneumolysin sequence is expected to result in simultaneous incorporation of the plasmid (encoding erythromycin resistance) into the chromosome and interruption of the pneumolysin-coding sequence (Fig. 1).

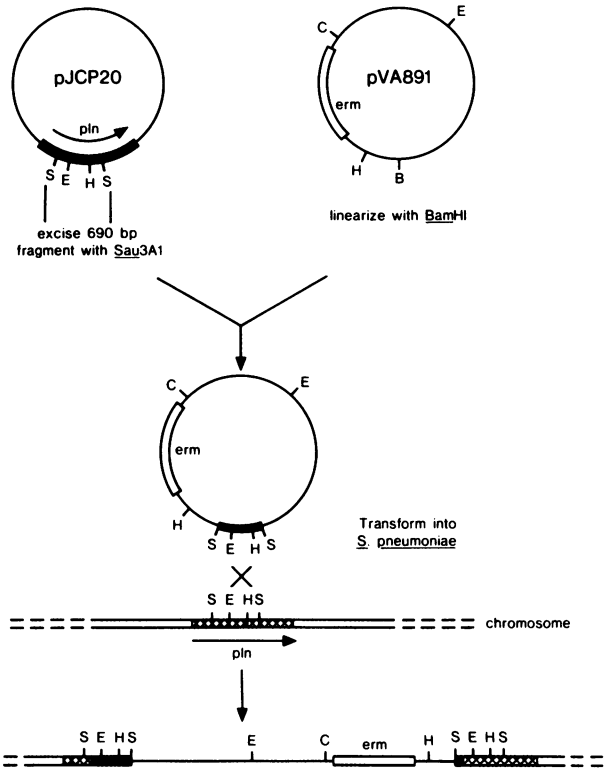

FIG. 1. Scheme for insertion-duplication mutagenesis of the *S. pneumoniae* chromosomal pneumolysin gene. The region in pJCP20 labeled pln represents the complete structural gene for pneumolysin. The homologous region in the *S. pneumoniae* chromosome is cross-hatched. The region in pVA891 labeled erm indicates the location of the erythromycin resistance gene. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; and S, *Sau*3A1.

Several attempts to directly transform the encapsulated type 2 strain D39 to erythromycin resistance with the recombinant plasmid, however, were not successful, even in the presence of competence factor derived from the highly transformable D39 derivative Rx1. To circumvent this problem, we adopted a two-step approach. First, we transformed the nonencapsulated strain Rx1 with the recombinant plasmid and isolated a single erythromycin-resistant transformant (MIC, >1 μg/ml; cf. 0.06 μg/ml for Rx1). This isolate did not produce detectable levels of pneumolysin (i.e., less than 0.5 hemolytic units per ml of culture). A similar culture of Rx1, by comparison, contained in excess of 100 hemolytic units of pneumolysin per ml.

To confirm that the pneumolysin gene in the transformant was inactivated by insertion of the plasmid, chromosomal DNA was analyzed by Southern blot hybridization. DNA from Rx1 and the transformant was digested with *Cla*I, electrophoresed, and transferred to nitrocellulose, as described in Materials and Methods. Filters were then probed with ^{32}P-labeled pVA891 or a 2.9-kilobase (kb) fragment excised from pJCP20, which contains the complete pneumolysin gene (Fig. 2). The pneumolysin probe hybridized with a single DNA band in the Rx1 digest with an approximate size of 5.1 kb. However, the digest of the pneumolysin-negative transformant contained two species with homology to the pneumolysin probe, with approximate sizes of 7.0 and 4.3

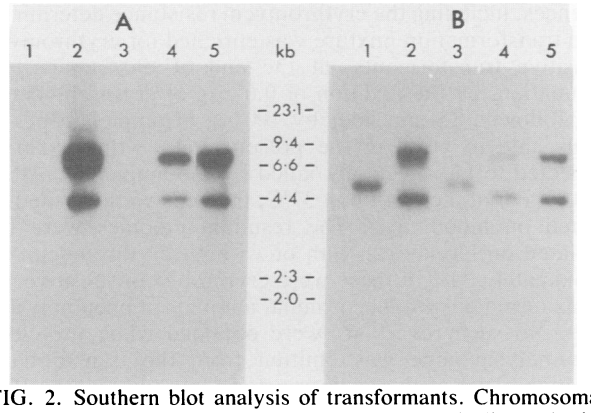

FIG. 2. Southern blot analysis of transformants. Chromosomal DNA was extracted from the various pneumococci, digested with *Cla*I, and subjected to Southern blot analysis using ^{32}P-labeled pVA891 (A) or the cloned pneumolysin gene (B) as the probe. Lanes: 1, Rx1; 2, pneumolysin-negative Rx1 transformant; 3, D39; 4, PLN-A; 5, PLN-B. The mobilities of various DNA size markers are also indicated.

kb. This is consistent with insertion of the 6.1-kb recombinant plasmid (which contains a single *Cla*I site) into the middle of the pneumolysin gene (Fig. 1). The pVA891 probe labeled fragments of identical size in the transformant digest (7.0 and 4.3 kb) but did not label any DNA fragments in the Rx1 digest (Fig. 2), which is also consistent with the proposed model of insertion-duplication mutagenesis. Further Southern blot hybridization studies (not shown) using *Eco*RI and *Hin*dIII digests suggested that the recombinant plasmid had become incorporated into the *S. pneumoniae* chromosome as a result of a crossover somewhere between the first *Sau*3A1 site and the *Eco*RI site in the pneumolysin gene.

To construct an encapsulated pneumolysin-negative pneumococcus, the encapsulated strain D39 was then transformed with DNA extracted from the pneumolysin-negative, erythromycin-resistant transformant of Rx1. One would expect a higher D39 transformation frequency in this experiment than that previously experienced with the recombinant plasmid, because the donor DNA was derived from the *S. pneumoniae* chromosome. Two independent transformation experiments were carried out to minimize the possibility of cotransformation of spurious *S. pneumoniae* sequences along with the interrupted pneumolysin-erythromycin resistance locus. The first experiment yielded a single erythromycin-resistant D39 transformant, while the second transformation yielded 12. All 13 isolates produced a type 2 capsule (confirmed by Quellung reaction) and required for inhibition an erythromycin MIC >1 μg/ml, but the isolates failed to produce pneumolysin activity. Also, Western blot (immunoblot) analysis using antipneumolysin serum failed to detect any truncated antibody-reactive protein species in any of the isolates (result not shown).

The transformant from the first experiment (designated PLN-A) and two transformants selected at random from the second experiment (designated PLN-B and PLN-C) were chosen for further analysis. *Cla*I digests of chromosomal DNA extracted from these three strains and from D39 were subjected to Southern blot analysis as described above for the Rx1 transformant. The hybridization pattern with the two probes (pVA891 and the pneumolysin gene from pJCP20) for D39 was identical to that seen for Rx1 (Fig. 2). Similarly, PLN-A, PLN-B, and PLN-C had hybridization patterns identical to that observed for the pneumolysin-negative Rx1 transformant (results for PLN-A and PLN-B are shown in Fig. 2).

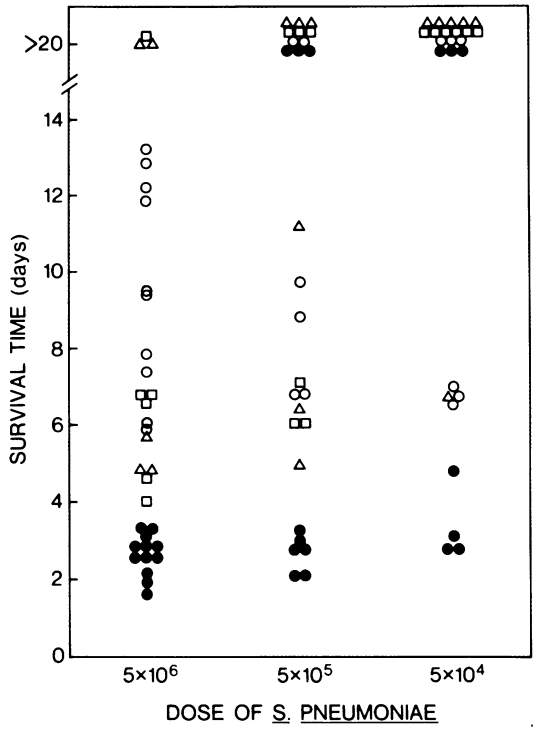

FIG. 3. Intranasal challenge. Groups of mice were anaesthetized and challenged via the intranasal route with the indicated doses of D39 (●), PLN-A (○), PLN-B (□), or PLN-C (△). The survival time of each mouse is shown.

Comparative virulence of D39, PLN-A, PLN-B, and PLN-C. To determine the effect of the inactivation of the pneumolysin gene on virulence, mice were challenged via the intranasal route with various doses of D39, PLN-A, PLN-B, and PLN-C (Fig. 3). At the maximum dose tested (5×10^6 CFU) all mice challenged with D39 died within 3.1 days (median survival time, 2.8 days). Mice challenged with the same dose of PLN-A, PLN-B, or PLN-C all survived significantly longer ($P < 0.001$, Mann-Whitney U test); median survival times were 9.4, 6.8, and 5.8 days, respectively (these median survival times are not significantly different from each other). Pneumococci isolated from the heart blood of these mice immediately after death retained erythromycin resistance and the inability to produce pneumolysin. As the dose was reduced, the overall survival rate for mice challenged with PLN-A, PLN-B, and PLN-C increased from 14% at 5×10^6 CFU to 44% at 5×10^5 CFU and 78% at 5×10^4 CFU, compared with 0, 33, and 42% for mice challenged with the respective doses of D39. Thus, the intranasal LD_{50} for PLN-A, PLN-B, and PLN-C was approximately 10 times that for D39.

The i.p. LD_{50} was also determined using BALB/c mice as described in Materials and Methods. PLN-A, PLN-B, and PLN-C had an i.p. LD_{50} of approximately 3×10^4 CFU compared with approximately 3×10^2 CFU for D39.

Blood clearance studies were also performed on D39, PLN-A, PLN-B, and PLN-C. Blood samples were collected from CBA/N mice at various times after administration of approximately 10^6 organisms via the tail vein, and the number of viable pneumococci per milliliter of blood was

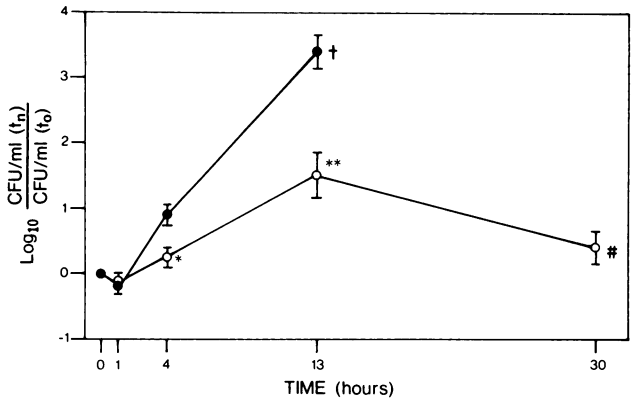

FIG. 4. Reduced multiplication of pneumolysin-negative pneumococci in vivo. CBA/N female mice were inoculated intravenously with approximately 10^6 pneumococci. Five mice received D39 (●), and six mice received the pneumolysin-negative derivatives (two mice each for PLN-A, PLN-B, and PLN-C) (○). Blood samples were collected after 1 min, 1 h, 4 h, 13 h, or 30 h, and the number of viable bacteria was determined. For each mouse, bacterial counts were normalized with respect to the 1-min time point (t_0) (i.e., CFU per ml at t_n/CFU per ml at t_0). The data shown are geometric means plus or minus the standard error for the two groups. Symbols: *, significantly different from D39, $P < 0.02$ (Student's t test); **, significantly different from D39, $P < 0.001$ (Student's t test); †, five of five mice dead in <24 h (mean, 18.2 h); #, two of six mice dead at 130 and 208 h, respectively (significantly different from D39, $P < 0.005$, Mann-Whitney U test).

determined (Fig. 4). For the mice challenged with D39, the number of bacteria had increased 8-fold after 4 h and 2,600-fold after 13 h; all five of these mice died within 24 h. For mice challenged with PLN-A, PLN-B, or PLN-C, the multiplication rate of the pneumococci in the blood was significantly lower. The number of bacteria per milliliter of blood had increased only 1.8-fold and 33-fold after 4 and 13 h, respectively. At 30 h after challenge, bacterial counts were only 2.5-fold greater than at zero time. Duplicate plating of all blood samples on erythromycin plates confirmed that erythromycin resistance was being stably maintained in the absence of selection throughout the experiment. In contrast to the results for D39, only two of the six mice challenged with pneumolysin-negative pneumococci died (one at 130 h and the other at 208 h; $P < 0.005$, Mann-Whitney U test). Again, pneumococci isolated from the heart blood had retained the erythromycin-resistant, pneumolysin-negative phenotype.

Isolation of a pneumolysin-positive revertant. It could be argued that the obviously reduced virulence of the pneumolysin-negative D39 derivatives was due to cotransformation with other DNA sequences from the pneumolysin-negative Rx1 donor. This was unlikely, since D39 transformants from independent experiments showed an identical change in the structure of the pneumolysin locus and an identical reduction in virulence. The objection was quashed, however, by constructing a pneumolysin-positive, erythromycin-sensitive revertant of PLN-A. This was achieved by transforming PLN-A with a purified 2.9-kb *Cla*I DNA fragment from pJCP20. This fragment contains 350 base pairs of vector DNA and 2.55 kb of cloned pneumococcal DNA, including the complete (1.5-kb) pneumolysin-coding sequence. Homologous double recombination between this fragment and the PLN-A chromosome could result in reconstitution of a complete copy of the pneumolysin gene in the chromosome, with simultaneous elimination of the pVA891-derived sequences, including the erythromycin resistance determinant. The transformation mixture was enriched for erythromycin-sensitive transformants, at the end of the standard 2-h incubation, by the addition of 0.07 μg of erythromycin per ml, followed 15 min later by 100 μg of ampicillin per ml. Cells able to grow in the presence of erythromycin are expected to be selectively killed by the ampicillin. After a further 6 h of incubation at 37°C, the cells were washed and plated on blood agar. The resultant colonies were then replated on blood agar with or without erythromycin. Approximately 20% of these were erythromycin sensitive, and of six tested, all produced similar amounts of pneumolysin to D39. No such revertants were obtained when the cloned pneumolysin gene was omitted from the transformation reaction. Two of the back-transformants (designated PLN-A-1 and PLN-A-2) were subjected to Southern blot analysis. *Cla*I digests of chromosomal DNA exhibited a hybridization pattern identical to D39, using either pVA891 or the pneumolysin gene as a probe (result not shown). This confirmed the reconstitution of the pneumolysin gene and the elimination of the pVA891-derived sequences. The virulence of PLN-A-1 and PLN-A-2 was then compared with that of D39 by challenging groups of seven mice by the intranasal route with 5×10^6 organisms. No mice survived longer than 3 days, and the median survival times were 2.7, 2.1, and 2.1 days for PLN-A-1, PLN-A-2, and D39 respectively. Thus, full virulence had been restored by reconstitution of the pneumolysin gene.

DISCUSSION

Previous studies in this laboratory (19) provided the first direct evidence for the involvement of pneumolysin in pneumococcal pathogenesis by showing that mice immunized with the purified toxin survived significantly longer than did control mice after intranasal challenge with virulent *S. pneumoniae*. The protection observed in that study was not complete, however, which may have been due to failure to achieve sufficient antibody levels of the appropriate immunoglobulin class at the appropriate site (i.e., blood or mucosae). Alternatively, the incomplete protection imparted by immunization with pneumolysin may have been attributable to production of other toxic virulence factors by the invading pneumococci.

In the present study, we have used molecular genetic techniques to directly assess the role of pneumolysin in pneumococcal virulence. Insertion-duplication mutagenesis was used to construct an encapsulated strain of *S. pneumoniae* which was unable to produce pneumolysin. This resulted in a reduction in virulence for mice, as judged by survival time after intranasal challenge and by i.p. LD_{50} and blood clearance studies. Full virulence was restored when pneumolysin production was reconstituted by back-transformation with the cloned pneumolysin gene. Random insertions of pVA891 into the chromosome have previously been shown not to alter the virulence of D39 (15). The effects observed in the present study are therefore directly attributable to the pneumolysin locus itself, which clearly establishes its importance in the pathogenesis of pneumococcal disease. However, the possibility cannot be eliminated that our observations might be due in part to polar effects on sequences downstream of the toxin-coding sequence. The sequence of the 30 nucleotides immediately downstream of the pneumolysin gene has been reported (23), but it does not appear to contain sequences resembling any known transcription termination signals. Notwithstanding this element

of uncertainty, the fact that the survival times of mice challenged with our pneumolysin-negative derivatives are similar to those previously reported for pneumolysin-immunized mice challenged with D39 (19) strongly supports the conclusion that our observed effects are indeed a consequence of inactivation of the pneumolysin structural gene.

The fact that the pneumolysin-negative pneumococcus was not completely avirulent for mice (a result which is consistent with our earlier immunization-challenge experiments [19]) is interesting. Inactivation of the pneumolysin gene increased the intranasal and i.p. LD_{50}s approximately 10^1-fold and 10^2-fold, respectively. This increase in LD_{50} is less dramatic than that observed for *Listeria monocytogenes* when the gene for its related thiol-activated toxin (listeriolysin) is inactivated by transposon mutagenesis (8). In a previous study (15), D39 mutants deficient in the production of pneumococcal surface protein A were generated by the same technique as that used here (pVA891 insertion). Interestingly, the surface-protein-A-negative pneumococci are also reduced in virulence, having an elevated intravenous LD_{50} and increased survival time, but nevertheless, like the pneumolysin-negative mutants, they are still capable of eventually killing mice. Pneumococcal surface protein A and pneumolysin are, to our knowledge, the only pneumococcal products other than the capsule whose role in virulence has been confirmed by genetic means. It would be of great interest to examine the virulence of pneumococci lacking both these proteins. Nevertheless, the possibility remains that *S. pneumoniae* is capable of producing other potentially lethal factors. Pneumococcal neuraminidase (10) and purpura-producing principle (5) are possible candidates. We have recently shown that immunization of mice with neuraminidase results in increased survival time after intranasal challenge (11). We have also cloned the pneumococcal neuraminidase gene in *E. coli* (2), and this will facilitate the genetic assessment of its contribution to virulence.

While the involvement of pneumolysin in pathogenesis has now been established beyond reasonable doubt (at least in a mouse model), its precise in vivo function has yet to be elucidated. The rate of growth of the pneumolysin-negative pneumococcus and its parental type are identical in vitro, but the blood clearance studies presented here have shown clear differences in the rate of in vivo multiplication after intravenous inoculation into mice. This may reflect more efficient phagocytic clearance of the pneumolysin-negative pneumococcus compared with clearance of its toxin-producing parent. Such an interpretation would be consistent with earlier in vitro studies which showed that very low doses of purified pneumolysin significantly inhibited the bactericidal activity of human polymorphonuclear leukocytes and macrophages (16, 18). The generation of strains of encapsulated *S. pneumoniae* which differ genetically only at the pneumolysin locus will be a useful tool for further in vivo studies of the role of the toxin in pneumococcal pathogenesis.

ACKNOWLEDGMENTS

We thank Stephen Gregory and Julie Goldfinch for technical assistance and Rob Lock, Eric Glare, and David Catcheside for fruitful discussions.

This work was supported by grants from the National Health and Medical Research Council of Australia and the Adelaide Children's Hospital Research Trust and Public Health Service grants T32H007300, AI8557, and AI21548 from the National Institutes of Health.

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