

Comparative Virulence of *Streptococcus pneumoniae* Strains with Insertion-Duplication, Point, and Deletion Mutations in the Pneumolysin Gene

ANNE M. BERRY, A. DAVID OGUNNIYI, DAVID C. MILLER AND JAMES C. PATON*

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

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Pneumolysin is a 471-amino-acid toxin produced by *Streptococcus pneumoniae* which has both cytolytic and complement activation properties. We have constructed a derivative of the type 2 *S. pneumoniae* strain D39 in which the portion of the pneumolysin gene encoding amino acids 55 to 437 has been deleted in-frame. The virulence of this strain (Δ Ply) was compared with those of wild-type D39, a pneumolysin insertion-duplication mutant (PLN-A), and a derivative (PdT) carrying a toxin gene with three point mutations known to abolish both cytolytic activity and complement activation. PdT was intermediate in virulence between D39 and either PLN-A or Δ Ply in a mouse intraperitoneal challenge model. This provides unequivocal evidence that pneumolysin has an additional property that is not abolished by point mutations which reduce cytotoxicity and complement activation to virtually undetectable levels.

Pneumolysin, a potent 53-kDa thiol-activated cytolytic factor of *Streptococcus pneumoniae* (12, 13). Its cytolytic activity involves interaction with cholesterol in target cell membranes and insertion into the lipid bilayer, followed by oligomerization to form transmembrane pores, which bring about cell lysis. Erythrocytes are particularly susceptible to pneumolysin-induced lysis, but theoretically, the toxin can interact with any cell that has cholesterol in its plasma membrane. Indeed, in vitro studies with purified toxin have demonstrated detrimental effects on a wide range of cells and tissues, even at sublytic concentrations (12, 13). However, pneumolysin is a bifunctional toxin, and in addition to its cytotoxic properties, it is capable of directly activating the classical complement pathway in the absence of specific antibody, with a concomitant reduction in serum opsonic activity (14). This is mediated by its capacity to bind directly to the Fc region of human immunoglobulin G (IgG) (10).

Structure-function analysis of pneumolysin has demonstrated that a domain towards the C terminus of the toxin which includes the unique cysteine residue (amino acids [aa] 427 to 437) is critical for cytotoxicity. This cysteine motif is highly conserved among other members of the thiol-activated cytolytic family. Several single amino acid substitutions within this region reduce the cytotoxicity of pneumolysin by up to 99.9% (8, 17). A separate region, which has a degree of amino acid homology with human complement (C)-reactive protein, is responsible for IgG binding and complement activation, and a mutation in this domain (Asp385→Asn) inhibits IgG binding and abolishes complement activation (10).

Inactivation of the pneumolysin gene (*ply*) by insertion-duplication mutagenesis in either a type 2 or a type 3 strain reduced virulence of *S. pneumoniae* for mice challenged by either the intranasal (i.n.) or intraperitoneal (i.p.) route (6, 7). Subsequent studies demonstrated that i.n. or intratracheal (i.t.)

challenge with *ply*-negative pneumococci resulted in a less-severe inflammatory response, a reduced rate of multiplication within the lung, a reduced capacity to injure the alveolar-capillary barrier, and a delayed onset of bacteremia compared with those of the wild-type strain (9, 16). Intravenously (i.v.) administered *ply*-negative pneumococci also exhibited a reduced rate of multiplication in the blood relative to that of the wild-type strain (7). Benton et al. (3) subsequently reported that i.v. challenge of mice with the *ply*-negative mutant resulted in a chronic bacteremia, with numbers of pneumococci in the blood remaining at, or below, 10^7 CFU per ml for a week in some mice. i.v. administration of an identical dose of the wild-type pneumococcus, however, resulted in fulminant infection; mice invariably died within 28 h, at which time there were approximately 10^9 to 10^{10} CFU per ml of blood. Benton et al. concluded that during the first few hours of bacteremia pneumolysin plays a critical role by preventing the generation of inflammation-based immunity, thereby permitting continued exponential net growth of pneumococci (3).

We have also constructed a series of derivatives of the type 2 *S. pneumoniae* strain D39 in which the wild-type *ply* gene has been replaced by mutated genes encoding toxins with defined point mutations affecting either or both of the cytotoxic and complement activation properties (5). In an i.p. challenge model, pneumococci carrying two *ply* point mutations (Cys428→Gly and Trp433→Phe) resulting in production of a toxin with only 0.001% residual cytolytic activity was much less virulent than wild-type D39. A D39 derivative producing pneumolysin with just the Trp433→Phe mutation (with 0.1% residual cytolytic activity) was only marginally (albeit statistically significantly) less virulent than the wild-type strain. Interestingly, in this model system, pneumococci carrying the Asp385→Asn *ply* mutation, which abolishes complement activation, was as virulent as the wild-type strain. However, by using the same pneumococcal mutants, distinct roles have been demonstrated for both toxin properties in the pathogenesis of bronchopneumonia and lobar pneumonia with mouse i.n. and i.t. challenge models. In both models, strains producing pneumolysin lacking either property were less virulent than the wild type (1, 15). In the i.t. challenge model, cytotoxic activity was required for

* 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. E-mail: patonj@wch.sa.gov.au.

damage to the alveolar-capillary barrier and for optimal bacterial multiplication in the alveoli and lung tissue during the first 6 h of infection. However, the complement activation property was associated with increased numbers of pneumococci in lung tissue and the blood 24 h after infection (15). In the i.n. challenge model, the complement activation property was important for growth of pneumococci in both the lungs and the blood 6 to 24 h after challenge, while the cytotoxic property was associated with increased numbers of pneumococci in the lungs after 24 h (1).

An *S. pneumoniae* D39 derivative carrying three *ply* point mutations (Asp385→Asn, Cys428→Gly and Trp433→Phe) abrogating both complement activation and cytolytic properties of the toxin has been shown to be less virulent than strains carrying either Asp385→Asn or Cys428→Gly and Trp433→Phe substitutions in both i.n. and i.v. challenge models (1, 4). However, in both cases, the triple point mutant was significantly more virulent than a D39 derivative in which *ply* was disrupted by insertion-duplication mutagenesis, leading to the suggestion that pneumolysin has an additional property which contributes to pathogenesis.

The above observations could, of course, be explained by polar effects caused by integration of the insertion-duplication mutagenesis vector pVA891 into *ply*, although there are no open reading frames immediately downstream of *ply* in the *S. pneumoniae* genome. Moreover, construction of the various D39 *ply* point mutants also involved insertion of pVA891 immediately downstream of the intact *ply* open reading frame; these constructs included a control strain in which insertion of pVA891 reconstituted a wild-type *ply* gene, and this strain was fully virulent (1, 5). An alternative explanation for the lower virulence of the *ply* insertion-duplication mutant could be the production of a truncated gene product, or a *ply*-vector encoded fusion protein, which is toxic for the pneumococcus. The insertion-duplication mutant was constructed with a derivative of pVA891 containing a 695-bp *Sau3A* fragment from the middle of *ply* cloned into the *Bam*HI site of the vector. This was transformed into *S. pneumoniae* and integrated into the chromosome by a single recombination event (7). Translation of the interrupted gene would be expected to result in a fusion protein containing the first 322 aa of pneumolysin plus an indeterminate number of vector-encoded amino acids. Theoretically, fusions containing vector-encoded amino acids plus the C-terminal 380 aa of pneumolysin could also result from readthrough from vector sequences into the 3' portion of *ply*. Furthermore, smaller truncated pneumolysin C-terminal fragments could result from initiation at internal ATG codons in the distal portion of *ply*.

In the present study we have eliminated these complications by constructing a D39 derivative with an in-frame *ply* deletion, and we compared its virulence to that of the wild-type strain and derivatives carrying insertion-duplication and point mutations in *ply*.

Bacterial strains. The virulent type 2 *S. pneumoniae* strain D39 (NCTC 7466) has been described previously (2). D39 derivatives in which *ply* has been disrupted by insertion-duplication mutagenesis (designated PLN-A) or in which the wild-type *ply* has been replaced by a mutated gene encoding pneumolysin with three amino acid substitutions (Asp385→Asn, Cys428→Gly and Trp433→Phe) abrogating both complement activation and cytolytic properties of the toxin (designated PdT) have also been described previously (5, 7).

The *ply* deletion derivative of D39 (designated ΔPly) was constructed as follows. First, *S. pneumoniae* D39 genomic DNA was digested with *Cla*I and recircularized, and the region immediately upstream of *ply* was isolated by inverse PCR (11)

with the primers 5'-GGGATCCTGTTTCGTAATCTCTCTGT CA-3' and 5'-GAGGAGCTACCTTGACTCC-3', which were designed on the basis of the published *ply* sequence (18). The inverse PCR product was digested with *Cla*I and *Sal*I, and the resultant 3,050-bp fragment, which contains DNA from the *Cla*I site 2,883 nucleotides (nt) upstream of the *ply* initiation codon to the *Sal*I site in codon 55 of *ply*, was cloned into pBluescript SK (obtained from Stratagene, La Jolla, Calif.). This construct was transformed into *Escherichia coli* DH5α (Gibco-BRL, Gaithersburg, Md.). The 3' portion of *ply* and flanking sequences were isolated by PCR amplification of D39 chromosomal DNA with primers 5'-TGGTGGTTCGACGGTTTATG AAAAACC-3' and 5'-CCTTTGGCTCGAGCAATCGCTT TATCG-3'. The former primer (plus strand) creates a *Sal*I site (underlined) by changing 3 nt (double underlined); *ply* codon 438 is shown in boldface. The latter primer (minus strand) anneals to a site approximately 1,100 nt 3' to the *ply* termination codon and creates a *Xho*I site (underlined) by changing 3 nt (double underlined). The resultant PCR product was then digested with *Sal*I and *Xho*I, and the 1,212-bp fragment was cloned into the similarly restricted pBluescript SK derivative containing the 5' terminus of *ply* and flanking sequences. This procedure results in an in-frame fusion of the 5' region of *ply*, encoding the 54 N-terminal amino acids, with the 3' region encoding the 34 C-terminal amino acids (residues 438 to 471). Plasmid DNA was extracted from *E. coli* DH5α which had been transformed with the above construct, and the complete 4,262-bp pneumococcal DNA insert was then excised by digestion with *Cla*I and *Xho*I. This was used to transform *S. pneumoniae* PLN-A. A derivative in which a double recombination had resulted in replacement of the pVA891-interrupted *ply* locus with the deleted *ply* locus (with concomitant loss of the pVA891-encoded erythromycin resistance marker) was isolated after enrichment in the presence of erythromycin and ampicillin, as previously described (7). In the previous study we demonstrated that PLN-A does not contain additional mutations affecting virulence, as back-transformation with an intact copy of *ply* reconstituted wild-type virulence. To confirm that the transformant isolated in the present study contained the *ply* deletion mutation, chromosomal DNA was amplified by PCR by using a plus-strand primer annealing 200 nt upstream of the *ply* initiation codon (5'-TTACAAGACCAACCTTGATTG-3') and the minus-strand primer annealing downstream of the *ply* termination codon described above. The PCR product was then sequenced by dye-terminator chemistry on an Applied Biosystems model 373A automated DNA sequencer. The sequence was analyzed using DNASIS and PROSIS Version 7.0 software (Hitachi Software Engineering, South San Francisco, Calif.), and this analysis confirmed that the *ply* locus of strain ΔPly encodes an in-frame deletion derivative of pneumolysin lacking amino acids 55 to 437.

Virulence studies. In order to compare the virulence of the various *S. pneumoniae* strains, D39, PLN-A, PdT, and ΔPly were grown overnight on blood agar (supplemented with 0.2 μg of erythromycin/ml in the case of PLN-A and PdT), 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 by using antisera obtained from Statens Serum Institut, Copenhagen, Denmark. In vitro growth rates of the three strains were also identical (result not shown). Cultures were then diluted to a density of 5×10^4 CFU/ml, and 0.1-ml volumes were injected i.p. into groups of 12 to 13 BALB/c mice. The survival time of each mouse was recorded (Fig. 1), and differences in median survival time between groups were analyzed using the Mann-Whitney U test (two tailed). Eleven of the 12 mice challenged

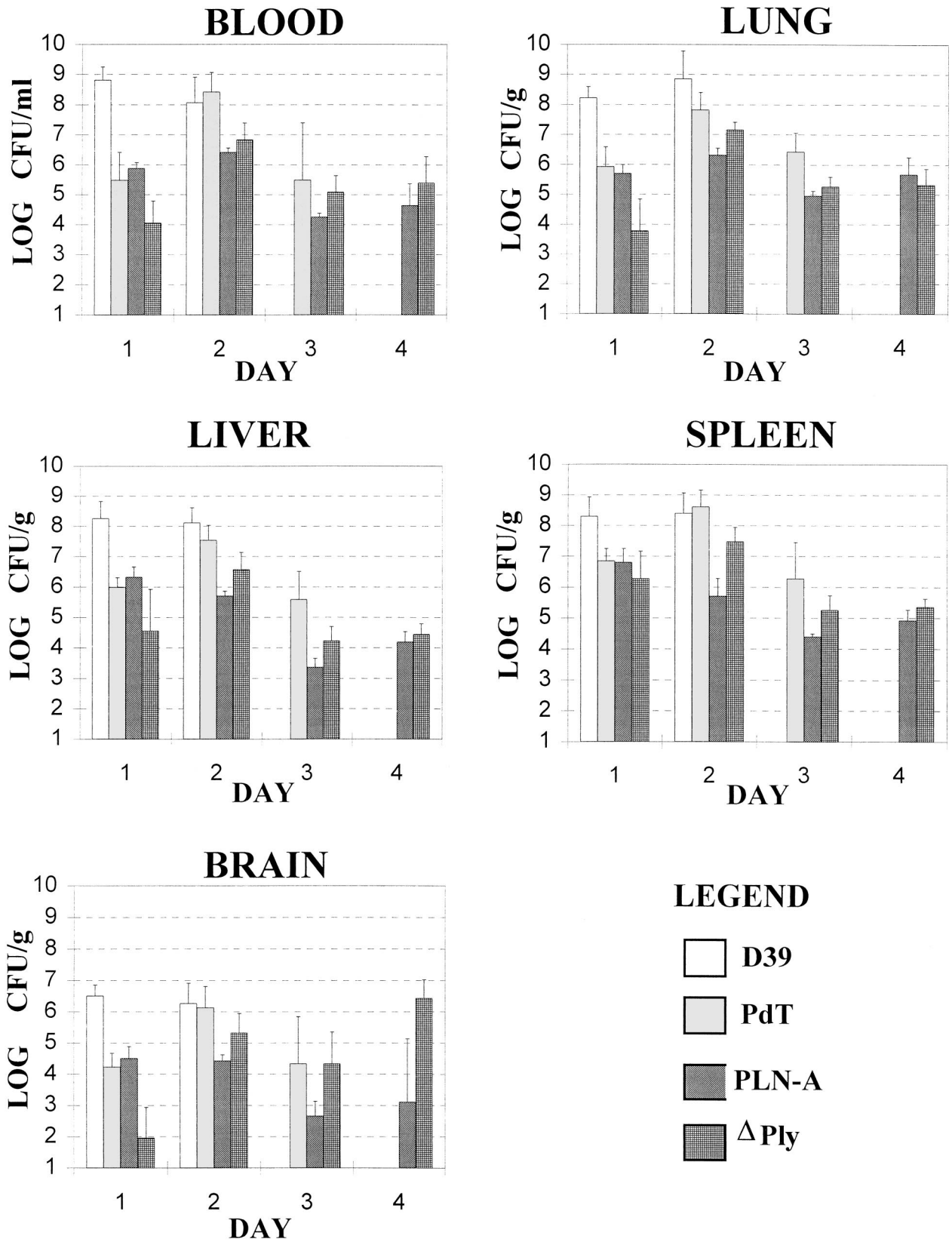


FIG. 2. Numbers of *S. pneumoniae* in tissues after i.p. challenge. Groups of 16 BALB/c mice were challenged i.p. with 5×10^2 CFU of D39, PLN-A, PdT, or Δ Ply. Four mice chosen at random from each group were sacrificed 1, 2, 3, or 4 days after challenge (where there were sufficient survivors). Numbers of each strain in blood (expressed as \log_{10} CFU/ml) or in homogenates of lungs, liver, spleen, or brain (expressed as \log_{10} CFU/g of tissue) were determined as described in the text. Data are the means for each group, with the standard errors indicated by the bars.

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