INFECTION AND IMMUNITY, Oct. 2011, p. 4122–4130 Vol. 79, No. 10
0019-9567/11/$12.00 doi:10.1128/IAI.05418-11

Pneumolysin with Low Hemolytic Activity Confers an Early Growth Advantage to *Streptococcus pneumoniae* in the Blood[▽]

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Received 20 May 2011/Returned for modification 22 June 2011/Accepted 11 July 2011

Streptococcus pneumoniae **is a leading cause of human diseases such as pneumonia, bacteremia, meningitis, and otitis media. Pneumolysin (Ply) is an important virulence factor of *S. pneumoniae* and a promising future vaccine target. However, the expansion of clones carrying *ply* alleles with reduced hemolytic activity has been observed in serotypes associated with outbreaks of invasive disease and includes an allele identified in a highly virulent serotype 1 isolate (*ply*4496). The virulence of Ply-deficient and *ply* allelic-replacement derivatives of *S. pneumoniae* D39 was compared with that of wild-type D39. In addition, the protective immunogenicity of Ply against pneumococci with low versus high hemolytic activity was also investigated. Replacement of D39 *ply* with *ply*4496 resulted in a small but statistically significant reduction of virulence. However, both native Ply- and Ply4496-expressing strains were significantly more virulent than a Ply-deficient mutant. While the numbers of both Ply- and Ply4496-expressing isolate cells were higher in the blood than the numbers of Ply-deficient mutant cells, the growth of the Ply4496-expressing strain was superior to that of the wild type in the first 15 h postchallenge. Ply immunization provided protection regardless of the hemolytic activity of the challenge strain. In summary, we show that low-hemolytic-activity Ply alleles contribute to systemic virulence and may provide a survival advantage in the blood. Moreover, pneumococci expressing such alleles remain vulnerable to Ply-based vaccines.**

Streptococcus pneumoniae (the pneumococcus) is the world's foremost bacterial pathogen, is responsible for 1 million deaths of children <5 years of age each year, and is a leading cause of diseases such as pneumonia, bacteremia, meningitis, and otitis media (29). The cholesterol-dependent cytolysin pneumolysin (Ply) is important for pneumococcal pathogenesis, as the virulence of Ply-deficient derivatives is severely attenuated in systemic and intranasal animal models of infection (5). Due to the important role of Ply in pneumococcal pathogenesis and the relatively conserved nature of the toxin, considerable research has investigated the use of nontoxic Ply derivatives in future serotype-independent vaccine formulations (1, 2, 9, 18, 30, 32, 33). The central property of Ply is its ability to form pores in cholesterol-containing cell membranes, resulting in cytolysis and potent induction of inflammation (4–6, 8, 13, 16). However, recent epidemiological studies have detected the clonal expansion of isolates expressing Ply with either low or undetectable hemolytic activity associated with serotype 1, 7F, and 8 isolates, which are considered to be highly invasive (8, 14, 17, 21). Therefore, the reduced hemolytic activity of Ply in isolates readily capable of causing invasive pneumococcal disease (IPD) has cast doubt on the importance of Ply to the pathogenesis of highly invasive isolates. However, Ply possesses a number of immunomodulatory properties that act independently of the toxin's cytolytic activity (4, 26). These properties include antibody-independent activation of the classical complement pathway (34), activation of Toll-like receptor 4 (TLR4) (22), and TLR4-independent activation of the NLRP3 inflammasome (25). However, it is not clear whether the noncytolytic properties of Ply are sufficient or essential for IPD or whether a drastic reduction in hemolytic activity diminishes Ply's overall contribution to IPD. Furthermore, it is not clear whether Ply with low hemolytic activity enables escape from vaccines that include Ply-specific antigen.

Recently, our laboratory identified a highly virulent serotype 1 clinical strain, 4496 (ST3018; lineage C [7]), that exhibits significantly reduced hemolytic activity relative to a range of other serotype 1 isolates, including the highly virulent strain Menzies[1]-1861 (ST3079; lineage B [7]) (11). Given that strain 4496 is hypervirulent, the low hemolytic activity of strain 4496 raises the question of whether the Ply variant expressed by this strain is important for IPD and whether this strain remains vulnerable to Ply-specific immunization. In this study, we aimed to address these questions using the strain 4496 Ply variant (Ply4496) as a representative model of other naturally occurring derivatives of Ply with anomalous hemolytic activity. The contribution of Ply4496 to IPD was compared with that of normally cytolytic Ply in a murine model of IPD, and protection against a strain 4496 challenge was confirmed following immunization with Ply-specific antigen.

MATERIALS AND METHODS

Ethics statement. This study was conducted in compliance with the Australian code of practice for the care and use of animals for scientific purposes (7th edition, 2004) and South Australian Animal Welfare Act 1985. All animal experiments were approved by the Animal Ethics Committee of the University of Adelaide.

Strains and media. The *S. pneumoniae* serotype 1 clinical isolate strains, 4496 (ST3018) and 1861 (ST3079), used in this study were obtained from the Women's and Children's Hospital, North Adelaide, Australia, and the Menzies School of Health Research, Darwin, Australia, respectively. Virulent serotype 2 strain D39 (NCTC 7466) was also used in this study. Opaque-phase variants of all strains were selected on Todd-Hewitt broth supplemented with 1% yeast extract (THY)-catalase plates (35), and these were used in all animal experiments. Before infection, the bacteria were grown in serum broth (SB) {nutrient broth

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▽ Published ahead of print on 25 July 2011.

TABLE 1. Oligonucleotides used in this study

Oligonucleotide application and name	Sequence
Sequencing of *ply*	
AD20	GGA ACT TAT TAG GAT CCA GAA GAT GGC
AD21	TTG TCG CGA GCT CTC TCC TCT CCT A
RHplygapF	ACG GCT CAC AGC ATG GAA CAA CTC
RHplyfor1	GGG ATT ATT CTC TAA CAA GGT CTC
RHplyrev1	CAT AGT GGT GCC TAT GTT GCC C
Construction of mutants	
$RHPlyF_{(5)}$	GGT GGT GCT TAT GCT TTG TCG
$RHPlyR_{(5)}$	GTG GGC AAT GAC AAA GGA TGT G
RHplyΔspecR	TAC AGT CGG CCG CT ACCT CCT AAT AAG TTC CTG G
RhplyΔspecF	GAC TAT CTC GAG GGA GAG GAG AAT GCT TGG GAC
RHcatF	TAT AAT CGG CCG CAA CAG CGT GAC CGA AAA TTG
RHcatR	TAT AAT CTC GAG GGG TTC CGA GGC TCA ACG TC
Real time RT-PCR	
$RH16SF_{(3)}$	CAT GCA AGT AGA ACG CTG AA
$RH16SR_{(3)}$	TGT CAT GCA ACA TCC ACT CT
RHrtplyF	AGC TCC TCA GAC AGA GTG GA
RHrtplyR	GTA AAG CGA TGC CTT CTT G

[10 g/liter peptone (Oxoid), 10 g/liter Lab Lemco powder (Oxoid), 5 g/liter NaCl] and 10% (vol/vol) donor horse serum} to an A_{600} of 0.16, which is approximately 1×10^8 CFU/ml, and then diluted to the relevant challenge dose.

PCR. Chromosomal DNA for PCR was extracted and purified using the Wizard genomic DNA purification kit (Promega Corporation, Madison, WI), with the exception of cell lysis, which was performed by incubating cells at 37°C for 10 min with 0.1% (wt/vol) sodium deoxycholate. PCRs were performed using a G-STORM GS482 thermal cycler (Gene Technologies). Standard reactions were performed using *Taq* DNA polymerase (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The Expand Long Template or High Fidelity PCR system was used when high-fidelity amplification was required. Overlap-extension PCR was carried out essentially as previously described (12, 27), using the Expand Long Template PCR system. *ply* DNA sequencing reactions were carried out using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA), using the primers listed in Table 1. Amino acid sequences were aligned using the ClustalW algorithm available at http://align.genome.jp/.

Construction of Ply mutants. Generation of competent *S. pneumoniae* cells and subsequent transformation were performed using the complete transformation medium method (10, 23). Mutants were constructed using the primers in Table 1. D39ΔPly was constructed by replacing the *ply* open reading frame (ORF) with the gene encoding chloramphenicol acetyltransferase (*cml*) and subsequent selection of chloramphenicol-resistant recombinants. D39::Ply4496 was constructed by replacing *cml* in D39ΔPly with *ply*4496, which was amplified from strain 4496. D39::Ply4496 recombinants were selected for the loss of chloramphenicol resistance as previously described (28). The desired recombination was verified by direct sequencing. These manipulations do not affect DNA sequences flanking the *ply* ORF.

Protein analyses. Bacteria were grown to mid-exponential phase ($A_{600} = 0.5$) in THY broth, concentrated 10-fold, and lysed by treatment with 0.1% sodium deoxycholate. The total protein in these lysates was determined by using the BCA Protein Assay kit according to the manufacturer's instructions (Thermo Scientific). Approximately 17 μg of protein was subjected to SDS-PAGE by standard methods. Separated proteins were electroblotted onto nitrocellulose (Protran, Melbourne, Australia) as described previously (24). After transfer, the membrane was probed with mouse anti-Ply polyclonal antiserum at a dilution of 1/3,000 and then reacted with a blotting grade donkey anti-mouse IRDye 800 CW secondary antibody (LI-COR Biosciences) at a dilution of 1/30,000. The blot was scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences). Relative quantification of Ply between strains was performed using the Odyssey Infrared Imaging System application software v 3.0.21 by comparing units of fluorescence (LI-COR Biosciences).

Real-time relative qRT-PCR. RNA for *ply* expression analysis was extracted from 10 ml of a mid-exponential-phase THY broth culture using acid phenol-chloroform-isoamyl alcohol (125:25:1; Ambion) as described elsewhere (31). The abundance of *ply* mRNA was measured by real-time quantitative reverse transcription (qRT)-PCR. *ply*-specific primers were designed using OligoPerfect software (Invitrogen), and primers specific for 16S rRNA were used as internal controls for data normalization (Table 1). Real-time RT-PCR was performed using a LightCycler 480 II (Roche) and the Superscript III One-Step RT-PCR kit (Invitrogen) according to the manufacturers' instructions. Quantitative differences for each transcript were calculated using the $2^{-\Delta\Delta CT}$ method (20).

Hemolysis assay. The hemolytic activity of pneumococcal lysates was determined as described previously (32), using phosphate-buffered saline-washed human erythrocytes. Approximately 85 μg of total lysate supernatant protein (prepared as described above) was used for each sample in triplicate.

Animal studies. Outbred 5- to 6-week-old female CD1 (Swiss) mice were used in all challenge experiments. Mice were challenged intraperitoneally with 100 μl of a bacterial suspension containing approximately 2×10^4 CFU in SB. The challenge dose was confirmed retrospectively by serial dilution and plating of the inocula on blood agar. For survival experiments, mice were closely monitored for signs of illness for 14 days and humanely sacrificed when terminal infection was reached. Heart blood was taken from sacrificed mice and plated on blood agar to confirm the presence of *S. pneumoniae*. For quantitation of pneumococci, blood was taken from the submandibular veins of infected mice at 3-h intervals over the course of the experiment. A 20-μl aliquot of blood immediately underwent serial 10-fold dilutions in SB. All 8 dilutions were plotted in duplicate on blood agar to determine the number of CFU. Blood plates were incubated at 37°C in 95% air–5% CO_2 overnight.

Immunization of mice with Ply toxoid. Fifteen CD1 mice were immunized intraperitoneally (i.p.) with a genetically obtained Ply toxoid derivative, PdT (4). Each mouse received three doses of 10 μg of PdT in 100 μg alum adjuvant (Imject Alum; Pierce, Rockford, IL) at 14-day intervals. Sera were collected from the mice by submandibular bleeding 1 week after the third immunization, and enzyme-linked immunosorbent assay (ELISA) titers of pooled sera were determined as described previously (30). Mice were challenged 2 weeks after the third immunization with 10^4 CFU of strain 4496 or 1.5×10^3 CFU of strain 1861. These doses are approximately 100 times the 50% lethal dose of the respective strain. Survival time was recorded as described above.

Statistical analyses. Differences in median survival time between challenge groups were analyzed by the (one-tailed) Mann-Whitney U test. Differences in the geometric mean number of pneumococci in the blood between challenge groups were calculated on log-transformed data using the unpaired (two-tailed) *t* test. Differences between the relative levels of *ply* expression in strains were analyzed using the unpaired (two-tailed) *t* test. All analyses were performed using GraphPad Prism version 5.01. A *P* value of <0.05 was considered significant.

Nucleotide sequence accession number. The complete nucleotide sequence of the gene for Ply4496 was deposited in GenBank under accession number HQ711618.

RESULTS

Alignment of Ply4496 with D39 Ply, Ply306, and Ply8. The *ply* ORF in strain 4496 was sequenced, and the deduced amino

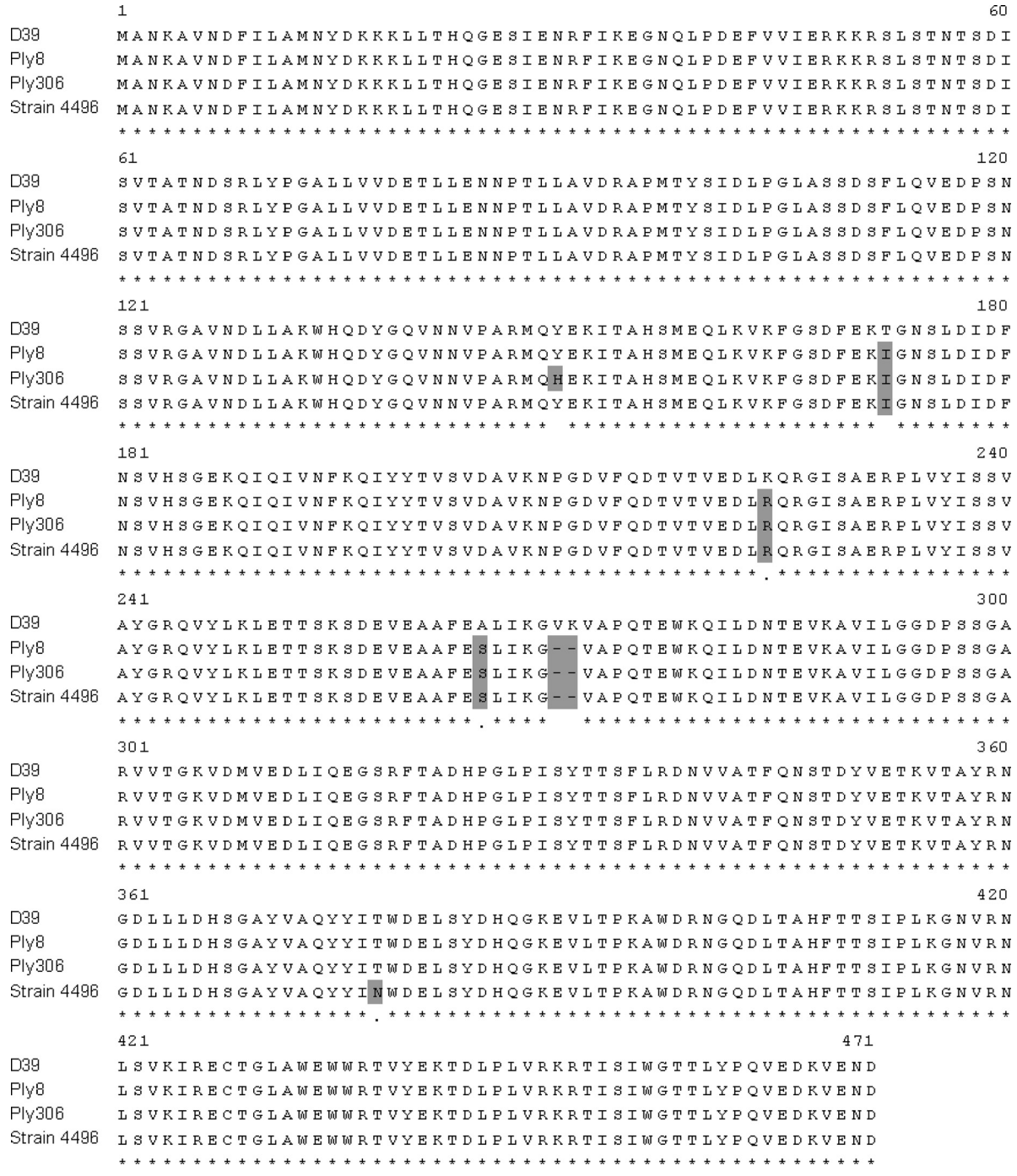

FIG. 1. Amino acid sequence alignment of Ply variants. The amino acid sequences of D39 Ply (GenBank accession no. CP1000410), Ply8, Ply306, and Ply4496 (GenBank accession no. HQ711618) were aligned using the ClustalW algorithm available at http://align.genome.jp/. An asterisk indicates an identical amino acid, a colon indicates a conservative mutation, and a period indicates a semiconservative mutation. Shading indicates key amino acid differences relative to D39 Ply.

acid sequence of Ply4496 was aligned with the published sequences of D39 Ply, Ply8, and Ply306 (Fig. 1). Ply8 and Ply306 are previously described alleles that exhibit low and undetectable hemolytic activity, respectively (17, 21). The alignment shows that Ply4496 contains T172I, K224R, A256S, ΔV270, and ΔK271 mutations relative to D39, which are also shared with both the Ply8 and Ply306 variants (Fig. 1). In contrast to the Ply306 variant, Ply4496 lacked the Y150H substitution, which, in combination with the mutations described above, is responsible for the abrogation of hemolytic activity in ST306 (17). The T378N mutation in Ply4496 has not been reported previously. However, the low hemolytic activity of Ply4496 is most likely due to the T172I mutation, as was previously reported for Ply8 (21).

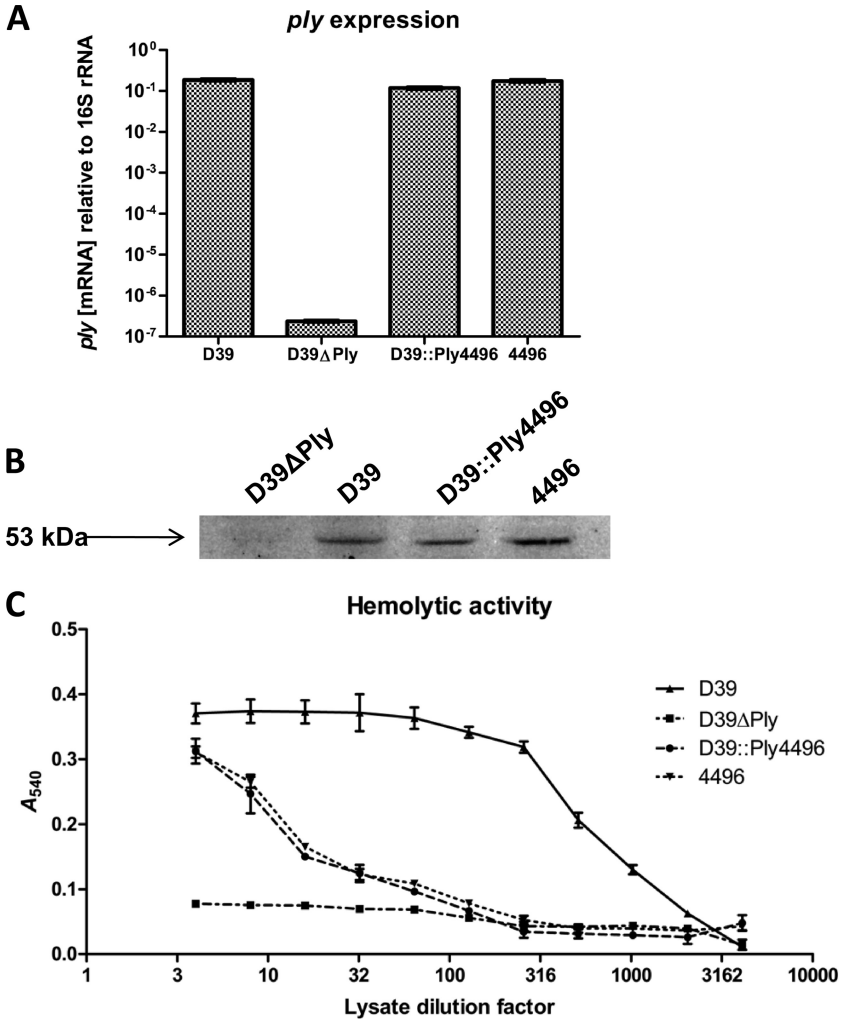

FIG. 2. Ply expression and activity analysis. (A) Ply expression was compared among wild-type D39, Ply-deficient D39 (D39ΔPly), D39 expressing strain 4496-derived Ply (D39::Ply4496), and serotype 1 strain 4496 at both the mRNA (A) and protein (B) levels. *ply* expression was determined by real time qRT-PCR and is expressed relative to that of the 16S rRNA gene. Error bars indicate the standard error of the mean of triplicate reactions. (B) Total amounts of Ply protein produced by D39ΔPly, D39, D39::Ply4496, and strain 4496 determined by quantitative Western blotting using Ply-specific mouse antiserum. The molecular mass of Ply (53 kDa) is indicated. (C) Lysis of human red blood cells (detected by A_{540}) in serial 2-fold dilutions of 850 μg/ml total protein from mid-exponential-phase cell lysates. The hemolytic activities of D39, D39ΔPly, D39::Ply4496, and serotype 1 strain 4496 were compared. Hemoglobin release is expressed as the A_{540} of assay supernatants (mean ± the standard error of the mean of triplicate assays). The specific hemolytic activity was defined as the dilution factor at which 50% hemolysis was detected.

Characterization of D39 Ply mutants. The most direct way of assessing the role of Ply4496 in virulence would be to construct mutants with changes in the respective gene in the serotype 1 strain 4496 background. However, repeated attempts to transform this strain failed, requiring the adoption of an indirect method. Therefore, in order to investigate the impact of the low hemolytic activity of Ply4496 on pathogenesis, D39 derivatives were constructed that either lacked *ply* (designated D39ΔPly) or had the endogenous gene replaced with *ply*4496 (designated D39::Ply4496). All four strains (including strain 4496) were cultured in THY broth, and aliquots were taken for mRNA, protein, and hemolytic activity analyses. Gene expression analysis performed by qRT-PCR confirmed that *ply* transcription was the same among D39, D39::Ply4496, and strain 4496 (Fig. 2A). In addition, quantitative Western blotting of equal quantities of total lysate protein confirmed that there were no differences in the amounts of Ply protein produced by D39, D39::Ply4496, and strain 4496, with 2,100, 2,200, and 2,200 U of fluorescence, respectively (Fig. 2B). Finally, a Ply activity assay was used to compare the specific hemolytic activities of D39, D39ΔPly, D39::Ply4496, and strain 4496. This confirmed that the specific hemolytic activities of D39::Ply4496 and strain 4496 were identical at approximately 13 hemolytic units (HU)/mg total protein (Fig. 2C). In contrast, the specific hemolytic activity of wild-type D39 was approximately 800 HU/mg total protein, which showed that the hemolytic activity of Ply4496 was approximately 1.6% of that of D39Ply. In addition, D39ΔPly was confirmed to be Ply deficient by mRNA, protein, and activity measurements (Fig. 2). Thus, there were no differences in Ply expression at either the mRNA or the

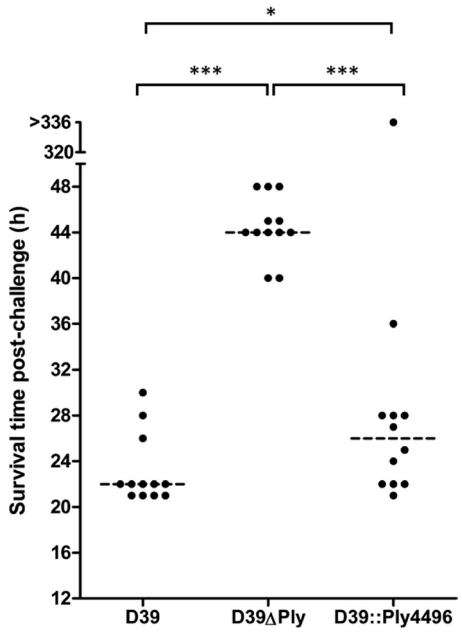

FIG. 3. Survival times of mice challenged with D39 and its derivatives. Groups of 12 mice were infected intraperitoneally with 2×10^4 CFU of *S. pneumoniae* D39, Ply-deficient D39 (D39ΔPly), and D39 expressing strain 4496-derived Ply (D39::Ply4496). The broken lines indicate the median survival times of the groups. The statistical significance of differences between survival times was calculated by the (one-tailed) Mann-Whitney U test. Brackets indicate statistical comparisons. *, $P < 0.05$; ***, $P < 0.001$.

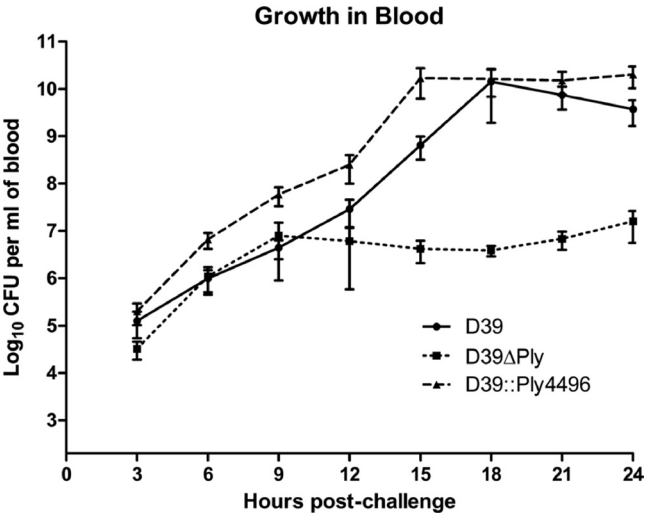

FIG. 4. Time course of growth of D39 and its derivatives in blood. Groups of mice were infected intraperitoneally with 2×10^4 CFU of *S. pneumoniae* of D39, D39ΔPly, or D39::Ply4496 (n = 8, 7, and 5, respectively). Blood was taken from the submandibular vein of each mouse at each time point and serially diluted before plating on blood agar for enumeration. Data are the geometric mean number of CFU/ml of blood (± the standard error of the mean) for each group.

protein level among all three Ply-expressing strains. Moreover, the total hemolytic activity of D39::Ply4496 is identical to that of strain 4496.

Contribution of Ply hemolytic activity to systemic disease. The impact of the hemolytic activity of Ply4496 on virulence was investigated using a murine model of systemic disease in which the survival times of D39-, D39ΔPly-, and D39::Ply4496-infected mice were compared over 14 days (Fig. 3). There was a small but nevertheless statistically significant difference in median survival time between D39- and D39::Ply4496-infected mice, which indicated that the reduced hemolytic activity of Ply4496 was responsible for a small increase of approximately 4 h in median survival time ($P < 0.05$). This showed that the hemolytic activity of Ply does make a demonstrable albeit modest contribution to systemic virulence. In contrast, the median survival time of D39ΔPly was approximately 12 h and 8 h longer than those of D39- and D39::Ply4496-infected mice, respectively ($P < 0.001$), which indicated that the effect on systemic virulence of completely deleting *ply* far exceeded the impact of a 98% reduction in specific hemolytic activity. Therefore, despite the low hemolytic activity of Ply4496, this variant makes an important contribution to IPD.

Contribution of Ply hemolytic activity to the proliferation of *S. pneumoniae* in blood. Since the systemic virulence of D39::Ply4496 was slightly less than that of the wild type, we investigated whether this difference was due to a deficiency in the ability of D39::Ply4496 to proliferate in blood relative to that of D39. Therefore, the level of bacteremia was determined in D39-, D39ΔPly-, and D39::Ply4496-infected mice at 3-h intervals over a 24-h period following i.p. challenge.

For D39ΔPly, the geometric mean number of CFU/ml of blood plateaued at approximately 5×10^6 after 9 h, whereas for both wild-type D39 and D39::Ply4496, exponential growth continued for 15 to 18 h and then plateaued between 10^9 and 10^{10} CFU/ml of blood (Fig. 4). At all time points, D39::Ply4496 maintained bacterial numbers in the blood that were significantly higher than those of D39ΔPly (Fig. 5). In addition, D39 bacteria reached numbers in the blood that were significantly greater than those of D39ΔPly bacteria from 15 h (Fig. 5). However, of particular interest was the significantly larger numbers of bacteria in the blood of D39::Ply4496-infected mice than in that of wild-type D39-infected mice between 6 and 15 h postchallenge (Fig. 5). The numbers of D39::Ply4496 bacteria in the blood were also significantly greater than those of D39 bacteria at 24 h. These data suggest that in the early hours of systemic disease, the Ply variant with significantly reduced cytolytic activity provides a growth advantage within the blood. Therefore, it appears that *ply* alleles with reduced cytolytic activity may have a growth advantage in the early stages of bacteremia, which could be particularly significant in highly invasive serotypes, such as 7F, 8, and 1.

Immunization with a Ply derivative protects against pneumococci with low and high hemolytic activities. Immunization of mice with a genetically detoxified Ply derivative, PdT, that lacks both cytolytic and complement-activating properties has been shown previously to protect mice against an *S. pneumoniae* challenge (4, 33). To investigate whether protection against challenge strains with low Ply hemolytic activity was maintained, groups of mice were immunized with PdT and then challenged with either 4496 or similarly virulent strain 1861, which produces wild-type Ply. Immunization of mice with PdT elicited a strong Ply-specific antibody response, as deter-

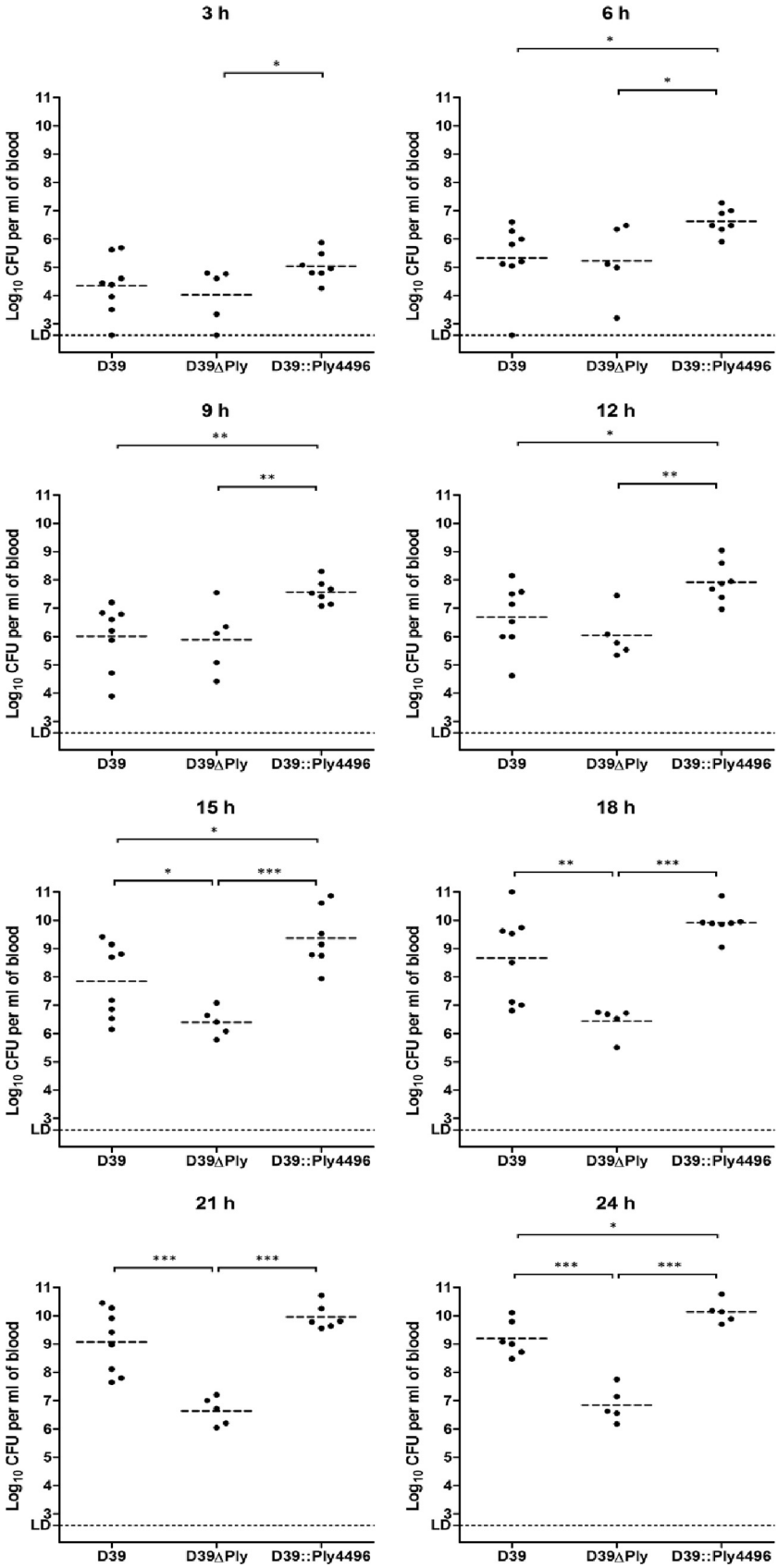

FIG. 5. Numbers of D39 bacteria and their derivatives in the blood of individual mice. Mice were challenged with D39, D39ΔPly, or D39::Ply4496 as detailed in the legend to Fig. 3. The number of CFU/ml blood is indicated for each mouse. Broken lines indicate the geometric mean number of CFU/ml blood. The limit of detection (LD) was 2×10^2 CFU/ml blood. Statistical significance was calculated on log-transformed data using the unpaired (two-tailed) *t* test. Brackets indicate statistical comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

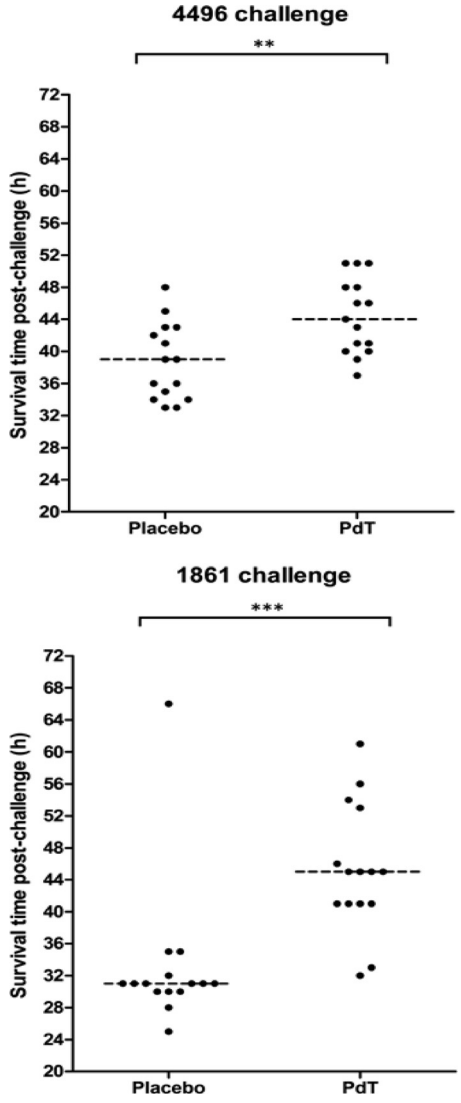

FIG. 6. Immunization challenge. Groups of 15 CD1 mice were immunized with either PdT or alum placebo and challenged 2 weeks after the third immunization with either 10^4 CFU of strain 4496 or 1.5×10^3 CFU of strain 1861. Data are the survival times of the mice; the broken lines denote the median survival times of the groups. **, $P < 0.01$; ***, $P < 0.001$.

mined by ELISA, but immunization with the alum placebo did not (the geometric mean titers were approximately 10,000 and <200, respectively). PdT-immunized mice given either of the challenge strains survived significantly longer than those in the respective placebo group ($P < 0.01$ and $P < 0.001$, respectively, for 4496 and 1861) (Fig. 6).

DISCUSSION

Considerable research is under way to develop serotype-independent pneumococcal vaccines. Candidate formulations include combinations of detoxified Ply derivatives with surface-exposed proteins such as PspA and CbpA (30). However, epidemiological studies in the United Kingdom and elsewhere in Europe have detected the expansion of clonal types expressing *ply* alleles that either lack hemolytic activity or exhibit very low levels thereof (17), casting doubt on the importance of Ply in the pathogenesis of IPD due to these emerging clonal types, and raised the possibility that such strains may escape Ply-based vaccines. In addition, it is possible that naturally occurring mutations in Ply could interfere with the antigenic recognition of Ply, also potentially reducing the effectiveness of Ply-based vaccination. Therefore, in this study, we used a Ply variant from a highly virulent serotype 1 isolate (strain 4496) that exhibits low hemolytic activity as a model for the role of such Ply variants in IPD.

Sequencing of *ply*4496 confirmed that the gene included mutations that have been previously reported in serotype 1, 7F, and 8 isolates (17, 21). However, the additional semiconservative T378N mutation present in Ply4496 has not been previously described. In contrast, Ply4496 lacked the Y150H mutation, which is likely to explain the residual hemolytic activity of strain 4496 relative to that of Ply306-expressing strains. Therefore, the reduced hemolytic activity of Ply4496 is most likely due to the T172I mutation (21).

In order to investigate whether Ply with low hemolytic activity is important for IPD, we constructed a derivative of D39 where the endogenous *ply* gene was exchanged for *ply*4496 (D39::Ply4496) and another that was Ply deficient (D39ΔPly). While *ply* exchange mutants would ideally be constructed in the serotype 1 background, the inability to genetically transform these isolates made such comparisons impossible. Nevertheless, in this study, we constructed the relevant Ply mutants in a single genetic background to eliminate the risk that other differences in the genetic background between different clonal types could unduly influence the outcome of subsequent virulence comparisons.

Differences in Ply expression were eliminated as a potential source of variability in virulence by confirming at both the mRNA and protein levels *in vitro* that there were no differences in expression among D39, strain 4496, and D39::Ply4496. Furthermore, the specific hemolytic activities of D39::Ply4496 and strain 4496 were identical and approximately 1.6% of that of the wild type. Similarity of expression levels *in vitro* is likely to reflect the *in vivo* situation, since flanking (regulatory) sequences are identical. Also, at the mRNA, protein, and activity levels, the D39ΔPly strain was confirmed to be Ply deficient.

In this study, a mouse model of pneumococcal bacteremia was used to compare the virulence of the hemolytic, low-hemolytic-activity, and Ply-deficient derivatives of D39. We showed that the low hemolytic activity of Ply4496 relative to that of the wild type is responsible for a small but statistically significant reduction in virulence, which is consistent with previously reported studies that compared the virulence of D39 derivatives expressing targeted active-site Ply mutants with extremely low hemolytic activity relative to that of the wild type (4). In contrast, a difference in virulence between the serotype 1 ST227 and ST306 clonal types, which express hemolytic and nonhemolytic Ply, respectively, was not detected (17). However, it is possible that any small differences in virulence due to the absence of hemolytic activity might have been obscured by other clonally related differences between the ST227 and ST306 genomes. In contrast to the small reduction in virulence caused by the low hemolytic activity of Ply4496, the virulence of D39ΔPly was significantly attenuated relative to that of both

Ply-expressing strains. This large reduction in virulence confirms that despite the very low hemolytic activity of Ply4496, this naturally occurring variant toxin makes an important contribution to IPD.

In addition to significant differences in survival time, the attenuated virulence of D39ΔPly correlated with attenuated proliferation in blood. The numbers of Ply-deficient pneumococci in blood reached a plateau between 10^6 and 10^7 CFU/ml at only 8 h postchallenge, which is consistent with the chronic bacteremia previously attributed to abrogation of Ply expression (3). The fact that the *in vivo* growth of D39ΔPly was significantly less than that of both the wild type and the Ply4496-expressing strain demonstrates that despite the low hemolytic activity of Ply4496, this Ply variant is essential for efficient proliferation in blood. In addition, we showed that Ply4496 conferred a growth advantage relative to not only the Ply-deficient strain but also the wild type. This unexpected finding suggests that the full hemolytic activity of wild-type D39 may actually hinder early proliferation in blood. The contrast between the increased eventual lethality of D39 expressing fully cytolytic Ply versus the growth advantage of D39::Ply4496 in blood suggests that the cytolytic activity of Ply contributes largely to disease severity at high levels of bacteremia, whereas the noncytolytic properties of Ply are important for the initial survival and proliferation of *S. pneumoniae* within blood. These findings are not unprecedented, as previous studies using strains expressing Ply with defined active-site mutations have demonstrated the independent contributions of the complement-activating properties and cytolytic activity of Ply to pneumococcal pathogenesis (4, 15, 26).

Differences in behavior between D39 and D39::Ply4496 during IPD are supported by previous reports showing differences in the immune response directed against pneumococci expressing either hemolytic or nonhemolytic Ply. The uptake of *S. pneumoniae* by human dendritic cells (DCs) is drastically reduced by the expression of nonhemolytic Ply compared to that of bacteria expressing the fully toxic version. In addition, nonhemolytic Ply induced a greater proinflammatory cytokine response by DCs but significantly less DC apoptosis (19). A very recent study has also shown that pneumococci expressing wild-type Ply activated the NLRP3 inflammasome, whereas those expressing low-hemolytic-activity Ply, similar to strain 4496, or Ply knockout mutants did not. Moreover, the proinflammatory cytokine responses triggered by NLRP3 inflammasome activation were protective to the host in a pneumonia model (36). These findings are consistent with those of the present study, where we show that while reduced hemolytic activity leads to a marginal reduction in virulence, it is responsible for an early growth advantage in blood compared to fully hemolytic Ply. Thus, differences in the immunomodulatory properties of Ply alleles and associated growth advantages in blood during the early stages of infection for strains that produce them may account for the apparent clonal expansion of certain invasive sequence types.

As further confirmation that Ply4496 is important to the pathogenesis of parent strain 4496, it was shown that immunization with the Ply toxoid PdT provides protection against an i.p. challenge with strain 4496. Furthermore, PdT immunization elicited similar degrees of protection against challenges with low-hemolytic-activity strain 4496 and fully hemolytic type 1 strain 1861. Thus, allelic variation in Ply that affects hemolytic activity does not diminish the susceptibility of pneumococci to Ply-based vaccines.

ACKNOWLEDGMENTS

This research was funded by program grant 565526 and project grant 627142 from the National Health and Medical Research Council (NHMRC) of Australia. J.C.P. is an NHMRC Australia Fellow.

The funders had no role in study design, data collection and analysis, the decision to publish, or preparation of the manuscript.

We thank Uwe Stroeher for sequencing assistance and Andrew Lawrence from the Women's and Children's Hospital, North Adelaide, South Australia, Australia, for the provision of strain 4496.

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Editor: A. Camilli