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Identification of Invasive Serotype 1 Pneumococcal Isolates That Express Nonhemolytic Pneumolysin

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Recently, there has been an increase in invasive pneumococcal disease (IPD) caused by serotype 1 *Streptococcus pneumoniae* throughout Europe. Serotype 1 IPD is associated with bacteremia and pneumonia in Europe and North America, especially in neonates, and is ranked among the top five most prevalent pneumococcal serotypes in at least 10 countries. The currently licensed pediatric pneumococcal vaccine does not afford protection to this serotype. Upon screening of 252 clinical isolates of *S. pneumoniae*, we discovered mutations in the pneumolysin gene of two out of the four serotype 1 strains present in the study group. Analysis of an additional 28 serotype 1 isolates from patients with IPD from various Scottish Health Boards, revealed that >50% had mutations in their pneumolysin genes. This resulted in the expression of nonhemolytic forms of pneumolysin. All of the strains producing nonhemolytic pneumolysin were sequence type 306 (ST306), whereas those producing "wild-type" pneumolysin were ST227. The mutations were in a region of pneumolysin involved in pore formation. These mutations can be made in vitro to give the nonhemolytic phenotype. Pneumolysin is generally conserved throughout all serotypes of *S. pneumoniae* and is essential for full invasive disease; however, it appears that serotype 1 ST306 does not require hemolytically active pneumolysin to cause IPD.

Streptococcus pneumoniae (the pneumococcus) is the predominant cause of fatal infections such as bacterial pneumonia and meningitis. Pneumococci can be divided into 90 serotypes depending on the immunochemistry of their capsular polysaccharide (27), but less than 20% are the major cause of disease (25). Although there is an overlap in strains that cause disease and those associated with carriage, some serotypes are more likely to be recovered from invasive disease (serotypes 1, 4, 14, and 18C) and others are more commonly isolated from nasopharyngeal swabbing of healthy individuals (serotype 3 sequence type 180 [ST180], 6B, 19F, and 23F) (10). Serotype 1 *S. pneumoniae*, initially classified in 1913 (18), has remained one of the most prevalent invasive serotypes, with reports of increases in serotype 1 invasive pneumococcal disease (IPD) in Scotland (48), Sweden (28), and Denmark (39) and a high prevalence of serotype 1 disease throughout Europe, South America, Africa, and Asia (25, 26). Interestingly, serotype 1 disease has been reported to be decreasing in North America (21) (ranked 17th, per reference 11) and Egypt (69). Serotype 1 is associated with complicated pneumonia, pulmonary empyema, peritonitis, and salpingitis (19, 49, 65, 66, 72) and has been directly linked to mortality, irrespective of factors such as age, environment, and leukocyte count of patients (47). Along with serotypes 5 and 7, serotype 1 is also associated with a higher ratio of hospitalization versus ambulatory care compared with pneumococcal infections from other serotypes (2). Serotype 1 is one of the few serotypes linked with non-hospital outbreaks of pneumococcal disease; such outbreaks occur in overcrowded institutions and/or where alcoholism is a problem (14, 16, 24, 50). Serotype 1 is said to have a high attack rate, as it has a global association with IPD and is rarely isolated from nasopharyngeal swabbing of healthy patients (11).

The recently licensed seven-valent polysaccharide conjugate vaccine (7PCV) is highly efficacious against pneumococcal disease caused by the seven vaccine serotypes (6, 34, 73). However, the decrease observed with otitis media from serotypes included in 7PCV has coincided with a marked increase in disease caused by non-vaccine serotypes (36). Capsule polysaccharide from serotype 1 pneumococci is not included in the licensed 7PCV, and with the rise in non-vaccine serotypes, this situation needs to be closely monitored. Although 9-valent and 11-valent formulations of conjugate vaccines including serotype 1 capsule polysaccharide have undergone trials (38, 55), capsule polysaccharide from all 90 pneumococcal serotypes cannot be included in one vaccine (37). As a result of this, research is focusing on alternatives such as using common immunogenic pneumococcal proteins to be included in future vaccines (1, 9, 51, 56).

Pneumolysin, a cytoplasmic cholesterol-dependent cytolysin, is of particular interest as a vaccine candidate as it is produced by all strains of *S. pneumoniae* (33) and is protective in animal models of vaccination (1, 42, 51, 59). The cholesterol-dependent cytolysins form large pores in cholesterol-containing membranes and are therefore cytotoxic to mammalian cells (23, 58). In addition to cytolytic (hemolytic) activity, pneumolysin is known to induce inflammatory responses in the host lung similar to that caused by *S. pneumoniae*, activate comple-

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TABLE 1. Sequence type, hemolytic activity, and pneumolysin expression levels of 29 serotype 1 *S. pneumoniae* isolates with D39 as a reference strain

Sample identification	Patient age (yr)	Diagnosis[c]	ST	Hemolytic activity (+/−)[a]	Ply expression (ng/ml)[b]	Specific activity (10^5 HU/mg)[b]
D39 (reference)			128	+	989	3.2
00-3645	40		227	−	0	
01-1956	5	Bacteremia	306	−	1,203	0
03-1769	58		306	−	1,663	0
03-1828	63		306	−	2,214	0
03-1986	65		306	−	1,654	0
03-2412	84	Pyrexia	306	−	1,608	0
03-2473	38	Pyrexia	306	−	1,454	0
03-2672	25	Pneumonia	306	−	1,976	0
03-2759	66	Pneumonia	306	−	1,695	0
03-3425	22	Pyrexia	306	−	1,516	0
03-3431	12	Pyrexia	306	−	1,701	0
03-3485	56	Pyrexia	306	−	982	0
03-3582	36		306	−	1,651	0
03-3788	35	Pneumonia	306	−	1,245	0
03-3828	32	Pneumonia	306	−	1,326	0
03-3921	64	Pneumonia	306	−	1,355	0
03-1063	18		306	−	2,621	0
01-2696	33		227	+	1,155	4.2
03-2135	38	Pneumonia	227	+	1,520	4.2
03-2219	76		227	+	1,401	4.6
03-2234	46		227	+	1,230	5.2
03-2406	75	Septicemia	227	+	1,447	4.4
03-2920	42		227	+	1,606	2.0
03-2809	9	Pyrexia	227	+	1,431	2.2
03-3629	67	Pyrexia	227	+	1,063	1.5
03-3920	28	Pneumonia	227	+	1,329	4.8
01-2526	6		227	+	670	7.2
03-1510	14	Pneumonia	227	+	1,518	4.2
03-3856	20		227	+	901	7.1

[a] Hemolytic activity assessed with human erythrocytes.
[b] Cell lysates were diluted in 1× PBS to 300 μg/ml total protein prior to analysis.
[c] All isolates were from blood cultures and therefore caused bacteremia.

ment in the absence of antibody, induce apoptosis, and activate Toll-like receptor 4 (TLR-4) (8, 13, 46, 52, 63). Generally, the amino acid sequence of pneumolysin is thought to be highly conserved throughout all pneumococcal serotypes with little variance over time and geographic distance (53). Serotypes 7 and 8 have been reported to possess a threonine-to-isoleucine substitution at amino acid position 172 that reduces the specific activity of the toxin (45), but this is the only reported naturally occurring mutation within pneumolysin that affects function. It is important to note that residual hemolytic activity of pneumolysin in an animal model is adequate for full virulence of the pneumococcus as demonstrated by the chromosomal replacement of pneumolysin with pneumolysin carrying a mutation that reduces the specific activity of the toxin to 0.1% of wild-type pneumolysin (5).

As part of an ongoing study of pneumococcal virulence genes (Jefferies et al., manuscript in preparation), we identified a number of clinical isolates with mutations in their pneumolysin gene (*ply*). The mutations were predominantly in the *ply* gene of serotypes 1, 7, and 8. The serotype 1 isolates had additional mutations in the *ply* gene to those previously described for serotypes 7 and 8 (45) and were chosen for further investigation due to their high attack rate and recent reports of an increase in serotype 1 disease. From the initial 252 Scottish clinical pneumococcal isolates (32) (chosen to represent all serotypes received by the Scottish Meningococcus and Pneumococcus Reference Laboratory; SMPRL), 4 were serotype 1, and of these, 2 had mutations within the *ply* gene. Further analysis of an additional 28 serotype 1 isolates revealed that more than half had mutations within the *ply* gene, which resulted in the abrogation of the toxin's hemolytic activity. Multilocus sequence typing (MLST) of the serotype 1 isolates revealed a correlation between mutations in the *ply* gene and sequence type.

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MATERIALS AND METHODS

To investigate whether the *ply* genes of serotype 1 pneumococci often contain variations from published sequences (45, 53, 68); 28 serotype 1 IPD isolates from the collection held at SMPRL were selected for further study to give a study collection of 32 isolates. These additional clinical isolates represent all of the serotype 1 isolates received by SMPRL for the first 9 months of 2003. MLST was performed using previously described primers (32) and a semiautomated method (20). The sequence type of each serotype 1 isolate was designated according to the MLST website (http://www.mlst.net). Isolates were from various Scottish Health Boards and from a wide patient age range (Table 1), minimizing the study of clonally identical isolates from possible outbreaks. Out of the 28 isolates from 2003, 25 were available for further study. This resulted in a collection of 29 serotype 1 samples plus *S. pneumoniae* D39 (serotype 2), which was included as our laboratory reference strain.

Bacterial strains, storage, and growth conditions. The serotype 1 pneumococcal strains used in this study are listed in Table 1. Strains were grown in brain

TABLE 2. Primers used for DNA sequencing of the pneumolysin gene and cloning of 00-3645 pneumolysin

Primer	Sequence (5′ to 3′)
4V	CAATACAGAAGTGAAGGCGG
4T	GTTGATCGTGCTCCGATGAC
4W	GATCATCAAGGTAAGGAAGTC
27R	CTTGGCTACGATATTGGC
27S	TACTTAGTCCAACCACGG
27T	ATAAGTCATCGGAGCACG
9Y	CGGGATCCGGCAAATAAAGCAGTAAATGACTTT[a]
9Z	GACGGAGCTCGACTAGTCATTTTCTACCTTATC[b]

[a] The BamHI restriction site is underlined.
[b] The SacI restriction site is underlined.

heart infusion broth (BHI; Oxoid, Basingstoke, United Kingdom) and stored as frozen stocks with Protect beads (Technical Service Consultants, Lancashire, United Kingdom). For genomic DNA (gDNA) preparations, cells were grown statically overnight in 10 ml BHI at 37°C. Cultures were harvested by centrifugation for 10 min at 5,000 × *g* using a benchtop centrifuge. DNA was extracted using QIAGEN Midi columns (QIAGEN, West Sussex, United Kingdom), and the quality and quantity of DNA were assessed by agarose gel electrophoresis.

Amplification of *ply* gene by PCR and DNA sequencing. Pneumococcal *ply* genes (1.4 kb) were amplified by PCR from gDNA preparations using *Taq* polymerase (Promega, Southampton, United Kingdom) and primers 27R and 27S (Table 2) to give an amplicon size of 2,406 bp. PCR products were cleaned using PCR purification columns (QIAGEN). DNA sequencing was performed by DBS Genomics (Durham, United Kingdom) using primers 27R, 4T, 4V, 4W, 9Y, and 27T (Table 2). Sequence data for each *ply* gene were assembled, aligned, and translated using Vector NTI software (Invitrogen, Paisley, United Kingdom). DNA sequences have been deposited at NCBI under accession numbers DQ251177, DQ251178, DQ251179, DQ251180, and DQ251181.

Preparation of cell extracts from serotype 1 *S. pneumoniae* isolates. Single colonies of each isolate were selected from blood agar base plates (Oxoid, Basingstoke, United Kingdom) supplemented with 5% horse blood (E & O Laboratories, Bonnybridge, United Kingdom) and grown to mid-log phase in 15 ml or 50 ml BHI. Ten milliliters of each 15-ml culture and 40 ml of each 50-ml culture were centrifuged at 3,000 × *g* for 20 min at 4°C, and the cell pellet was stored overnight at −20°C. The cell pellets were resuspended in 1.5 ml 1× phosphate-buffered saline (PBS; Oxoid) and sonicated on ice using a high-intensity ultrasonic processor (Jencons Scientific Ltd., Bedfordshire, United Kingdom). Cell lysates were centrifuged at 20,000 × *g* for 30 min at 4°C. The soluble fraction (cell extract) was transferred to a separate tube, and the insoluble fraction (pellet) was washed in PBS and resuspended in 1.5 ml PBS. The total protein content of the cell extract and pellet was determined using Bradford's assay (7). Cell extract samples were diluted to either 300 μg/ml total protein or 2.35 mg/ml total protein for the concentrated samples to give a baseline from which pneumolysin expression levels and hemolytic activity could be measured.

Hemolytic activity of pneumolysin in clinical isolates. Cell extract from the serotype 1 lysates was assessed by a hemolysis assay (68) using a 2% (vol/vol) human blood suspension (Scottish National Blood Transfusion Service) in 1× PBS.

Western blotting. Pneumolysin was detected using standard Western blotting techniques. Briefly, samples were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, which were transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, United Kingdom) and blotted for 90 min at 80 V. Blots were incubated in 3% skim milk with polyclonal rabbit antipneumolysin serum (54), washed four times in Tris-NaCl (pH 7.4), and then incubated with anti-rabbit horseradish peroxidase (HRP)-linked antibody (Amersham Biosciences) in 3% skim milk, washed four times, and developed with 30 mg 4-chloro-naphthol (Sigma, Haverhill, United Kingdom) dissolved in 10 ml methanol plus 30 μl H_2O_2 (Sigma) in 40 ml Tris-NaCl, pH 7.4. The reaction was stopped with distilled water.

Detection of pneumolysin expression by ELISA. The pneumolysin enzyme-linked immunosorbent assay (ELISA) developed by Cima-Cabal et al. (12) was used to quantify pneumolysin expression from the serotype 1 isolates with the following modifications: wells were coated with 100 μl of 2.5 μg/ml capture antibody, PLY 7 (15). Plates were blocked with 10% fetal calf serum (Invitrogen) in PBS; washes were with 0.05% Tween 20 (Sigma) in 1× PBS and dilutions were made in assay buffer (blocking buffer plus 0.05% Tween 20). Pneumococcal cell extracts were diluted to 1:500 for the 300 μg/ml total protein samples (or 1:2,500 for the 2.35-mg/ml total protein samples) and added in duplicate at 100 μl/well. Purified pneumolysin, prepared as described previously (54), was used to provide a standard curve with a range from 2,000 pg/ml to 31.25 pg/ml. One hundred microliters/well rabbit polyclonal antipneumolysin antibody (54) was then added at a 1:2,000 dilution in assay buffer, and plates were shaken for 30 min at 37°C. Following four washes, 100 μl/well of biotinylated anti-rabbit immunoglobulin G (IgG) (Amersham Biosciences) was added at 1:500 and the mixture was shaken for 30 min at 37°C. Plates were washed four times and incubated with a 1:2,000 dilution of streptavidin-HRP (KPL, Gaithersburg, Md.) and developed with tetramethylbenzidine substrate (KPL) according to the manufacturer's instructions.

Analysis of cell binding and pore formation. Pneumococcal cell extracts were incubated with an equal volume of 2% erythrocyte suspension, washed to remove unbound toxin, and run on SDS-PAGE for Western blotting with antipneumolysin antibody. This allowed analysis of pneumolysin's ability to bind to erythrocytes as previously described (57). Transmission electron microscopy was used to

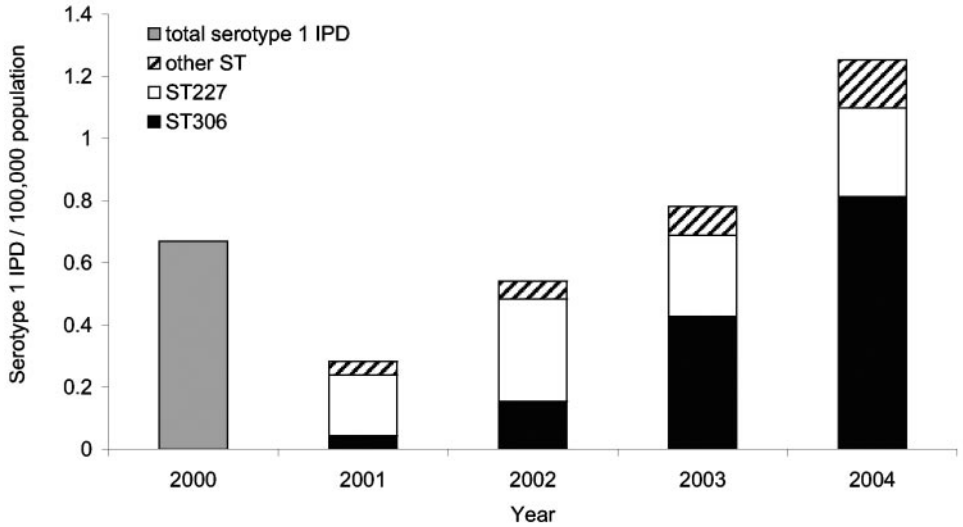

FIG. 1. Incidence of serotype 1 IPD in Scotland from 2000 to 2004. MLST data for 2001 to 2004 allow the sequence type distribution within serotype 1 isolates to be recorded. Black bars represent ST306, white bars represent ST227, and diagonally striped bars represent all other sequence types. The gray bar represents the total serotype 1 IPD in the year 2000.

assess the toxin's ability to form pores on host cell membranes. Briefly, cell extract from two strains representing two pneumolysin alleles was prepared as described earlier and then filtered using 0.2-μm syringe filters (Sartorius, Goettingen, Germany). Equal volumes of cell extract containing equivalent total protein levels were mixed with a 2% erythrocyte suspension and incubated for 30 min at 37°C. The cells were lysed and membranes were washed five times with distilled water to remove unbound toxin. The membranes were then resuspended in a quarter of the total volume. Five microliters of sample was placed onto glow-discharged carbon-coated grids and negatively stained with NanoVan (Nanoprobes, Yaphank, N.Y.) according to the manufacturer's instructions. Grids were viewed at a magnification of ×25,000 using an LEO 912 energy filter transmission electron microscope.

Expression of recombinant 00-3645 pneumolysin. The *ply* gene was amplified by PCR from gDNA of strain 00-3645 using primers 9Y and 9Z, which encode restriction enzyme sites for subsequent digests (Table 2). The 00-3645 pneumolysin PCR product (1,418 bp) was cut with BamHI and SacI (Promega) and ligated into BamHI/SacI-digested pET33b (Merck Biosciences, Nottingham, United Kingdom) to give pETply00-3645. This plasmid was transformed into *Escherichia coli* XL-1 cells (Stratagene, Amsterdam, The Netherlands), and the sequence of the insert was confirmed. The plasmid was then transformed into *E. coli* BL21(DE3) (Stratagene) for expression of 00-3645 pneumolysin as a histidine-tagged fusion protein. Recombinant 00-3645 pneumolysin (rPly00-3645) was expressed in HySoy J medium (Sigma) with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma) induction. Cells were harvested at 4,000 × *g* and disrupted using a One Shot cell disruptor (Constant Systems Ltd., Warwick, United Kingdom). The cell lysate was then centrifuged as described earlier for the pneumococcal lysates. The insoluble membrane fraction was resuspended in 1× PBS, to the same volume as the soluble fraction, for analysis of protein expression by SDS-PAGE, Western blotting, and hemolytic activity.

Infection of mice with serotype 1 pneumococci. Female C3-deficient mice ($C3^{-/-}$) (71) bred in house) and outbred MF1 mice (Harlan Olac, Bicester, United Kingdom) were used at approximately 9 weeks of age. Three serotype 1 strains were chosen for in vivo analysis: 00-3645, 01-1956, and 01-2696 (Table 1). To obtain bacteria virulent for mice, pneumococci were passaged intraperitoneally through mice as previously described (1) and stored at −80°C. To compare virulence of the strains during pneumococcal pneumonia, mice were lightly anesthetized with 1.5% (vol/vol) halothane, and 10^7 CFU were introduced intranasally in a 50-μl volume of fetal calf serum (Invitrogen). Signs of disease were frequently monitored until the animals became moribund (35), when they were humanely sacrificed. Mice displaying no signs of illness by 336 h were considered to have survived the infection. Blood samples were removed 24 h postintranasal infection from a superficial tail vein for bacterial load analysis (35). All in vivo experiments were carried out in accordance with UK Animals (Scientific Procedures Act) 1986.

RESULTS

The incidence rate of serotype 1 IPD in Scotland has increased from 0.67 cases/100,000 population in 2000 to 1.25 cases/100,000 population in 2004 (Fig. 1). It is important to note that surveillance in Scotland improved in 2001, resulting in 90 to 95% of all cases in Scotland being reported to SMPRL. The incidence of ST306 serotype 1 IPD increased from 0.04/100,000 population in 2001 to 0.813/100,000 population in 2004, whereas the incidence of ST227 remained fairly unchanged with 0.196 cases/100,000 population in 2001 and 0.286 cases/100,000 population in 2004 (Fig. 1). In 2003, there were 41 cases of serotype 1 IPD in Scotland (48), resulting in an incidence rate of 0.76/100,000 population. MLST for the 2003 serotype 1 isolates revealed that 23 of these isolates were ST306 and 13 were ST227, and there were 5 others (ST217 [1 isolate], ST1310 [2 isolates], ST1247 [1 isolate], and ST1239 [1 isolate]). Our study sampled the isolates from the first 9 months of 2003, of which there were 28 cases. Twelve of the 28 were ST227, and 16 were ST306. From this group size of 28, 25 were available for further study. This gave a final sample group of 29 isolates (25 from 2003 and 4 from 2000 to 2001): 16 were ST306, and 13 were ST227 (Table 1). ST306 (allelic profile 12 8 13 5 16 4 20) is a double-locus variant of ST227 (allelic profile 12 5 13 5 17 4 20), sharing five of the seven housekeeping genes assessed by MLST.

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        1                                                   50
PlyD39  MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK
Ply227  MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK
Ply306  MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK

        51                                                  100
PlyD39  RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS
Ply227  RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS
Ply306  RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS

        101                                                 150
PlyD39  IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY
Ply227  IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY
Ply306  IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQH

        151                                                 200
PlyD39  EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY
Ply227  EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY
Ply306  EKITAHSMEQ LKVKFGSDFE KIGNSLDIDF NSVHSGEKQI QIVNFKQIYY

        201                                                 250
PlyD39  TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL
Ply227  TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL
Ply306  TVSVDAVKNP GDVFQDTVTV EDLRQRGISA ERPLVYISSV AYGRQVYLKL

        251                                                 300
PlyD39  ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA
Ply227  ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA
Ply306  ETTSKSDEVE AAFESLIKG. .VAPQTEWKQ ILDNTEVKAV ILGGDPSSGA

        301                                                 350
PlyD39  RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY
Ply227  RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY
Ply306  RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY

        351                                                 400
PlyD39  VETKVTAYRN GDLLLDHSGA YVAQYYITWD ELSYDHQGKE VLTPKAWDRN
Ply227  VETKVTAYRN GDLLLDHSGA YVAQYYITWN ELSYDHQGKE VLTPKAWDRN
Ply306  VETKVTAYRN GDLLLDHSGA YVAQYYITWD ELSYDHQGKE VLTPKAWDRN

        401                                                 450
PlyD39  GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEWWRTVY EKTDLPLVRK
Ply227  GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEWWRTVY EKTDLPLVRK
Ply306  GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEWWRTVY EKTDLPLVRK

        451                470
PlyD39  RTISIWGTTL YPQVEDKVEN D
Ply227  RTISIWGTTL YPQVEDKVEN D
Ply306  RTISIWGTTL YPQVEDKVEN D
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FIG. 2. Alignments of the amino acid sequence for ST227 and ST306 pneumolysin compared with the D39 pneumolysin sequence. The mutations are highlighted in gray. ST227 pneumolysin differs from D39 pneumolysin by 1 amino acid substitution (D380N). ST306 pneumolysin differs from D39 pneumolysin by 4 amino acid substitutions (Y150H, T172I, K224R, and A265S) and 2 amino acid deletions (V270K271).

The *ply* genes from the 29 serotype 1 pneumococci were sequenced and analyzed alongside the pneumolysin sequence from the serotype 2 laboratory strain D39 (68). At the DNA level, five different alleles of the *ply* gene were present in this collection; on translation, this resulted in two protein alleles. The two pneumolysin protein alleles correlated with the ST of the serotype 1 pneumococci in that all isolates of ST306 (n = 16) contained mutations at six amino acid positions (Y150H, T172I, K224R, A265S, ΔV270, and ΔK271) when compared with the D39 pneumolysin sequence (Fig. 2). Five of these mutations, T172I, K224R, A265S, ΔV270, and ΔK271, have previously been reported in serotype 7 and 8 strains (45). We refer to this protein allele as Ply306. Twelve of the 13 isolates of ST227 differed from the D39 sequence at only one residue, D380N. We refer to this protein as Ply227 (Fig. 2). One ST227 isolate, strain 00-3645, was found to have a 24-bp insertion in

the *ply* gene, which was a direct repeat of the preceding sequence and resulted in an 8-amino-acid insertion in the protein (NVRNLLKG, at residues 417 to 424).

Hemolytic assays were carried out on lysates from the 29 serotype 1 pneumococcal isolates and D39 (Table 1). Fifty-five percent (16/29) of the serotype 1 isolates produced pneumolysin that did not lyse human erythrocytes: all of these isolates were ST306. Lysates prepared from serotype 1 pneumococci of ST227 and the positive control, D39, were hemolytic. Western blots were run with the cell extract from each sample, and all, except 00-3645, were positive for pneumolysin expression (data not shown). A pneumolysin ELISA revealed that all ST306 nonhemolytic lysates contained titers comparable to, and in some cases higher than, those of lysates expressing hemolytically active pneumolysin (Table 1). The specific activity of pneumolysin can be calculated from the hemolytic activity and expression levels to give the number of hemolytic units (HU) per mg of toxin. The specific activities of pneumolysin produced by D39 and ST227 isolates were similar; however, pneumolysin expressed by ST306 strains had no specific activity (Table 1). Pneumolysin expression by strain 00-3645 was not detectable by ELISA. Large-scale preparations were made of representative ST306 (01-1956) and ST227 (01-2696) strains and also of 00-3645 to give samples that were eight times more concentrated. Extracts of strain 01-1956 (ST306) contained 6.4 μg/ml pneumolysin, which had no hemolytic activity, and extracts of strain 01-2696 (ST227) contained 5.8 μg/ml pneumolysin with a specific activity of 2.8×10^7 HU/mg. Pneumolysin expression by 00-3645 was still not detected in the concentrated sample; however, a faint band was detected by Western blotting with antipneumolysin antibody. The insoluble fraction from 00-3645 lysis was negative for the presence of pneumolysin by Western blotting and ELISA. Although the levels of pneumolysin expression from 00-3645 were too low for ELISA detection, the cell extract from this strain was found to have residual hemolytic activity in the first well of the hemolytic assay. Recombinant expression of 00-3645 pneumolysin was achieved, but it was almost exclusively found in the insoluble cell fraction. The r00-3645Ply that was soluble possessed some hemolytic activity but at levels ~100 times lower than that of recombinant "wild-type" pneumolysin.

Pneumolysin from both ST306 and ST227 bound to erythrocytes; however, ST306 pneumolysin did not bind as well (data not shown). Analysis of pore formation by transmission electron microscopy revealed that pneumolysin expressed by ST306 was unable to form pores on erythrocyte membranes (Fig. 3b) in comparison with ST227 pneumolysin (Fig. 3a). Arcs were observed on membranes treated with cell extract from both STs, but the Ply 306 toxin did not assemble to form functional pores in the host cell membrane. PBS-treated membrane was included as a negative control and was similar in appearance to ST306-treated membrane without the presence of arc structures.

Intraperitoneal injection of MF1 mice with 5×10^7 CFU 00-3645 did not result in infection. In order to establish infection, complement-deficient $C3^{-/-}$ mice were given the same dose but still did not succumb to infection. The mice did not display any clinical signs of illness following challenge. Thus, 00-3645 was deemed to be avirulent in mice. In contrast, intraperitoneal injection of 5×10^7 CFU 01-1956 (ST306) or 01-2696 (ST227) resulted in bacteremia within 24 h in MF1 and $C3^{-/-}$ mice. Intranasal inoculation of MF1 mice with 5×10^7 CFU 01-1956 or 01-2696 resulted in transient signs of infection in all animals. These signs ranged from a hunched stance to lethargy, but all animals survived the challenge. At 24 h postchallenge, 3/5 mice inoculated with either 01-2696 or 01-1956 were bacteremic. There was no difference in the levels of bacteremia caused by 01-2696 or 01-1956.

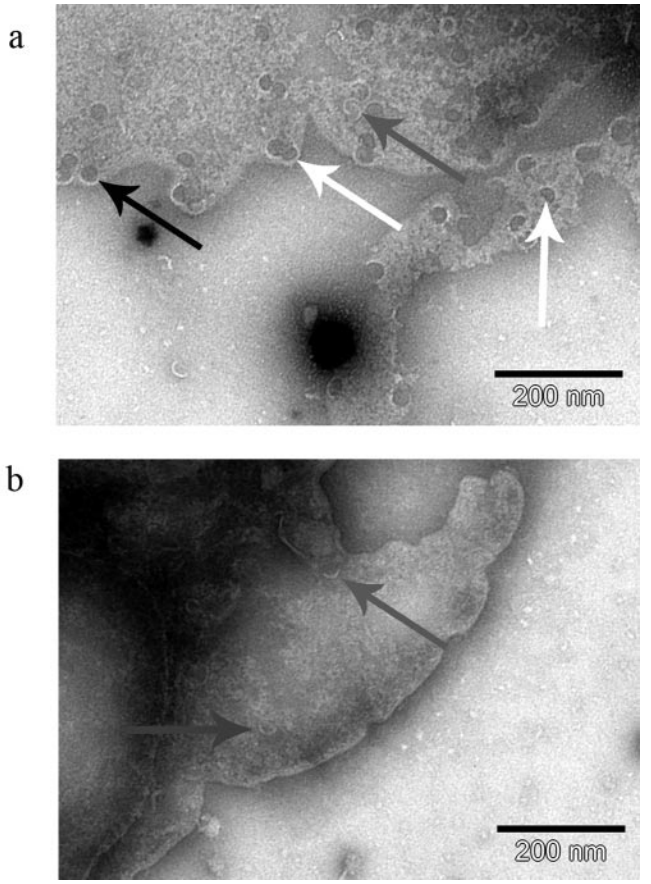

FIG. 3. Transmission electron microscopy of negatively stained erythrocyte membrane treated with filtered cell extract from an ST227 isolate (a) or cell extract from an ST306 isolate (b). Original magnification, ×25,000. Bar, 200 nm. The black arrow indicates a pore, white arrows show arcs that are inserted into the membrane, and gray arrows highlight arcs of toxin that have not inserted into the membrane.

DISCUSSION

Serotype 1 has remained one of the most prevalent IPD-causing serotypes and has recently been ranked among the top five IPD-causing serotypes in England, Germany, Spain, Quebec, Chile, and South Africa (11). Data collected in Scotland from 1999 to 2001 ranked serotype 1 as the 10th most prevalent serotype, causing 3.5% of IPD (43). When data from 2002 were included, serotype 1 ranked 9th, causing 3.8% of IPD in Scotland (17), and by 2003, serotype 1 ranked as the 4th most prevalent serotype, causing 6.7% of IPD (48). MLST data reveals that this increase in serotype 1 disease in Scotland, beginning in 2001, is from the expansion of ST306 (Fig. 1). This

has previously occurred in Sweden, where serotype 1-caused IPD increased from 1% in 1992 to 10% in 1997, and this was shown to be solely due to the emergence of ST306 (28).

ST227 and ST306 are from the same lineage and clonal group (11) and are almost exclusive to serotype 1 pneumococci, although ST227 has been shown to switch capsule (32). ST306 IPD is predominant in continental Europe, whereas ST227 is the most prevalent serotype 1 ST in England, North America, and Canada (11). The reasons for this distribution are unclear, and larger sample groups would be required for such analysis. Brueggemann and Spratt (11) explained the differences in ST distribution between England and the rest of Europe to be a result of the low carriage rate of serotype 1, which thereby reduces the probability of transfer of clones between populations in comparison with more carriage-associated serotypes. We were surprised by the ST distribution between England and Scotland, with ST306 only present in the Scottish samples. Isolates in the two studies were from different years, and it may be that ST306 is now also found in England; however, there are no ST306 isolates from England in the MLST database. We are possibly witnessing the clonal expansion of serotype 1 with the emergence of ST306 in Scotland.

All of the ST306 isolates were shown to have mutations in their *ply* gene that resulted in the expression of nonhemolytic pneumolysin, yet these clinical isolates were from patients with IPD. Despite serotype 1 pneumococci being traditionally highly virulent in mice (41), the strains in this study were found to cause mild disease in MF1 mice. For this reason, high infectious doses were used in infection studies. Strain 00-3645, expressing low levels of pneumolysin, was avirulent in both wild-type and immune-compromised $C3^{-/-}$ mice. Intraperitoneal injection of strains 01-2696 and 01-1956, producing wild-type and nonhemolytic pneumolysin, respectively, caused terminal septicemia. These strains were less virulent intranasally, causing only transient infection. There was no difference observed in the survival times or 24-h bacteremia of mice infected with either ST227 (strain 01-2696) or ST306 (01-1956). Thus, the hemolytic activity of pneumolysin does not have an effect on the virulence of these serotype 1 isolates in our model. This correlates with the fact that all of the strains analyzed caused IPD in patients irrespective of pneumolysin phenotype.

Pneumolysin has long been known as an important virulence factor of the pneumococcus, but a number of studies with a *ply*-knockout mutant of *S. pneumoniae* have revealed that the toxin is not essential for pneumococcus-induced inflammation during meningitis (22, 70, 74). D39 *S. pneumoniae* carrying the *ply* gene was found to cause acute sepsis in mice following intravenous challenge compared with chronic bacteremia if the *ply* gene was absent (3). Studies with defined point mutations in the *ply* gene showed that the hemolytic activity of pneumolysin was important during sepsis (5). The hemolytic property of pneumolysin has also been shown to be involved in the initial stages of pneumonia, including invasion of the lung tissue and neutrophil recruitment; however, hemolytic activity is not important in facilitating pneumococcal growth in the alveoli (62, 63). As serotype 1 disease is associated with pneumonia rather than meningitis in Europe and the United States (26, 30) (Table 1), it may be that hemolytically active pneumolysin is not essential for the pathogenesis of certain serotype 1 sequence types, such as ST306. This may mean that there will be less selective pressure to conserve the nucleotide sequences that encode the hemolytic activity of pneumolysin and may allow the mutations we have identified to arise. Recently, an increase in pneumococcal meningitis in Ghana has been attributed to serotype 1 clonal complex ST217 (44), which the authors propose may be better equipped to cause meningitis. ST227 and ST306 were not found in the Ghana study, but it would be interesting to analyze the *ply* gene from serotype 1 isolates that have caused meningitis, in particular ST217, to see if the Ply227 allele is required. This highlights the need for closer investigation of not only serotype-specific virulence factors but the possibility of variation within serotypes and underlines the importance of MLST surveillance.

Malley and colleagues (46) have shown that pneumolysin interacts with TLR-4. Using a pneumolysin mutant, the presence of pneumolysin but not its hemolytic activity was shown to be important for activation of TLR-4-dependent inflammatory responses. This interaction between TLR-4 and pneumolysin is thought to be essential in allowing pneumococcal colonization of the host but preventing progression to invasive disease. However, it is not clear what happens when the host gets IPD. In the same study, mice lacking functional TLR-4 were more likely to succumb to type 3 pneumococcal infection than mice with functional TLR-4. It is possible that TLR-4 interaction with pneumolysin helps to retain the pneumococci in the nasopharynx due to subtle inflammatory responses; however, this may become irrelevant if the bacteria can reach the lower respiratory tract. As ST227 and ST306 both cause IPD, hemolytic activity appears to be irrelevant and some other activity of pneumolysin may be important for causing disease. Strains carrying pneumolysin lacking hemolytic activity and complement binding activity are still more virulent than strains with the *ply* gene knocked out, indicating that pneumolysin has an additional function yet to be determined (4).

The majority of the mutations observed in Ply306 are those previously implicated in the reduction of hemolytic activity for certain serotype 7 and serotype 8 strains (45). These mutations have not, until now, been associated with serotype 1 strains. Further analysis of these serotype 7 and 8 strains revealed that replacement of threonine with isoleucine at residue 172 was responsible for reducing the hemolytic activity of the pneumococcal lysates from 10^6 HU/mg pneumolysin to 10^4 HU/mg pneumolysin and was also attributable for anomalously slow migration of the toxin on SDS-PAGE gels. From more recent studies, the T172I mutation probably affects insertion of domain 3 of the toxin into the host cell membrane (60, 61). Ply306 also ran anomalously on SDS-PAGE (data not shown), but in contrast with the serotype 7 and 8 isolates, there was no hemolytic activity observed even when the samples were concentrated. The abrogation of hemolytic activity of Ply306 is probably due to the combination of the T172I substitution with a further mutation, Y150H, not observed in the serotype 7 or 8 strains. The Y150H mutation in pneumolysin is comparable to the Y181A mutation made in the related toxin perfringolysin O (PFO) (29). PFO^{Y181A} is unable to form pores in cell membranes, thus reducing hemolytic activity to less than 1% activity of wild-type toxin. We propose that mutation of Y150 in pneumolysin to a histidine residue would have a similar effect. Indeed, we have replaced this residue in pneumolysin with an alanine; this reduced the hemolytic activity to 0.2% of wild-

type pneumolysin (data not shown). We have also deleted this residue and the adjacent glutamic acid (ΔY150E151) in pneumolysin, and this resulted in a nonhemolytic form of pneumolysin (L.-A. S. Kirkham et al., submitted for publication).

Ply306's reduced binding ability and therefore inability to form pores is likely to be a result of the combination of mutations in the *ply* gene. Functional pores were not observed on membranes treated with Ply306; however, arc structures were observed on the surface of membrane treated with Ply306 and Ply227 (Fig. 3). Arcs remain a subject of contention within cholesterol-dependent cytolysin research (67), and there is debate as to whether arcs can insert into the membrane and cause membrane damage. Here, Ply306 was not found to be cytolytic and thus may provide evidence that arcs are nonfunctional structures formed by the toxin.

The 8-amino-acid insertion present in pneumolysin from strain 00-3645 occurs just upstream of the highly conserved undecapeptide region involved in membrane binding (31). A single amino acid substitution in this region reduces the hemolytic activity of pneumolysin by 100-fold (40). The residual hemolytic activity of r00-3645Ply indicates that it can bind to eukaryotic cells and form pores but not as effectively as the native toxin. This residual activity may mean that the 8-amino-acid repeat, which results in the extension of the loop involved in host cell binding, does not completely disrupt folding and therefore functioning of the toxin. Due to the low levels of expression, pore formation was not investigated with the 00-3645 strain.

The fact that pneumolysin may be less conserved than previously assumed should not affect its use as the protein component of next generation conjugate vaccines. Antibodies raised against wild-type pneumolysin still recognized the pneumolysin expressed by all serotype 1 pneumococci in Western blots and ELISA.

The Ply306 allele may be of some benefit to the pneumococci, possibly prolonging carriage in the nasopharynx compared with other serotype 1 STs. Indeed in Sweden, ST306 was found in the nasopharynx of two healthy siblings when ST306 was at its most prevalent (64) and was suggested to either be adapting to colonization or possibly the short carriage time of this ST had been captured. We suggest that the increase in ST306 observed in Scotland and Sweden may be due to an increased ability to colonize the host; however, there are insufficient MLST data of serotype 1 isolates from carriage, and we do not know whether the *ply* gene of the Swedish ST306 isolates carries the same mutations. We may be witnessing some serotype 1 strains evolving to become less invasive and more carriage associated. This highlights the requirement for global MLST surveillance of both carriage- and IPD-associated pneumococci.

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