INFECTION AND IMMUNITY, June 1999, p. 2776–2782 Vol. 67, No. 6
0019-9567/99/$04.00+0

Molecular Characterization of Equine Isolates of *Streptococcus pneumoniae*: Natural Disruption of Genes Encoding the Virulence Factors Pneumolysin and Autolysin

ADRIAN M. WHATMORE,[1]* SAMANTHA J. KING,[1] NEIL C. DOHERTY,[1] DANIEL STURGEON,[1] NEIL CHANTER,[2] AND CHRISTOPHER G. DOWSON[1]

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL,[1] and Animal Health Trust, Newmarket CB8 7DW,[2] United Kingdom

Received 6 January 1999/Returned for modification 12 February 1999/Accepted 5 March 1999

Although often considered a strict human pathogen, *Streptococcus pneumoniae* has been reported to infect and cause pneumonia in horses, although the pathology appears restricted compared to that of human infections. Here we report on the molecular characterization of a group of *S. pneumoniae* isolates obtained from horses in England and Ireland. Despite being obtained from geographically distinct locations, the isolates were found to represent a tight clonal group, virtually identical to each other but genetically distinguishable from more than 120 divergent isolates of human *S. pneumoniae*. A comprehensive analysis of known pneumococcal virulence determinants was undertaken in an attempt to understand the pathogenicity of equine pneumococci. Surprisingly, equine isolates appear to lack activities associated with both the hemolytic cytotoxin pneumolysin, often considered a major virulence factor of pneumococci, and the major autolysin gene *lytA*, also considered an important virulence factor. In support of phenotypic data, molecular studies demonstrated a deletion of parts of the coding sequences of both *lytA* and *ply* genes in equine pneumococci. The implications of these findings for the evolution and pathogenicity of equine *S. pneumoniae* are discussed.

Streptococcus pneumoniae is a common and important human pathogen causing a wide variety of diseases, including pneumonia, otitis media, meningitis, and bacteremia. *S. pneumoniae* was reported to be pathogenic in horses as long ago as 1928 (22). Since that time there have been sporadic reports of isolations of *S. pneumoniae* from the equine respiratory tract (1, 2, 11), although until recently, there was little direct evidence associating these isolates with disease. However, *S. pneumoniae* has been isolated from pneumonic foals (17, 29) and has been associated with lower airway disease in training thoroughbreds (4, 16). Blunden et al. (3) provided the first direct evidence that *S. pneumoniae* can act as a primary pathogen in horses. Experimental infection of ponies was shown to result in the development of clinical respiratory disease with fever, cough, and ocular and nasal discharge, and evidence of a lobar pneumonia, from which *S. pneumoniae* could be isolated in pure culture or demonstrated by immunostaining, was seen post mortem. The disease experimentally reproduced in ponies by *S. pneumoniae* challenge (3) was less severe than the typical lobar pneumonia seen in humans, although the histopathological features of the lesions were very similar. However, natural disease can vary in severity. A more severe pneumonia with bacteremia is infrequently seen in foals (17), whereas upper and lower respiratory tract inflammation is more commonly observed in thoroughbreds, mainly affecting 2 year olds, where it compromises training (29). There is no evidence of otitis media caused by *S. pneumoniae* in the horse.

Little is known regarding the relationship of equine *S. pneumoniae* isolates to their human counterparts. Interestingly, all equine isolates reported to date are of capsular serotype 3 (5), but there is no data regarding the relationship of these strains to human serotype 3 pneumococci. It is still unclear whether equine isolates can infect humans, whether human isolates can infect horses, or whether equine isolates form an entirely distinct population. However, only *S. pneumoniae* serotype 9 was isolated from the staff of a training yard that contained *S. pneumoniae*-infected horses (4). To address these issues, we have performed a preliminary molecular characterization of equine pneumococcus isolates in order to examine the genetic and evolutionary relationships of various equine *S. pneumoniae* isolates among themselves and to human *S. pneumoniae* isolates and to examine the molecular basis of the pathogenicity of this bacterium in horses.

* Corresponding author. Mailing address: Dept. of Biological Sciences, University of Warwick, Coventry, CV4 7AL, United Kingdom. Phone: 44-1203-528359. Fax: 44-1203-523701. E-mail: a.m.whatmore@warwick.ac.uk.

MATERIALS AND METHODS

Culture conditions. Isolates were cultured on brain heart infusion agar supplemented with 5% sheep blood at 37°C and 5% CO_2. The species designations of isolates were confirmed by serotyping and optochin susceptibility and by a species-specific 16S RNA gene probe (GenProbe Inc., San Diego, Calif.).

Strains. The equine *S. pneumoniae* strains used in this study, isolated from either the nasopharynx of horses with upper respiratory tract infection or the tracheal washes of horses showing poor training performance, are listed in Table 1. The details of human serotype 3 isolates, used in restriction fragment length polymorphism (RFLP) analysis of housekeeping genes, are given in Table 2. Ten human isolates of *S. pneumoniae*, selected on the basis of a population genetic study (21) to represent divergent members of the species, were used in phylogenetic analyses. The strains, geographic origins, sites and years of isolation, and serotypes of these isolates were as follows: 1012, Manchester (United Kingdom), throat, 1993, 35; Sp8, Spain, blood, 1988, 13; E226, Uruguay, blood, 1995, 1; Sp9, Spain, blood, 1988, 8; KD12, Kenya, sputum, 1991, 7; 951, Oxford (United Kingdom), throat, 1994, 6A; 912, Oxford (United Kingdom), throat, 1996, 15B; 873, Kenya, throat, 1990, 8; 1011, Manchester (United Kingdom), throat, 1993, 23; and R6, United States, cerebrospinal fluid, 1930s, nonserotypeable. The type strains of *Streptococcus mitis* and *Streptococcus oralis*, NCTC12261 and NCTC11427, respectively, were also included in this analysis. The well-characterized *S. pneumoniae* strain D39 (NCTC7466), used as a control in hemolytic titer assays, is a reference serotype 2 strain.

Preparation of chromosomal DNA. Chromosomal DNA was prepared from all isolates used, including 10 diverse human isolates of *S. pneumoniae* and the type strains of *S. oralis* and *S. mitis*, as described previously (28).

Clonal analysis. RFLP analyses of both virulence factor- and housekeeping-protein-encoding genes were performed following the amplifications of the ap-

TABLE 1. Equine isolates used in this study[a]

Warwick strain no.	Supplier's strain no.	Yr isolated	Site of isolation	Country/county of isolation[e]	Source
495/Pn138	409	1993	Nasopharynx	United Kingdom/West Suffolk	N. Chanter[b]
496/Pn139	7731	1992	Nasopharynx	United Kingdom/West Suffolk	N. Chanter
497/Pn140	600142	1996	Nasopharynx	United Kingdom/West Suffolk	N. Chanter
498/Pn141	3307	1992	Nasopharynx	United Kingdom/West Sussex	N. Chanter
499/Pn142	4826	1992	Nasopharynx	United Kingdom/West Suffolk	N. Chanter
500/Pn143[d]	4778	1992	Tracheal wash	United Kingdom/East Suffolk	N. Chanter
501/Pn144	5330	1992	Nasopharynx	United Kingdom/West Suffolk	N. Chanter
502/Pn145	21	1987	Tracheal wash	Ireland	J. F. Timoney[c]
503/Pn146	5	1987	Tracheal wash	Ireland	J. F. Timoney
504/Pn147	15	1987	Tracheal wash	Ireland	J. F. Timoney
505/Pn148	17	1987	Tracheal wash	Ireland	J. F. Timoney

[a] All strains tested were optochin sensitive and bound a 16S RNA, species-specific gene probe.
[b] Animal Health Trust, Newmarket, United Kingdom.
[c] Gluck Equine Research Center, University of Kentucky.
[d] Isolate 500 was not examined in RFLP studies of housekeeping genes and virulence factors.
[e] All West Suffolk isolates originated from distinct stables.

propriate products by PCR. For housekeeping gene analysis, products representing *hexA* (DNA mismatch repair protein), *recP* (transketolase), *polI* (DNA polymerase I), *hexB* (DNA mismatch repair protein), *ddl* (D-alanine-D-alanine ligase), and *trpB-trpA* (tryptophan biosynthetic cluster) were used. PCR products were obtained by using the following primer sets: *hexA*up (5′CCGACAATCCG ATCCCTATGG3′) and *hexA*dn (5′TATCAGCTGGTCCCGGTTCAATC3′), *recP*up (5′ACCGCGACCGCTTTATTCTTTC3′) and *recP*dn (5′ATGCTGAC TACGCGGGATTTTTC3′), *polI*up (5′TCGGGCCAAGACTCCTGATGA3′) and *polI*dn (5′CGCCTCCCGCACCACTTC3′), *hexB*up (5′CCATTGACGCGG GCTCTA3′) and *hexB*dn (5′CCTGAATACGTCGGAACATCTTT3′), *ddl*up (5′CTCCGGGGGCAAGAAGAC3′) and *ddl*dn 5′CAATGGCACGGAAGGC TGT3′, and *trpB*up (5′CTAGTAGCCTGTGTTGGT3′) and *trpA*dn (5′TCGAA CCGACGATAACAC3′). Sequences of *hexA*, *recP*, *polI*, and *hexB* were available in GenBank, while sequences of *ddl* and *trpB-trpA* were kindly provided by Martin Burnham of SmithKline Beecham. PCR products corresponding to virulence factor genes encoding two neuraminidases (*nanA* and *nanB*), the pneumococcal surface protein (*pspA*), and hyaluronidase (*hyl*) were obtained by using the primer sets *nanA*up (5′TCAACTTTCGGGGGAGAGC3′) and *nanA*dn (5′T GGAGCGAATTATAGGCAAACT3′), *nanB*up (5′TTCTTGTGTAGGCAT TAGTCTTTT3′) and *nanB*dn (5′ATCGCAGAATAGGCATAACCAT3′), *pspA*up (5′AAAGAGATTGATGAGTCTGA3′) and *pspA*dn (5′TTAAACCCA TTCACCATTGG3′), and *hyl*up (5′TGCAACGACGTCAGGAACAAAG3′) and *hyl*dn (5′ACGGAATAAATAAAACGCCCCAAGTA3′), respectively. PCR was performed under standard conditions with 32 cycles of 95°C for 1 min, X°C for 1 min, and 72°C for 1 min, where X°C represents an annealing temperature appropriate for the particular primer set used. PCR products were digested with a number of frequently cutting restriction enzymes as described below, and the resulting profiles were resolved on 4 and 8% polyacrylamide gels.

Phylogenetic analysis. Fragments carrying the housekeeping genes *hexB*, *recP*, and *xpt* (encoding xanthine phosphoribosyltransferase) were amplified by PCR by using the *hexB* and *recP* primer sets described above and the *xpt* primer set *xpt*up (5′GAAATTATTAGAAGARCGCATC3′) and *xpt*dn (5′TTAGAGAT CTGCCTCCWTRAA3′), respectively (where R and W are A or G and A or T, respectively), with standard PCR conditions. Fragments were purified through Qiagen PCR purification columns and sequenced directly by using the same primers and an ABI373 automated sequencing system. Sequences of 395 bp (*xpt*up), 288 bp (*hexB*up), 351 bp (*hexB*dn), 327 bp (*recP*up), and 339 bp (*recP*dn) were obtained from each isolate and combined for phylogenetic analyses performed with the MEGA software (15). Phylogenetic trees were constructed by the neighbor-joining method with the Jukes-Cantor correction, and the bootstrap confidence level of internal branches was calculated with 500 resamplings of the data.

Genetic and phenotypic analyses of the *ply* locus. Three primer sets, *ply*up (5′TTGTTGTTATCGAAAGAAAGAAGCGGA3′) and *ply*dn (5′AAACCGT ACGCCACCATTCCCA3′) (located within the pneumolysin-coding sequence [*ply*]), *ply*conup (5′CGAAAGAAAGAAGC3′) and *ply*condn (5′CGTACGCCA CCATT3′) (located outside *ply*), and *ply*degup (5′TGGMATSAIRAITAT3′) and *ply*degdn (5′CCACCATTCCCAIGC3′) (designed on the basis of conserved sequences identified within members of the thiol-activated toxin family) (where M, S, R, and I are A or C, C or G, A or G, and deoxyinosine, respectively), were used to attempt to amplify *ply* from equine pneumococci.

The presence of the *lytA-ply* fusion in equine isolates was confirmed by using primer C (5′TTGGGGGCGGTTGGAATGC3′), corresponding to bases 231 to 249 of the *lytA* coding sequence, in conjunction with primer INT (5′TGGTAG AGGACTTGATTCA3′), corresponding to bases 926 to 944 of *ply*.

Southern blot analyses were performed with the Boehringer digoxigenin (DIG) system according to the manufacturer's instructions. Pneumolysin probes, representing internal fragments of the *ply* gene (obtained by using primer set *ply*up and *ply*dn or *ply*up and hor*ply* [5′ATTATCCTCTACCGTTACAGT3′]), were obtained by using DIG-High Prime to label a *ply* fragment amplified from *S. pneumoniae* R6. These probes were used to probe *Eco*RV- and *Pvu*II-digested chromosomal DNA from equine pneumococci.

Hemolytic titer assays were performed by using cultures grown in brain heart infusion broth to an optical density at 600 nm (OD_{600}) of approximately 0.3. Cells were washed in phosphate-buffered saline (PBS) and resuspended to an OD_{600} of 0.6. The cell suspension was then sonicated and lysis of pneumococci was confirmed both by clearance of the cell suspension and by the absence of viable cells as determined by plating sonicant onto blood agar. Cell debris was removed by centrifugation, and serial doubling dilutions of lysate in PBS were prepared. A standard erythrocyte suspension was prepared by diluting washed cells in PBS to an OD_{600} of 0.8. For each assay, 40 μl of erythrocytes was added to 160 μl of lysate and incubated for 1 h at 37°C. Cell debris and unlysed erythrocytes were removed by centrifugation, and the degree of hemolysis was determined by measuring released heme spectrophotometrically at 410 nm. The reciprocal of the lowest dilution showing hemolytic activity was taken as the titer.

Analysis of *lytA* activity. Bile solubility tests and assays monitoring degradation of choline-labelled cell walls were performed as described previously (8, 12). Southern blot analyses were performed with the Boehringer DIG system according to the manufacturer's instructions. A probe was constructed by amplifying a *lytA* fragment from *S. pneumoniae* R6 by using primers *lytA*up (5′ GGAGTAG AATATGGAAATTGATGTGAGTAA3′) and *lytA*dn (5′TTTATTTTACTGT AATCAAGCCATCTGGCTC3′), and it was used to probe for the presence of *lytA* in *Eco*RV-digested chromosomal DNA.

Analysis of competence genes. PCR and the sequencing of genes in the *com* locus were performed as described previously (28).

Nucleotide sequence accession numbers. All sequences used in the phylogenetic analyses performed with MEGA software have been deposited in EMBL and assigned accession no. AJ240606 to AJ240674. The sequence of the *lytA* to

TABLE 2. RFLP analysis of housekeeping genes from equine and human clinical isolates of serotype 3 pneumococci isolated from the United Kingdom

Strain	Place of isolation	Allele designation of:					
		hexA	*recP*	*polI*	*hexB*	*ddl*	*trpB-trpA*
480	Portsmouth[a]	1	1	1	1	1	1
476 (GBO5)	Leicester[b]	2	2	2	2	2	2
471 (SPO1)	Leicester[b]	2	2	2	2	3	2
470 (WU2)	Leicester[b]	2	3	2	2	2	2
494	Liverpool[a]	2	4	3	2	3	3
483	Sheffield[a]	1	5	3	2	4	4
481	Tooting[a]	2	5	3	2	3	5
Equine isolates[c]		2	5	3	2	5	6

[a] Generously provided by A. Efstratiou, Central Public Health Laboratory, Colindale, United Kingdom.
[b] Generously provided by T. Mitchell, University of Glasgow, Glasgow, United Kingdom.
[c] All equine isolates gave identical RFLP profiles.

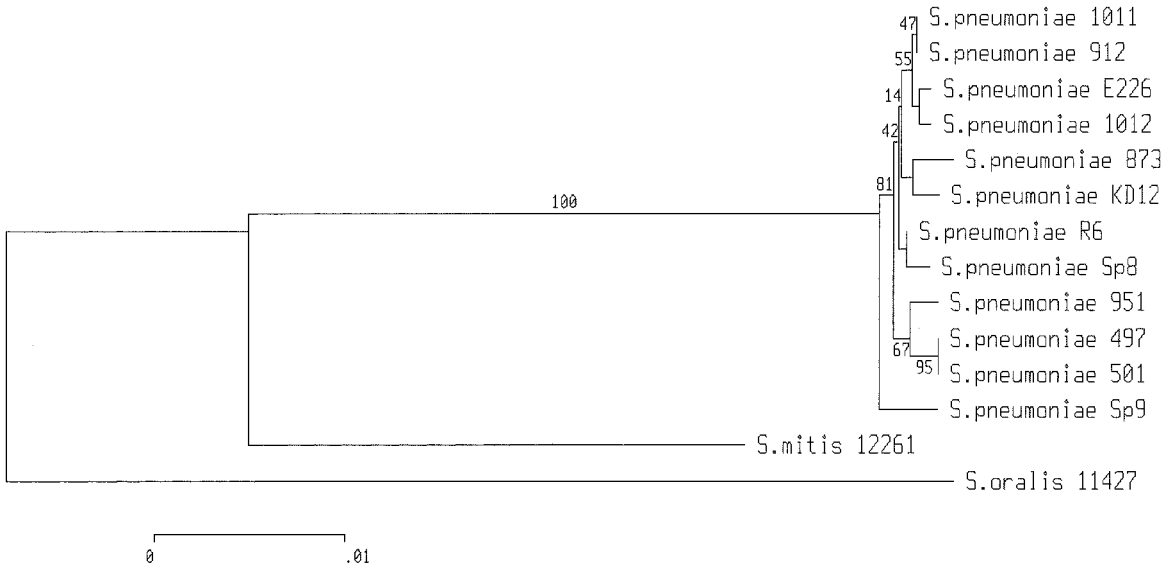

FIG. 1. Phylogenetic tree of the relationships between equine *S. pneumoniae* isolates 497 and 501, 10 diverse human *S. pneumoniae* isolates, and the type strains of *S. oralis* and *S. mitis*.

ply region of the equine pneumococcus has been assigned accession no. AJ240675.

RESULTS

Confirmation of species designation. All 11 isolates were examined on receipt, as summarized in Table 1, to confirm their presumptive identification as *S. pneumoniae*. When cultured on blood agar, all isolates were found to yield optochin-sensitive colonies with a highly mucoid appearance characteristic of serotype 3 pneumococcal isolates. All isolates were confirmed as *S. pneumoniae* by using the Accuprobe *S. pneumoniae* culture identification test, a highly sensitive test based on a probe to an rRNA gene (6). However, atypically for *S. pneumoniae*, all isolates were found to be bile insoluble.

Equine pneumococci represent a tight clonal group. In order to examine the relationships of the equine pneumococci to each other, PCR products representing six distinct housekeeping genes (*recP*, *polI*, *ddl*, *trpB-trpA*, *hexA*, and *hexB*) were obtained. Each PCR product was digested with at least four distinct frequently cutting restriction enzymes (*recP* was digested with *Tsp*509I, *Mnl*I, *Hin*fI, *Hsp*92II, and *Mae*III; *polI* was digested with *Alu*I, *Mse*I, *Mbo*II, *Hha*I, *Hin*fI, and *Dde*I; *ddl* was digested with *Hsp*92II, *Hae*III/*Dde*I, *Bsr*I, and *Mwo*I; *trpB-trpA* was digested with *Hin*fI, *Tsp*509I, *Mse*I, and *Hsp*92II; *hexA* was digested with *Alu*I, *Hha*I, *Hin*fI, *Tsp*509I, and *Hsp*92II; and *hexB* was digested with *Alu*I, *Sty*I, *Fok*I, *Mnl*I, *Bfa*I, and *Aci*I) and subjected to RFLP analysis. In all cases the equine isolates showed identical RFLP patterns, suggesting that equine pneumococci represent a tight clonal group. A group of seven human serotype 3 *S. pneumoniae* clinical isolates were subjected to the same RFLP treatment. These isolates were selected to represent genetically diverse strains on the basis of preliminary studies of genetic variation of neuraminidase-encoding genes and surrounding regions in over 20 serotype 3 isolates (14). The results of this analysis are shown in Table 2 with each variant allele, determined by RFLP studies using multiple restriction enzymes, given a distinct arbitrary number. All seven human serotype 3 isolates gave distinct overall allelic profiles when compared by allelic variation at six loci. Although the equine isolates contain *hexA*, *recP*, *polI*, and *hexB* alleles which are also seen in some of the human type 3 isolates, the *ddl* and *trpB-trpA* alleles are unique, resulting in an equine allelic profile distinct from that of any of the human serotype 3 isolates examined in this study.

Equine pneumococci are closely related to human isolates. In order to further examine the relationship of equine to human *S. pneumoniae*, phylogenetic analysis was performed with data obtained by sequencing fragments of three distinct housekeeping genes (*recP*, *hexB*, and *xpt*) amplified from equine isolates 497 and 501 and from a diverse range of human pneumococci. The type strains of *S. oralis* and *S. mitis* were also included in this analysis. All the *S. pneumoniae* isolates examined appeared to be closely related to each other, with the maximum nucleotide divergence between any two isolates being only 0.77%. In agreement with the RFLP data described above, the two equine *S. pneumoniae* isolates were found to be identical to each other. The equine nucleotide sequence is distinct from those of all the human isolates examined here, although it is still closely related, being between 0.29 and 0.65% divergent from any of the human isolates. In all, only two polymorphic sites (one in *xpt* and one in *recP*) were unique to the two equine pneumococcal isolates. Phylogenetic analysis performed with combined sequence data from the three housekeeping genes (Fig. 1) clearly places the equine isolates in a group including the human isolates of *S. pneumoniae*.

Analysis of virulence factors. (i) Pneumolysin. Pneumolysin, a thiol-activated cytotoxin, is generally considered to be an important virulence factor of *S. pneumoniae* (23). No PCR product could be amplified from any of the equine isolates by using the primer set *ply*up and *ply*dn, previously found to amplify *ply* from all examined human *S. pneumoniae* isolates. In order to allow for possible polymorphism in equine *ply*, alternative primer sets located outside the pneumolysin-coding sequence and degenerate primers were designed based on the consensus sequence of the thiol-activated toxin group. However, the use of these additional primer sets and all possible combinations of the three primer sets under nonstringent annealing conditions resulted in the amplification of only nonspecific PCR products.

In light of the failure to detect *ply* by PCR, hemolytic activity

TABLE 3. Comparison of hemolytic titers of human and equine pneumococci

Strain	Source	Hemolytic titer in blood from:	
		Human	Horse
480	Human	>4,096	256
D39	Human	>4,096	512
504	Horse	0	0
497	Horse	0	0
501	Horse	0	0
500	Horse	0	0

assays were performed to determine whether the isolates also lack the normal pneumolysin phenotype. Sonicated extracts of four equine isolates (497, 500, 501, and 504) and two human isolate controls (D39 and human serotype 3 clinical isolate 480) were tested for hemolytic activities against horse and human blood (Table 3). There was no evidence of hemolytic activity associated with the equine isolates when extracts were tested against either horse or human blood, while, in contrast, the two human isolates showed high levels of hemolytic activity against both human and horse blood.

In order to confirm the absence of *ply* in equine pneumococci, Southern blot analyses were performed by using probes representing both a 500-bp 5′ fragment and a central 1,191-bp fragment of *S. pneumoniae* R6 *ply*. In contrast to PCR results, both of these probes hybridized with equine pneumococcal DNA, producing a single band of a size distinct from that seen in human pneumococcal controls (data not shown). Thus, it appeared that, despite both the apparent lack of hemolytic activity and the inability to amplify the *ply* gene by PCR with primers located at either end of *ply*, at least a fragment of *ply* is present in the chromosome of equine pneumococci.

(ii) Autolysin. As previously mentioned, the equine pneumococci are unusual in that they are all bile insoluble. Bile solubility is considered a characteristic feature of pneumococci, and the basis of this phenotype is the activation of autolysin by bile. In order to confirm these observations more stringently, extracts prepared from two of the equine isolates, 497 and 501, were assayed for autolytic activity on choline-labelled cell walls. No evidence of autolytic activity was detected. Furthermore, microscopic examination of exponentially growing equine pneumococci revealed a chain-like morphology (Fig. 2) and not the diplococcal structure characteristic of *S. pneumoniae*. The use of a *lytA* probe in a Southern blot analysis demonstrated the presence of multiple bands (data not shown) with homology to the probe. Southern blot analyses performed with *lytA* probes are potentially difficult to interpret because of the frequent presence of homologous, bacteriophage lytic genes and proteins with homologous choline-binding domains. However, the blots provided no evidence that the *lytA* gene is absent from equine pneumococci.

(iii) Detection of a *lytA-ply* fusion in equine pneumococci. The genes encoding pneumolysin and autolysin are known to be closely linked in the genome, with *ply* situated some 7 kb downstream of *lytA* (12a). It was therefore considered possible that the apparent lack of the pneumolysin and autolysin phenotypes could be linked. While investigating this possibility, we amplified and fully sequenced a 730-bp PCR product. The PCR product was found to represent a fusion of the 5′ region of *lytA* with a sequence corresponding to *ply*, located some 450 bp downstream of the start codon (Fig. 3). The first 482 bp of the PCR product were over 97% identical with bases 700 to 1181 of the previously published *lytA*-containing sequence (GenBank accession no. M13812), while the remaining 3′ sequence showed over 98% identity with bases 667 to 910 of the previously published *ply*-containing sequence (GenBank accession no. X52474). The presence of this apparent deletion of 7 kb of DNA, including the 3′ region of *lytA*, the 5′ region of *ply*, and the entire intervening sequence, was confirmed by using primers to *lytA* and *ply* (C and INT) to amplify the junction region from all of the equine pneumococci. An identical product could not be amplified from human pneumococci.

(iv) Other virulence factors. *nanA* and *nanB*, *hyl*, and *pspA* were all detected by PCR without difficulty. PCR products corresponding to these genes were amplified from 10 equine isolates and subjected to RFLP analyses with multiple, frequently cutting restriction enzymes. Restriction enzymes used were as follows: *nanA* was digested with *Dde*I, *Mnl*I, *Taq*I, and *Tsp*509I; *nanB* was digested with *Dra*I, *Nla*III, *Tfi*I, *Tsp*509I, and *Hae*III/*Dde*I; *pspA* was digested with *Mse*I and *Tsp*509I; and *hyl* was digested with *Dde*I, *Hae*III, *Hin*fI, *Mse*I, and *Rsa*I. RFLP analyses of these loci revealed no allelic variation (Table 4), with the single exception of one allelic variant of *pspA* from strain 501 which was slightly smaller than that of the remaining strains and resulted in one smaller band in the RFLP profile. It is possible that this size variation may reflect the loss of a

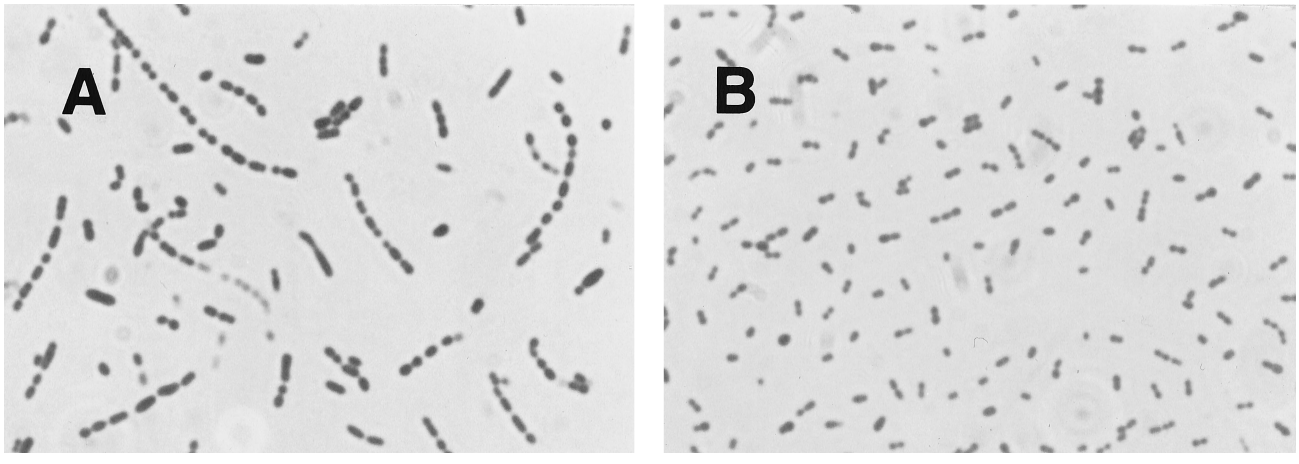

FIG. 2. Gram-stained exponential-phase culture of equine isolate 501 of *S. pneumoniae* (A) and human clinical serotype 3 *S. pneumoniae* isolate 1128 (B) cultured under identical conditions (magnification, ×100).

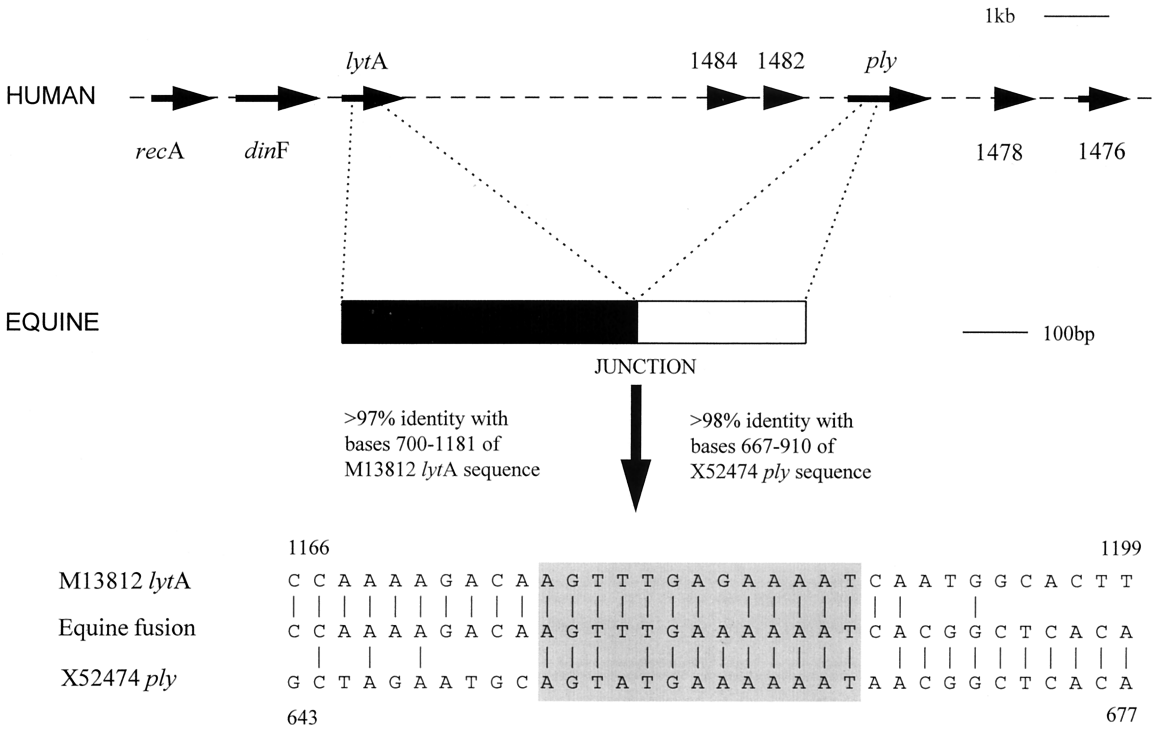

FIG. 3. Diagrammatic representation of the *lytA-ply* fusion generated by the deletion of the intervening sequence. The arrangement of the region in human pneumococci as determined by genome sequencing (26a) is shown at the top. Below, the sequence detected in equine pneumococci with a fusion of the *lytA* and *ply* sequences generated by a deletion event is illustrated. At the bottom, the sequence of the junction region is compared with the corresponding regions of the *lytA* and *ply* sequences. Bases identical to those seen in the equine sequence are marked (|) and illustrate the switch from *lytA* sequence at the 5′ end of the sequence shown to *ply* sequence at the 3′ end. The boxed region represents a sequence block conserved between *lytA* and *ply* coding sequences (11 of 13 bases) which corresponds to the *lytA-ply* junction.

repeat unit, as the *pspA* gene is known to be highly repetitive (31) and such bacterial surface proteins are prone to variation by the gain or loss of repeats (13). However, the alleles found at all these virulence factor loci in equine isolates were distinct from those seen in any of up to 122 human isolates (with diverse serotypes and isolated from different places on different dates) examined in the same manner (Table 4). These findings therefore confirm that equine isolates of pneumococci appear to consist of a clone distinct from any isolate seen to date in our extensive studies of the human pneumococcal population.

Competence genes of equine pneumococci. The *comC* gene of naturally transformable streptococci encodes a competence-stimulating peptide (CSP) which induces competence in the bacterial population at a critical extracellular concentration. The associated *comD* and *comE* genes encode a transmembrane histidine kinase and response regulator protein, respectively, of a two-component regulator, with the *comD*-encoded protein being a receptor for the CSP (20). The *comC* and 5′ *comD* genes were sequenced from two equine *S. pneumoniae* isolates, 496 and 505. Both were found to contain the CSP-1 allele which is characteristic of approximately 50% of the human pneumococci examined to date. The corresponding *comD* sequences were found to be identical to each other, characteristic of CSP-1-producing strains, and to contain only a single polymorphic site which distinguished them from all other known human pneumococci (28).

DISCUSSION

The results presented in this paper represent the first molecular characterization of *S. pneumoniae* isolates infecting nonhuman hosts. Serotype 3 pneumococci have been reported to cause respiratory tract infections in various breeds of horse, including hunters, Arabs, standardbreds, and thoroughbreds, for at least the past 25 years (1, 11). Eleven isolates of pneumococci were obtained from thoroughbred racehorses with respiratory tract infections from different stables in both England and Ireland over a period of almost 10 years. Extensive RFLP and sequencing analyses of both housekeeping-protein- and virulence factor-encoding genes in these organisms suggest that equine pneumococci represent a very tight clonal group closely related to human pneumococci. Phylogenetic analysis clearly shows that equine pneumococci cluster within the normal human-infecting population and do not form a separate, deeply branching group. The apparent uniformity among isolates could suggest that the movement of pneumococci from

TABLE 4. Analysis of the nature of putative virulence factors of equine pneumococci

Gene	Virulence factor	Virulence factor present	Identical allele possessed by all equine isolates	Identical allele seen in human isolates (no. of human strains compared)
lytA	Autolysin	—[a]		
ply	Pneumolysin	—[a]		
nanA	Neuraminidase	Yes	Yes	No (20)
nanB	Neuraminidase	Yes	Yes	No (94)
pspA	Surface protein A	Yes	Yes[b]	ND[c]
hyl	Hyaluronidase	Yes	Yes	No (122)

[a] —, truncated inactive copy.
[b] Except 501.
[c] ND, not done.

humans to horses was a relatively recent event and that the isolates examined are part of a recent epidemic clone that has spread throughout thoroughbreds.

***ply* of equine pneumococci.** It seems clear that, in spite of the apparently close genetic relationship between equine and human pneumococci, two loci, *ply* and *lytA*, often considered crucial to virulence in humans, are clearly different in equine pneumococci. Although pneumolysin is regarded as a diagnostic feature for pneumococci (25–27) and specific and degenerate PCR primers targeted to regions of the gene known to encode conserved domains within pneumolysin always resulted in amplification of *ply* from a diverse collection of human isolates, we were unable to amplify *ply* from any of the equine *S. pneumoniae* isolates. Neither was there any evidence for hemolytic activities among the isolates examined, even when they were assayed with horse blood. However, Southern blotting clearly demonstrated that *ply*-specific probes do hybridize to the genomic DNA of equine pneumococci. The reason for this discrepancy became clear with the identification of the *lytA-ply* fusion. Only a nonfunctional fragment of *ply* is present, explaining both the inability to amplify the gene by PCR and the lack of hemolytic activity.

***lytA* of equine pneumococci.** The major autolysin of pneumococci, LytA, responsible for the cleavage of peptidoglycan, is also considered a virulence factor. LytA may be important, both directly and indirectly, in the pathogenic process by mediating inflammation and by allowing the release of other nonexported virulence factors, such as pneumolysin, from the cell (19). Although no evidence for the absence of the *lytA* gene was obtained by Southern blotting, phenotypic evidence indicated no autolysin-associated activity. Microscopic examination of exponentially growing cultures showed that the equine pneumococci grew as chains rather than as characteristic diplococci. Interestingly, this equine chain phenotype is characteristic of *lytA* mutants previously constructed in human isolates (24). Furthermore, sonicated extracts prepared from two equine isolates and assayed for degradation of choline-labelled cell walls did not show any LytA activity.

A deletion event has disrupted both *lytA* and *ply*. The discrepancy between the apparent presence of *lytA* and *ply* detected by Southern blotting and the lack of the associated phenotypes was resolved with the identification of an apparent deletion in equine pneumococci resulting in the loss of substantial fragments of both of these genes and the intervening sequence. The basis of this deletion becomes clear when the sequences of *lytA* and *ply* are compared with the sequence of the fusion junction, as illustrated in Fig. 3. The junction corresponds to a highly similar sequence (11 of 13 bases identical) seen in the previously published sequences of both *lytA* and *ply*. Therefore, it appears that the presence of this repeat sequence has mediated a recombination event resulting in the deletion of the sequence between the repeats. This event, resulting in the loss of substantial fragments of both *lytA* and *ply* as well as the intervening sequence, would clearly result in the inactivation of these genes. Indeed, it is of interest that the *lytA* sequence upstream of the junction has a G-to-T transversion at the equivalent of base 142 in *lytA*, generating a stop codon. This event may have occurred subsequent to the deletion event, preventing the production of a potentially toxic or metabolically wasteful fusion protein, since despite the deletion event, the fused truncated pneumolysin gene remains in frame with the autolysin gene.

A number of PCR primers were designed to confirm the absence of the region between *lytA* and *ply*. This region contains a number of predicted open reading frames (ORFs) (26a), although none, other than one (RPN1484) predicted to encode transposase, have been assigned any function based on sequence similarity. Thus, there appear to be no metabolically crucial genes in the region apparently deleted from equine pneumococci. Primers designed on the basis of regions corresponding to potential ORF RPN1482 and a region intergenic to potential ORFs RPN1484 and *lytA* did not result in amplification of PCR products from equine pneumococci. A product was obtained when a primer set corresponding to the predicted transposase ORF RPN1484 was used, but there are likely to be multiple copies of such an ORF within the chromosome. In contrast to the apparent absence of DNA corresponding to the regions between *lytA* and *ply*, primer sets corresponding to *dinF* and *recA* (upstream of *lytA*) and two potential ORFs, RPN1478 and RPN1476 (downstream of *ply*), produced PCR products with equine pneumococcal DNA. All of these findings are consistent with the deletion of the ca. 7-kb region between *lytA* and *ply* in equine pneumococci.

Phylogeny of equine isolates. It is unclear whether the equine isolates retain the capacity to infect humans, although previous studies have indicated that farmworkers are usually colonized with pneumococci serotypically distinct from those infecting horses (4). Although the equine isolates retain a normal human CSP and receptor protein, illustrating that they could be induced to competence in the presence of human pneumococci, and human pneumococci possess an epidemic population structure (10, 21), suggesting that recombination is relatively frequent, the data presented here suggests that frequent transfer of DNA between equine and human pneumococci may not occur. Polymorphisms seen in the housekeeping genes *xpt* and *recP* were found to be unique to equine isolates when compared with pneumococcal isolates selected to represent the breadth of genetically divergent isolates and in *comD* when compared with the sequences of some 60 pneumococcal isolates (28). Interestingly, a recent study of the genetic variation of almost 300 isolates of invasive human pneumococci reported the presence of 35 distinct *xpt* alleles (7). None of the 35 alleles contained the unique polymorphism associated with the equine isolates found in this study. Additionally, equine isolates appear to possess distinct alleles of virulence factor genes for neuramindases A and B and hyaluronidase. Although the sampling in this study is clearly not exhaustive and the possibility of the equine alleles being found in some human pneumococci cannot be ruled out, the results suggest that equine pneumococci may represent a subpopulation ecologically isolated from other pneumococci.

Pathogenicity of equine pneumococci. The implications of the alterations in genes believed to encode two of the major virulence factors of *S. pneumoniae* for that organism's pathogenicity in horses remain unclear. Although functional pneumolysin and autolysin are important in several murine models of infection and a functional pneumolysin is responsible for damage to the ciliated cells in the respiratory tract, there is evidence that the inactivation of pneumolysin in some human *S. pneumoniae* isolates has little impact upon their virulence in some models of infection (18). Although the extent of lung pathology seen in horses is generally less than that seen in lobar pneumonia in humans, the overall severity of pneumococcal disease seen in the two hosts may be similar. Subclinical infections and, more frequently, asymptomatic carriage are common in humans, and the very high incidence of infection in 2-year-old thoroughbreds (ca. 95%), although very strongly associated with lower airway inflammation, is frequently subclinical (30). Whether or not there are real differences in the pathologic severity of human and equine pneumococcus infections, it is interesting that *Streptococcus equi*, the cause of strangles (a severe upper respiratory tract infection in the

horse) also lacks a thiol-activated toxin equivalent to streptolysin O (9). Equine *S. pneumoniae* and *S. equi* are two pathogenic streptococci that, by comparison to other pathogenic members of the genus, would be expected to produce a thiol-activated toxin but do not. This deficiency poses the question of whether the thiol-activated toxins provide a selective disadvantage in the horse and so have been lost with time.

ACKNOWLEDGMENTS

We are grateful to Rubens López for performing the analysis of autolytic activity and to Paul Pickerill for valuable technical assistance.

This work was supported by grants from the Wellcome Trust, the BBSRC and the UK MRC.

REFERENCES

1. **Benson, C. E., and C. R. Sweeney.** 1984. Isolation of *Streptococcus pneumoniae* type 3 from equine species. J. Clin. Microbiol. **20:**1028–1030.
2. **Blunden, A. S., and M. E. Mackintosh.** 1991. The microflora of the lower respiratory tract of the horse: an autopsy study. Br. Vet. J. **147:**238–250.
3. **Blunden, A. S., D. Hannant, G. Livesay, and J. A. Mumford.** 1994. Susceptibility of ponies to infection with *Streptococcus pneumoniae* (capsular type 3). Equine Vet. J. **26:**22–28.
4. **Burrell, M. H., M. E. Mackintosh, and C. E. D. Taylor.** 1986. Isolation of *Streptococcus pneumoniae* from the respiratory tract of horses. Equine Vet. J. **18:**183–186.
5. **Chanter, N.** 1994. *Streptococcus pneumoniae* and equine disease. Equine Vet. J. **26:**5–6.
6. **Denys, G. A., and R. B. Carey.** 1992. Identification of *Streptococcus pneumoniae* with a DNA probe. J. Clin. Microbiol. **30:**2725–2727.
7. **Enright, M. C., and B. G. Spratt.** 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology (Reading) **144:**3049–3060.
8. **Fenoll, A., J. V. Martinez-Suarez, R. Muñoz, J. Casal, and J. L. Garcia.** 1990. Identification of atypical strains of Streptococcus pneumoniae by a specific DNA probe. Eur. J. Clin. Microbiol. Infect. Dis. **9:**396–401.
9. **Flanagan, J., N. Colin, J. Timoney, T. Mitchell, J. A. Mumford, and N. Chanter.** 1998. Characterisation of the haemolytic activity of *Streptococcus equi*. Microb. Pathog. **24:**211–221.
10. **Hall, L. M. C., R. A. Whiley, B. Duke, R. C. George, and A. Efstratiou.** 1996. Genetic relatedness within and between serotypes of *Streptococcus pneumoniae* from the United Kingdom: analysis of multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and antimicrobial resistance patterns. J. Clin. Microbiol. **34:**853–859.
11. **Hofer, B. F., H. Steck, J. Gerber, J. Lohrer, J. Nicolet, and M. F. Paccaud.** 1972. An investigation of the aetiology of viral respiratory disease in a remount depot, *In* p. 527–545. *In* Proceedings of the 3rd International Conference on Equine Infectious Diseases.
12. **Höltje, J. V., and A. Tomasz.** 1976. Purification of the pneumococcal *N*-acetylmuramyl-L-alanine amidase to biochemical homogeneity. J. Biol. Chem. **251:**4199–4207.

12a. **The Institute for Genomic Research.** 1999, copyright date. Sequences. [Online.] http://tgr.org. [8 April 1999, last date accessed.]

13. **Kehoe, M. A.** 1994. Cell wall associated proteins in Gram-positive bacteria. New Compr. Biochem. **27:**217–261.
14. **King, S. J., and C. G. Dowson.** Unpublished data.
15. **Kumar, S. K., K. Tamura, and M. Nei.** 1993. MEGA: molecular evolutionary genetic analysis software for microcomputers. CABIOS **10:**189–191.
16. **Mackintosh, M. E., S. T. Grant, and M. H. Burrell.** 1989. Evidence for *Streptococcus pneumoniae* as a cause of respiratory disease in young thoroughbred horses in training, p. 41–44. *In* D. G. Powell (ed.), Equine infectious diseases, no. 5: Proceedings of the Fifth International Conference. University Press of Kentucky, Lexington.
17. **Meyer, J. C., A. Koterba, G. Lester, and B. L. Purich.** 1992. Bacteremia and pneumonia in a neonatal foal caused by *Streptococcus pneumoniae* type 3. Equine Vet. J. **24:**407–410.
18. **Mitchell, T. J., and P. W. Andrew.** 1997. Biological properties of pneumolysin. Microb. Drug Resist. **3:**19–26.
19. **Mitchell, T. J., J. E. Alexander, P. J. Morgan, and P. W. Andrew.** 1997. Molecular analysis of virulence factors of *Streptococcus pneumoniae*. J. Appl. Microbiol. **83:**62S–71S.
20. **Morrison, D. A.** 1997. Streptococcal competence for genetic transformation: regulation by peptide pheromones. Microb. Drug Resist. **3:**27–37.
21. **Müller-Graf, C. D. M., A. M. Whatmore, S. J. King, A. P. Pickerill, N. C. Doherty, and C. G. Dowson.** Unpublished data.
22. **Neufeld, F., and R. Schnitzler.** 1928. Pneumokokken Handbuch der Pathogenen Mikro-organismen, 3rd ed. Gustav Fischer, Berlin, Germany.
23. **Paton, J. C.** 1996. The contribution of pneumolysin to the pathogenicity of *Streptococcus pneumoniae*. Trends Microbiol. **4:**103–106.
24. **Ronda, C., J. L. García, E. García, J. M. Sánchez-Puelles, and R. López.** 1987. Biological role of the pneumococcal amidase. Cloning of the *lytA* gene in *Streptococcus pneumoniae*. Eur. J. Biochem. **164:**621–624.
25. **Rudolph, K. M., A. J. Parkinson, C. M. Black, and L. W. Mayer.** 1993. Evaluation of polymerase chain reaction for diagnosis of pneumococcal pneumonia. J. Clin. Microbiol. **31:**2661–2666.
26. **Salo, P., A. Ortqvist, and M. Leinonen.** 1995. Diagnosis of bacteremic pneumococcal pneumonia by amplification of a pneumolysin gene fragment in serum. J. Infect. Dis. **171:**479–482.

26a. **Selkov, E.** Metabolic reconstructions. [Online.] WIT Program, version 2. http://wit.mcs.anl.gov/wit2. [8 April 1999, last date accessed.]

27. **Virolainen, A., P. Salo, J. Jero, P. Karma, J. Eskola, and M. Leinonen.** 1994. Comparison of PCR assay with bacterial culture for detecting *Streptococcus pneumoniae* in middle ear fluid of children with acute otitis media. J. Clin. Microbiol. **32:**2667–2670.
28. **Whatmore, A. M., V. A. Barcus, and C. G. Dowson.** 1999. Genetic diversity of the streptococcal competence (*com*) gene locus. J. Bacteriol. **181:**3144–3154.
29. **Wood, J. L. N., M. H. Burrell, C. A. Roberts, N. Chanter, and Y. Shaw.** 1993. Streptococci and *Pasteurella* spp. associated with disease of the equine lower respiratory tract. Equine Vet. J. **25:**314–318.
30. **Wood, J. L. N., J. R. Newton, N. Chanter, J. A. Mumford, H. G. G. Townsend, K. H. Lakhani, S. M. Gower, M. H. Burrell, R. C. Pilsworth, M. Shepherd, R. Hopes, D. Dugdale, B. M. B. Herinckx, J. P. M. Main, H. M. Windsor, and G. D. Windsor.** Longitudinal epidemiological study of respiratory disease in race horses: disease definitions, prevalence, and incidence. *In* U. Wernery, J. F. Wade, J. A. Mumford, and O.-R. Kaarden (ed.), Proceedings of the 8th Conference on Equine Infectious Diseases, in press. R & W Publications, Newmarket, United Kingdom.
31. **Yother, J., and D. E. Briles.** 1992. Structural properties and evolutionary relationships of PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence analysis. J. Bacteriol. **174:**601–609.

Editor: E. I. Tuomanen