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Evidence of Recombination and an Antigenically Diverse Immunoglobulin A1 Protease among Strains of *Streptococcus pneumoniae*

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The genetic relationships among 114 isolates of *Streptococcus pneumoniae* representing mainly nine serotypes that frequently cause severe childhood disease in Northern Europe were examined by use of multilocus enzyme electrophoresis. A comparison was made of the corresponding antigenic variations of excreted immunoglobulin A1 (IgA1) proteases detected by enzyme neutralization assays. Allelic variation at 13 gene loci among 70 electrophoretic types disclosed a comparatively low mean genetic diversity per locus ($H = 0.319$). In contrast, IgA1 proteases showed extensive antigenic diversity as 17 different inhibition types were distinguished. A lack of overall clonality was apparent from the linkage equilibrium of alleles harbored by 28 isolates chosen to represent the genetic diversity of the study population. However, certain clones, such as those marked by identical electrophoretic type, serotype, and IgA1 protease type, persisted for a sufficiently long time to enable clonal spread between distant geographic areas. Among clonally related isolates, examples illustrating a shift of capsular serotype or IgA1 protease type supported the view that recombination occurs in vivo in corresponding genes. In conclusion, over time, horizontal genetic exchange appears to be sufficiently frequent to disrupt the clonal structure otherwise generated by binary fission in natural populations of *S. pneumoniae*. The clonal instability combined with considerable antigenic heterogeneity renders the pneumococcal IgA1 protease less attractive as a potential component of future vaccines.

Encapsulated strains of *Streptococcus pneumoniae* produce one of 84 antigenically distinct capsular polysaccharides, 23 of which constitute the basis for the currently available vaccine (18). The vaccine fails to protect against serotypes not included, and protection is inadequate for certain high-risk groups, including children less than 2 years of age (18). In view of the recent widespread appearance of multiresistant pneumococci, there is an urgent need to develop more efficient preventive strategies (40). In this respect, it is of importance to elucidate the role played by putative proteinaceous virulence factors, such as pneumolysin, neuraminidase, autolysin, pneumococcal surface protein A (PspA), and immunoglobulin A1 (IgA1) protease (34) in the ecological maintenance and infectious pathogenesis of the organism and to evaluate these proteins as to their potential use in future vaccine formulations.

The pneumococcal IgA1 protease has been hitherto only scarcely characterized. The enzyme is highly specific for the proline-threonine bond at positions 227 and 228 in the hinge region of human IgA1, which is the only known substrate (12). Since IgA1 is the principal mediator of specific immunity in the upper airways, IgA1 protease activity conceivably allows pneumococci to evade the local mucosal defense system (13). The biological significance of the enzyme is supported by the fact that the three leading causes of bacterial meningitis, *S. pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae*, all produce IgA1 protease, while closely related nonpathogenic species do not (14, 25, 30). (For reviews on bacterial IgA1 proteases, see references 15, 29, and 35.)

The IgA1 protease excreted by *S. pneumoniae* apparently is immunogenic in humans since pooled normal human serum and colostrum (17) as well as saliva (37) neutralize the activity of selected *S. pneumoniae* IgA1 proteases. However, the extent of antigenic heterogeneity among *S. pneumoniae* IgA1 proteases has not been studied systematically. Interestingly, epitopes recognized by neutralizing antibodies are shared by some IgA1 proteases of *S. pneumoniae* and IgA1 proteases of the related species *Streptococcus sanguis* (20). The *S. sanguis* IgA1 protease is predicted to be a metalloproteinase because of its amino acid sequence homology with a common zinc binding site (7) and a high sensitivity, shared with the *S. pneumoniae* IgA1 protease, to inhibition by the metal chelating agent EDTA (12, 36). Taken together, these data point to a common origin of these proteases, although the cloned *iga* gene of *S. sanguis* does not hybridize to chromosomal DNA of pneumococci (6).

Besides information on crucial antigens, knowledge of the genetic population structure and dynamics of the pneumococci is important for a more profound understanding of the interplay between the pathogen and the host defense system. For epidemiologic purposes, multilocus enzyme electrophoresis has been employed in several studies to yield information on genetic relationships among selected groups of *S. pneumoniae* organisms in relation to penicillin resistance (2, 11, 31, 32, 41, 44, 45). Combined with DNA sequence analysis of penicillin-binding proteins (4, 19, 26, 42), these studies corroborate the notion that horizontal genetic exchange has occurred in vivo in genes encoding penicillin-binding proteins 1a, 2b, and 2x as well as in genes specifying capsular serotype. However, the extent to which such successful recombination occurs in the population is not known. The population structure of different bacterial species may range from strictly clonal to virtually panmictic, i.e., from significant linkage disequilibrium to linkage equilibrium (43). An intermediate type of population structure, termed epidemic, may arise as a result of rapid

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spread of certain epidemic clones spawned from a population that is fully sexual in the long term (43). The genetic structure of natural populations of *S. pneumoniae* has not been determined.

The objective of the present study of selected serotypes of *S. pneumoniae* that frequently cause childhood disease in Europe and North America was to examine and compare genomic relatedness, as revealed by electrophoresis of enzymes presumably not subjected to an external selection pressure, with the antigenic variation of the excreted IgA1 protease and the capsular polysaccharide, both conceivably subjected to immune selection by the host.

MATERIALS AND METHODS

Bacterial isolates. The present study included 114 isolates of *S. pneumoniae* representing the nine serotypes that most frequently cause childhood infections in Denmark, i.e., serotypes 1, 3, 6A, 6B, 7F, 14, 18C, 19F, and 23F (33), each by 7 to 11 isolates. In addition, one or two isolates of serotypes 4, 8, 9V, 10A, 10F, 11A, 12F, 16F, 19A, 22F, 33F, and 35F were included. The vast majority of isolates were recovered during 1991 to 1993 and originated as follows: 54 from Denmark, 2 from Australia, 14 from Belgium, 9 from France, 19 from Norway, 2 from The Netherlands, 2 from Yugoslavia, and, finally, 12 of unknown origin. Serotyping was performed with antisera produced by Statens Seruminstitut, Copenhagen, Denmark, by use of procedures recommended by the manufacturer.

IgA1 protease preparations. Sixteen crude preparations of IgA1 proteases used for immunization of rabbits were made from the supernatant of two 24-h cultures in 1,250 ml of Todd-Hewitt broth incubated at 37°C. After precipitation of the supernatant by 60% saturation with ammonium sulfate, the precipitate was redissolved in 10 ml of a Tris-HCl buffer (0.05 M Tris-HCl [pH 7.4], 0.85% NaCl, 0.05 M NaN_3). Dialysis was performed twice for 24 h against 1 liter of this Tris-HCl buffer, and subsequently, the preparation was concentrated by use of positive-pressure filtration (Centriprep-10; Amicon, Beverly, Mass.) to a final volume of approximately 10 ml.

IgA1 proteases used for inhibition typing were prepared as supernatants of 20-ml Todd-Hewitt broth cultures grown overnight at 37°C. Preparations were stored at 4°C with the addition of NaN_3 (0.05 M).

Enzyme-neutralizing antibodies. Every 2 weeks, rabbits were immunized subcutaneously with 200 μl of IgA1 protease preparation mixed with adjuvant essentially as described previously (9). Freund's complete adjuvant was used for the initial immunization, and Freund's incomplete adjuvant was used for subsequent ones. When the inhibitory response to the homologous IgA1 protease was between titers of 2^3 and 2^8 (see below), the immunoglobulin fraction was isolated from serum as described previously (9) but excluding the final ion-exchange step.

Assays for determination of activity and inhibition of IgA1 protease. For initial screening, IgA1 protease activity and enzyme inhibition were determined by an enzyme-linked immunosorbent assay (ELISA) essentially as described previously (37). Either 20 μl of twofold dilutions of IgA1 protease (activity assays) or 10 μl each of IgA1 protease and antiserum (inhibition assays) were incubated overnight at 37°C with 20 μl of IgA1 substrate (10 μg of IgA1 per ml, purified from a patient [Fri] with multiple myelomas). Subsequently, 200 μl of washing buffer (0.5 M NaCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 0.15% [wt/vol] Tween 20 [pH 7.2]) was added to the sample before transfer to an ELISA well coated with rabbit anti-mouse immunoglobulin (1:2,000; DAKO, Glostrup, Denmark) and a second layer of monoclonal anti-human Fc_α (1:400; DAKO). After incubation for 2 h and washing, peroxidase-conjugated rabbit anti-human kappa light chain (1:1,000; DAKO) was used for detection of intact IgA1 in the sample. Finally, *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) was added, and after the color reaction was stopped with 1 M H_2SO_4, the optical density at 492 nm was monitored. The assay was performed in doublets.

Immunoelectrophoresis was used for titrations of enzyme-neutralizing activity of antibody preparations against the various IgA1 proteases as described previously (22, 27). In short, equal volumes of IgA1 protease adjusted to titer 4 (see below) and twofold dilutions of antibody preparations were incubated for 1 h at room temperature. Substrate IgA1 was added subsequently to a concentration of 0.5 mg/ml, and the mixture was incubated overnight at 37°C. Cleavage was finally detected by immunoelectrophoresis.

The titer of IgA1 protease activity was defined as the reciprocal of the highest dilution of IgA1 protease that caused complete cleavage of substrate IgA1. Correspondingly, the titer of enzyme inhibition was defined as the reciprocal of the highest dilution of antiserum that abolished activity of a protease adjusted to a titer of 2 in the final reaction.

IgA1 protease inhibition typing. Initially, IgA1 proteases in culture supernatants from 102 pneumococcal strains were screened for inhibition by nine of the antibody preparations by the ELISA. On the basis of different combinations of IgA1 protease inhibition by the battery of antibodies and capsular serotype, IgA1 proteases from 61 representative strains were selected for further characterization. Against these, the neutralizing activity of the nine antibody preparations was titrated by immunoelectrophoresis. Differences of three or more twofold dilutions were considered significant for discrimination between antigenic types. Each protease was assigned to an inhibition type on the basis of the combined pattern of neutralization by these nine antibody preparations.

With the dual purpose of further confirming the different IgA1 protease inhibition types and identifying possible widely distributed common epitopes, an additional seven antisera were titrated against one IgA1 protease representing each of 38 combinations of capsular serotype and inhibition type.

Multilocus enzyme electrophoresis. Bacteria were harvested from 200 ml of 24-h cultures grown in Todd-Hewitt broth and transferred to 1.5 ml of Tris-HCl buffer (50 mM Tris-HCl [pH 8], 5 mM EDTA). Lysates were prepared by freezing at −20°C followed by thawing and sonication twice for 5 min. After the final round of freezing and thawing, the supernatant was stored at −70°C until use. Electrophoresis in starch gels and selective staining for each of 13 metabolic enzymes was performed as described by Selander et al. (39) except for carbamate kinase (see below). The enzymes assayed were esterase, carbamate kinase, adenylate kinase, glutamate dehydrogenase NADP, alcohol dehydrogenase, phosphoglucose isomerase, glucose 6-phosphate dehydrogenase, L-lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, nucleoside phosphorylase, 6-phosphogluconate dehydrogenase, leucine aminopeptidase, and aldolase. Carbamate kinase was electrophoresed in buffer I (39) and visualized with the staining solution for adenylate kinase with the addition of 100 mg of carbamyl phosphate (C-4135; Sigma).

Distinctive mobility variants of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding structural gene locus. Each combination of alleles at the 13 enzyme loci defined a multilocus electrophoretic type (ET), with an absence of enzyme activity (null allele) considered as missing data. The detailed data set is available on request. Genetic diversity at an enzyme locus (h) among strains was calculated from allele frequencies by the formula $h = 1 - \Sigma x_i^2 [n/(n-1)]$, where x_i is the frequency of the ith allele of the locus, and n is the number of strains. Mean genetic diversity per locus (H) was calculated as the arithmetic average of h values for all loci. Computerized cluster analysis was performed by the average-linkage method from a matrix of pairwise genetic distances between ETs. Genetic distance between ETs was expressed as the proportion of enzyme loci at which dissimilar electrophoretic mobilities occurred. The software used was the ETCLUS program kindly provided by T. S. Whittam, Institute of Molecular Evolutionary Genetics, University of Pennsylvania. The index of association (I_A) was calculated as described previously (43) from the formula $I_A = (V_o/V_e) - 1$, where V_o is the variance in mismatches between pairs of isolates observed for the sample, and V_e is the corresponding variance expected in a population at linkage equilibrium. The error variance of I_A, assuming no association between loci, was calculated as $I_A = 0 \pm \text{var}(V_E)^{-1}/V_E$, where $\text{var}(V_E) = 1/n\,[\Sigma h_j - \Sigma h_j^2 + 12\,\Sigma h_j^3 - 6\Sigma h_j^4 + 2(\Sigma h_j - \Sigma h_j^2)]$ (43). For each allele of a particular enzyme, Fisher's exact test was used in two-locus comparisons to determine whether the distribution of paired alleles was in agreement with that expected in a situation of random assortment. The expected distribution was calculated from observed allele frequencies.

RESULTS

IgA1 protease activity. For 107 of the 114 isolates, IgA1 protease activity was examined. Of these, three showed no IgA1 protease activity and two showed extremely low activity in the supernatant even after precipitation with ammonium sulfate and subsequent concentration. The remaining 102 isolates showed IgA1 protease activity at titers ranging from 2 to 8 in the supernatant.

Serological analysis of IgA1 proteases. Enzyme-neutralizing antibodies were prepared from the sera of 16 rabbits, each immunized with IgA1 protease from a different pneumococcal strain. Three rabbits were immunized with an IgA1 protease antigenically similar to one of the remaining 13 proteases, and accordingly, antibodies from these rabbits showed a spectrum of enzyme inhibition similar to that of their doublets. Thus, 13 antibody preparations showed a distinct spectrum of enzyme inhibition. Table 1 provides details on these antibody preparations and the corresponding strains. The combined pattern of inhibition by these 13 antibody preparations allowed distinction between 17 different antigenic variants, termed inhibition types, among the pneumococcal IgA1 proteases. These 17 IgA1 protease inhibition types are illustrated in Table 2. All 102 IgA1 proteases examined were neutralized by at least one antibody preparation. Notably, none of the 13 antibody preparations recognized epitopes that were common to all of the

TABLE 1. Characteristics of thirteen antibody preparations raised against pneumococcal IgA1 proteases

Antibody prepn	Strain no.[a]	Capsular serotype	Homologous inhibition titer[b]	Inhibition type[c]
A	PK75	1	2^6	2
B	PK77	3	2^{10}	3
C	PK87	23F	2^7	4
D	PK88	23F	2^8	4
E	PK79	6B	2^8	6
F	PK80	7F	2^8	8
G	PK81	14	2^8	9
H	PK82	14	2^8	10
I	PK12	6B	2^{10}	ND[d]
J	PK26	18C	2^3	ND
K	PK84	18C	2^5	13
L	PK85	19F	2^6	15
M	PK86	19F	2^7	16

[a] Strain excreting the IgA1 protease used for immunization.
[b] Inhibition titer against the homologous IgA1 protease.
[c] Inhibition type of the IgA1 protease used for immunization (see Table 2).
[d] ND, not determined.

pneumococcal IgA1 proteases (Table 2), indicating a high degree of antigenic heterogeneity. Antibody preparations C and D, both raised against IgA1 proteases displaying the inhibition pattern designated type 4, showed different spectra of inhibition. This suggests the existence of further antigenic variation not disclosed by the inhibition typing scheme.

The relationships between IgA1 protease inhibition type, capsular serotype, and origin of the isolates are given in Table 3. Of 14 IgA1 protease inhibition types represented by more than one isolate, 5 were confined to isolates of a single capsular serotype, whereas the remainder were associated with up to 8 different serotypes (Table 3). Among capsular types represented by more than one isolate, serotypes 3 and 7F were unique in being associated with only a single IgA1 protease inhibition type (Table 3). It is noteworthy that 15 of the 17 inhibition types were observed among isolates recovered in Denmark, thereby revealing an extensive antigenic variation among *S. pneumoniae* IgA1 proteases even within a confined geographic area (Table 3).

ETs and genetic diversity. The 114 isolates of *S. pneumoniae* examined by multilocus enzyme electrophoresis for allelic diversity at 13 enzyme loci comprised 70 distinct ETs. Of these, 21 ETs were represented by more than a single strain encompassing from 2 to 7 isolates. Twelve of the enzymes were polymorphic, with two or three alleles per locus (Table 4). The mean genetic diversity per locus (H) among the 70 ETs was 0.319, and among the 114 isolates, it was 0.300. The genetic diversity and the number of ETs identified among isolates of the same IgA1 protease inhibition type are given in Table 3.

Tests of linkage disequilibrium. ETs were assigned to clusters by the average-linkage method, and 28 clusters were defined at a genetic distance of approximately 0.15. To test whether the population studied showed a clonal structure, one representative isolate of each cluster was chosen as a unit for calculating the index of association (43). This index allows a crude quantification of multilocus linkage disequilibrium of gene alleles based on the distribution of allelic mismatches between pairs of isolates. Thus, the ratio of the observed to the expected variance in allelic mismatches is calculated. For bacterial populations in linkage equilibrium, the expected value of I_A is zero, whereas in the absence of recombination between lineages, the expected value of I_A differs significantly from zero. With complete panmixia, the expected value of I_A, calculated with ETs as the unit instead of single isolates, is negative (43). In the present study, the value of I_A based on representatives of the 28 clusters of *S. pneumoniae* was -0.14 ± 0.26, suggesting that the population studied is effectively sexual in the long term.

As a further verification that the 28 ETs showed linkage equilibrium, the 66 possible two-locus comparisons showed no significant linkage disequilibrium for any pairs, when analyzed by Fisher's exact test ($P > 0.5$). In addition, six arbitrarily selected four-allele combinations were observed in numbers close to what would be expected under conditions of random assortment (Table 5). Expected numbers were calculated as the product of allele frequencies multiplied by the 28 isolates. As a consequence of the apparent linkage equilibrium, a phy-

TABLE 2. Seventeen inhibition types of *S. pneumoniae* IgA1 proteases detected among 102 strains by use of 13 antibody preparations

IgA1 protease inhibition type	Inhibition by antibody prepn[a]												
	A	B	C	D	E	F	G	H	I	J	K	L	M
1	−	++	−	−	−	−	−	−	−	−	−	−	−
2	+++	−	+	−	−	−	+	−	−	(+)	+++	−	+
3	−	+++	++	−	−	−	+	−	−	−	−	−	++
4	+	+	+++	+++	+	−	+++	+	−	−	+++	−	++
5	−	−	−	−	−	+++	−	+++	−	−	++	−	−
6	−	−	−	−	+++	−	−	−	−	(+)	−	−	+++
7	−	++	++	+	−	−	+	−	−	−	−	+	+++
8	−	−	−	−	−	+++	−	+	−	−	++	−	−
9	−	+	+++	+	−	−	+++	+	−	−	+	−	+
10	−	−	−	−	−	++	−	+++	+++	−	−	+	−
11	−	++	+	−	+	−	+	−	−	+++	−	−	+++
12	−	+	++	+	−	−	−	+	−	−	+	−	+
13	+++	−	(+)	+	−	−	++	−	−	−	+++	−	+++
14	−	++	++	−	+	−	+	−	−	−	++	++	++
15	−	++	++	+	−	−	+	−	−	−	+	+++	++
16	−	++	++	++	++	−	++	−	−	++	−	++	+++
17	++	+	+	−	+	−	−	−	−	−	++	−	−

[a] Symbols: +++, ≥25% homologous inhibition titer (fewer than two doubling dilutions below homologous reaction); ++, 3 to 12.5% homologous inhibition titer (three or four doubling dilutions below homologous reaction); +, ≤1.5% homologous inhibition titer (five or more doubling dilutions below homologous reaction); (+), partial inhibition by undiluted antibodies; −, no inhibition.

TABLE 3. IgA1 protease inhibition type in relation to other strain characteristics

IgA1 protease inhibition type	No. of isolates	Serotype(s)[a]	Origin(s)[b]	*H*[c]	No. of ETs
1	11	1 (4), 10A, 10F, 12F, 19A, 19F, 23F, 35F	Au, DK (2), Fra (6), ND (2)	0.232	7
2	8	1 (7), 6A	Bel (3), DK (4), Nor	0.181	6
3	9	3 (7), 6A (2)	Bel (4), DK (5)	0.124	4
4	16	6A (5), 6B (2), 23F (9)	Bel (3), DK (10), Nor (3)	0.187	8
5	1	8	DK		1
6	6	6B (6)	DK (3), Nor (3)	0.185	4
7	2	6B, 18C	Yugo, Nor	0.154	2
8	13	7F (9), 9V, 11A, 12F, 33F	DK (8), Fra, Nor (4)	0.213	9
9	8	14 (8)	Bel, DK (5), Nor, ND	0.151	4
10	6	6A, 14 (3), 19F (2)	Bel (2), DK, Nl, Nor, ND	0.205	6
11	5	18C (5)	DK (2), Nor (2), ND	0.131	4
12	1	22F	DK		1
13	2	18C (2)	DK, Nor	0.154	2
14	8	18C (3), 19F (4), 22F	DK (6), Nor, Yugo	0.280	6
15	1	19F	DK		1
16	3	19F (3)	Au, Fra, Nor	0.051	2
17	2	4, 16F	DK (2)	0.385	2

[a] Numbers in parentheses following a serotype indicate the numbers of isolates for the serotype if more than one.
[b] Numbers in parentheses following an origin indicate the numbers of isolates recovered in that country if more than one. Abbreviations: Au, Australia; Bel, Belgium; DK, Denmark; Fra, France; Nl, The Netherlands; Nor, Norway; Yugo, Yugoslavia; ND, not determined.
[c] Mean genetic diversity among isolates of each IgA1 protease inhibition type.

logenetic dendrogram based on the cluster analysis would be meaningless.

Multiply represented ETs in relation to IgA1 protease type and capsular serotype. After omission of strains containing null alleles, 17 ETs comprised more than one isolate. With a few exceptions, isolates with identical multilocus genotypes also showed identical capsular serotypes and identical IgA1 protease inhibition types (Table 6). Therefore, such isolates are most likely members of a common clone. Some clones proved to be widely distributed. An example was the serotype 19F, IgA1 protease inhibition type 16, clone ET11 isolated from Australia in 1991 and subsequently from Norway in 1992 (Table 6). Strains assigned to ET57 were isolated 5 years apart in Norway (Table 6), while the time of recovery of the two ET7 strains differed by 51 years. Thus, distinct clones seem to exist for a considerable length of time, allowing for a clonal spread to different geographic locations.

A shift of capsular type among members of a single clone was indicated by the finding that ET64 comprised isolates with identical IgA1 protease types but with different capsular serotypes (Table 6). Conversely, a shift of IgA1 protease type probably occurred among members of ET30, since these isolates shared serotype but not IgA1 protease type (Table 6). Finally, ET11 and ET32 were composed of isolates differing in both capsule and IgA1 protease type (Table 6), consistent with a shift in both traits. Alternatively, these latter isolates may, coincidentally, show identical enzyme electromorphs while being clonally unrelated.

TABLE 4. Genetic diversity and allele frequency at 13 enzyme loci in 114 isolates and 70 ETs

Enzyme locus[a]	Frequency of isolates with alleles numbering:				*h*	
	0	1	2	3	Isolates	ETs
EST	0.07	0.26	0.67		0.410	0.343
CDK	0.01	0.16	0.83		0.270	0.328
ADK	0.00	0.89	0.07	0.04	0.210	0.235
GD2	0.00	0.83	0.17		0.280	0.325
ADH	0.12	0.63	0.25		0.407	0.403
PGI	0.00	1.00			0.000	0.000
G6P	0.04	0.46	0.39	0.11	0.594	0.606
LDH	0.10	0.02	0.75	0.13	0.286	0.294
G3P	0.02	0.70	0.28		0.412	0.430
NSP	0.02	0.86	0.12		0.221	0.328
6GP	0.04	0.80	0.16		0.278	0.295
LAP	0.00	0.95	0.05		0.101	0.135
ALD	0.00	0.32	0.68		0.437	0.426
Mean (*H*)					0.300	0.319

[a] Abbreviations: EST, esterase; CDK, carbamate kinase; ADK, adenylate kinase; GD2, glutamate dehydrogenase NADP; ADH, alcohol dehydrogenase; PGI, phosphoglucose isomerase; G6P, glucose 6-phosphate dehydrogenase; LDH, L-lactate dehydrogenase; G3P, glyceraldehyde 3-phosphate dehydrogenase; NSP, nucleoside phosphorylase; 6PG, 6-phosphogluconate dehydrogenase; LAP, leucine aminopeptidase; ALD, aldolase.

DISCUSSION

Inferences from studies on the structure of bacterial populations are clearly dependent on the pattern and extent of sampling. Since isolates of *S. pneumoniae* display a vast number of different capsular serotypes, each of which may be associated with numerous clones, a comprehensive pneumococcal framework study would require an immense number of isolates. For practical purposes, the present study was re-

TABLE 5. Observed versus expected numbers of six four-locus allele combinations among 28 representative ETs

Combination no.	Allele no.[a]				No. of allele combinations	
	a	b	c	d	Expected	Observed
1	EST"2"	CDK"2"	ADK"1"	GD2"1"	9.6	8
2	G6P"2"	LDH"2"	G3P"2"	NSP"1"	1.8	1
3	NSP"1"	6GP"2"	LAP"1"	ALD"2"	3.4	3
4	ADK"1"	GD2"2"	ADH"1"	PGI"1"	3.3	5
5	GD2"2"	ADH"2"	G6P"2"	NSP"1"	0.5	0
6	LDH"2"	G3P"1"	NSP"1"	6GP"1"	8.8	8

[a] For enzyme abbreviations, see Table 4, footnote *a*. Numbers set off by quotation marks indicate allele numbers corresponding to Table 4.

TABLE 6. Multilocus genotypes represented by more than one isolate without null alleles

ET	No. of isolates	Origin(s)[a,b]	Serotype(s)[b]	IgA1 protease inhibition type(s)[b]
ET7	2	Nor, ND	18C	11
ET9	3	DK (2), Nor	19F	14
ET11	3	Au, Bel, Nor	19F (2), 14	16 (2), 10
ET13	3	DK (2), Bel	3	3
ET14	3	DK	7F	8
ET29	3	DK	6A	4
ET30	4	DK (3), Fra (1)	1	1 (3), 2
ET32	2	DK	18C, 23F	11, 4
ET33	3	DK (2), Nor	6B	6
ET35	2	DK, Nl	6A	3
ET36	4	DK (3), Nor	14	9
ET37	2	DK, Bel	23F	4
ET44	2	DK	14	9
ET52	2	Bel, Nor	23F	4
ET57	3	Nor	7F	8
ET61	2	Bel	1	2
ET64	2	Fra	10A, 10F	1

[a] Abbreviations: ND, not determined; Au, Australia; Bel, Belgium; DK, Denmark; Fra, France; Nl, The Netherlands; Nor, Norway.

[b] For several origins, serotypes, and inhibition types, numbers in parentheses indicate the number of isolates if more than one.

stricted to isolates recovered mainly during 1991 to 1993 in Denmark and, to a lesser extent, in Norway, Belgium, France, The Netherlands, Yugoslavia, and Australia. The serotypes that most often cause disease vary with age, geographical area, and chronological time (33). This study focused on serotypes frequently recovered from diseased children because these patients constitute a serious health problem of high priority in the respective countries.

The electrophoretically demonstrable allelic variation among 70 ETs at the 13 enzyme loci examined in this study suggested a relatively limited genetic diversity ($H = 0.319$) in the pneumococcal population in comparison with most other bacterial species previously studied (43). This may be due in part to the spatially and temporarily restricted sampling of the isolates. A slightly lower diversity ($H = 0.247$) was calculated for 28 ETs identified among 66 pneumococcal isolates recovered worldwide, including both penicillin-susceptible and -resistant strains (41). Furthermore, a number of reports on DNA sequence comparisons, including amylomaltase genes (4), pneumolysin genes (28), and genes of penicillin-susceptible strains encoding penicillin-binding proteins 1a, 2b, and 2x as well as their flanking regions (4, 19, 26, 42), have shown highly uniform sequences, leading to the suggestion that *S. pneumoniae* is a relatively young species phylogenetically (4). Alternatively, extensive recombination may tend to decrease diversity in the absence of a selective pressure driven by host-immune forces.

To estimate the frequency of recombination in natural populations of pneumococci, the index of association (43), which provides a measure of clonality, was calculated for 28 ETs chosen to represent clusters separated at a genetic distance of approximately 0.15. For populations that evolve primarily as separate clonal lineages, the index value significantly exceeds zero. However, the calculated index value for the examined pneumococci was -0.14 ± 0.26. Combined with the finding that all alleles were at linkage equilibrium in pairwise analysis, the data suggest that the population examined is panmictic in the long term as a result of a high frequency of horizontal genetic transfer relative to that of the mutation rate. Nevertheless, in the short term, specific clones seem to exist long enough for considerable spread, since isolates of identical ET, capsular serotype, and IgA1 protease inhibition type were recovered from widely different geographic areas. In addition, repeated sampling of related strains was indicated by the correlation observed between certain capsular serotypes and IgA1 protease inhibition types when all isolates were considered. Taken together, these findings point to the conclusion that the population of *S. pneumoniae* studied possesses an epidemic genetic structure such as that previously proposed for the population of another important human pathogen, *N. meningitidis* (43).

The concept of an epidemic structure in natural populations of pneumococci is easily reconciled with results of a number of previous studies. Altered penicillin-binding proteins appear to have arisen on multiple occasions from different recombinational events (8, 19, 32, 41, 45). Several reports suggest that capsular serotype is subjected to recombination in natural populations (2, 10, 11, 32, 41). Members of certain penicillin-resistant clones have been recovered independently in different countries and continents (2, 10, 31, 32, 41, 44), implying that epidemic spread of pneumococcal clones does occur. A likely mechanism for the proposed frequent horizontal genetic transfer is provided collectively by the following findings: the pneumococcus is naturally competent for DNA uptake (24), the organism autolyses under certain growth conditions, thereby possibly providing an abundance of DNA for uptake (34), and concomitant colonization by more than one serotype of pneumococci is a relatively frequent event (1). Thus, the stage seems set for frequent transformation and subsequent homologous recombination.

The structural relatedness among IgA1 proteases excreted by *S. pneumoniae* was assessed with enzyme-neutralizing antibodies raised in rabbits as probes. Such antibodies have been shown previously to be very sensitive tools in disclosing heterogeneity among IgA1 proteases within single species (5, 16, 20–23, 38). The neutralizing response of rabbits to bacterial IgA1 proteases apparently reflects very well the corresponding response in humans (20, 21). Moreover, a qualitatively similar neutralizing response seems to be induced in individual rabbits immunized with the same bacterial IgA1 protease (20). Pneumococci produce an autolysin, and the extent to which the enzyme preparations were a mixture of IgA1 proteases excreted and others released by autolysis is not clear. No data are available on the nature of the excretion pathway and the possible existence of antigenic differences between such versions of the IgA1 protease.

In the present study, extensive antigenic heterogeneity was observed among *S. pneumoniae* IgA1 proteases even within a restricted geographic region like Denmark. This finding markedly contrasts with those for IgA1 proteases excreted by members of the related streptococcal species *S. sanguis* and *Streptococcus oralis*, which are antigenically homogeneous (38). However, another surface-exposed protein of the pneumococcus, PspA, exhibits a similarly high level of antigenic diversity (3). Host-immune selective forces may preserve a high level of variation provided by recombination and thereby foster diversity in crucial epitopes. The antigenic polymorphism conceivably serves to manipulate and evade specific host defenses as previously suggested for IgA1 proteases of *Haemophilus influenzae* (23). The ensuing lack of common epitopes recognized by neutralizing antibodies and the apparent potential for rapid evolution of new antigenic variants make the use of the pneumococcal IgA1 protease as a component of future vaccines less attractive. However, the extensive change in the protease sug-

gests that it is important for the pneumococcus to not have the activity of this enzyme inhibited by the host.

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