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The Autolysin-Encoding Gene (*lytA*) of *Streptococcus pneumoniae* Displays Restricted Allelic Variation despite Localized Recombination Events with Genes of Pneumococcal Bacteriophage Encoding Cell Wall Lytic Enzymes

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The *lytA*-encoded autolysin (*N*-acetylmuramoyl-L-alanine amidase) of *Streptococcus pneumoniae* is believed to play an important role in the pathogenesis of pneumococcal infection and has been identified as a putative vaccine target. Allelic diversity of *lytA* in an extensive collection of clinical isolates was assessed by restriction fragment length polymorphism and confirmatory sequencing studies. Genetic diversity within *lytA* is limited, especially compared to the high levels of diversity seen in other pneumococcal virulence factor genes, although small blocks generating mosaic structure were identified. Sequence comparisons with genes encoding cell wall lytic enzymes of pneumococcal bacteriophage suggest that localized recombination events have occurred between host *lytA* and these bacteriophage genes. These results confirm earlier suggestions that recombination between DNA encoding bacteriophage autolytic enzymes and chromosomally encoded *lytA* might be important in the evolution of *lytA*. The implications of these findings for understanding the evolution of *lytA* and the potential utility of LytA as a vaccine target are discussed.

The *lytA*-encoded major autolysin (*N*-acetylmuramoyl-L-alanine amidase) of *Streptococcus pneumoniae* is a member of a widely distributed group of cell wall-degrading enzymes located in the cell envelope and postulated to play roles in a variety of physiological functions associated with cell wall growth, wall turnover, and cell separation in microorganisms (27). The pneumococcal autolysin has a modular organization; the catalytic function is located in the N-terminal domain, and the C-terminal domain, composed of six repeat units and a short tail, acts as a binding arm attaching the enzyme to the choline residues of pneumococcal cell walls (5). Many bacteriophage infecting pneumococci also possess cell wall lytic enzymes which can show high similarity to either or both domains of the host *lytA*. In recent years it has become clear that these cell wall lytic enzymes provide one of the clearest examples among prokaryotic proteins of a two-domain structure whereby similarity between bacteriophage and bacterial DNA allows shuffling of domains by recombination restructuring both viral and bacterial genomes (9, 17).

Although there remains some controversy regarding the importance of autolysin in pathogenesis, both direct and indirect roles in the pathogenic process have been postulated. Autolysin may play a direct role in virulence by mediating the release of cell wall components shown to be highly inflammatory in animal models (29, 30). In addition, it has been suggested that autolysin plays an indirect role in pathogenesis by mediating cell lysis and the subsequent release of virulence factors, such as pneumolysin, not actively exported from the cell (12, 20). In support of a role for *lytA* in virulence, isogenic *lytA* mutants have been found to be significantly less virulent than the parent strain in some animal models (1, 2), and when inoculated into the mouse lung in a model of pneumonia, *lytA* mutants are cleared rapidly and do not invade the bloodstream (3). However, there are contradictory reports claiming no role for autolysin in virulence (28). Findings that mice immunized with autolysin survived significantly longer than control mice following intranasal challenge identified autolysin as a possible vaccine candidate (1, 15). However, the degree of protection was similar to that seen in those immunized with pneumolysin, with no increased protection apparent in animals immunized with both pneumolysin and autolysin. In association with data showing that survival time was not increased in animals challenged with a pneumolysin-negative strain, these findings indicate that at least in the mouse model, antibodies against autolysin appear to mediate their effects primarily by preventing the release of pneumolysin. In contrast, in a chinchilla otitis media model, autolysin induced release of cell wall components plays a key role in middle ear inflammation whereas pneumolysin appeared to have a limited role (26). A recent study using a signature-tagged mutagenesis approach to facilitate a large-scale identification of virulence-associated genes appeared to demonstrate an important role for autolysin in establishing pneumonia, while intraperitoneal inoculation of the same mutant demonstrated no role for autolysin in septicemia (23). Thus, there remains some controversy about the relevance of autolysin in pathogenesis, with the relative contribution of particular virulence factors appearing to vary between both different disease states and different animal models (22).

As part of a systematic study investigating the allelic variation of virulence determinants of *S. pneumoniae* (8) examining both the molecular evolution and the potential utility of these proteins as vaccine targets, we have performed a detailed analysis of the genetic diversity of *lytA*. Little is known about the allelic diversity of *lytA* in pneumococci, although the gene from an atypical clinical isolate (101/87) shows only 81% identity with *lytA* (6). However, recent studies in our laboratory, involving extensive sequencing of housekeeping genes, have shown that strain 101/87 is genetically distant from clinical isolates of typical pneumococci (31). A recent study, based on

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TABLE 1. Allelic profiles of bacterial isolates used in this study as determined by RFLP of *lytA*

Strain	City and/or country of isolation	Source	Date of isolation	Serotype	Allelic profile with indicated restriction enzyme:				Overall *lytA* profile
					*Rsa*I	*Bsr*I	*Aci*I	*Hsp*92II	
Selected for *lytA* sequencing									
494	Liverpool, UK[a]	NK[a]	1995	3	1	1	1	1	1
7751	Spain	NK	NK	6	1	1	1	1	1
670	Spain	NK	1988	6B	1	1	1	1	1
PN8	Oldham, UK	NK	1987	23	1	1	1	1	1
Pn107	Oxford, UK	NK	1995	1	1	1	1	2	2
CL2	Spain	Vagina	1987	1	1	1	1	2	2
Pn58	Spain	NK	1993	19A	2	1	1	2	3
VA1	US[a]	NK	1983	19	1	2	1	3	4
472	Leicester, UK	NK	NK	3	1	1	1	4	5
29044	Czechslovakia	NK	1987	14	1	1	1	4	5
860	NK	NK	1994	NK	1	1	1	4	5
CL18	Kenya	Blood	1991	10	3	1	1	2	6
1012	Manchester, UK	Throat	1993	35	1	1	2	1	7
PN15	Papua New Guinea	NK	1969	12	1	1	1	5	8
233	Poland	Throat	1995	23F	1	1	3	4	9
234	Poland	Throat	1995	23F	1	1	3	4	9
Others									
PN109	Middlesbrough, UK	Ear	1995	1	1	1	1	1	1
969	Manchester, UK	Throat	1993	3	1	1	1	1	1
940	Oxford, UK	Throat	1995	3	1	1	1	1	1
886	Kenya	Throat	1991	3	1	1	1	1	1
PN112	Cambridge, UK	Ear	1995	3	1	1	1	1	1
1002	Manchester, UK	Throat	1994	3	1	1	1	1	1
PN111	Ashford, UK	Sputum	1995	3	1	1	1	1	1
CL4	NK	NK	NK	4	1	1	1	1	1
CL5	Spain	Blood	1988	4	1	1	1	1	1
PN12	Papua New Guinea	NK	1992	6	1	1	1	1	1
CL31	Spain	Peritoneal	1993	15	1	1	1	1	1
CL42	Spain	Sputum	1988	19	1	1	1	1	1
CL45	Spain	Blood	1988	20	1	1	1	1	1
SP1	Spain	Blood	1988	23	1	1	1	1	1
Pn24	Spain	NK	NK	23	1	1	1	1	1
Pn25	Spain	NK	NK	23	1	1	1	1	1
967	Oxford, UK	Throat	1994	35F	1	1	1	1	1
Pn16	Papua New Guinea	NK	1970	42	1	1	1	1	1
Pn11	UK	NK	1988	NK	1	1	1	1	1
PN108	Oxford, UK	NK	1995	1	1	1	1	2	2
900	Oxford, UK	Throat	1994	1	1	1	1	2	2
951	Oxford, UK	Throat	1994	6A	1	1	1	2	2
990	Manchester, UK	Throat	1994	10	1	1	1	2	2
CL21	Kenya	Sputum	1991	11	1	1	1	2	2
CL25	Kenya	NK	1991	12	1	1	1	2	2
CL26	Spain	Blood	1988	13	1	1	1	2	2
PN13	Papua New Guinea	NK	1973	14	1	1	1	2	2
912	Oxford, UK	Throat	1995	15B	1	1	1	2	2
PN60	Spain	NK	NK	19A	1	1	1	2	2
1011	Manchester, UK	Throat	1994	23	1	1	1	2	2
PN27	NK	NK	NK	35	1	1	1	2	2
9858	Brighton, UK	NK	1988	NK	1	1	1	2	2
R6	US	NK	ca. 1930	NT[a]	1	1	1	2	2
PN5	Brighton, UK	NK	1988	NK	1	1	1	2	2
PN110	Norwich, UK	Sputum	1995	3	1	1	1	4	5
CL44	Kenya	NK	1991	7	1	1	1	4	5
CL13	Spain	Blood	1988	8	1	1	1	4	5
873	Kenya	Throat	1990	8	1	1	1	4	5
998	Manchester, UK	Throat	1994	9	1	1	1	4	5
880	Kenya	Throat	1990	13	1	1	1	4	5
CL35	Spain	Vagina	1987	16	1	1	1	4	5
CL43	Kenya	Sputum	1991	19	1	1	1	4	5
CL41	Kenya	Throat	1991	19	1	1	1	4	5
PN6	Brighton, UK	NK	1988	NK	1	1	1	4	5
CL20	Kenya	Blood	1991	10	3	1	1	2	6
878	Kenya	Throat	1990	10F	1	1	2	1	7

[a] UK, United Kingdom; US, United States; NK, not known; NT, nontypeable (acapsular).

single-strand conformational polymorphism (SSCP) analysis of a small number of clinical isolates, suggested that *lytA* is a heterogeneous gene subject to continual variation (11). This was in contrast to preliminary data obtained by us which showed only five closely related alleles of *lytA* in a limited collection of strains (32). Here we confirm and extend our findings and report on both restriction fragment length polymorphism (RFLP) and nucleotide sequencing studies which demonstrate that in contrast to many other genes encoding virulence factors of *S. pneumoniae*, *lytA* is a rather highly conserved gene.

MATERIALS AND METHODS

Purification of chromosomal DNA. Chromosomal DNA was purified as described previously (33) from 62 strains of *S. pneumoniae* selected to represent a diverse range of isolates in terms of serotype, clinical association, and time and place of isolation (Table 1).

PCR analysis. A *lytA* PCR product was amplified by using primers *lytA*up (5′ GGAGTAGAATATGGAAATTGATGTGAGTAA 3′ and *lytA*dn 5′ TTTATT TTACTGTAATCAAGCCATCTGGCTC 3′), corresponding to the extreme 5′ and 3′ regions of the *lytA* coding sequence. PCR conditions used were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, repeated for 30 cycles.

RFLP analysis. Approximately 5 μl of PCR product was digested with restriction enzymes according to the manufacturer's instructions in a total volume of 25 μl. Digests were then separated on 4 and 8% polyacrylamide gels and visualized under UV illumination following staining for 15 min in ethidium bromide (0.3 μg ml^{-1}).

Direct sequencing of PCR products. A fraction of the PCR products used in RFLP analysis were purified by passage through QiaQuick PCR product purification columns and sequenced directly, using both primers *lytA*up and *lytA*dn and a series of internal primers. Sequencing was performed with an ABI 373 system.

Sequence analysis. Preliminary sequence analysis was performed with the DNAStar package. Comparisons of polymorphic sites and calculations of the ratio of synonymous to nonsynonymous change were constructed by using the MEGA package (14).

Nucleotide sequence accession numbers. The sequences of the *lytA* alleles have been submitted to GenBank and assigned accession no. AJ243399 to AJ43414.

RESULTS AND DISCUSSION

Assessment of allelic diversity of *lytA* by RFLP. Chromosomal DNA was purified from 62 strains of *S. pneumoniae* selected to represent a diverse range of isolates in terms of serotype, clinical association, and time and place of isolation (Table 1). A PCR product representing the entire *lytA* gene was successfully amplified from each chromosomal DNA preparation. Allelic diversity of *lytA* was then assessed by digesting each PCR product independently with four frequently cutting restriction enzymes, *Rsa*I, *Bsr*I, *Aci*I, and *Hsp*92II, resulting in coverage of at least 10% of the *lytA* sequence. The numbers of distinct alleles detected with each of these restriction enzymes were three, two, three, and five, respectively resulting in nine distinct overall allelic profiles of *lytA* (Table 1). The vast majority of isolates (85.5%) possessed one of the three most common alleles, *lytA1*, *lytA2*, or *lytA5*, with all other alleles present in no more than two isolates (Table 2). By applying the equation of Nei and Li (21) to the RFLP data, it is possible to obtain an estimate of the genetic diversity between alleles, which ranged from a minimum of 0.13% (between *lytA5* and *lytA8*) to a maximum of 2.31% (between *lytA4* and *lytA6*), indicating that genetic diversity in *lytA* is rather limited.

Sequencing of *lytA* RFLP allelic variants confirms limited genetic diversity. To confirm our understanding of the extent and nature of *lytA* genetic diversity, a PCR product representative of each of the nine RFLP allelic variants was sequenced directly. In addition, multiple representatives of the most common alleles (*lytA1*, *lytA2*, and *lytA5*) and *lytA9* were sequenced to examine diversity within an allele as defined by RFLP analysis (Table 1). As illustrated in Fig. 1, sequencing directly confirmed the limited sequence diversity suggested by RFLP analysis. Sequence diversity ranges from a minimum of 0.11% (equivalent to one base difference) to a maximum of 3.20% between *lytA4* and *lytA6*. The previously published *lytA* sequence from strain Rst7 (10) is also included in Fig. 1 for comparison; this sequence represents a distinct allele on the basis of one polymorphism not seen in any other sequence. Isolates which are largely geographically, temporally, and clinically distinct but found to possess the same RFLP allele are also closely related in terms of sequence. The *lytA* sequences of the three *lytA5*-, two *lytA2*-, and two *lytA9*-containing strains are identical, while a maximum of two base substitutions are seen between the four *lytA1*-containing strains sequenced. The extent of genetic diversity is broadly similar to that estimated by RFLP, and sequences are entirely consistent with the profiles obtained for each enzyme by RFLP analysis.

TABLE 2. Distribution of *lytA* allelic variants as determined by RFLP

Allele	No. (%) of isolates
lytA1	23 (37.1)
lytA2	17 (27.4)
lytA3	1 (1.6)
lytA4	1 (1.6)
lytA5	13 (21.0)
lytA6	2 (3.2)
lytA7	2 (3.2)
lytA8	1 (1.6)
lytA9	2 (3.2)

Mosaic structure of *lytA* resulting from localized recombination with pneumococcal bacteriophage. Comparison of only sites polymorphic between the sequences, shown in Fig. 2, reveals that genetic variation in *lytA* is not randomly distributed. Three apparent blocks of diversity can be seen at bases 441 to 465 in *lytA2*, *lytA3*, *lytA6*, and the previously published Rst7 sequence, bases 681 to 699 in *lytA4*, and bases 747 to 758 in *lytA6*. The remaining 19 (<50%) polymorphic sites are scattered throughout the remaining ca. 95% of the *lytA* sequence. The predicted amino acid sequences of each allelic variant, also shown in Fig. 2, illustrate that the vast majority of the nucleotide variation seen in *lytA* is synonymous. The proportion of synonymous changes per synonymous site (0.0347 ± 0.008) to nonsynonymous changes per nonsynonymous site (0.0036 ± 0.0012) calculated by using the Jukes-Cantor correction in the MEGA suite of programs (14) approaches 10:1, implying that purifying selection is preferentially eliminating amino acid changes.

The mosaic distribution of polymorphic sites suggests that very localized recombination events may have occurred, resulting in the "pock-marked" structure of the *lytA* genes in some pneumococci. In light of previous reports (5, 7, 9, 25), likely donors of DNA in such recombination events are genes of pneumococcal bacteriophage encoding cell wall lytic enzymes which share considerable sequence similarity with *lytA*. We therefore compared the available sequences of these bacteriophage genes with the mosaic *lytA* genes. Figure 3 shows an alignment of the polymorphic sites, comparing the *lytA* sequences of strains CL18 and VA1 containing putative blocks with the amidase genes *hbl* and *ejl* of the bacteriophage HB-3 and EJ-1, respectively (7, 25). The sequences are compared with that of the *lytA* gene of 7751 as a background strain which does not appear to have a mosaic structure. In general, the bacteriophage genes show divergence from the pneumococcal *lytA* sequences throughout their entire length. However the

	7751 Pn8	494	670	Pn107 CL2	RST7	Pn58	VA1	29044 860, 472	CL18	1012	Pn15	233, 234
	lytA1	*lytA1*	*lytA1*	*lytA2*		*lytA3*	*lytA4*	*lytA5*	*lytA6*	*lytA7*	*lytA8*	*lytA9*
7751, Pn8	-	0.11	0.11	1.43	1.43	1.43	1.43	0.22	1.98	0.33	0.11	0.22
494		-	0.22	1.54	1.54	1.54	1.54	0.33	2.09	0.44	0.22	0.33
670			-	1.54	1.54	1.54	1.32	0.33	2.09	0.44	0.22	0.33
Pn107, CL2				-	0.22	0.22	2.64	1.43	0.77	1.76	1.54	1.43
RST7					-	0.22	2.64	1.43	0.77	1.76	1.54	1.43
Pn58						-	2.64	1.43	0.77	1.76	1.54	1.43
VA1							-	1.43	3.20	1.76	1.54	1.43
860,29044,472								-	1.98	0.55	0.33	0.22
CL18									-	2.32	2.09	1.98
1012										-	0.44	0.55
Pn15											-	0.33
233, 234												-

FIG. 1. Percent nucleotide divergence of *lytA* sequences as determined by direct sequencing. The previously published sequence from strain Rst7 (10) is included for comparison. Where more than one strain is shown in a column, the *lytA* sequences of these strains were found to be identical. The allele designations refer to alleles identified by RFLP.

polymorphic sites which define the blocks described above for both CL18 and VA1 correspond almost perfectly with the sequence of the equivalent region of one or other of the bacteriophage genes. Thus, the CL18 block from bp 441 to 462 is identical to the equivalent region in *ejl*, the CL18 block from bp 753 to 758 is identical to sequence of *hbl*, and the VA1 block from bp 681 to 699 is also identical with the equivalent sequence of *hbl*. Although these blocks of identity are small, they contrast strongly with the variation seen between the bacteriophage and bacterial sequences over the rest of the alignment and strongly suggest that localized recombination events with bacteriophage DNA have been involved in the evolution of the pneumococcal *lytA* gene in nature. The presence of these blocks was confirmed by using the maximum chi-squared procedure with sites polymorphic over the potential recombinant strain and both potential parent strains (e.g., CL18/EJ-1/7751) to test the statistical significance of the observed mosaic structure (18). Both the larger blocks (CL18 block at bp 441 to 462 and VA1 block at bp 681 to 699) reach statistical significance ($P < 0.0001$).

Molecular evolution of *lytA*. In summary, our data show that *lytA* is essentially a rather conserved gene displaying limited genetic variation (0.11 to 3.2%). If the putative recombinant blocks are discounted from sequence comparisons, *lytA* genes from all strains except VA1 differ from that seen in strain 7751 by only one to three base changes. Even the maximal seven base changes seen in VA1 compared to 7751 (excluding recombinant blocks) is equivalent to variation of only 0.8%, which is similar to that reported for pneumococcal housekeeping genes (34). However, there is substantial evidence that localized recombination events with bacteriophage have occurred in the evolution of pneumococcal *lytA*. These findings confirm earlier reports that recombination with resident bacteriophage autolytic genes may play a role in driving the evolution of *lytA*. Shuffling of distinct C- and N-terminal domains is thought to have played a role in the evolution of this gene family (5, 9), and the potential for recombination to occur between bacteriophage DNA and *lytA* was demonstrated in the laboratory by the repair of a number of distinct point mutations following transformation with a plasmid containing the *hbl* gene (25). However, evidence for the occurrence of such small localized recombination events seen in clinical isolates of pneumococci not subject to laboratory manipulations has not been reported previously. It is also of interest that one of the mosaic blocks (identified in strain CL18) displays most similarity with the equivalent region of *ejl* from bacteriophage EJ-1. This bacteriophage was isolated from the atypical pneumococcal isolate (101/87) and reported to be unable to infect any of the typical pneumococcal strains tested (7).

Even with the occurrence of recombination, the variation

```
                 1111222344444444444456666666777777778899     12222233
                 4267804804445555666962889999034555563411   6663345500
                 5627823291470369025671190379587346881318   3860362346

7751   CCCTAGGTCTGTACTCTGATTCCGGCACCTCCACACCTAA   QDYWNDNEKE   lytA1
494    ......A.....................................   ..........   lytA1
670    ............................G..........   .....E....   lytA1
PN107  .......C.CTCTTATCTTC...................C   ..H......D   lytA2
PN58   .......C.CTCTTATCTTC.T..................   ..H.......   lytA3
VA1    TT.A.A............C..TACTCA.G......A...   .NHYQE....   lytA4
29044  ..................CC.....................   ..H.......   lytA5
CL18   ..T....C.CTCTTATCTTC..........TTGTT.....   ..H...DV..   lytA6
1012   ........T...................T........C..   ..........   lytA7
PN15   ....................................A....   ..........   lytA8
233    ....G.............C.....................   R.H.......   lytA9
RST7   .......C.CTCTTATCTTC...................G.   ..H.....R.   GenBank

                 3333213333333333133133323313333313233323
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FIG. 2. Illustration of the diversity of *lytA* alleles showing only the polymorphic sites in both nucleotide and predicted amino acid sequence alignments. Numbering begins at the first residue of the ATG start codon, although residues 1 to 20 and 922 to 951 of *lytA* were not sequenced in this study since they correspond to the sequences of the PCR primers. Residues identical to those in strain 7751 are indicated by dots. Numbers below the nucleotide sequence represent codon positions of the changes. Allele designations refer to alleles identified by RFLP.

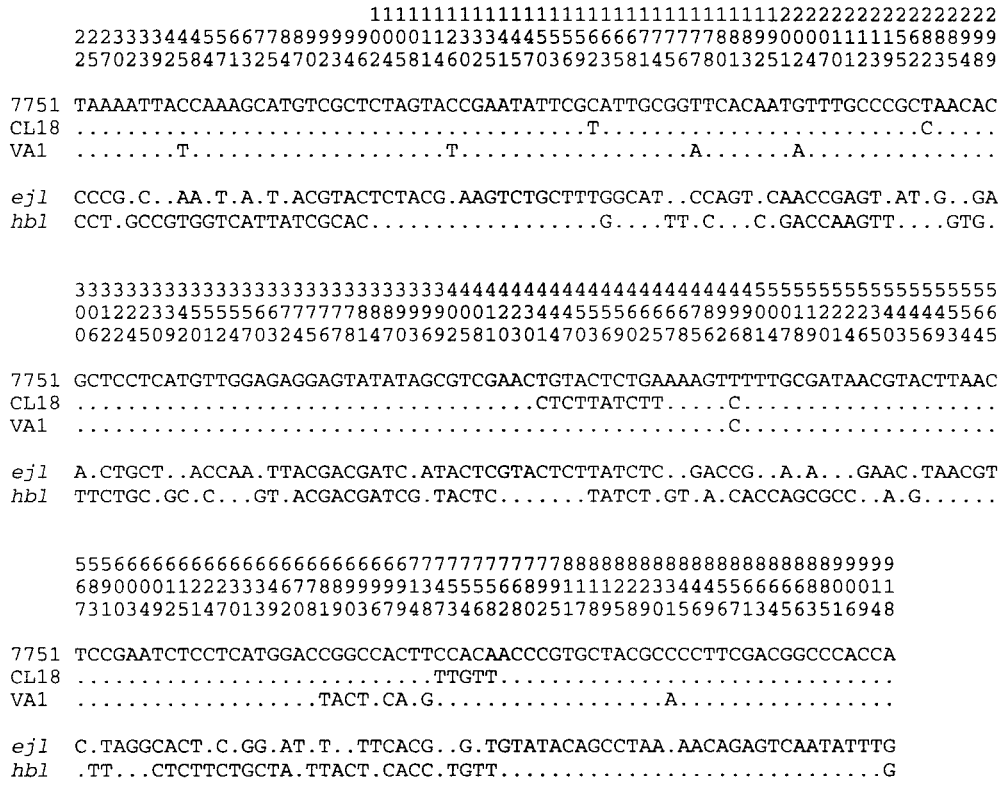

FIG. 3. Alignment of polymorphic sites in *lytA* alleles with mosaic structure represented by strains CL18 and VA1 in comparison with an allele containing no blocks represented by 7751 and the amidase genes of pneumococcal bacteriophage HB-3 (*hbl*) and EJ-1 (*ejl*). Numbering begins at the first residue of the ATG start codon, although residues 1 to 20 and 922 to 951 of *lytA* were not sequenced in this study since they correspond to the sequences of the PCR primers.

seen in *lytA* is substantially lower than that reported for other putative pneumococcal virulence factor genes such as those encoding PspA (4, 19), immunoglobulin A protease (16, 24), or NanA (8, 13) which have been shown to be highly heterogeneous, with up to 30% diversity at the nucleotide level. The data presented here contrast with previous assertions (11), based on a small SSCP study, that *lytA* is a heterogeneous gene "subject to continual variation." We suspect that the existence of these small recombinant blocks, illustrated in Fig. 3, resulted in SSCP providing a misleading picture of the evolution and overall genetic diversity of *lytA*. Gillespie et al. (11) sequenced only one small fragment deemed variable by SSCP. However, in support of our hypothesis, many of the changes reported in their study were also seen in this study (using a much more extensive strain collection) and found to correspond to one of the potential recombinant blocks identified in CL18. In spite of the small number of coding changes identified in this study, it remains possible that some of these could significantly alter autolysin functioning; further studies assessing the activity and affinity of purified allelic variants are required to address this issue.

This work has been driven by limited knowledge of allelic diversity of pneumococcal virulence determinants and the desire to identify conserved vaccine targets (8). Autolysin appears to represent such a conserved target. The relative infrequency of nonsynonymous change suggests that there is not a strong positive selection pressure for diversity on autolysin. This implies either that the function of the protein is unable to accommodate substantial change or that there may be little role for antibody driven evolution of autolysin in natural infections. Despite this, immunization with LytA can produce a protective response in mice and can induce antibodies capable of inhibiting spontaneous autolysis even in encapsulated organisms (1). As a protein found in all strains, associated with virulence, and apparently highly conserved, autolysin might appear to be a suitable target for inclusion in a potential vaccine. However, the results presented here suggest that any future selective pressure imposed by the use of such a vaccine could drive the selection of novel *lytA* alleles resulting from recombination with bacteriophage DNA. Although antisera prepared against some of the lytic enzymes of pneumococci and their bacteriophage may cross-react with one another, such events could theoretically lead to the rapid generation of immune escape mutants to a LytA-containing vaccine. Further laboratory studies are required to address this possibility.

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