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NOTES

Allelic Variation in *Streptococcus pneumoniae* Autolysin (*N*-Acetyl Muramoyl-L-Alanine Amidase)

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The *lytA* gene encoding the autolysin of *Streptococcus pneumoniae* may be a virulence determinant. Single-strand conformational polymorphism analysis demonstrated heterogenicity throughout the gene in clinical isolates and strains from the clonal serotypes 7 and 14. Sequence analysis of part of the choline-binding domain showed that in two isolates four amino acid substitutions occurred.

The cell wall of *Streptococcus pneumoniae* has an important role in generating inflammatory mediators and consequent pathological changes (13). Injection of teichoic acid-containing cell wall preparations generates a strong inflammatory response in chinchilla rabbits (16).

The components of the complement cascade generated by interaction with C polysaccharide are thought to be crucial in generation of an inflammatory reaction in the alveoli (14), the meninges (16), and the middle ear (1). Organisms with high cell wall turnover induce a greater inflammatory response in the rabbit meninges (12), and we have demonstrated a trend to higher mortality in patients with detectable C polysaccharide in their serum (7).

N-Acetyl muramoyl-L-alanine amidase is responsible for cellular autolysis at the end of log phase and has a role in cell division and peptidoglycan remodelling (15). The C-terminal domain binds the choline phosphate residue of the teichoic acid, and the catalytic amidase domain is located in the N terminus (9).

A *lytA* deletion mutant, M31, with a normal growth rate but no static-phase lysis has been described (10). The *lytA* gene from clinical isolate Lyta 101 shows only 81% identity with the published *lytA* sequence and has lower autolytic activity with a LytA$^-$ phenotype (2). A single amino acid change from glutamic acid to glycine in a *lyt-4* mutant strain altered the hydropathic profile of the protein and produced thermosensitive activity (3).

Isolates of *S. pneumoniae* were obtained from the clinical laboratories of the Royal Free, Whittington, and University College London Hospitals. All were invasive strains from patients with pneumonia, septicemia, and meningitis. Isolates of serotypes 7 and 14 were obtained from the Central Public Health Laboratory.

PCR amplification of the whole *lytA* gene was performed on DNA obtained from a few colonies emulsified in 50 μl of distilled water and boiled for 5 min by using primers Lyt1 (5′ GCC TAA TCG TGA CTA AGA 3′) and Lyt2 (5′ ACC TAA TAA TAT GCG CTG 3′) as described previously (6).

Amplified DNA was purified by using the Wizard PCR preparation kit (Promega) and digested with *Hin*fI and *Taq*I to produce four fragments of 377, 319, 299, and 190 bp.

Digested DNA (7 μl) was denatured at 90°C for 5 min with 5 μl of single-strand conformational polymorphism analysis (SSCP) loading buffer and 2 μl of SSCP stop dye (Promega). Samples were loaded directly onto a 0.5× mutation detection enhancement acrylamide analog sequencing format gel (FMC Bioproducts, Rockport, Maine) and run for 16 h at 6 W by using 0.6× Tris-borate-EDTA. Bands were visualized by the silver staining method by using the manufacturer's instructions

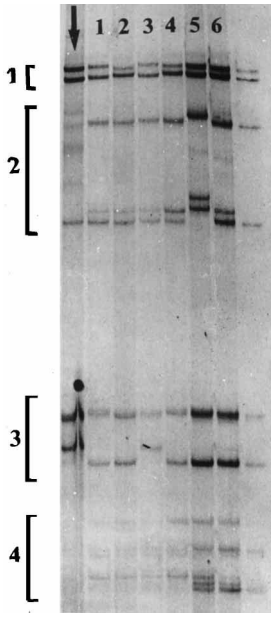

FIG. 1. SSCP of the *lytA* gene from representative clinical isolates of *S. pneumoniae*. The SSCP pattern of the published *lytA* sequence (4) is indicated by an arrow.

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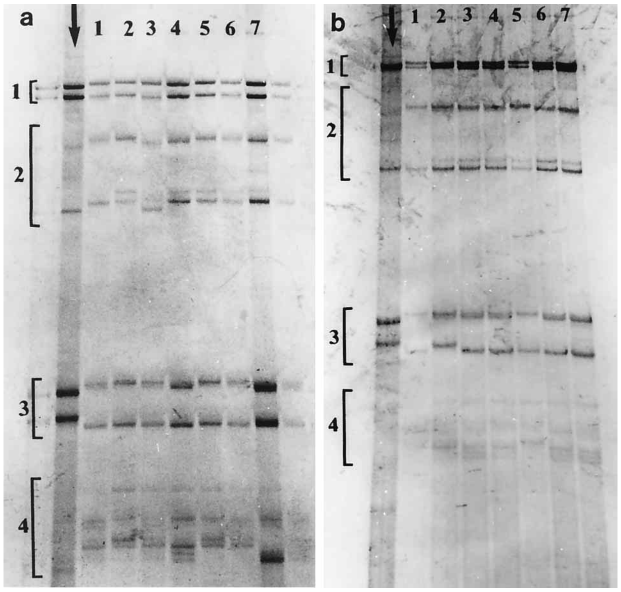

FIG. 2. (a) SSCP of *lytA* from serotype 7 isolates of *S. pneumoniae*. (b) SSCP of *lytA* from serotype 14 isolates of *S. pneumoniae.*

(Promega, Southampton, United Kingdom). DNA from *lytA* in pGL100 (provided by P. Garcia and colleagues) was run in all SSCP gels (4).

Variation was found among all of the clinical isolates by SSCP, and only two isolates (isolates 3 and 18) had a pattern similar to the published *lytA* sequence. The variation occurred in all of the four fragments studied, including fragments 2 and 4, the choline-binding and amidase domains of the gene, respectively. Although there is apparently less variation in the first fragment, the small changes here are very significant because of the short resolving distance. In fragment 4, there are multiple bands, indicating that there are a number of different conformations possible for the sequence of that fragment. Typical SSCP patterns for seven of the clinical isolates are illustrated in Fig. 1.

Variation was found in strains of serotype 7 (Fig. 2a), and none of the serotype 7 strains had a fragment 2 or fragment 3 which was similar to the published *lytA* sequence. Among the serotype 14 isolates, the most variation was found in fragments 3 and 4 (Fig. 2b). None of the strains had a fragment 3 homologous with the published sequence. There appeared to be less variation in the serotype 7 and serotype 14 strains than in the other clinical isolates.

Direct nucleotide sequencing of a 360-bp fragment of the choline-binding domain of *lytA* was performed on a purified, PCR-generated, double-stranded template by using the sequencing primers 5′ TATCCAAAAGACAAG 3′ and 5′ GTCGCGTATAATAATCCA 3′ with an ABI 377 automated DNA sequencer. Sequence analysis was carried out by using the GCG software package, version 8 (Genetics Computer Group, Madison, Wis.).

The two clinical isolates sequenced (Fig. 1, lanes 1 and 3) had an SSCP pattern similar but not identical in fragment 2 to the published *lytA* sequence. One isolate had an identical sequence, and the other had two point mutations which resulted in a change in the amino acid sequence from arginine to lysine.

The two serotype 7 strains sequenced (Fig. 2a, lanes 4 and 7) had similar SSCP patterns which differed from the published sequence. There were nine base pair substitutions and identity between the two strains, confirming the SSCP data. Two changes were noncoding, but the others resulted in changes to the amino acid sequence: aspartate to glutamate, asparagine to aspartate, glutamate to valine, and arginine to lysine (Fig. 3).

An association between cell wall turnover and virulence in a rabbit meningitis model has been demonstrated (12). Such variation may arise because of differences in synthesis or degradative processes. Autolysin is not the only enzyme capable of digesting *S. pneumoniae* peptidoglycan (5), but it is responsible for the major portion of cell wall degradation. Variation in autolysin activity might arise because of differences in the number of copies of functional amidase due to the presence of lysogenic phage DNA, and this possibility was not directly addressed in this study. Alternatively, the chromosomally encoded autolysin may have variable activity because of variation in the sequence of the gene coding for significant amino acid differences and strains with a LytA$^-$ phenotype are known to occur naturally (2, 3, 10).

Only 2 of the 18 clinical isolates had a *lytA* gene similar to the published sequence, and variation was found throughout the gene, including the amidase and choline-binding domains. Variation appeared more pronounced in the fragments furthest away from the choline-binding and amidase sites, suggesting a degree of evolutionary constraint. This result differs from those of previous studies, which used restriction fragment length polymorphism methodology and demonstrated only two alleles for autolysin (17), calling into question the validity of using restriction fragment length polymorphism methods to determine gene variation, as important differences may be missed.

Two strains had nine base pair substitutions, and these substitutions resulted in alterations in the predicted amino acid sequence. It opens intriguing possibilities that the differences in *lytA* demonstrated here might translate into significant differences in the function of transcribed autolysin. Isolates with altered pneumolysin proteins with three and four amino acid substitutions and two deletions in the gene have been reported. The mutant genes were shown to have reduced hemolytic activity (8). The variation we have found in autolysin exceeds that

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         1          134                                               311                 360
LytA     ------------ ATC GCT GAT AAG TGG TAC TAT TTC AAC GAA ------------ GAC AGG CCA GAA ------------
                       I   A   D   K   W   Y   Y   F   N   E               D   R   P   E

1        ------------ ATT GCT GAT AAG TGG TAC TAT TTC AAC GAA ------------ GAC AAG CCA GAA ------------
                       *   *   *   *   *   *   *   *   *   *               *   K   *   *

3        ------------ ATC GCT GAT AAG TGG TAC TAT TTC AAC GAA ------------ GAC AGG CCA GAA ------------
                       *   *   *   *   *   *   *   *   *   *               *   *   *   *

13       ------------ ATC GCT GAG AAG TGG TAC TAT TTT GAT GTA ------------ GAT AAA CCA GAG ------------
                       *   *   E   *   *   *   *   *   D   V               *   K   *   *

16       ------------ ATC GCT GAG AAG TGG TAC TAT TTT GAT GTA ------------ GAT AAA CCA GAG ------------
                       *   *   E   *   *   *   *   *   D   V               *   K   *   *
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FIG. 3. Sequence comparison of the 360-bp fragment encompassing the *S. pneumoniae* choline-binding domain of *lytA*. LytA, published *lytA* sequence (4); 1, 3, 13, and 16, clinical isolates. Nucleotides that diverge from the published nucleotide sequence are in boldface type. Conserved amino acids are denoted by asterisks.

demonstrated in pneumolysin, but any effect on virulence must await the result of activity and affinity studies on purified variant proteins, although our isolates were phenotypically normal.

Serotype 14 strains fall into a small number of highly related strains which constitute a clone by multilocus enzyme electrophoresis (11). If our data were confirmed in a larger group of isolates, it would suggest that the variation demonstrated in our studies has arisen relatively recently and that *lytA* is a gene which is subject to continual variation. This is in contrast to penicillin-binding protein genes from sensitive isolates of *S. pneumoniae*, which demonstrated very little variation by SSCP (data not shown).

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