Implications of Physiological Studies Based on Genomic Sequences: Streptococcus pneumoniae TIGR4 Synthesizes a Functional LytC Lysozyme

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Received 31 March 2005/Accepted 13 June 2005

The Streptococcus pneumoniae LytC lysozyme is responsible for autolysis at 30°C (a temperature close to that of the upper respiratory tract), promotes DNA release in competent cultures, and participates in nasopharyngeal colonization. We show that the virulent pneumococcal TIGR4 strain encodes an active LytC enzyme, in contrast with genome-based predictions.

Streptococcus pneumoniae is a major gram-positive human pathogen. Currently, the pneumococcus is the main cause of pneumonia, meningitis, and bloodstream infections in the elderly, the young, and the immunocompromised and is responsible for middle ear infections in children. In the United States, more than 500,000 cases of pneumococcal pneumonia occur each year, causing the deaths of almost 40,000 adults and children (18). The pneumococcal cell wall hydrolases are located on the cell surface and participate in many fundamental biological functions. These grounds make murein hydrolases good candidates in the search for new targets for fighting pneumococcal infections (10). To date, four pneumococcal murein hydrolases have been described (9, 11): the LytA amidase that is the major pneumococcal autolysin; the LytC lysozyme, which acts as an autolysin at 30°C; the LytB glucosaminidase responsible for cell separation at the end of cell division; and the Pce phosphorylcholine esterase, which most possibly regulates the number of choline residues available at the pneumococcal cell surface.

It is currently recognized that the autolytic enzymes LytA and LytC contribute to pneumococcal virulence. Cell wall components released after bacterial lysis are highly inflammatory in animal models (22). Moreover, cytoplasmic virulence proteins like pneumolysin and other intracellular toxins are liberated upon lysis. In addition, LytC also plays a role in the colonization of the rat nasopharynx by the pneumococcus (7). As well, the hypothesis focusing on autolysis as a possible explanation for the mechanism of competence-induced DNA release was favored, since mutants in the hylA gene exhibited a four- to eightfold decrease in the amount of DNA available in the medium (13, 19, 20). In fact, it has been demonstrated that a combined action of the autolytic amidase and lysozyme is required to promote DNA release in competent cultures of S. pneumoniae (13). DNA exchanges have been shown to take place in natural environments (16), and LytC might contribute to the liberation of DNA in habitats like the upper respiratory tract, where the temperature is close to 30°C.

Predictions of gene functionality based exclusively in computer analysis can lead to annotation errors (see reference 6 and the references therein). This may be of primary importance in those cases in which the gene product is involved in important functions, such as virulence. The complete genome sequence of S. pneumoniae TIGR4, a virulent serotype 4 isolate, has been reported (accession number AE005672) (21) and analysis of the gene SP1573 (corresponding to lytC) revealed the presence of an authentic frameshift mutation (i.e., it is not the result of a sequencing artifact) that was assumed to interrupt gene translation (Fig. 1). However, we show here that, actually, TIGR4 synthesizes an enzymatically active LytC protein.

The pneumococcal strains used were R6 (15), TIGR4 (21), M31 (ΔhylA) (17), R924 (hylA::kan) (13), and the hylC mutant strains R6C (hylC::ermC), M31C (ΔhylA hylC::ermC) (5), and R6CD (hylC::tet pce::ermC) (2). Strains P026 (hylA::kan), P036 (hylC::tet), and P031 (hylA::kan hylC::tet) were constructed by transformation of the TIGR4 strain with chromosomal DNA from R924 or R6CD. S. pneumoniae strains were grown in C medium (8) supplemented with yeast extract (0.8 mg/ml; Difco Laboratories) at 37°C without shaking. DNA sequencing was carried out by using an ABI Prism 3700 DNA sequencer (Applied Biosystems, Inc.). Antiserum against LytC lysozyme was purchased from Alcaligenes (760117) and anti-LytC serum (Fig. 2B). In agreement with previous findings (5), extracts prepared from strain TIGR4 and some of its derivatives showed higher hydrolytic activities on [3H]choline-labeled pneumococcal cell walls when tested at 30°C than at 37°C (data not shown). Additional evidence for the presence of an enzymatically active LytC lysozyme is pre-
sented in Fig. 3. As previously reported (5), the M31 strain that lacks the major autolysin (LytA) did not autolyze at the stationary phase of growth at 37°C but lysed when incubated at 30°C. This lysis was due to the activity of the LytC lysozyme, since the M31C \textit{lytC} mutant strain did not lyse (Fig. 3A). The alternative initiation codon (TTG) proposed here for \textit{lytC}\_TIGR4 is boxed. Another possible initiation codon (GTG) is underlined and in boldface. Putative RBSs are doubly underlined. G54 and Smi correspond to preliminary genomic sequences from \textit{S. pneumoniae} G54 (3) (http://bioinfo.cnio.es/data/Spneumo) and \textit{S. mitis} NCTC 12261 (type strain), respectively (http://tigrblast.tigr.org/ufmg).

**FIG. 1.** Sequence alignment of the DNA region surrounding the signal peptide of \textit{lytC}. Black and gray boxes show the ATG initiation codon of \textit{lytC} and in-frame stop codons located upstream of \textit{lytC}, respectively. The N-terminal residues of the mature form of LytC were experimentally determined in a previous work (5) and are in boldface. The arrow indicates the cleavage site of the signal peptide of LytC. The alternative initiation codon (TTG) proposed here for \textit{lytC}\_TIGR4 is boxed. Another possible initiation codon (GTG) is underlined and in boldface. Putative RBSs are doubly underlined. G54 and Smi correspond to preliminary genomic sequences from \textit{S. pneumoniae} G54 (3) (http://bioinfo.cnio.es/data/Spneumo) and \textit{S. mitis} NCTC 12261 (type strain), respectively (http://tigrblast.tigr.org/ufmg).

**FIG. 2.** Immunodetection of the LytC lysozyme in the cell envelope of the TIGR4 strain. (A) Western blot analysis of cell extracts prepared from the following strains (indicated at the top): M31 \((\Delta\textit{lytA})\), M31C \((\Delta\textit{lytA} \textit{lytC}_{::}\textit{ermC})\), R6 (wild type), R6C (R6 but \(\Delta\textit{lytC}_{::}\textit{ermC})\), TIGR4 (capsular serotype 4 strain), and P036 (TIGR4 but \(\Delta\textit{lytC}_{::}\textit{tet}\)). The \(M_r\) of the size standards (S) are indicated on the left. (B) The double-immunodiffusion experiment was performed on an agarose-coated microscope slide with anti-LytC antiserum in the center well (7) and cell envelope preparations from M31, P026 (TIGR4 but \(\Delta\textit{lytA}_{::}\textit{kan}\)), P031 (TIGR4 but \(\Delta\textit{lytC}_{::}\textit{kan}\)), and M31C in wells 1, 2, 3, and 6, respectively. In wells 4 and 5, phosphate-buffered saline and 1 \(\mu\)g of pure LytC, respectively, were used.

**FIG. 3.** Autolysis at 30°C of the \textit{S. pneumoniae} TIGR4 strain is due to the production of enzymatically active LytC lysozyme. Growth (and lysis) curves of the different pneumococcal strains in C medium at 30°C are shown. (A) Open circles correspond to strain M31 \((\Delta\textit{lytA})\), and solid circles and squares correspond to the same strain incubated with 0.5% anti-LytC and preimmune sera, respectively. The growth and lysis curve of the \textit{lytC} mutant of strain M31 (M31C) is indicated by triangles. (B) Open circles correspond to strain P026 (TIGR4 \(\Delta\textit{lytA}_{::}\textit{kan}\)), and solid circles and squares correspond, to the same strain incubated with 0.5% anti-LytC and preimmune sera, respectively. The growth curve of the P031 strain (P026 \(\Delta\textit{lytC}_{::}\textit{tet}\)) is indicated by triangles.
lytA mutant of *S. pneumoniae* TIGR4 (strain P026) also exhibits this autolysis-prone behavior when incubated under the same experimental conditions (Fig. 3B), in contrast to what was expected according to the genomic sequence analysis (21). Autolysis of P026 was due to the action of LytC on the basis of two independent criteria: (i) anti-LytC serum (but not preimmune serum) completely blocked culture autolysis and (ii) no lysis was found when strain P031 (a lytC mutant of strain P026) was incubated at 30°C (Fig. 3B). Similar results were found when the control strains M31 and M31C were used (Fig. 3A).

Figure 1 shows a multiple sequence alignment of the DNA region adjacent to that encoding the signal peptide of lytC in three different pneumococcal isolates as well as in the homologous region of the type strain of *Streptococcus mitis*. A preliminary sequence of the *S. pneumoniae* strain 670 is also available at http://tigrblast.tigr.org/ufmg but, since it was identical to that of the TIGR4 strain, is not considered further. It is conceivable that the lytC<sub>TIGR4</sub> allele might be translated from the GTG codon located upstream of the predicted ATG one. This possible action would render a signal peptide with a different N-terminal amino acid sequence (Fig. 1). Nevertheless, a closer examination of the sequence did not reveal a clear ribosome binding site (RBS) located upstream of the alternative GTG codon. On the contrary, a potential RBS (AGGAG) with a spacing of eight bases preceded a putative TTG initiation codon. Although a spacing of 9 to 11 bases has been proposed as optimal for low-G+C-content gram-positive bacteria (12), it is conceivable that this potential RBS could be used for translation initiation of the lytC<sub>TIGR4</sub> gene. Although in the case of R6, a potential RBS (GGAA) is located 11 bases upstream of the originally proposed ATG initiation codon (Fig. 1), the TTG codon mentioned above might be used for the translation initiation of the lytC<sub>R6</sub> gene since it is preceded by a good potential RBS (AGGAG) with an optimal spacing of nine bases from the start codon (5).

Previous studies have shown that most signal peptides have a high pI (the average value was 10.59 for gram-positive proteins) (see reference 1 and the references therein). The predicted pI values for signal peptides translated from the ATG or TTG codons were 7.98 and 9.31, respectively. Besides, two different computer programs, PSORT (14) and SignalP 3.0 (1), were used for signal peptides prediction. By using PSORT, signal scores of 1.06 and 4.27 were obtained for translation from the ATG and TTG codons, respectively, suggesting that the most probable signal peptide translates initiates at the later codon. This assumption was confirmed with SignalP since the values of the D score (the mean S score for discrimination of signal peptides versus nonsignal peptides) were 0.524 and 0.735 for LytC proteins starting at the ATG and TTG codons, respectively. Both programs correctly predicted the cleavage site. Different experimental approaches were used to get experimental confirmation of the initiation codon of the lytC gene. Briefly, we first attempted to purify the complete, unprocessed form of LytC from the cytoplasmic fraction of pneumococcal cultures. Besides, several experiments were carried out to express in *Escherichia coli* the part of the lytC gene encoding the N-terminal domain of the protein (including the signal peptide), since it had been previously reported that the complete lytC gene could not be cloned in *Escherichia coli* (5). Also, an in vitro, coupled transcription-translation kit (*E. coli* S30 extract system for circular DNA; Promega) was employed. Unfortunately, none of these approaches provided evidence of detectable synthesis of the unprocessed LytC protein and, consequently, a determination of the N-terminal amino acid sequence could not be accomplished.

Genome sequencing is establishing the grounds to understand gene expression patterns. However, even in the most optimistic scenario, genomic analysis requires the valuable information provided by physiological analyses to achieve a real biological picture and to open the way for a better understanding of the in vivo regulation of relevant proteins. LytC, a pneumococcal choline-binding protein that is known to be important for colonization and liberation of DNA, provides a case study that illustrates annotation problems.

This work was supported by grants from the Dirección General de Investigación Científica y Técnica (BMC2003-00074) and from Redes Temáticas de Investigación Cooperativa (G03/103 and C03/14).

The advice from J. P. Claveries on sequence alignments of the region upstream of the lytC gene has been very valuable. We are grateful to P. García for helpful comments and critical reading of the manuscript. We thank E. Cano for skillful technical assistance.

**REFERENCES**


