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Role of the Major Pneumococcal Autolysin in the Atypical Response of a Clinical Isolate of *Streptococcus pneumoniae*

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The autolytic enzyme (an *N*-acetylmuramyl-L-alanine amidase) of a clinical isolate, strain 101/87, which is classified as an atypical pneumococcus, has been studied for the first time. The *lytA101* gene coding for this amidase (LYTA101) has been cloned, sequenced, and expressed in *Escherichia coli*. The LYTA101 amidase has been purified and shown to be similar to the main autolytic enzyme (LYTA) present in the wild-type strain of *Streptococcus pneumoniae*, although it exhibits a lower specific activity, a higher sensitivity to inhibition by free choline, and a modified thermosensitivity with respect to LYTA. Most important, in contrast with the LYTA amidase, the activity of the LYTA101 amidase was inhibited by sodium deoxycholate. This property is most probably responsible of the deoxycholate-insensitive phenotype shown by strain 101/87. Phenotypic curing of strain 101/87 by externally adding purified LYTA or LYTA101 amidase restored in this strain some typical characteristics of the wild-type strain of pneumococcus (e.g., formation of diplo cells and sensitization to lysis by sodium deoxycholate), although the amount of the LYTA101 amidase required to restore these properties was much higher than in the case of the LYTA amidase. Our results indicate that modifications in the primary structure or in the mechanisms that control the activity of cell wall lytic enzymes seem to be responsible for the characteristics exhibited by some strains of *S. pneumoniae* that have been classically misclassified and should be now considered atypical pneumococcal strains.

The recent development of DNA probes for the rapid and reliable identification of *Streptococcus pneumoniae* from clinical specimens has facilitated the recognition of pneumococcal isolates showing atypical responses in conventional identification tests (11, 28). One of the best-documented examples of such behavior is the sensitivity of pneumococci to bile (deoxycholate [DOC]-sensitive [DOC^+] phenotype), which is considered a distinctive characteristic of this species (24). It has been demonstrated that there are laboratory pneumococcal strains for which the atypical responses to this test can be easily ascribed to a genetic defect, the most common being DOC^- mutants designated $LYTA^-$, i.e., lacking the main autolytic enzyme (an *N*-acetylmuramyl-L-alanine amidase designated the LYTA amidase) characteristic of this species (20, 23, 34).

We report here the characterization of the autolytic enzyme of a clinical isolate, strain 101/87, that has been identified as an atypical pneumococcus on the basis of the use of highly specific DNA probes. Strain 101/87 apparently contains a normal level of active autolytic amidase, although it is not bile sensitive, a phenomenon classically considered autolysin dependent (26). Detailed study of the autolytic system of pneumococcal clinical isolates like the one reported here, using recently developed molecular tools (6–9, 12–16, 23, 29–32, 34), should contribute to a better knowledge of the factor(s) that leads to the appearance of more sophisticated phenotypes of atypical pneumococcal strains in nature and should help to develop strategies for dealing with such varieties of pathogenic strains. In addition, the availability of such mutants can provide new ways to study the molecular mechanisms that regulate the activity of the autolytic enzymes as well as the evolution of the genes coding for these enzymes since, to the best of our knowledge, this is the first time that the autolysin of a clinical isolate of *S. pneumoniae* has been studied at a molecular level.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *Escherichia coli* strains used were HB101 [F^- *hsdS20* ($r_B^-m_B^-$) *recA13 ara-14 proA2 leuB lacY1 galK2 rpsL20 xyl5 mtl-1 supE44*] (33), C600 (F^- *thi-1 leuB6 thr-1 lacY6 tonA21 supE44*) (33), and JM83 [Δ(*lac-proAB*) *ara rpsL* φ80 *lacZ* ΔM15] (25). *S. pneumoniae* strains used were wild-type R6 (Rockefeller University), M31 (Δ*lytA*) (34), and 101/87 (11). Plasmids used were pUC19 (Ap^r) (25), pGL30 (*lytA* Ap^r) (13), and pED1 (*lytA* Tc^r) (7). *E. coli* C600 or *S. pneumoniae* M31 was used as the host for pED1 and derivative plasmids. *E. coli* was grown in LB medium (33) at 37°C with shaking, and *S. pneumoniae* was grown in C medium (38) supplemented with yeast extract (0.8 mg/ml; Difco Laboratories) (C + Y medium) or in Cden-EA medium (41) at 37°C without shaking. Growth of *S. pneumoniae* was monitored with a Coleman nephelometer.

Plasmid isolation and transformation. Plasmid DNA was prepared by the rapid alkaline method (2). Transformation of *E. coli* was carried out by using the RbCl method (33). Transformation of competent cells of *S. pneumoniae* was performed according to a procedure previously described (38).

DNA sequence analysis. DNA sequencing was carried out by the dideoxy-chain termination method (35), using the Sequenase kit from United States Biochemical. Oligonucleotide primers were synthesized in a Pharmacia LKB Gene Assembler Plus DNA synthesizer.

Purification of enzymes. The LYTA101 amidase was purified from extracts of *E. coli* HB101(pNH101) (see below) by affinity chromatography on DEAE-cellulose, using the sin-

gle-step procedure described elsewhere (36). The apparent M_rs of the enzymes were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (21).

Assay for cell wall lytic activity. Assays for cell wall lytic activity were carried out according to standard conditions described elsewhere (18), using as the substrate ethanolamine-containing cell walls labeled with [^{3}H]lysine or choline-containing cell walls labeled either with [*methyl*-^{3}H]choline or [^{3}H]lysine. One unit of lytic activity was defined as the amount of enzyme that catalyzed the hydrolysis (solubilization) of 1 μg of cell wall material in 10 min (18). Assays for enzymatic conversion (activation) by choline or choline-containing pneumococcal cell walls of the low-activity form of the lytic enzymes were performed as previously described (3, 18), using crude extracts prepared by sonication of *E. coli* transformants. The use of crude extracts was required to avoid the conversion of the enzymes by choline during the purification process by affinity chromatography (3, 36).

Characterization of cell wall lytic activity. The type of enzymatic activity (lysozyme or amidase) was determined by analysis on a Sephadex G-75 column of the degradation products resulting from the hydrolysis of choline-containing pneumococcal cell walls labeled either with [*methyl*-^{3}H]choline or [^{3}H]lysine (14, 39).

Immunoblot and Southern blot analyses. Immunoblots were carried out by using anti-pneumococcal amidase serum as described previously (30). Extracts were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). After incubation with the primary antiamidase serum, the blots were incubated with a peroxidase-conjugated AffiniPure goat anti-rabbit serum (Jackson Immunoresearch), and the protein bands were visualized by using 4-chloro-1-naphthol (Sigma). Southern blot analyses were carried out by using radioactive probes labeled by the random-primer method in the presence of [α-^{32}P]dCTP (33).

DOC-induced lysis of pneumococcal cells. Susceptibility of the bacteria to sodium DOC or Triton X-100 was tested as previously described (41). In short, 1-ml portions of a culture in the exponential phase of growth were pipetted into test tubes containing 0.1 ml of 1 M sodium phosphate buffer (pH 8.0). After the initial light-scattering value of the suspension was recorded, 0.1 ml of a 10% sodium DOC or Triton X-100 solution was added, the suspension was incubated at 37°C for 5 min, and the light scattering of the treated suspension was recorded again.

Sensitization to externally added autolytic enzyme. Sensitization to externally added lytic amidase was tested as previously described (40). Exponentially growing cultures of pneumococcal M31 or 101/87 cells received different amounts of purified LYTA101 or LYTA amidase and were incubated at 37°C. A control culture received no addition. Cultures were tested for cell autolysis and for susceptibility to lysis by detergents.

RESULTS

Isolation of *S. pneumoniae* 101/87. In the course of an epidemiological survey carried out in Spain, we paid special attention to strain 101/87, isolated at the Hospital de San Juan de Dios (Barcelona) from the blood of a patient suffering from pneumonia and originally identified as *Streptococcus mitis* on the basis of biochemical tests indicating that it was an α-hemolytic strain resistant to bile and optoquin and nontypeable. Nevertheless, the use of a highly specific DNA probe recently developed to identify pneumococcal species (11, 28) allowed us to classify this isolate as an atypical *S. pneumoniae* strain. In addition, strong hybridization bands were detected by using another recently developed pneumococcus-specific DNA probe on the basis of the presence of repeated sequences present upstream the structural regions of several well-characterized pneumococcal genes (5).

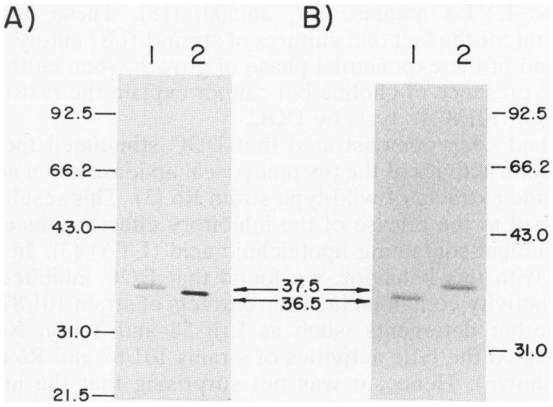

FIG. 1. SDS-PAGE. (A) Immunoblot analysis. Lanes: 1, total crude extracts of *S. pneumoniae* 101/87 (180 μg); 2, purified LYTA amidase (2 μg). (B) Gel stained with Coomassie blue. Lanes: 1, purified LYTA; 2, purified LYTA101. One microgram of each enzyme was electrophoresed on an SDS–10% polyacrylamide gel. Molecular masses of standard markers are indicated in kilodaltons. Protein standards (Bio-Rad) were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor. Arrows show the molecular masses of the proteins present in the samples.

Physiological and biochemical characterization of strain 101/87. *S. pneumoniae* 101/87 grows in C + Y medium at a rate similar to that showed by wild-type strain R6, but it forms long chains and ultimately gives rise to large clumps of cells (data not shown). Remarkably, the culture lysed at the end of the stationary phase of growth (LYT$^+$ phenotype) even though it was resistant to lysis by DOC (DOC$^-$ phenotype). The presence of 2% choline in the growth medium prevents cultures of strain 101/87 from lysing at the end of the stationary phase of growth, a characteristic that has been postulated to be due to a blockage in the activity of the main autolytic enzyme (LYTA amidase) present in *S. pneumoniae* R6 (3, 9, 17), suggesting the presence in strain 101/87 of an autolytic enzyme that shares some characteristics with the pneumococcal amidase. Finally, cultures of strain 101/87 can incorporate, as insoluble material, (*methyl*-^{3}H)choline that has been added to the culture medium, indicating that this strain also shares with the pneumococcal species the peculiarity of a cell wall that contains choline as a structural component (26, 37).

On the other hand, crude extracts prepared from strain 101/87 revealed the existence of a lytic activity, constitutively produced and able to degrade efficiently pneumococcal cell walls containing choline but not ethanolamine, in a fashion similar to that previously found with extracts prepared from wild-type strain R6 (not shown). Furthermore, immunoblot analyses of crude extracts of strain 101/87 with use of polyclonal antibodies prepared against the pneumococcal LYTA amidase showed the presence of a protein with an apparent M_r of 37,500 (Fig. 1A), slightly higher than that

of the LYTA amidase (M_r 36,500) (18). These findings account for the fact that cultures of strain 101/87 autolysed at the end of the exponential phase of growth when cultivated in the presence of choline but cannot explain the resistance of strain 101/87 to lysis by DOC.

It had been demonstrated that DOC stimulated the cell wall lytic activity of the pneumococcal amidase when added to crude extracts of wild-type strain R6 (3). This result was ascribed to the release of the inhibitory effect produced by the choline-containing lipoteichoic acid (LTA) (3). In contrast with this behavior, we found that DOC inhibited the lytic activity contained in crude extracts of strain 101/87 and that other detergents, such as Brij 58 and Triton X-100, stimulated the lytic activities of strains 101/87 and R6 (data not shown). Hence, it was not surprising that the use of Triton X-100 instead of DOC immediately induced the lysis of cultures of strain 101/87.

The data presented above demonstrated the presence in strain 101/87 of a lytic enzyme similar, but apparently not identical, to that of the LYTA amidase present in wild-type strain R6 (15). We speculated that differences at the level of the primary structures between the two enzymes might account for the inhibitory effect of DOC and the altered characteristics of the pneumococcal strain described in this report. Hence, we decided to clone, sequence, and express the gene encoding the autolytic enzyme of strain 101/87 in *E. coli*.

Cloning and expression in *E. coli* of the *lytA101* gene of *S. pneumoniae* 101/87. Since the DNA from strain 101/87 hybridized with the *lytA* gene used as a specific pneumococcal probe in dot blot hybridization experiments (11), and given that the complete structural *lytA* gene coding for the pneumococcal amidase of the wild-type strain had been localized to a 1.2-kb *Hin*dIII fragment (15), we constructed in *E. coli* JM83 a *Hin*dIII library with the DNA of strain 101/87, using pUC19 as the vector. A clone containing a plasmid of 4.4-kb was isolated from this library by using the *lytA* gene as a probe. The plasmid, designated pNH101, was used to transform *E. coli* HB101, and crude extracts obtained from one of the transformants, *E. coli* HB101(pNH101), exhibited lytic activity when tested on choline-containing pneumococcal cell walls in an in vitro assay, suggesting that the cloned fragment contained the *lytA101* gene coding for the autolytic enzyme of *S. pneumoniae* 101/87. It is interesting that the enzyme responsible for this activity requires the process of conversion to achieve full enzymatic activity, as reported for the pneumococcal host and phage amidases described so far (3, 17, 29, 36). It was observed that the primary translation product of the *lytA* gene, an inactive form of the pneumococcal amidase (E form), can be converted to the active C form in the presence of choline-containing cell walls or 2% free choline (3, 36).

Nucleotide sequence analysis of the *lytA101* gene. Remarkably, the size of plasmid pNH101 indicated that the *lytA101* gene was contained in a 1.7-kb *Hin*dIII fragment, in contrast to the *lytA* gene, which was localized to a 1.2-kb *Hin*dIII fragment (15). The nucleotide sequence of the 1.7-kb *Hin*dIII fragment present in plasmid pNH101 is shown in Fig. 2. The *lytA101* gene codes for a protein (LYTA101) of 316 amino acids with a deduced M_r of 36,472. As expected, the LYTA101 enzyme shows a modular organization similar to that previously reported for the lytic enzymes of *S. pneumoniae* and its bacteriophages, i.e., an N-terminal domain responsible for the specific enzymatic activity and a C-terminal domain containing a set of six repeated sequences or motifs (P1 to P6) that should be involved in the recognition of the cell wall substrate (8, 9, 12, 16). Overall comparison of the sequences of the structural *lytA* and *lytA101* genes revealed a remarkable similarity (81% identity), although the latter contains 6 bp less than the former. This deletion was located in the P6 motif of the C-terminal domain of the LYTA101 enzyme, as in the case of the P6 motif of the CPL1 and CPL9 lysozymes of pneumococcal bacteriophages Cp-1 and Cp-9, respectively (12, 16).

Comparisons of the flanking regions of *lytA* and *lytA101* revealed a high similarity upstream from the ATG initiation codon, whereas the similarity was lost downstream from the termination codon. This observation could account for the cloning of a 1.7-kb *Hin*dIII fragment instead of the 1.2-kb *Hin*dIII fragment found in the wild-type strain. To determine whether this finding was due to a cloning artifact introduced during preparation of the *Hin*dIII library of the strain 101/87 genome or to a deletion or insertion characteristic of this strain, we carried out a series of Southern blot hybridizations. Comparisons of the hybridization patterns of chromosomal DNAs from strain 101/87 and from strain R6 digested with *Hin*dIII by using the *lytA* gene as a probe demonstrated the existence of a 1.7-kb *Hin*dIII band in strain 101/87 (Fig. 3). Another remarkable difference between these strains was the detection in the genome of strain 101/87 of a second 9.4-kb *Hin*dIII hybridization band that was not present in the genome of R6 (see below). Additional comparative Southern blot hybridizations of chromosomal DNA from R6 (Fig. 4A) and plasmid pGL30 (Fig. 4B), which contains a 7.5-kb *Bcl*I fragment of R6 DNA encompassing the *lytA* gene (Fig. 4C), digested with *Hin*dIII, *Eco*RI, *Acc*I or *Pvu*II, and probed with a 0.35-kb *Dra*I-*Hin*dIII fragment of plasmid pNH101, a fragment that is located 197 bp downstream from the termination codon of the *lytA101* gene, allowed us to determine the existence of a 1- to 2.1-kb-long deletion in the vicinity of the termination codon of the *lytA101* gene (Fig. 4D), affecting the hairpin that participates in termination of the transcript of the *lytA* gene (7).

Purification and biochemical characterization of the LYTA101 protein. We purify the lytic activity present in extracts obtained from *E. coli* HB101(pNH101) in a single step, using DEAE-cellulose (36). The LYTA101 enzyme is slightly lower in mobility than the LYTA wild-type amidase (Fig. 1B), as observed for the crude extracts of strain 101/87 (Fig. 1A); this mobility corresponds to an apparent M_r of 37,500, slightly higher than that deduced from the amino acid sequence. Table 1 summarizes the most relevant biochemical properties of the LYTA101 enzyme. Analyses of the soluble degradation products of pneumococcal cell walls radioactively labeled with either choline or lysine indicated that this enzyme is an *N*-acetylmuramyl-L-alanine amidase (data not shown). The purified enzyme can degrade cell walls containing choline but not ethanolamine, indicating that it is a choline-dependent enzyme. DOC inhibited the activity of the enzyme, as found when crude extracts containing LYTA101 were tested in vitro. However, DOC does not alter the activity of the purified wild-type LYTA enzyme, since LTA is not present in the assay. Triton X-100 did not change the activities of the purified LYTA or LYTA101 amidase. Free choline, a noncompetitive inhibitor of pneumococcal lytic enzymes (3, 14, 16, 17, 29), inhibited about 50% of the activity of the LYTA101 amidase at a concentration of 7 mM, lower than that required to inhibit the LYTA amidase (24 mM). This characteristic was reminiscent of that of the CPL1 lysozyme encoded by the pneumococcal phage Cp-1; 3 mM choline was sufficient to inhibit 50% of this lysozyme activity (8). The specific activity of the purified

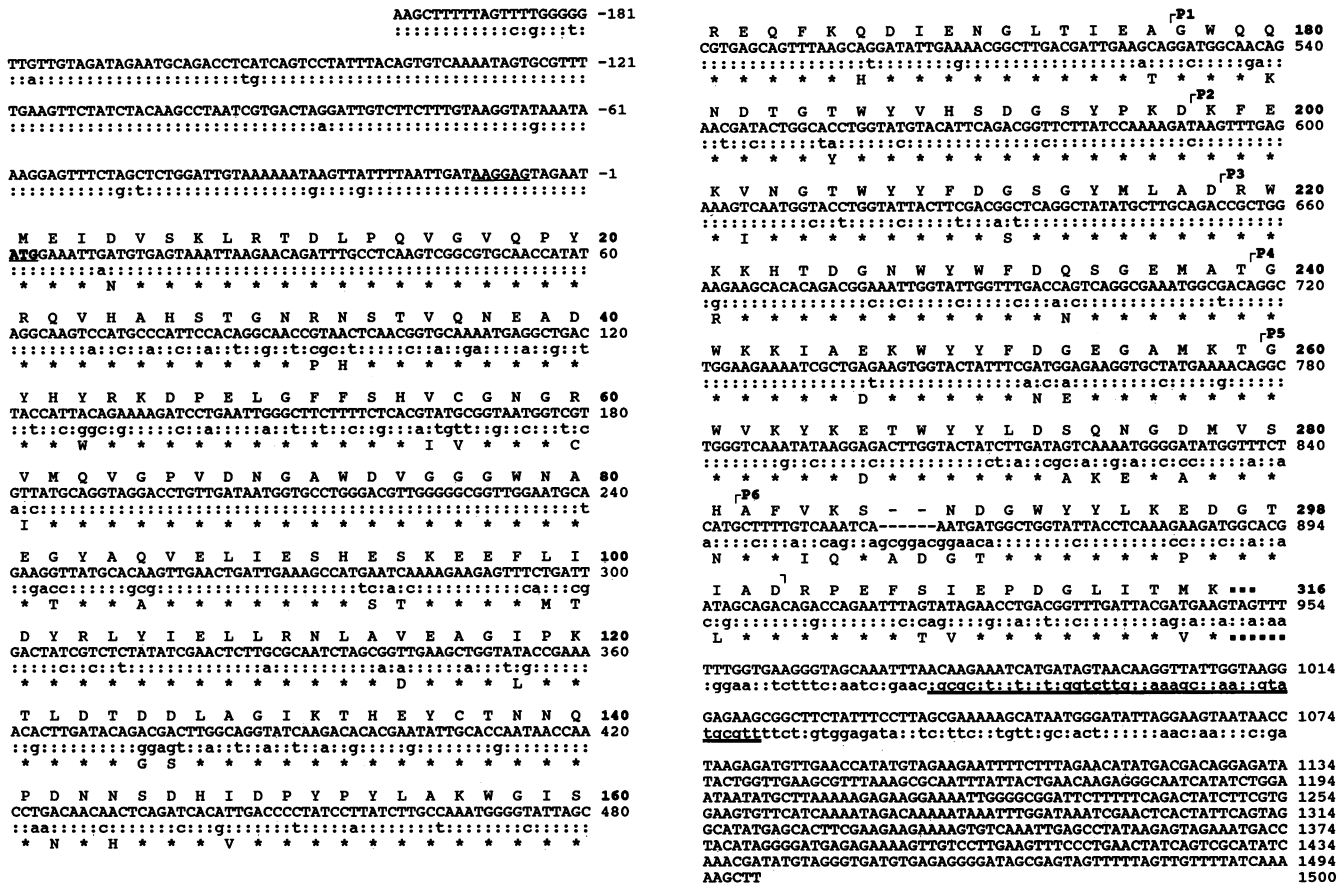

FIG. 2. Comparison of the nucleotide and derived amino acid sequences of the LYTA and LYTA101 enzymes. Only the DNA strand corresponding to the mRNA sequence is shown; uppercase letters correspond to *lytA101*; lowercase letters correspond to *lytA* (15). Numbering of the sequences starts with the predicted initiation codons. Boldface numbers correspond to the amino acid sequences. Identical nucleotides are indicated by colons in the sequence of the *lytA* gene. The top and bottom lines are the deduced amino acid sequences (in one-letter code) for the LYTA101 and LYTA enzymes, respectively. Where identical amino acids coincide, those corresponding to the LYTA amidase are indicated by asterisks. P1 to P6 are sets of repeated sequences. The underline represents the putative ribosome-binding site. The double underline indicates the transcription terminator sequence identified in the *lytA* gene (7). Dashes are gaps introduced to maximize homology. The translation termination codons are represented by filled squares.

LYTA101 amidase was lower than that of the LYTA amidase. In addition, the LYTA101 amidase appeared to be more unstable than the LYTA amidase, as judged by its lower thermostability at 37°C. Although both LYTA101 and LYTA amidases showed an optimal pH of 6.5, the former was three times less active than the latter when assayed at an alkaline pH, a fact that should be considered if the clinical DOC assay used to identify pneumococcal strains is performed at pH 8.0.

Biological test of the LYTA101 amidase. The purified LYTA101 amidase was also tested for its capacity to restore in strain M31, a pneumococcal mutant that is deleted of the *lytA* gene, a series of biological and biochemical properties pertinent to the wild-type amidase (LYTA). We have found that addition of 300 U of the LYTA101 or LYTA amidase per ml to the growth medium for strain M31 makes these cells lyse at the end of the exponential phase of growth when incubated at 37°C as a result of the phenotypic curing (40) achieved by M31, which leads to a behavior that mimics the spontaneous lysis found for wild-type strain R6 at the end of the exponential phase of growth. However, sensitization to DOC required the addition of much higher amounts of

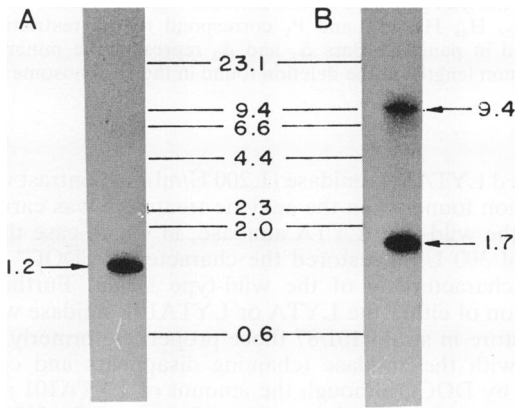

FIG. 3. Southern blot hybridization analyses of DNAs from pneumococcal strains R6 and 101/87. R6 (A) and 101/87 (B) DNAs were digested with *Hin*dIII and hybridized with a ^{32}P-labeled *lytA* gene. Arrows show the positions and sizes (in kilobases) of the *Hin*dIII fragments that hybridize with the ^{32}P-labeled *lytA* gene. The sizes (in kilobases) of the restriction fragments of *Hin*dIII-digested DNA are indicated.

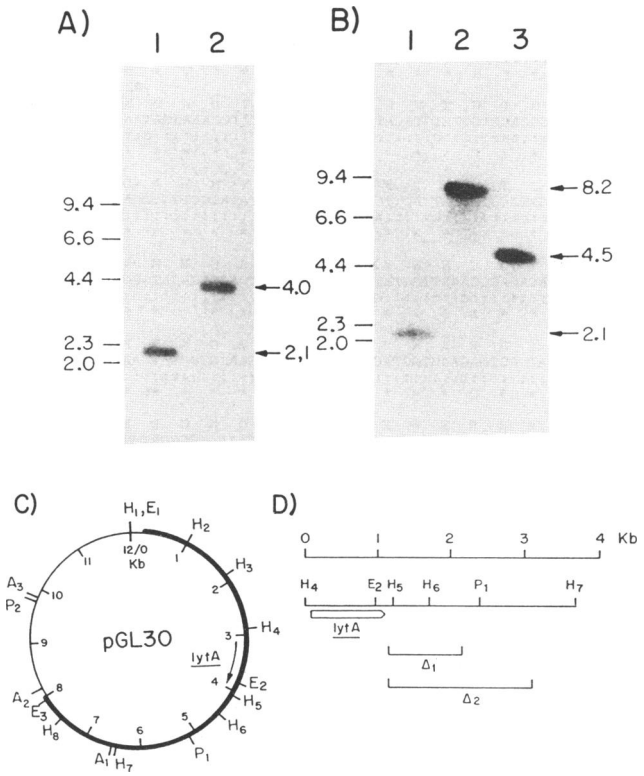

FIG. 4. Characterization of a deletion in the DNA of *S. pneumoniae* 101/87. (A) Southern blot hybridization analyses of R6 DNA digested with *Hin*dIII (lane 1) or *Eco*RI (lane 2). (B) Southern blot hybridization analyses of pGL30 digested with *Hin*dIII (lane 1), *Acc*I (lane 2), or *Pvu*II (lane 3). Both Southern blots were probed with the 0.35-kb ^{32}P-labeled *Dra*I-*Hin*dIII restriction fragment from plasmid pNH101. The sizes (in kilobases) of the restriction fragments of *Hin*dIII-digested DNA are indicated. Arrows show the positions and sizes (in kilobases) of the fragments that hybridize with the probes used. (C) Physical map of plasmid pGL30. The heavy line represents the 7.5-kb *Bcl*I pneumococcal insert. The *lytA* gene is showed by an arrow. Abbreviations: A_1 to A_3, *Acc*I restriction sites; E_1 to E_3, *Eco*RI restriction sites; H_1 to H_8, *Hin*dIII restriction sites; P_1 and P_2, *Pst*I restriction sites. (D) Physical map showing the deletion in the DNA of strain 101/87. The diagrams shows the *lytA* gene and its downstream region in the DNA of strain R6. E_2, H_4, H_5, H_7, and P_1 correspond to the restriction sites showed in panel C. Bars Δ_1 and Δ_2 represent the minimum and maximum lengths of the deletion found in the chromosome of strain 101/87.

purified LYTA101 amidase (1,200 U/ml), in contrast with the situation found when the enzyme treatment was carried out with the wild-type LYTA amidase, in which case the addition of 300 U/ml restored the characteristic DOC$^+$ phenotype characteristic of the wild-type strain. Furthermore, addition of either the LYTA or LYTA101 amidase was able to restore in strain 101/87 those properties formerly associated with the amidase (chaining disappears and cells are lysed by DOC), although the amount of LYTA101 amidase required to restore these properties to strain 101/87 was again much higher than in the case of the LYTA amidase. The fact that strain 101/87 returned to a typical diplo morphology when it was phenotypically cured with either amidase also confirmed that we were dealing with true pneumococcal cells.

Cloning of the *lytA101* gene in *S. pneumoniae*. To test in a direct experimental way the assumption that a high level of LYTA101 activity can restore the DOC$^+$ phenotype, we decided to clone the *lytA101* gene in *S. pneumoniae* M31. To do this, we placed this gene under the control of the *lytA* promoter in a plasmid adapted to pneumococcus, using a strategy previously reported (7), to create plasmid pED4 (not shown). Competent cells of strain M31 were transformed with pED4, and one of the tetracycline-resistant transformants was identified as *S. pneumoniae* M31(pED4). When this strain was cultured in C + Y medium, the cells grew at a normal rate, although they lysed rapidly at the end of the exponential phase of growth, in contrast with the noticeable stationary phase normally found in wild-type strain R6 (Fig. 5). This characteristic can be ascribed to a high level of amidase activity due to the presence of several copies of plasmid pED4, as previously reported for M31 transformed with similar high-copy-number plasmids containing the *lytA* gene (7, 31, 32). In fact, we found that crude extracts from *S.*

TABLE 1. Comparison of the biochemical properties of the purified LYTA101 and LYTA amidases[a]

Amidase	Activity (U/mg of protein) on choline-containing walls[b]	Choline 50% inhibitory concn (mM)	% Activity[c]	
			After 15 min at 37°C	After treatment with 0.1% DOC
LYTA101	580	7	15	40
LYTA	1,000	24	58	100

[a] Although the two enzymes had the same optimal pH (6.5), the residual activity of the LYTA101 amidase choline-containing cell walls at pH 8.0 was 16%, whereas the LYTA amidase retained 45% of activity. However, the residual activity of the LYTA101 amidase at pH 5.5 was 84%, whereas the activity of the LYTA amidase was 55%. Both enzymes exhibited conversion by choline. Conversion was determined by using crude extracts of *E. coli* recombinants expressing the LYTA101 or LYTA amidase after 5 min of preincubation at 0°C with choline-containing cell walls or 2% choline chloride, since the purified enzymes were converted after elution from DEAE-cellulose with 2% choline (36).

[b] Both enzymes had activity of <1 U/mg on ethanolamine-containing cell walls.

[c] Both enzymes showed 100% activity after treatment with 0.1% Triton X-100.

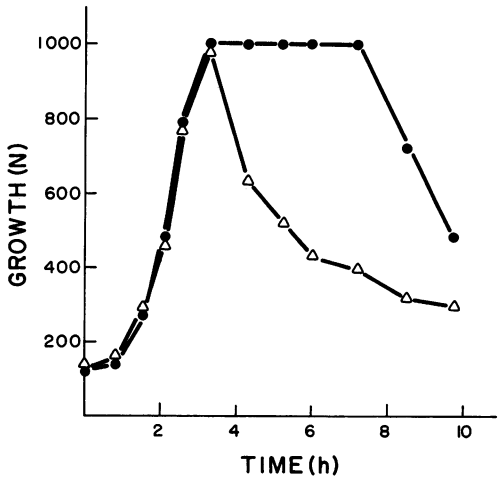

FIG. 5. Growth curves of *S. pneumoniae* R6 and M31(pED4). Cultures of strains R6 (●) and M31(pED4) (△) were incubated at 37°C in C + Y medium. Growth and lysis were monitored by nephelometry (N).

pneumoniae M31(pED4) were about ninefold more active than those prepared from wild-type strain R6. Interestingly, strain M31(pED4) was very prone to lysis by DOC.

Identification of a second fragment in the genome of strain 101/87 with homology to the *lytA* gene. As pointed out above, a second hybridization band was found when the DNA of strain 101/87 was probed with the *lytA* gene (Fig. 3B), suggesting that the genome of this strain contains a second sequence of DNA with a high degree of similarity to the *lytA* gene coding for the pneumococcal amidase. It has been recently reported (29) that a lysogenic strain of *S. pneumoniae* contains two genes coding for cell wall lytic enzymes; on the other hand, it has been suggested that there is a relationship between the lysogenic state and loss of the capacity to develop competence for genetic transformation (27), a characteristic also found in strain 101/87. Attempts to transform strain 101/87 by inducing genetic competence in the presence of competence factor prepared from pneumococcal strain R6 have been unsuccessful, which hindered attempts to test the effect of plasmid pED4 in strain 101/87. These observations led us to look for the presence of temperate phages in the genome of strain 101/87. When strain 101/87 growing exponentially was treated with mitomycin, the cultures rapidly lysed, liberating to the supernatant phage particles showing a peculiar morphology (10).

DISCUSSION

The resistance of *S. pneumoniae* to lysis by DOC (DOC^- phenotype) has been associated with the absence of the autolytic activity of the LYTA amidase (20, 23, 26, 31, 34). However, the mechanistic basis of the morphological characteristics and the DOC^- phenotype of the clinical isolate, strain 101/87, could not be associated merely with the absence or low level of amidase activity in crude extracts of this strain. Although extracts from strain 101/87 contained an autolytic activity lower than that present in wild-type strain R6, this reduced activity was far above that of other pneumococcal strains which exhibited a DOC^+ phenotype but had an autolytic activity much lower than that found in R6 (22). On the other hand, the observation that the LYTA and LYTA101 amidases were able to reestablish the normal diplo morphology in cultures of strain 101/87 strongly indicated that this strain has a normal choline-containing cell wall. However, the finding that the lytic activity present in crude extracts from strain 101/87 was inhibited by DOC rather than stimulated, as occurs in wild-type strain R6 (Table 1), indicated that the DOC^- phenotype was correlated with the atypical inhibition of the LYTA101 amidase by DOC. Interestingly, the effect of DOC could be ascribed to an intrinsic property of the LYTA101 amidase, since DOC inhibition was also observed for the purified enzyme (Table 1). Moreover, given that the lytic activity of the LYTA101 amidase was considerably reduced at pH 8.0, it seems reasonable to assume that the enzymatic level required to induce cell lysis was not achieved in the presence of DOC at pH 8.0.

Biochemical studies and biological tests for phenotypic curing of mutant M31 performed with the purified LYTA101 amidase suggest that in addition to the effects of DOC and pH, other properties of this amidase, i.e., its lower specific activity and lower thermostability, render this enzyme less efficient than the LYTA amidase. In turn, these properties could also contribute to the phenotypic characteristics exhibited by strain 101/87 and help to explain why amounts of the LYTA101 amidase higher than those of the wild-type LYTA amidase were required to restore the sensitivity of strains M31 and 101/87 to lysis by DOC when the enzymes were exogenously added. This conclusion was reinforced by the observation that the presence in M31(pED4) of several copies of the *lytA101* gene, expressing higher amounts of the LYTA101 amidase, reintroduced sensitivity to DOC (Fig. 5). Nevertheless, we cannot rule out the possibility that the DOC^- phenotype and the long-chain morphology of strain 101/87 might be consequences of a lysis-defective mechanism that could involve some aspects of autolysin control due to an alteration in the pathway that triggers the amidase. It appears reasonable to consider that the structural changes observed in the LYTA101 amidase might generate a stronger atypical interaction of the enzyme with the components of the cell envelope that seem to regulate its activity (membrane, LTA, etc.) (6). This interaction could prevent an adequate level of lytic activity from reaching the cell wall substrate when cells are growing normally as well as in the presence of DOC. Two observations appear to support this assumption: (i) the fact that the nonionic detergent Triton X-100 was able to induce the lysis of strain 101/87, probably because of a more efficient release of the LYTA101 enzyme than of DOC from the cellular envelope, as deduced from the stimulating effect of Triton X-100 on the lytic activity determined in crude extracts (Table 1), and (ii) the finding that the LYTA101 amidase was more sensitive to the inhibition by free choline than was the LYTA amidase (Table 1), which may account for the more stable interaction between the LYTA101 amidase and its natural inhibitor, the choline-containing LTA present in the pneumococcal membrane.

The sequences of the *lytA* and *lytA101* genes were found to differ by about 19% (Fig. 2). It has been proposed that some essential genes, like the lytic genes, have arisen in phages from host genes (4), and recombination between genes coding for cell wall lytic enzymes of phages and bacteria in *S. pneumoniae* has been demonstrated in the laboratory (30). This recombination was facilitated by the close relationship between these genes, which exhibit the highest similarity described so far between phages and bacteria (88% base identity) (30). This type of genetic event might be the mechanism for introducing diversity in the genes coding for lytic enzymes in pneumococci. The observation that most pathogenic strains isolated from pediatric populations yielded phages when induced by mitomycin (1), as in the case reported in the accompanying report (10), is very suggestive in this respect. Furthermore, some differences between the *lytA101* and *lytA* genes, such as a deletion of six nucleotides in the P6 motif of the *lytA101* gene, have been found (Fig. 4); interestingly, the CPL1 lysozyme encoded by the pneumococcal bacteriophage Cp-1, which also shows a deletion of six nucleotides in the P6 motif of the *cpl*-1 gene, shares with the LYTA101 amidase a high sensitivity to inhibition of activity by low concentrations of choline (8). The sum of alterations found in the coding sequence of the *lytA101* gene with respect to the wild-type *lytA* gene might account for the structural changes responsible for the biochemical properties of the LYTA101 amidase pointed out above.

In contrast to the relatively high divergence (19%) observed between the structural regions of the *lytA* and *lytA101* genes, the 5′-end flanking regions of these genes, encompassing the leader sequence previously reported for the *lytA* gene (7), remain quite similar (6% divergence). The fact that the *lytA* and *lytA101* genes differ completely in the region downstream from the termination codon affecting the hairpin found in the *lytA* gene, which functions as a transcription termination site (7), appears to be due to a deletion event

that originated the 1.7-kb *Hin*dIII fragment containing the *lytA101* structural gene and its flanking regions. The assumption that this lack of similarity was due to a deletion in this region rather than to an insertion was based on comparative Southern blot hybridizations between the chromosomal DNA from R6 and DNA from plasmid pGL30, using the 0.3-kb *Dra*I-*Hin*dIII fragment of plasmid pNH101 as a probe (Fig. 3A). The results strongly suggest that the DNA from strain 101/87 resumes homology with the DNA of wild-type strain R6 immediately after a deletion of 1 to 2.1 kb which starts just in the termination codon of the *lytA101* gene. Furthermore, a survey of a large number of clinical isolates of pneumococci always revealed the presence of a 1.2-kb *Hin*dIII fragment that hybridized with the *lytA* gene in Southern blot tests in a fashion similar to that of wild-type strain R6, except in the case of the 1.7-kb *Hin*dIII fragment found in the DNA from strain 101/87 (11).

Hence, the regulatory systems of the autolytic enzymes are of interest with respect to both chemotherapy and morphogenesis of bacteria (19). The findings reported in this communication illustrate how alterations in the activity of the main autolytic enzyme or in its regulation can give rise to dramatic changes affecting the phenotypic characteristics of a clinical isolate of *S. pneumoniae* and, in turn, might also influence the pathogenic properties of such strains if, as has been recently pointed out (42), autolysis plays a role in shaping the course of pneumococcal meningitis in vivo, since strains that exhibit alterations in their lytic systems appear to contribute to higher morbidity and mortality from this type of infection. As exemplified in this work, much can be learned from molecular studies of the autolysins of clinical isolates of pneumococci.

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