DNA Probe for Identification of *Streptococcus pneumoniae*

GIANNI POZZI,^{†*} MARCO R. OGGIONI,[‡] AND ALEXANDER TOMASZ

Laboratory of Microbiology, The Rockefeller University, 1230 York Avenue, New York, New York 10021

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A total of 287 clinical isolates of *Streptococcus pneumoniae* (pneumococcus) were tested for their ability to undergo autolysis when treated with sodium deoxycholate. The test was positive for all but one isolate, strain DOC-1. This autolysis required the activity of an enzyme which is unique and characteristic of *S. pneumoniae*: a choline-dependent *N*-acetylmuramoyl-L-alanine amidase, the gene product of the *lytA* gene. We used *lytA* as a DNA probe to test the distribution of the autolysin gene among clinical isolates of *S. pneumoniae*. In dot blot hybridization experiments our probe reacted with the DNA of 60 of 60 strains tested, including the autolysis-deficient clinical isolate DOC-1. No hybridization occurred when strains of *Streptococcus sanguis*, *Streptococcus mutans*, *Streptococcus pyogenes*, *Streptococcus* (*Enterococcus*) *faecalis*, *Streptococcus* (*Enterococcus*) *faecium*, *Streptococcus agalactiae*, and *Streptococcus bovis* were tested. The *lytA* gene appears to be an ideal candidate for use as a DNA probe for the identification of *S. pneumoniae*.

There is a need for the development of a simple and selective method for the rapid identification of *Streptococcus pneumoniae* (pneumococcus) in clinical specimens. The methods currently available, counterimmunoelectrophoresis and coagglutination, are based on the immunological detection of the capsular polysaccharides (at least 83 different types are known); however, these immunological methods are not totally satisfactory, mainly because of sensitivity problems. In this paper we describe a candidate DNA probe that allows the detection of a single, common, and unique component of all pneumococcal strains.

The gene *lytA* (1, 3) codes for the major autolysin of *S. pneumoniae*, an *N*-acetylmuramoyl-L-alanine amidase (amidase; 6, 7, 10). Unlike some other bacterial autolytic enzymes, the pneumococcal amidase has a high degree of specificity for homologous cell walls (4); its hydrolytic activity has an absolute requirement for the presence of choline residues in the cell wall teichoic acid (5, 17), and choline, to our best knowledge, can be found only in the cell walls of pneumococci (16) and of some rare viridans group streptococci (15). While a tendency toward sodium deoxycholate (DOC)-induced autolysis (an amidase-dependent process; 10) is generally believed to be a property of all pneumococci, actual data on the distribution of the amidase among clinical isolates are not available. In this work we investigated the distribution of the pneumococcal amidase and of its gene among clinical isolates to establish whether *lytA* could be used as a DNA probe for the identification of *S. pneumoniae*.

We investigated 287 clinical isolates of *S. pneumoniae* and, as controls, 2 laboratory strains deficient in autolysis: deletion mutants M31 ($\Delta lytA$) (14) and RUP1 (G. Pozzi, P. Moreillon, and A. Tomasz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D90, p. 86). The strains of *S. pneumoniae* utilized in this study were from a variety of geographical sources and represented a wide range of serotypes, isolation dates, and clinical sources (see Table 1). The following strains of other streptococcal species were also tested:

Streptococcus sanguis V288 and 685; *Streptococcus pyogenes* B788, D471, and J17E; *Streptococcus* (*Enterococcus*) *faecalis* JH2-2; *Streptococcus* (*Enterococcus*) *faecium* ATCC 9790, CH-1, and CH-3; *Streptococcus mutans* GS5; *Streptococcus bovis* BS-1 and BS-5; and *Streptococcus agalactiae* 090-S.

Lysis by DOC. Pneumococcal strains grown at 37°C to the late exponential phase in C medium supplemented with 0.4% yeast extract (Difco Laboratories, Detroit, Mich.) (8) were tested for lysis by adding 0.1% DOC to the cultures.

Crude lysates of pneumococcal strains. The presence of chromosomal DNA sequences homologous to *lytA* was assayed with crude lysates of *S. pneumoniae* strains prepared as follows. Pneumococci were grown in CAT broth (12) to a concentration of about 3.2×10^8 cells per ml. One milliliter of the culture was spun down and suspended in 0.180 ml of 0.01 M Tris hydrochloride–0.001 M EDTA (pH 8.0); 20 μ l of 1% DOC was added, and bacteria were incubated for 10 min at 37°C. Incubation at 37°C was continued for another hour after the addition of 5 μ l of 20% sodium dodecyl sulfate and 1 μ l of proteinase K (20 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Purified pneumococcal amidase (2 to 5 μ g/ml; kindly provided by Z. Markiewicz of this laboratory) was added along with DOC when autolysis-deficient strains were being lysed. Lysates were stored frozen at –20°C.

Lysis of protoplasts of other streptococci. Chromosomal DNA of streptococcal strains other than pneumococci was prepared from 1-ml cultures in Todd-Hewitt broth (Difco) containing about 10^8 to 10^9 cells. Bacteria were spun down and suspended in 0.2 ml of protoplast buffer (30% raffinose in 0.5 M K_2HPO_4 buffer [pH 6.1]) containing 50 μ g of *N*-acetylmuramidase (mutanolysin) from *Streptomyces globisporus* (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) per ml; incubation was done for 30 min at 37°C. Protoplasts were centrifuged and suspended in 0.2 ml of lysis buffer (0.01 M Tris hydrochloride, 0.01 M EDTA, 0.5% sodium dodecyl sulfate). Subsequent treatment with proteinase K was as described for crude lysates of pneumococci.

Dot blot hybridization. DNA to be probed for homology with *lytA* was denatured by the addition of an equal volume of 0.5 N NaOH at room temperature for 10 min and then kept on ice; when needed, dilutions were made in 0.125 N

* Corresponding author.

[†] Present address: Istituto di Microbiologia, Università di Verona, Strada le Grazie, I-37134 Verona, Italy.

[‡] Present address: Istituto di Microbiologia, Università di Siena, I-53100 Siena, Italy.

TABLE 1. Lysis by DOC and presence of the *lytA* gene in pneumococcal strains

Geographical source	No. of strains lysed by DOC/total no. tested	No. of strains hybridizing to <i>lytA</i> /total no. tested
United States	184/185 ^a	42/42
Italy	35/35	2/2
South Africa	34/34	7/7
Switzerland	30/30	7/7
Czechoslovakia	2/2	1/1
New Guinea	1/1	1/1

^a Strain DOC-1 cannot be lysed by DOC, but its DNA shows homology to *lytA*.

NaOH-0.125 SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Denatured DNA (0.1 ml) was applied to a 96-well Bio-Dot apparatus (Bio-Rad Laboratories, Richmond, Calif.) carefully assembled as recommended by the supplier. Gene Screen Plus membranes (New England Nuclear Corp., Boston, Mass.) were used, and the manufacturer's suggested procedures were followed both for the application of samples and for hybridization under conditions of high stringency.

DNA probe. Plasmid pRMB21 (3.9 kilobases) was constructed by standard recombinant DNA techniques (9) by subcloning the 1.2-kilobase *Hind*III fragment containing *lytA* into pUC19. The *lytA* gene was obtained from plasmid pGL80 (3); restriction enzymes, T4 DNA ligase, pUC19 (18), and competent cells of *Escherichia coli* were from Bethesda Research Laboratories, Inc., Gaithersburg, Md. A radiolabeled nick-translated probe was prepared by using a nick translation kit (Bethesda Research Laboratories) and [³²P]dCTP (New England Nuclear). Unincorporated nucleotides were removed by gravity flow on Sephadex G-50.

The capacity to undergo autolysis by treatment with 0.1% DOC was tested in 287 clinical isolates of *S. pneumoniae*. The test was positive for all the isolates, with the exception of strain DOC-1 (Table 1). Plasmid pRMB21 containing the *lytA* gene was used as a probe in dot blot hybridization experiments. Crude lysates of 60 clinical isolates of pneumococci, including DOC-1, were tested: they all proved to have DNA sequences homologous to *lytA* (Table 1). Positive results were also obtained for strain RUP1, a laboratory strain with an insertionally inactivated *lytA* gene; laboratory strain M31 was used as a negative control for our hybridization experiments, since *lytA* is deleted from it (14). No hybridization to *lytA* was detected when strains of *S. pyogenes*, *S. sanguis*, *S. agalactiae*, *S. mutans*, *S. faecalis*, *S. faecium*, and *S. bovis* were tested.

To test the sensitivity of our dot blot assay, we applied to the filters 10-fold dilutions of purified DNA from plasmids containing the *lytA* gene and from crude lysates of pneumococcal strains Rx1 (*lytA*) and M31 (Δ *lytA*). Radioactively labeled pRMB21 was used as a probe for hybridization. Three different exposures of the autoradiographic film are shown in Fig. 1. A total of 8×10^4 cell equivalents of Rx1, corresponding to approximately 0.14 ng of total chromosomal DNA, were detectable after 18 h of exposure; after 83 h, 8×10^3 cell equivalents were detectable. However, after 83 h of exposure, a background signal could be observed with the negative control M31 (Fig. 1, panel 83 h, lane D).

The work described here tested the feasibility of an approach for the identification of *S. pneumoniae* based on a DNA probe for the pneumococcal autolysin gene. Our

FIG. 1. Dot blot hybridization. [³²P]dCTP nick-translated pRMB21 (*lytA*) was used as a probe. Lanes: A and B, 10-fold dilutions (ranging from 0.1 μ g to 0.1 pg) of purified DNA of plasmids pGL80 (A) and pSMB21 (B); C and D, 10-fold dilutions of crude lysates (ranging from 8×10^7 to 8×10^2 cell equivalents) of pneumococcal strains Rx1 (*lytA*) (C) and M31 (Δ *lytA*) (D). Three different film exposures, 2, 18, and 83 h, are shown. After 18 h of exposure, autoradiography revealed a positive reaction for 0.1 pg of plasmid DNA (A and B) and 8×10^4 cell equivalents of strain Rx1 (C), while no reaction was observed with crude lysates of M31 (D). A background signal was observed with M31 after 83 h.

choice of this target was guided by the known high degree of selectivity in the substrate requirements of the pneumococcal autolytic amidase; however, the distribution of this enzyme and its gene among clinical isolates remained to be tested. Also to be tested was the possible cross-reactivity of the probe with DNA of other streptococcal species.

Lysis of pneumococci by relatively low concentrations of DOC is known to require the activity of an autolytic enzyme (10), and bile sensitivity is generally believed to be a universal taxonomic feature of pneumococci. Nevertheless, actual data for this notion are not available. In view of the relative ease with which autolysis-deficient mutants can be isolated in the laboratory, the existence of such DOC-insensitive strains among clinical isolates cannot be excluded. Indeed, one such strain (DOC-1) was identified among the 287 isolates tested. Although not lysed by DOC, this strain nevertheless could be identified as *S. pneumoniae* by the positive reaction with the *lytA* DNA probe. DOC-1 is probably a mutant similar to some laboratory-constructed autolysin-deficient strains (2). All the other 286 pneumococcal strains tested contained an active autolysin, as judged by quick lysis by DOC.

The 60 strains of *S. pneumoniae* randomly chosen for dot blot hybridization analysis were all positive for the presence of DNA sequences homologous to *lytA*. These strains included representatives of 20 different serotypes as well as nontypeable strains; they were isolated in different years (1916 to 1987), in different places (United States, Europe, South Africa, and New Guinea), and from a variety of clinical sources (nasopharynx, blood, cerebrospinal fluid, and middle ear). These 60 strains also displayed a wide range of MICs of benzylpenicillin (from 0.008 to 17.0 μ g/ml), and a number of strains also carried the recently detected penicillin lysis defect (P. Moreillon and A. Tomasz, J. Infect. Dis., in press). Irrespective of these differences, all pneumococcal strains contained DNA sequences homologous to *lytA*. No cross-reactivity with the pneumococcal probe was detected with any of the seven other streptococcal species tested, which included *S. sanguis*, a bacterium known to undergo interspecific transformation by pneumococcal DNA (13). Under our experimental conditions the limit of detection was

about 10^5 cell equivalents; however, sensitivity could be improved considerably by using *lytA* as a target for DNA amplification by the polymerase chain reaction (11). In conclusion, a DNA probe targeted to *lytA* appears to fulfill the most important criteria for use as a diagnostic tool for the identification of *S. pneumoniae*.

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