Demonstration of UDP-Glucose Dehydrogenase Activity in Cell Extracts of Escherichia coli Expressing the Pneumococcal cap3A Gene Required for the Synthesis of Type 3 Capsular Polysaccharide

CARLOS ARRECUBIETA, ERNESTO GARCÍA,* AND RUBENS LÓPEZ

Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain

Received 5 February 1996/Accepted 5 March 1996

The gene cluster of Streptococcus pneumoniae coding for the type 3 capsular polysaccharide contains four genes (cap3ABCD). A DNA fragment containing the cap3A gene was amplified by PCR and cloned under the control of a T7 RNA polymerase-dependent promoter. Overexpression of this gene in Escherichia coli resulted both in a 47-kDa protein in the cytoplasm of isopropyl-β-D-thiogalactopyranoside-induced bacteria and in high levels of UDP-glucose dehydrogenase activity. These data demonstrate, in a direct experimental way, that cap3A encodes the UDP-glucose dehydrogenase of pneumococcus type 3.

Ninety capsular polysaccharides on the surface of pneumococci have hitherto been described (10). Cellobiose, a disaccharide consisting of glucose (Glc) and galactose (Gal), and another disaccharide consisting of glucuronic acid (GlcA) and glucose (Glc), is the basic component of the capsular polysaccharide of Streptococcus pneumoniae serotype 3 (15). Most of the unencapsulated or rough strains of this serotype have been found to lack the enzyme UDP-glucose dehydrogenase (UDP-GlcDH), which catalyzes the formation of UDP-GlcA from UDP-Glc (4).

Early genetic analyses suggested that the genes coding for the pneumococcal capsules are closely linked in the chromosome and can be transferred as a unit during genetic transformation (for a review, see reference 13). Molecular studies have recently confirmed this finding, and a cassette-like organization for the genes responsible for type 3 capsular polysaccharide biosynthesis in S. pneumoniae has been described (2, 7). The nucleotide sequence of the cap3A gene (also named cps3D), which is directly responsible for the transformation of some unencapsulated serotype 3 mutants to the encapsulated phenotype, has been determined, and several mutants affecting this gene have been characterized (1, 7). Preliminary evidence (1) suggested that cap3A encodes a UDP-GlcDH: (i) the deduced amino acid sequence of Cap3A is similar to that of the HasB enzyme from Streptococcus pyogenes (8) and (ii) the wild-type cap3A gene restored encapsulation in a mutant previously described as deficient in UDP-GlcDH (3). Nevertheless, this evidence allowed the assignment of putative functions to the type 3-specific genes only until direct biochemical data were available.

The expression of cloned genes implicated in capsule formation in gram-positive bacteria has been hindered until recently, and this limitation was commonly ascribed to the toxicity of the gene products when tested in heterologous systems like that of Escherichia coli. Recently, some capsular genes of group A streptococci have been expressed in E. coli and biochemically identified, which has contributed to the definition of the proteins that participate in the polysaccharide production in this species (6, 8). In the present study, we have cloned and expressed in E. coli as well as biochemically characterized for the first time a gene product responsible for the synthesis of the pneumococcal type 3 polysaccharide.

Overexpression of the cap3A gene in E. coli. Several attempts to clone different pneumococcal genes possibly involved in capsular polysaccharide synthesis and express them in E. coli have been unsuccessful, probably because of the use of high-copy-number plasmids and/or poorly regulated promoters. The cluster determining the expression of the type 3 capsule has been proposed to contain four genes (Fig. 1). DNA from strain 406, a type 3 pneumococcal clinical isolate, was prepared from cells grown in C medium (23) according to a previously described procedure (9). To clone cap3A in the absence of its own promoter, this gene was first PCR amplified (16) with oligonucleotides based on the nucleotide sequence previously determined (1): PCAP20 (5′-GAAGAGGAGCTGTAGTCGATATGAAAATTG-3′) and PCAP22 (5′-TATAACCGGCCATGACATAAGAAAGAC-3′) (the underlined sequences indicate restriction sites for NdeI and SfiI, respectively). The amplified DNA was digested with NdeI and SfiI and ligated to pGEM-5Z(+)(Promega) previously cut with the same enzymes. The ligation mixture was used to transform E. coli DH10B [F- mcrA Δ(mrr hasRMS-mcrBC) φ80lacZΔM15 ΔluxX74 deoR recA1 araD139 (ara-leu)7697 galU galK λ- rpsL endA1 nupG; Life Technologies], and the recombinant plasmid pGDH1 was isolated among the ampicillin-resistant clones that showed white phenotypes in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indoly-β-D-galactoside. In pGDH1, neither the vector plasmid pGEM-5Zf(+) nor the insert contains a ribosome binding site for cap3A translation. Consequently, to express the

* Corresponding author. Mailing address: Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain. Phone: (1) 561-1801. Fax: (1) 562-7518. Electronic mail address: mio@pinar1.csic.es.
cap3A gene, the 1.4-kb NdeI-NsiI insert of pGDH1 was ligated to pT7-7 (22) previously digested with NdeI and PstI and introduced by transformation into the expression strain *E. coli* JM109(DE3) [endA1 recA1 gyrA96 hsdR17 supE44 relA1 thi Δ(lac-pro) F' (traD36 proAB' lacP1 lacZΔM15) acb857 ind1 Sam7 nin5 lacU5-17 gene 1; Promega]. The correct insertion of cap3A into the recombinant plasmid pTVU1 was verified by restriction analysis and DNA sequencing.

*E. coli* JM109(DE3) cells harboring pTVU1 were grown in LB broth (17) supplemented with ampicillin (100 μg ml⁻¹) at 37°C to an optical density at 600 nm of 0.6, the culture was shifted to 30°C, and expression of the cap3A gene was induced by adding IPTG to 0.5 mM (final concentration). After 1 or 3 h of induction, samples of the culture were harvested by centrifugation (10,000 x g, 5 min) and the pellets were washed in phosphate-buffered saline and disrupted by sonication. The insoluble fraction was separated by centrifugation (15,000 x g, 15 min), and this fraction and the supernatant were used to detect the presence of the Cap3A protein. Control cells containing the pT7-7 vector plasmid alone did not show expression over the 3-h time course analyzed, whereas expression of an approximately 47-kDa protein was apparent with JM109(DE3) cells harboring pTVU1, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12) (Fig. 2). This molecular mass is in good agreement with the *M₈* (44,646) deduced from the nucleotide sequence of the cap3A gene (1). After 3 h of incubation most of the 47-kDa band was detected in the pellet fraction (data not shown), probably because of the formation of inclusion bodies, which apparently leads to enzyme inactivation. After SDS-PAGE, proteins were blotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) and stained briefly with amido black (Sigma). Subsequently, the desired band was cut and the N-terminal amino acid sequence was determined as described elsewhere (21), yielding Met-Lys-Ile-Ala-Ile-Ala-Gly, which confirmed the sequence deduced from the nucleotide analysis of the gene (1).

**Enzymatic analysis of Cap3A.** As reported above, at least two lines of evidence have suggested that Cap3A might be a UDP-GlcDH. However, performing the standard spectrophotometric assay for UDP-GlcDH using extracts prepared from *S. pneumoniae* proved impossible because of the presence of an extremely active NADH oxidase (19) that copurifies with the enzyme (20). In addition, in vitro capsule synthesis assays (14) using genetically well-characterized UDP-GlcDH mutants do not allow the direct analysis of the biochemical product encoded by cap3A. Sonicated cell lysates prepared from *E. coli* JM109(DE3) (pTVU1) were assayed for UDP-GlcDH activity by following a modified protocol based on a method previously described (18). Unless stated otherwise, portions (5 to 40 μl, containing 20 to 160 μg of protein) of cytosol fractions were incubated in a buffer containing 100 mM Tris (pH 9.0), 10 mM sodium thioglycolate, 1 mM UDP-Glc, and 2 mM NAD, in a

![Image 1](https://example.com/image1.png)

**FIG. 1.** Genetic organization of *S. pneumoniae* type 3 capsular gene cluster and strategy for cloning the cap3A gene. The type 3-specific genes and the regions common to several serotypes are represented by hatched and white boxes, respectively. The length and direction of the mRNA transcript and the promoter corresponding to the cap3A and cap3B operon (2) are indicated by an open arrow and arrowhead, respectively. The cap3D gene appears to code for a glucose mutase (unpublished data). Transcription terminators are indicated by vertical loops, and the locations and directions of the oligonucleotides PCAP20 (solid triangle) and PCAP22 (open triangle) are also shown. The nucleotide sequence of PCAP20 is shown at the bottom of the figure. The ATG initiation codon of cap3A is indicated in italics. RBS, ribosome binding site. Abbreviations for restriction sites: A, AccI; E, EcoRV; H, HindIII; N, NdeI; Ns, NsiI; P, PvuII; S, ScaI.

![Image 2](https://example.com/image2.png)

**FIG. 2.** SDS-PAGE analysis of *E. coli* JM109(DE3) (pTVU1) cells for identification of Cap3A production. Lane 1, molecular mass markers; lane 2, crude, sonicated extract of an uninduced culture of *E. coli* JM109(DE3) (pTVU1); lane 3, sample of total sonicated IPTG-induced sample after 1 h of incubation at 30°C. The arrow indicates the induced protein. The gel was stained with Coomassie blue.

**TABLE 1.** UDP-GlcDH activity of *E. coli* lysates

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Induction condition(s)</th>
<th>pH of reaction mixture</th>
<th>Sp act/(U mg⁻¹)(10²)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT7-7</td>
<td>Uninduced</td>
<td>9.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>pT7-7</td>
<td>Induced (3 h)</td>
<td>9.0</td>
<td>2</td>
<td>5.5</td>
</tr>
<tr>
<td>pTVU1</td>
<td>Uninduced</td>
<td>9.0</td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td>pTVU1</td>
<td>Induced (1 h)</td>
<td>8.5</td>
<td>22</td>
<td>61.1</td>
</tr>
<tr>
<td>pTVU1</td>
<td>Induced (1 h)</td>
<td>8.0</td>
<td>19</td>
<td>52.8</td>
</tr>
<tr>
<td>pTVU1</td>
<td>Induced (1 h)</td>
<td>7.5</td>
<td>4</td>
<td>11.1</td>
</tr>
<tr>
<td>pTVU1</td>
<td>Induced (1 h)</td>
<td>9.0^c</td>
<td>3</td>
<td>8.3</td>
</tr>
<tr>
<td>pTVU1</td>
<td>Induced (1 h)</td>
<td>9.0^c</td>
<td>4</td>
<td>11.1</td>
</tr>
</tbody>
</table>

*E. coli* JM109(DE3) was the host strain.

b Induction was carried out at 30°C unless stated otherwise.

One unit of enzyme activity is designated as the amount of enzyme required to produce 2 μmol of NADH per min at 30°C. Values are the averages of three independent experiments.

—, below limit of detection.

^c This reaction mixture included 10 mM iodoacetate.
fraction that showed carbazole-positive reactions.

elution of pure UDP-Glc and UDP-GlcA, and the horizontal bar indicates
(100 s) of each fraction (0.5 ml) were determined. Arrows indicate the times of
elution of pure UDP-Glc and UDP-GlcA, and the horizontal bar indicates
fractions that showed carbazole-positive reactions.

The demonstration of enzyme activity, i.e., UDP-GlcA activity from UDP-GlcA. UDP-[U-14C]glucose was incubated for 1 h at 30°C with extracts prepared from uninduced (□) or IPTG-induced (○) cultures of E. coli JM109(DE3) harboring pTVU1. The samples were centrifuged (15,000 × g, 15 min), and 100-μl samples of the supernatant were analyzed on a Spherosorb-10 SAX column as indicated in the text. Levels of radioactivity for samples (100 μl) of each fraction (0.5 ml) were determined. Arrows indicate the times of elution of pure UDP-Glc and UDP-GlcA, and the horizontal bar indicates fractions that showed carbazole-positive reactions.

final volume of 1 ml. NAD reduction was monitored with a Shimadzu UV-260 spectrophotometer at 340 nm. In contrast with previous results (14) Mg²⁺ was apparently not required for full pneumococcal UDP-GlcDH activity, since the addition of 10 mM EDTA did not inhibit the reaction (not shown). Table 1 shows that E. coli JM109(DE3)(pTVU1) demonstrated UDP-GlcDH activity 1 h after IPTG induction at 30°C and that the optimal pH for activity appeared to be about 9.0. Interestingly, iodoacetic acid inhibited the enzyme activity, indicating that a cysteine residue is involved in the catalytic activity of the pneumococcal UDP-GlcDH, as previously suggested (1, 14). When the IPTG induction was carried out at 37°C for 1 h the extracts obtained showed a reduced level of enzymatic activity (Table 1), probably due to the formation of insoluble inclusion bodies as found by microscopical observations. E. coli cells harboring the pT7-7 expression plasmid did not show any UDP-GlcDH activity before or after induction.

To confirm that one of the products of the oxidation of UDP-GlcA in this assay reaction mixture was actually UDP-GlcA, cytosol fractions received a trace amount of UDP-[U-

14C]glucose (250 nCi; 309 mCi mmol⁻¹) and were incubated for 1 h and 100-μl samples were analyzed by high-performance liquid chromatography using a Spherosorb-10 SAX anion-exchange column (4.6 by 150 mm; Alttech) as previously described (11). Figure 3 shows the appearance of a radioactive peak which coeluted with authentic UDP-GlcA and shows a concomitant reduction of the UDP-Glc peak. Furthermore, a positive carbazole reaction (5) confirmed that the product of the reaction was indeed a uronic acid.

The demonstration of enzyme activity, i.e., UDP-GlcA production from UDP-Glc, confirms that cap3A code for a UDP-GlcDH involved in the synthesis of the pneumococcal type 3 capsule and represents the first time that such a pneumococcal gene has been cloned and expressed. Previous results showed that the cap3A gene is cotranscribed with cap3B, a gene possibly encoding a synthase (2), which should transfer alternate residues of GlcA and Glc to the growing S3 polysaccharide chain. This type of genetic organization is similar to that found in the group A streptococci for the synthesis of the hyaluronic acid capsule (6, 8) and to that found more recently in the operon kfiCD implicated in the production of the E. coli K5 capsule (18). Furthermore, in the case of type 3 pneumococcal strains it appears that the regions flanking the capsular cluster (cap3ABCD) do not participate in the formation of the capsular polysaccharide (2). A detailed biochemical analysis of the gene products of the pneumococcal operons directing the synthesis of capsular polysaccharides, similar to the one reported here for serotype 3, will allow the determination of the precise role of these genes in capsule biosynthesis.

We thank P. García, J. L. García, E. Diaz, and R. Muñoz for helpful comments and for the critical reading of the manuscript and M. Sheehan for correcting the English version. The technical assistance of M. Carrasco and E. Cano and the skillful work by A. Hurtado and V. Muñoz are greatly appreciated. This work was supported by grant PB-93-0115-C02-01 from the Programa Sectorial de Promocion General del Conocimiento. C.A. is a beneficiary of a predoctoral fellowship from Eusko Jaurlaritzia.

REFERENCES


