Characterization of the \textit{galU} Gene of \textit{Streptococcus pneumoniae} Encoding a Uridine Diphosphoglucose Pyrophosphorylase: A Gene Essential for Capsular Polysaccharide Biosynthesis

By Marta Mollerach,* Rubens López,‡ and Ernesto García‡

From the *D departamento de M i c r o b i o l o g í a, Facultad de F a r mf a y B i o q u í m i c a, U n i v e r s i d a d de B u e n o s A i r e s 1 1 1 3 B u e n o s A i r e s A r g e n t i n a; and the ‡ C e n t r o d e I n v e s t i g a c i ó n e s B i ó l o g i c a s, C o n s e j o S u p e r i o r d e I n v e s t i g a c i ó n e s C i e n t í f i c a s 2 8 0 0 6 M a d r i d, S p a i n

Summary

The \textit{galU} gene of \textit{Streptococcus pneumoniae} has been cloned and sequenced. \textit{Escherichia coli} cells harboring the recombinant plasmid pMMG2 (\textit{galU}) overproduced a protein that has been shown to correspond to a uridine 5'-triphosphateglucose-1-phosphate uridylyltransferase (udrige diphosphoglucose [UDP-Glc] pyrophosphorylase) responsible for the synthesis of UDP-Glc, a key compound in the biosynthesis of polysaccharides. A gene very similar to the \textit{S. pneumoniae} \textit{galU} has been found in a partial nucleotide sequence of the \textit{Streptococcus pyogenes} genome. Knockout \textit{galU} mutants of type 1 pneumococci are unable to synthesize a detectable capsule. An identical result was found in type 3 \textit{S. pneumoniae} cells in spite of the fact that these bacteria contain a type-specific gene (\textit{cap3C}) that also encodes a UDP-Glc pyrophosphorylase. Since eukaryotic UDP-Glc pyrophosphorylasess appear to be completely unrelated to their prokaryotic counterparts, we postulate that GalU may be an appropriate target for the search of new drugs to control the pathogenicity of bacteria like pneumococcus and \textit{S. pyogenes}.

Key words: pneumococcus • capsule • UDP-Glc pyrophosphorylase • virulence • galU

\textbf{S}treptococcus pneumoniae (pneumococcus) remains a major cause of morbidity and mortality throughout the world and its continuous increase in antimicrobial resistance is rapidly becoming a leading cause of concern for public health. Pneumococcal infection persists as the main causative agent of pneumonia, meningitis, and otitis media. These diseases remain as the most prevalent infections in many areas of the world, particularly in infants, the elderly, and in immunocompromised patients. As shown in a recent study (1), although a 68% reduction in total invasive diseases due to \textit{Haemophilus influenzae} type b was observed in the United States after licensure of the conjugated vaccine, a concomitant increase of 74% in the number of cases per 100,000 population for invasive pneumococcal diseases was also found. Although \textit{S. pneumoniae} produces several virulence factors (for review see reference 2), as early as 1928 Griffith reported that unencapsulated pneumococcal variants were avirulent (3), and that loss of the capsule is accompanied by a 105-fold reduction of the virulence of \textit{S. pneumoniae}. Uncapsulated pneumococci are readily phagocytized when added to a suspension of leukocytes in normal serum, whereas mucoid, capsulated organisms are resistant to phagocytosis and multiply rapidly. A quantitative relationship between the amount of type-specific polysaccharide and virulence has been found (4, 5), although the chemical composition of the capsule (6) as well as the cellular background in which the capsule is produced also appear to play an important role in virulence (7).

90 different pneumococcal types have been described (8). This remarkable phenotypic variability appears to be present also at the genetic level (9–11), which has precluded until now the search for drugs capable of inhibiting the synthesis of the capsular polysaccharides of \textit{S. pneumoniae}. The chemical structure of the repeat unit of these polysaccharides is known in more than half of the types described, most of them contain glucose (Glc) and/or galactose (Gal) or various derivatives of them in addition to other sugars (12). Early studies carried out by Mills and coworkers (for review see reference 13) showed that uridine diphosphoglucose (UDP-Glc) is a key component in

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1Abbreviations used in this paper: Gal, galactose; GAS, group A streptococci; Glc, glucose; Ln, lincomycin; UDP-GalA, uridine diphosphogalacturonic acid; UDP-Glc, uridine diphosphoglucosamine; UDP-GlcA, uridine diphosphoglucuronic acid; UDPG-PP, uridine diphosphoglucose pyrophosphorylase.
the biosynthetic pathway of pneumococcal capsular polysaccharides containing Glc, Gal, and/or UDP-glucuronic (UDP-GlcA) or UDP-galacturonic (UDP-GalA) acids (Fig. 1). The enzyme UTP:glucose-1-phosphate uridylyltransferase (UDP-Glc pyrophosphorylase, UDPG:PP) (EC 2.7.7.9) catalyzes the formation of UDP-Glc, which is the substrate for the synthesis of UDP-GlcA. UDP-Glc is also required for the interconversion of Gal and Glc by way of the Leloir pathway (14), and consequently, mutants of *E. coli* deficient in UDPG:PP activity cannot ferment Gal and fail to incorporate Glc and Gal into bacterial cell membranes, resulting in the incomplete synthesis of lipopolysaccharide (15). The gene cap3C of *S. pneumoniae*, one of the three genes located in the type 3-specific capsular operon, encodes a UDPG:PP that has been shown to be dispensable for capsule production (16, 17). This result strongly suggests that another different gene might also encode a UDPG:PP that, in addition, might be common to all of the pneumococci. In favor of this assumption is the fact that, apart from cap3C, a gene putatively encoding a UDPG:PP has not been found in any of the capsular clusters studied so far (9–11).

A partial (and still preliminary) nucleotide sequence of the genome of a type 4 pneumococcus has been released recently (ftp://ftp.tigr.org/pub/data/s_pneumoniae). This has allowed the search for genes coding for proteins similar to the Cap3C pyrophosphorylase and we report here the cloning, expression, and characterization of the galU gene of *S. pneumoniae*. We show that the pneumococcal galU mutants of types 1 and 3 did not synthesize detectable amounts of capsular polysaccharide.

### Materials and Methods

**Bacterial Strains, Plasmids, and Growth Conditions.** The bacterial strains and the plasmids used in this study are shown in Table 1. Unless otherwise stated, *S. pneumoniae* was grown in liquid C medium (18) containing 0.08% yeast extract (C + Y) without shaking or on reconstituted tryptose blood agar base plates (Difco Laboratories, Inc., Detroit, MI) supplemented with 5% defibrinated sheep blood. *E. coli* cells were grown in Luria-Bertani medium (19). When required, ampicillin was added to the medium at 100 μg/ml. Chromosomal DNA and plasmid purification, and transformation of *E. coli* and laboratory strains of *S. pneumoniae* were carried out as described elsewhere (20). *S. pneumoniae* clones obtained upon transformation with pUC18 or pMG1 (Table 1) were scored on blood agar plates containing 0.7 μg of lincomycin (Ln) per ml. MacConkey agar plates (Difco Laboratories, Inc.) containing 0.6% galactose were used for *E. coli* fermentation tests.

### Cloning and Sequencing

**DNA Techniques and Plasmid Construction.** Restriction endonucleases, T4 DNA ligase, and the Klenow (large) fragment of DNA polymerase I were obtained commercially and used according to the recommendations of the suppliers. Gel electrophoresis of plasmid restriction fragments, and PCR products was carried out in agarose gels as described (19). DNA was recovered from gel slices with the Gene Clean Kit (Bio 101, La Jolla, CA).

* S. pneumoniae DNA digested with either SmaI, SacI, or ApaI was analyzed by pulsed-field gel electrophoresis (PFGE) using a contour-clamped homogeneous electric field (CHEF) apparatus (Bio-Rad, Hercules, CA) as previously described (21).

**PCR amplifications were performed using 2 U of AmpliTaq™ DNA polymerase (Perkin-Elmer Applied Biosystems, Norwalk, CT).** 1 μg of chromosomal (or plasmid) DNA, 1 μM of each synthetic oligonucleotide primer, 200 μM of each deoxynucleotide triphosphate, and 2.5 mM of MgCl2 in the buffer recommended by the manufacturer. Conditions for amplification were chosen according to the G + C content of the corresponding oligonucleotide.

The oligonucleotides used were: (624), 5'-TTGGATCCGGAAACACTGGCATGC-3' (primer OGalU1); (1139/c) 5'-GCCAAGCGTCGTACGATCCCTGTG-3' (primer OGalU2); (1464/c), 5'-GGACAAATTGTTGGCCGAGTTTCTAGGC-3' (primer OGalU3); (323), 5'-TGAGTTGGACCTTACACCTCCCTATAGAAG-3' (primer OGalU4); (659/c), 5'-GAAATGAAAGGCGCATGCCCAGTTG-3' (primer IGalU1); (773), 5'-CGAGAAGGCCGGTCTCTCTGACT-3' (primer IGalU2). N numbers indicate the position of the first nucleotide of the primer in the sequence reported in this paper (see below), and /c means that the corresponding sequence corresponds to the complementary strand. Lowercase letters indicate nucleotides introduced to construct appropriate restriction sites. These are shown underlined. For inverse PCR, DNA prepared from strain 406 was digested with C11I and Clal, and the resulting fragments were diluted 10-fold and self-ligated. Afterwards, the DNA was concentrated by ethanol precipitation and amplified by PCR using oligonucleotides IGalU1 and IGalU2.

To construct pMMG2 (Table 1), DNA prepared from the type 3 strain 406 was amplified with oligonucleotides OGalU3 and OGalU4 and the 1.1-kb DNA fragment was purified, digested with MUnl and SalI, and ligated to EcoRI/Sall-digested pUC19. Plasmid pMMG1, which contains a 0.5-kb internal fragment of the gene galU (Table 1), was constructed as follows: DNA prepared from strain 406 was amplified with primers OGalU1 and OGalU2 and the 0.5-kb DNA fragment was purified, digested with KpnI and Clal, and ligated to pUC19 previously digested with KpnI plus Accl.

**NEBlot™ Phototope™ Kit** (Millipore Corp., Bedford, MA) was used to construct biotin-labeled probes and Phototope™ 6K Detection Kit (Millipore Corp.) for the chemiluminescent detection. Southern blots, dot blots, and hybridizations were carried out according to the manufacturer's instructions.
Nucleotide Sequence and Data Analysis. DNA sequencing was carried out by using an Abi Prism 377™ DNA sequencer (Applied Biosystems Inc., Foster City, CA). DNA and protein sequences were analyzed with the Genetics Computer Group software package (version 9.0) (22). Sequence similarity searches were done by using the EMBL/GenBank, SWISS-PROT, and PIR databases.

SDS-PAGE and N H₂-terminal Amino Acid Sequence Analysis. SDS-PAGE was carried out as described by Laemmli using 10% gels (23). After SDS-PAGE, proteins were blotted onto a polyvinylidene difluoride membrane (Immobilon-P™, Millipore Corp.), and stained briefly with Amido black (Sigma Chemical Co., St. Louis, MO). Subsequently, the desired band was cut and the NH₂-terminal amino acid sequence was determined as described elsewhere (24).

Miscellaneous Techniques. The test for carbohydrate fermentation by pneumococcal strains was carried out in Heart infusion broth (Difco Laboratories, Inc.) with the appropriate carbohydrate added at 1% (final concentration) as previously described (25). For immunoagglutination assays (26), S. pneumoniae cells were incubated on C medium containing 0.1% BSA and different amounts of anti-R antiserum. Anti-R (antisomatic) antiserum contains group-specific agglutinins that, at a convenient dilution, agglutinate only rough pneumococci, and was raised in rabbits as

Table 1. Bacterial Strains and Plasmids

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>M 25</td>
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</tr>
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<td>galU::pUCE191 lytA</td>
<td>T his study</td>
</tr>
<tr>
<td>406</td>
<td>S3⁺ LytA⁺</td>
<td>45</td>
</tr>
<tr>
<td>M 23</td>
<td>S3⁺ LytA⁺</td>
<td>45</td>
</tr>
<tr>
<td>M 23_c†</td>
<td>cap3C::pUCE191 lytA</td>
<td>16</td>
</tr>
<tr>
<td>M 23_g†</td>
<td>galU::pUCE191 lytA</td>
<td>T his study</td>
</tr>
<tr>
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<td>M 24_g‡</td>
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<td>1235/89</td>
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<td>E. coli strains</td>
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<tr>
<td>D H5x</td>
<td>(£801aZ∆M15) endA1 recA1 hsdR17 (r6K·mK−) supE44 ∆ladJ169 thi−1 leuB thr−1</td>
<td>47</td>
</tr>
<tr>
<td>T G1</td>
<td>supE ∆sdS· thi ∆(lac-proA B) F’ (traD36 proA B + ∆adA· laZ·∆M15)</td>
<td>19</td>
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<tr>
<td>FF4001</td>
<td>M C4100 galU 95</td>
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<td>Plasmids</td>
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<tr>
<td>pUC19</td>
<td>Cloning vector (Ap⁺)</td>
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<td>pUCE192</td>
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<td>pUCEK2</td>
<td>Internal fragment of cap3C cloned into pUCE191</td>
<td>16</td>
</tr>
<tr>
<td>pM M G1</td>
<td>0.45-kb internal fragment of S. pneumoniae galU cloned into pUCE191</td>
<td>T his study</td>
</tr>
<tr>
<td>pM M G2</td>
<td>galU gene from S. pneumoniae strain 406 cloned into pUC19</td>
<td>T his study</td>
</tr>
</tbody>
</table>

*Strain constructed by transformation of M 25 with DNA from strain M 24_g.
†Strain constructed by transformation of M 23 with pUCEK2.
‡Strain constructed by transformation of M 23 with DNA from M 24_g.
§Strain constructed by transformation of M 24 with pMMG1.
Results

Identification, Cloning, Sequencing, and Mapping of a Pneumococcal Gene Similar to cap3C. The deduced amino acid sequence of the cap3C gene was compared to the translated version of the nucleotide sequence of a type 4 pneumococcal strain that has been released recently. A gene (hereafter designated galU) encoding a protein 77% identical to Cap3C was located in the 12,128-bp contig No. 4225. Analysis of this gene and its surrounding regions (Fig. 2) revealed that contig No. 4225 does not contain the type 4-specific capsular cluster which appears to be localized in the contig No. 4108 (34,280 bp). The putative galU gene is preceded by a gene whose product showed strong similarity to the GpsA NAD(P)H-dependent dihydroxyacetone-phosphate reductase of B. subtilis (28) (Table 2). The galU and the gspA genes are only 21 bp apart. Other genes surrounding gspA-galU were preliminarily identified on the basis of sequence similarities with the exception of orf5 and orf6, which did not show any significant similarities to those available in the data banks.

Preliminary efforts to amplify by PCR the galU gene using DNA prepared from the S. pneumoniae type 3 strain 406 were unsuccessful when using oligonucleotide primers designed from the nucleotide sequence of genes gspA and orf5 from type 4 pneumococci. However, successful amplification was achieved with O GalU 3 and O GalU 4, which correspond to the 5' end of orf6 and to the 3' end of gspA, respectively. The 1.1-kb PCR fragment was cloned into pUC19 to create pMMG2 (Table 1). Moreover, inverse PCR using oligonucleotides IGalU1 and IGalU2 (designated from the nucleotide sequence of the SalI-MunI insert of pMMG2) produced a 2.3-kb DNA fragment that was partially sequenced. Determination of the sequence of 1,464 bp revealed that the orf5 gene was not present in 406 DNA, which accounts for the amplification failures discussed above. It should be noted that the nucleotide sequence of the galU 406 gene (900 bp) was nearly identical (89%) to that of the type 4 isolate (not shown).

The absence of orf5 in strain 406 prompted us to analyze whether other important differences exist among various pneumococcal isolates. Southern blot analysis of HindIII-digested chromosomal DNA prepared from a variety of pneumococcal strains using a 0.7-kb SalI-ClaI fragment of pMMG2 showed that all the strains tested contain at least one copy of the galU gene (Fig. 3). In the case of strains 6028/95 (S8+) and SSIP 33A/1 (S33A+), the pattern of hybridization might suggest the existence of a second copy of galU in their DNAs, although we favor the hypothesis that the 5.5-kb DNA band was incompletely cleaved in those two strains. Nevertheless, this point was not further investigated.

Fig. 4 shows a multiple alignment of the amino acid sequence of the galU 406 gene product with the proteins available in the databases. HasC, a UDPG:PP from the has operon required for expression of the hyaluronic acid capsule of Streptococcus pyogenes (29), showed the highest similarity to the pneumococcal GalU (>85% identical amino acids and 90.9% similarity). About 87% sequence similarity (77% identity) was found between the pneumococcal GalU and Cap3C proteins. Although not yet included in the data banks, a search of a partial nucleotide sequence of S. pneumoniae genome (http://www.genome.ou.edu/strep.html) for genes similar to galU (and hasC) showed that, as in S. pneumoniae, group A streptococci (GAS) also contain a galU homologue located in the contig No. 234 (8061 bp) (Fig. 2). The similarity between the GalU proteins of S. pneumoniae and S. pyogenes was even higher than that found between the former and the type 3-specific Cap3C UDPG:PP of S. pneumoniae (Fig. 4). Lower but significant similarities were also found between the pneumococcal GalU and UDPG:PPs from B. subtilis (30) and E. coli (31) as well as with the GalF protein of E. coli that modulates the activity of the GalF enzyme (32).

To determine the location of galU in the pneumococcal genome, chromosomal DNA of strain M24, a descendant of the classical laboratory strain R6 (33), was digested with SmaI, Apal, or SacII, subjected to PFGE, blotted, and hybridized with the 0.7-kb SalI-ClaI fragment of pMMG2 (see above). The results shown in Fig. 5 indicated that the galU gene resides in fragments S (SmaI, 235 kb), 4 (Apal, 235 kb), and 13 (SacII, 54 kb) of the physical map of the pneumococcal genome (34).

Overproduction and Characterization of the S. pneumoniae GalU Protein. E. coli DH5α cells harboring pMMG2 were incubated overnight at 37°C with shaking in LB me-
dium containing ampicillin (100 μg/ml). Crude extracts obtained by sonication were analyzed by 10% SDS-PAGE and a prominent protein band of ~37 kD was observed (Fig. 6, lane 2). This band was absent in crude extract prepared from *E. coli* DH5α cells containing the vector plasmid pUC19 (Fig. 6, lane 3). The molecular mass of the 37-kD overproduced protein was in fair agreement with that deduced from the nucleotide sequence of the *galU* gene (33,213 D). It should be mentioned that an apparent 38,000 M_r has been reported for the purified *E. coli* GalU whereas a molecular mass of 32,291 D was predicted from the sequence of the *galU* gene (31). The NH₂-terminal amino acid sequence of the protein was determined, yielding Met-Thr-Ser-Lys-Val-Arg-Lys-Ala-Val-Ile, which confirmed the sequence deduced from the nucleotide sequence of the gene (Fig. 4).

To ascertain that the pneumococcal *galU* gene codes for a UDPG:PP, the pGM M2 plasmid was introduced by transformation into the *galU* *E. coli* mutant strain FF4001. This mutant contains a T to C mutation causing the substitution of a proline residue by a serine one in the predicted amino acid sequence of the GalU protein and virtually lacks UDPG:PP activity (31). Ampicillin-resistant transformants scored on MacConkey-galactose plates grew as red colonies indicating that they were able to ferment the sugar (not shown). This result confirmed that the *galU* gene of *S. pneumoniae* encodes a UDPG:PP.

**Table 2.** The *galU* Genes of *S. pneumoniae* and *S. pyogenes* and Their Surrounding orfs: Properties and Similarities in the Database

<table>
<thead>
<tr>
<th></th>
<th>Predicted protein (aa/kD)</th>
<th>Proposed function</th>
<th>Most similar polypeptide</th>
<th>Database accession No.</th>
<th>Degree of identity/similarity (%)</th>
<th>Organism</th>
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<tbody>
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<td><em>S. pneumoniae</em> DNA (contig no. 4225, partial)</td>
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<td></td>
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<td></td>
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<tr>
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<td>78/8.2</td>
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<td>Mth1730</td>
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<td><em>Methanobacterium thermoautotrophicum</em></td>
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<td>250/28.1</td>
<td>Phosphate transport system ATP-binding protein</td>
<td>Mj1012</td>
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<td><em>Methanococcus jannaschii</em></td>
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<td>Negative regulator</td>
<td>PhoU</td>
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<td><em>Enterobacter cloacae</em></td>
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<td>Transposase</td>
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<td>GpsA</td>
<td>338/36.8</td>
<td>NAD(P)H-dependent dihydroxyacetone-phosphate reductase</td>
<td>GpsA</td>
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<td>299/33.2</td>
<td>UDP-Glc pyrophosphorylase</td>
<td>HasC</td>
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<td>YybA</td>
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<td>HasC</td>
<td>U33452</td>
<td>90.3/94.3</td>
<td><em>S. pyogenes</em></td>
</tr>
<tr>
<td>Orf5*</td>
<td>212/23.2</td>
<td>Unknown</td>
<td>YgRB</td>
<td>Z99116</td>
<td>24.1/44.9</td>
<td><em>B. subtilis</em></td>
</tr>
</tbody>
</table>

aa, amino acids.
*Partial ORF.
‡Interrupted reading frame.
A Ln-resistant transformant (designated as M24g) was isolated upon transformation of the pneumococcal strain M24 with pMMG1 isolated from E. coli TG1. Carbohydrate-fermentation tests showed that M24g fermented Glc but was unable to use Gal as a carbon source (not shown).

Chromosomal DNA prepared from strain M24g was used to transform the encapsulated strains M23 (S31) and M25 (S11) and Ln-resistant clones were picked for further study. As found for M24g, the transformant strains M23g and M25g were also unable to ferment Gal whereas M23c did ferment either Glc or Gal. Furthermore, when the M23g strain was grown in blood agar plates, small, rough-like colonies were formed, in sharp contrast with the big, smooth colonies typical of type 3 strains of S. pneumoniae (Fig. 7). As reported before (16), no major differences were observed between the colonies of M23c and M23g strains. Since type 1 pneumococcal strains form small colonies, no significant differences were found on the morphology of the colonies of M25 and M25g (not shown). Encapsulated pneumococci grow typically in suspension when incubated in broth, whereas unencapsulated mutants show a tendency to aggregate at the bottom of the tube. The pneumococcal galU mutants, but not the cap3C mutants (M24c), grew as true unencapsulated strains (Fig. 7) and were agglutinated with anti-R serum (not shown). Moreover, immunodiffusion analysis of cell extracts using either type 1- or type 3-specific antisera demonstrated that M23g and M25g did not synthesize any detectable capsular polysaccharide (Fig. 7). In contrast, M23c produced type 3 polysaccharide in amounts comparable to the M23 strain, which confirmed previous results (16). To further confirm that GalU is essential for the biosynthesis of capsular polysaccharides in S. pneumoniae, strains M23g and M25g were repeatedly subcultured in Ln-free C medium containing 0.1% BSA and anti-R antiserum (see Materials and Methods). After several passages, putatively encapsulated revertants that were not agglutinated by the serum and thus grew in suspension were isolated. They appeared to be true galU+ revertants as judged from the findings that they were no longer Ln-resistant and that the size (1.1 kb) of the PCR fragments obtained by using oligonucleotide primers OGalU3 and OGalU4
and DNA isolated from every revertant tested corresponded to that of the intact galU gene (see above). As expected, those revertants were able to synthesize a capsular polysaccharide corresponding to the original, encapsulated parental strain (type 1 or type 3) as demonstrated immunologically (data not shown).

Discussion

Duplicated genes appear to be rather unusual in bacteria and consequently type 3 pneumococci and S. pyogenes are somehow exceptional in having two genes coding for the same enzyme, namely, a UDPG:PP. The capsular polysaccharides of both bacteria contain glucuronic acid, and the genes responsible for their biosynthesis are organized in a similar fashion (16, 35), i.e., each operon contains three genes: the gene responsible for the synthesis of UDP-Glc (cap3C and hasC, in S. pneumoniae and GAS, respectively), the gene encoding a UDP-Glc dehydrogenase (cap3A and hasB), and the gene coding for the enzyme responsible for the synthesis of the type 3 polysaccharide in S. pneumoniae (cap3B) or hyaluronic acid in S. pyogenes (hasA). As shown for type 3 pneumococci where cap3C is not required for capsule formation, only HasA and HasB appear to be required for hyaluronic acid capsule production both in GAS and in heterologous bacteria as revealed by Tn916 mutagenesis (36, 37). Furthermore, a gene very similar to the pneumococcal galU gene described here has been found in the partial nucleotide sequence of the S. pyogenes genome that is currently available. It should be mentioned that, both in S. pneumoniae and S. pyogenes, the galU is immediately preceded by a gene (gpsA) that is presumably involved in the synthesis of membrane lipids (28). The significance of this finding is not currently understood but it is interesting to point out that gpsA and galU genes are completely unlinked in other bacteria such as B. subtilis or E. coli.
A relevant role of UDPG:PP for virulence has been recognized in various gram-negative bacteria (38–40). However, to the best of our knowledge, there are no data available in gram-positive organisms concerning the importance of this protein in pathogenicity. In this work, we have constructed galU mutants of two strains of S. pneumoniae of different serotypes, namely types 1 and 3. Both mutants were unencapsulated according to a series of criteria, i.e., colony morphology, growth in liquid medium, agglutinability with anti-R serum, and lack of recognition by type-specific antiserum. On the other hand, galU revertants of strains M 23 g and M 25 g synthesized type 1 and type 3 capsules, respectively. The unencapsulated phenotype of the galU mutants was expected in the case of the type 1 strain that apparently does not harbor any other gene encoding a UDPG:PP in addition to galU (20). However, in type 3 pneumococci the unencapsulated phenotype of the galU mutants was somehow surprising since these bacteria contain an active copy of cap3C that also codes for the same enzymatic activity (16). Since Cap3C UDPG:PP activity is not required for type 3 capsule formation and it cannot replace the activity lost in the galU mutants we conclude that, at least under laboratory conditions, either Cap3C is poorly translated or its enzymatic activity is very low. As the HasC protein of S. pyogenes is also not needed for hyaluronate biosynthesis (see above), it can be predicted that galU mutants of GAS will be also unencapsulated. This observation might be of remarkable clinical relevance since loss of capsule is associated with a 100-fold reduction in virulence of S. pyogenes (41).

The continuous dissemination of multiply resistant S. pneumoniae clones throughout the world is the cause of great concern, and much effort is currently dedicated to the search for new antibacterial drugs (42, 43). The polysaccharide capsule of pneumococcus is the main virulence factor of this bacterium (3) and drugs inhibiting its biosynthesis should potentially render S. pneumoniae virtually avirulent. Unfortunately, the noticeable genetic variability found in the genes responsible for the capsular polysaccharide biosynthesis has precluded until now the search for such drugs. Remarkably, the galU gene has been found in all the pneumococcal types tested so far (Fig. 3). The UDPG:PP, which is directly involved in the synthesis of the capsular polysaccharide in S. pneumoniae and other bacterial pathogens, might represent a suitable target for the search of inhibitors of such an important virulence factor. In this sense, it should be emphasized that eukaryotic UDPG:PPs appear to be completely unrelated to their prokaryotic counterparts (for review see reference 44). As depicted in Fig. 8, the structural arrangement of the domains found in prokaryotic UDPG:PP is remarkably similar. In contrast, the eukaryotic enzymes exhibit a completely different arrangement as well as a different amino acid sequence. This interesting feature suggests the possibility that putative inhibitors of the bacterial enzymes would not be harmful for the host.

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References

2. Frey, P.A. 1996. The Leloir pathway: a mechanistic impera-
4. van Dam, J.E., A. Fleer, and H. Snippe. 1990. Immunogenic-
5. Devereux, J., P. Haeberli, and O. Smithies. 1984. A compre-
10. van der Zeijst. 1997. Capsular polysaccharide synthesis in 
21. Arrecubieta, C., R. López, and E. García. 1996. Type 3–spe-
22. Devereux, J., P. Haeberli, and O. Smithies. 1984. A compre-
24. Speicher, D.W. 1994. Methods and strategies for the se-
26. R aivin, A.W. 1959. Reciprocal capsular transformation of 
27. Arrecubieta, C., R. López, and E. García. 1996. Type 3–spe-
32. Avery, O.T., C.M. MacLeod, and M. McCarty. 1944. Stud-
33. Avery, O.T., C.M. MacLeod, and M. McCarty. 1944. Stud-
39. Sandlin, R.C., K.A. Lampel, S.P. Keasler, M.B. Goldberg, 
41. Wessels, M.R., A.E. Moses, J.B. Goldberg, and T.J. Dic-
42. Arrecubieta, C., E. García, and R. López. 1995. Sequence 
44. Avery, O.T., C.M. MacLeod, and M. McCarty. 1944. Studies 
48. Avery, O.T., C.M. MacLeod, and M. McCarty. 1944. Studies 
52. Avery, O.T., C.M. MacLeod, and M. McCarty. 1944. Studies 
56. Avery, O.T., C.M. MacLeod, and M. McCarty. 1944. Studies 
60. Avery, O.T., C.M. MacLeod, and M. McCarty. 1944. Studies


