Characterization of the *galU* Gene of *Streptococcus pneumoniae* Encoding a Uridine Diphosphoglucose Pyrophosphorylase: A Gene Essential for Capsular Polysaccharide Biosynthesis

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

From the **Departamento de Microbiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 1113 Buenos Aires, Argentina; and the* ‡*Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain*

Summary

The *galU* gene of *Streptococcus pneumoniae* has been cloned and sequenced. *Escherichia coli* cells harboring the recombinant plasmid pMMG2 (*galU*) overproduced a protein that has been shown to correspond to a uridine 5′-triphosphate:glucose-1-phosphate uridylyltransferase (uridine 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 *S. pneumoniae galU* has been found in a partial nucleotide sequence of the *Streptococcus pyogenes* genome. Knockout *galU* mutants of type 1 pneumococci are unable to synthesize a detectable capsule. An identical result was found in type 3 *S. pneumoniae* cells in spite of the fact that these bacteria contain a type-specific gene (*cap3C*) that also encodes a UDP-Glc pyrophosphorylase. Since eukaryotic UDP-Glc pyrophosphorylases 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 *S. pyogenes.*

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

Streptococcus 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 *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 *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 10^5-fold reduction of the virulence of *S. pneumoniae.* Unencapsulated 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 *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)[1] and/or galactose (Gal) or various derivatives of them in addition to other sugars (12). Early studies carried out by Mills and co-workers (for review see reference 13) showed that uridine diphosphoglucose (UDP-Glc) is a key component in

[1]*Abbreviations used in this paper:* Gal, galactose; GAS, group A streptococci; Glc, glucose; Ln, lincomycin; UDP-GalA, uridine diphosphogalacturonic acid; UDP-Glc, uridine diphosphoglucose; UDP-GlcA, uridine diphosphoglucuronic acid; UDPG:PP, uridine diphosphoglucose pyrophosphorylase.

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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 *Escherichia 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 pUCEK2 or pMMG1 (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.

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 plasmids, 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, SacII, or ApaI was analyzed by pulsed-field gel electrophoresis (PFGE) using a contour-clamped homogeneous electric field DRII 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 deoxynucleoside triphosphate, and 2.5 mM of $MgCl_2$ 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′-TTGGTAccTGAAACAACTGGCATGC-3′ (primer OGalU1); (1139/c) 5′-CCCAACGTCGTAACGAGcTCCTG-3′ (primer OGalU2); (1464/c), 5′-GAGCAaTTGGTGGCGCATTTCTAGC-3′ (primer OGalU3); (323), 5′-TGAGTcGaCTTAACCCTCTATAGAAAG-3′ (primer OGalU4); (659/c), 5′-GAAATGAAGGCGCATGCCAGTTG-3′ (primer IGalU1); (773), 5′-CGAGAAGGCCGTTCCTTTGAC-3′ (primer IGalU2). 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 ClaI, 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 MunI and SalI, and ligated to EcoRI/SalI-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 ClaI, and ligated to pUCE191 previously digested with KpnI plus AccI.

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.

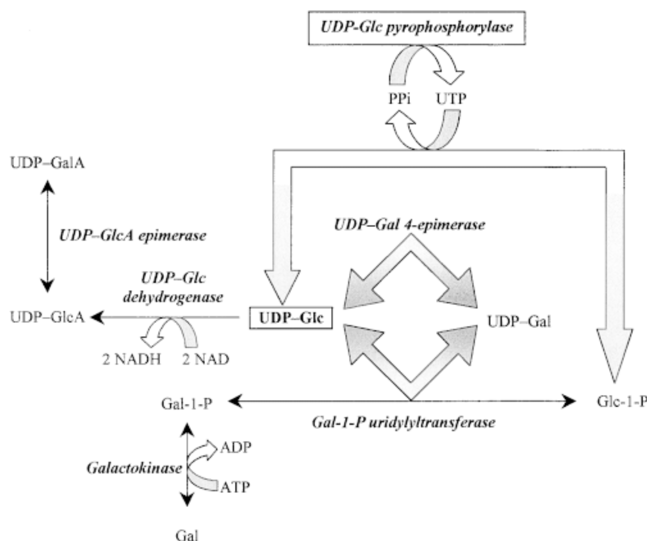


Figure 1. Metabolic pathway of UDP-Glc.

Table 1. *Bacterial Strains and Plasmids*

Strain or plasmid	Relevant genotype or phenotype	Source or reference
S. pneumoniae		
13868	$S1^+$ $LytA^+$	20
9060	$S1^+$ $LytA^+$	20
14388	$S1^+$ $LytA^+$	20
M25	$S1^+$ *lytA*	20
M25_g*	*galU*::pUCE191 *lytA*	This study
406	$S3^+$ $LytA^+$	45
M23	$S3^+$ *lytA*	45
M23_c‡	*cap3C*::pUCE191 *lytA*	16
M23_g§	*galU*::pUCE191 *lytA*	This study
M24	*cap3A* ($S3^-$) *lytA*	45
M24_g‖	*galU*::pUCE191 *lyt*A	This study
335/95	$S5^+$ $LytA^+$	A. Fenoll
6028/95	$S8^+$ $LytA^+$	A. Fenoll
536/96	$S9^+$ $LytA^+$	A. Fenoll
13783/90	$S14^+$ $LytA^+$	A. Fenoll
17A	$S17A^+$ $LytA^+$	Statens Seruminstitut
8249	$S19A^+$ $LytA^+$	46
436/96	$S31^+$ $LytA^+$	A. Fenoll
SSISP 33A/1	$S33A^+$ $LytA^+$	Statens Seruminstitut
1235/89	$S37^+$ $LytA^+$	A. Fenoll
E. coli strains		
DH5α	(φ80*lac*ZΔM15) *endA1 recA1 hsdR17* ($r_k^-m_k^-$) *supE44* Δ*lacU169 thi-1 leuB thr-1*	47
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lac*ZΔM15)	19
FF4001	MC4100 *galU95*	48
Plasmids		
pUC19	Cloning vector (Ap^R)	49
pUCE191	Cloning vector (Ap^R, Ln^R)	16
pUCEK2	Internal fragment of *cap3C* cloned into pUCE191	16
pMMG1	0.45-kb internal fragment of *S. pneumoniae galU* cloned into pUCE191	This study
pMMG2	*galU* gene from *S. pneumoniae* strain 406 cloned into pUC19	This study

*Strain constructed by transformation of M25 with DNA from strain M24_g.
‡Strain constructed by transformation of M23 with pUCEK2.
§Strain constructed by transformation of M23 with DNA from M24_g.
‖Strain constructed by transformation of M24 with pMMG1.

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 NH_2-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^SQ^, Millipore Corp.), and stained briefly with Amido black (Sigma Chemical Co., St. Louis, MO). Subsequently, the desired band was cut and the NH_2-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

previously described (26). Purification of capsular polysaccharides and double-diffusion experiments were performed as described previously (27). To estimate the amount of polysaccharide present in a sample, 5 μl of serial dilutions of the extract and known amounts of purified polysaccharide were analyzed by immunodiffusion against 5 μl of the same batch of antiserum.

Nucleotide Sequence Accession Number. The DNA sequence described here is deposited with the EMBL database under accession No. AJ004869.

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 *Bacillus subtilis* (28) (Table 2). The *galU* and the *gpsA* genes are only 21 bp apart. Other genes surrounding *gpsA-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 *gpsA* and *orf5* from type 4 pneumococci. However, successful amplification was achieved with OGalU3 and OGalU4, which correspond to the 5′ end of *orf6* and to the 3′ end of *gpsA*, respectively. The 1.1-kb PCR fragment was cloned into pUC19 to create pMMG2 (Table 1). Moreover, inverse PCR using oligonucleotides IGalU1 and IGalU2 (designed 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).

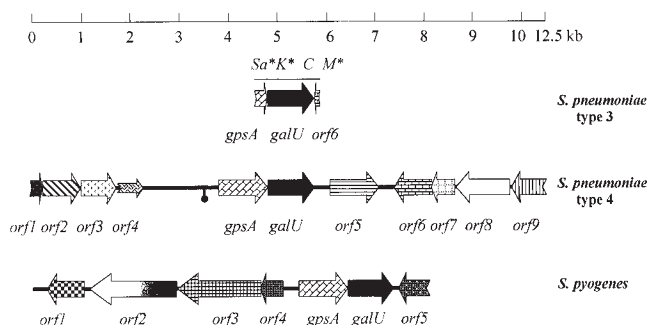


Figure 2. Genetic organization of two *S. pneumoniae* strain (types 3 and 4) DNAs containing the *galU* gene. The corresponding region of the *S. pyogenes* DNA is also shown. Thick and thin arrows represent complete or interrupted *orfs*, respectively. Identical shading represents DNA regions coding for the same putative protein. Relevant restriction sites are indicated (*C*, ClaI; *K*, KpnI; *M*, MunI; *Sa*, SalI). An asterisk indicates that the corresponding restriction site has been introduced using a synthetic oligonucleotide. *Filled key* represents a putative transcription terminator.

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 the *S. pyogenes* 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 GalU 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, ApaI, 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 5 (SmaI, 235 kb), 4 (ApaI, 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-

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

Protein	Predicted protein (aa/kD)	Proposed function	Most similar polypeptide	Database accession No.	Degree of identity/ similarity (%)	Organism
S. pneumoniae DNA (contig no. 4225, partial)						
Orf1*	78/8.2	Phosphate transporter permease	Mth1730	AE000929	40.5/68.9	*Methanobacterium thermoautotrophicum*
Orf2	250/28.1	Phosphate transport system ATP-binding protein	Mj1012	U67544	66.8/83.2	*Methanococcus jannaschii*
Orf3	216/24.2	Negative regulator	PhoU	D89963	30.7/53.2	*Enterobacter cloacae*
Orf4‡	159/18.5	Transposase	Transposase	X84038	25.0/49.3	*Xanthobacter autotrophycus*
GpsA	338/36.8	NAD(P)H-dependent dihydroxyacetone-phosphate reductase	GpsA	U32164	53.0/70.7	*B. subtilis*
GalU	299/33.2	UDP-Glc pyrophosphorylase	HasC	U33452	85.2/90.9	*S. pyogenes*
Orf5	313/34.7	Unknown				
Orf6	225/25.0	Unknown	Orf2	Z46863	30.4/59.9	*Acinetobacter calcoaceticus*
Orf7	179/20.6	5-Formyltetrahydrofolate cyclo-ligase	YqgN	Z99116	39.7/63.7	*B. subtilis*
Orf8	376/41.7	Amino acid hydrolase	YkuR	AJ222587	46.2/63.7	*B. subtilis*
Orf9*	232/23.9	Acetyltransferase	YkuQ	AJ222587	59.0/71.2	*B. subtilis*
S. pyogenes DNA (contig no. 234)						
Orf1	255/29.7	Unknown	HI0882	U32770	33.8/51.3	*H. influenzae*
Orf2	594/66.8	ABC transporter	YfiC	Z99108	36.5/60.0	*B. subtilis*
Orf3	568/63.0	ABC transporter	YfiB	Z99108	33.9/59.5	*B. subtilis*
Orf4	149/17.3	Transcriptional regulator	YybA	Z99124	20.8/48.6	*B. subtilis*
GspA	338/36.7	NAD(P)H-dependent dihydroxyacetone-phosphate reductase	GpsA	U32164	53.0/71.3	*B. subtilis*
GalU	299/33.3	UDP-Glc pyrophosphorylase	HasC	U33452	90.3/94.3	*S. pyogenes*
Orf5*	212/23.2	Unknown	YqgB	Z99116	24.1/44.9	*B. subtilis*

aa, amino acids.
*Partial ORF.
‡Interrupted reading frame.

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 NH_2-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 pGMM2 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.

Construction and Characterization of galU Mutants of Pneumococcus. To construct *galU* mutants of *S. pneumoniae* lacking UDPG:PP, insertion-duplication mutagenesis was carried out using plasmid pMMG1 that contains an internal fragment of the *galU* gene cloned into pUCE191 (Table 1).

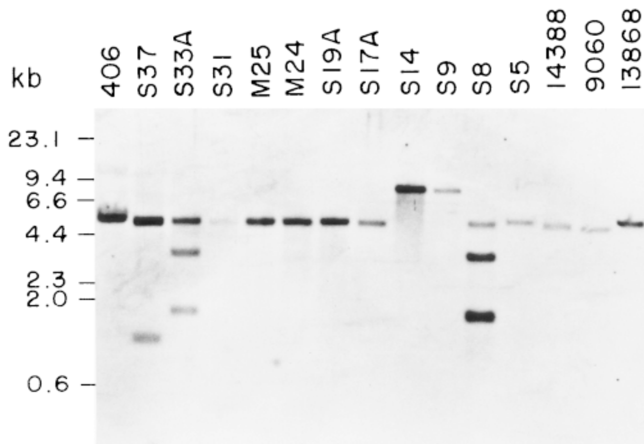
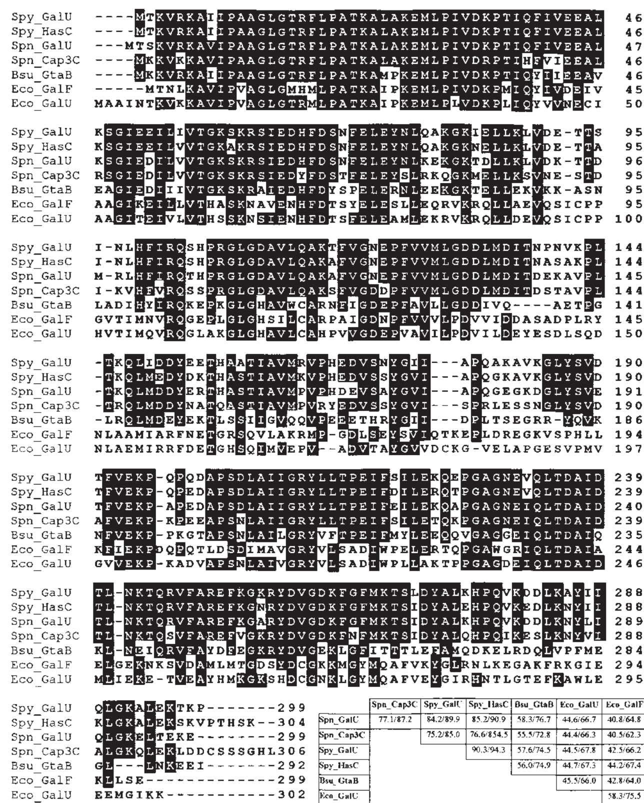


Figure 3. Identification of the *galU* gene of *S. pneumoniae* by DNA–DNA hybridization. Chromosomal DNA from 15 different pneumococcal strains of various types was digested with HindIII, electrophoresed, blotted, and hybridized with a 0.7-kb SalI-ClaI fragment of pMMG2 (see Table 1). As size standards, we used a HindIII digest of λ DNA.



	Spn_Cap3C	Spy_GalU	Spy_HasC	Bsu_GtaB	Eco_GalU	Eco_GalF
Spn_GalU	77.1/87.2	84.2/89.9	85.2/90.9	58.3/76.7	44.6/66.7	40.8/64.8
Spn_Cap3C		75.2/85.0	76.6/854.5	55.5/72.8	44.4/66.3	40.5/62.3
Spy_GalU			90.3/94.3	57.6/74.5	44.5/67.8	42.5/66.2
Spy_HasC				56.0/74.9	44.7/67.3	44.2/67.4
Bsu_GtaB					45.5/66.0	42.8/64.0
Eco_GalU						58.3/75.5

Figure 4. Computer-generated alignment (PILEUP) of the GalU protein of *S. pneumoniae* (*Spn_GalU*) with other similar proteins included in the databases. The following UDPG:PP were aligned: *S. pneumoniae* Cap3C (*Spn_Cap3C*) (reference 16), *S. pyogenes* HasC (*Spy_HasC*) (reference 29), *B. subtilis* GtaB (*Bsu_GtaB*) (reference 30), and *E. coli* GalU (*Eco_GalU*) (reference 31). Other proteins used were GalF of *E. coli* (*Eco_GalF*) (reference 32) and the putative UDPG:PP of *S. pyogenes*, the GalU protein described in this paper (*Spy_GalU*). The inset shows the percentages of identical/similar amino acid residues resulting from pairwise comparisons (BESTFIT).

A Ln-resistant transformant (designated as M24_g) was isolated upon transformation of the pneumococcal strain M24 with pMMG1 isolated from *E. coli* TG1. Carbohydrate-fermentation tests showed that M24_g fermented Glc but was unable to use Gal as a carbon source (not shown).

Chromosomal DNA prepared from strain M24_g was used to transform the encapsulated strains M23 (S3$^+$) and M25 (S1$^+$) and Ln-resistant clones were picked for further study. As found for M24_g, the transformant strains M23_g and M25_g were also unable to ferment Gal whereas M23_c did ferment either Glc or Gal. Furthermore, when the M23_g 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 M23 and M23_c strains. Since type 1 pneumococcal strains form small colonies, no significant differences were found on the morphology of the colonies of M25 and M25_g (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 (M24_c), 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 M23_g and M25_g did not synthesize any detectable capsular polysaccharide (Fig. 7). In contrast, M23_c 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 M23_g and M25_g 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 were no longer Ln-resistant and that the size (1.1 kb) of the PCR fragments obtained by using oligonucleotide primers OGalU3 and OGalU4

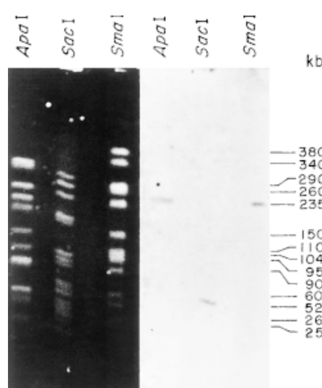


Figure 5. PFGE of the DNA prepared from strain M24 of *S. pneumoniae* digested with ApaI, SacI, or SmaI and blotted and hybridized with the 0.7-kb SalI-ClaI fragment of pMMG2. The sizes (in kb) of the SmaI fragments (reference 34) are indicated at the right.

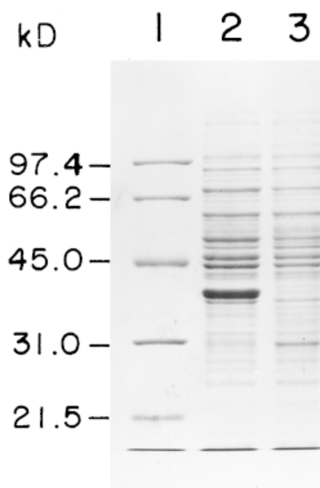


Figure 6. Expression of the GalU protein of *S. pneumoniae* by *E. coli* containing the recombinant plasmid pMMG2. *E. coli* DH5α cells containing either pMMG2 or pUC19 vector alone were incubated overnight on LB medium, and the crude sonicated extracts were analyzed by 10% SDS-PAGE. Lane *2*, extracts from bacteria containing pMMG2. Lane *3*, extracts from bacteria harboring pUC19. The molecular masses of the standards (lane *1*) are indicated on the left.

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

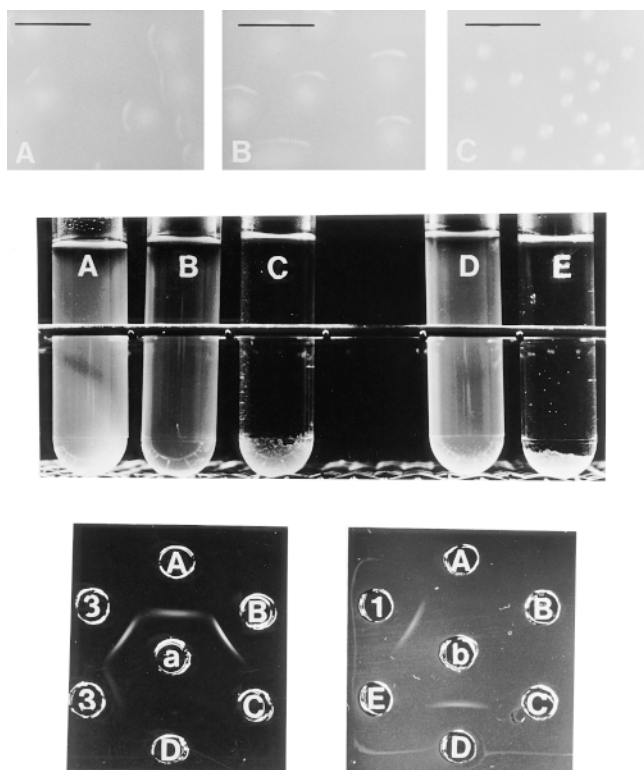


Figure 7. Unencapsulated phenotype of the pneumococcal *galU* mutants. (*Top*) Colony morphology on blood-agar plates. Bar, 1 mm. (*Middle*) Growth characteristics after overnight incubation at 37°C in C + Y medium. (*Bottom*) Double immunodifussion in agarose. The center wells received type 3 (*a*) or type 1 (*b*) antisera. The pneumococcal strains analyzed were: M23 (*A*); M23_c (Cap3C) (*B*); M23_g (GalU) (*C*); M25 (*D*); M25_g (GalU) (*E*). Purified pneumococcal polysaccharides from type 1 (*1*) and type 3 (*3*) were used as positive controls.

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 Tn*916* 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.*

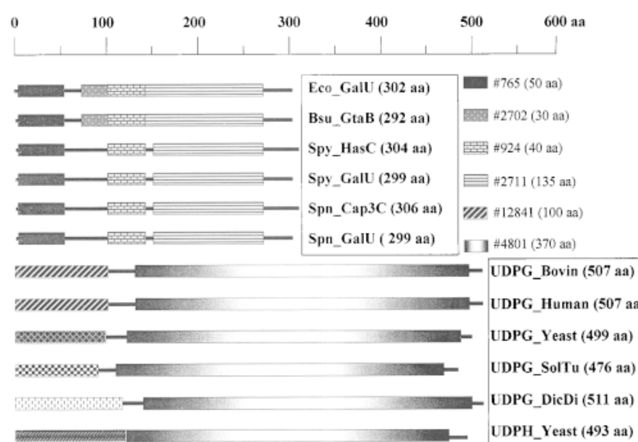


Figure 8. Domain arrangement of several UDPG:PP from prokaryotic and eukaryotic organisms. Bars represent domains and identical shading indicates high amino acid sequence similarity. The proteins were aligned according to programs available at the ProDom database (reference 50). The number assigned to each domain (*#*) as well as its length in amino acid residues (*aa*) are indicated at the top right. The UDPG:PP aligned correspond to the following species: *E. coli* (*Eco_GalU*); *B. subtilis* (*Bsu_GtaB*); *S. pyogenes* (*Spy_HasC* and *Spy_GalU*); *S. pneumoniae* (*Spn_Cap3C* and *Spn_GalU*); *Bos taurus* (*UDPG_Bovin*); *Homo sapiens* (*UDPG_Human*); *S. cerevisiae* (*UDPG_Yeast* and *UDPH_Yeast*); *Solanum tuberosum* (*UDPG_SolTu*); and *Dictyostelium discoideum* (*UDPG_DicDi*).

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 M23_g and M25_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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Address correspondence to Rubens López, Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, Velázquez 144, 28006 Madrid, Spain. Phone: 34-91-561-1800; Fax: 34-91-562-7518; E-mail: cibl220@fresno.csic.es

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