Immobilization-based isolation of capsule-negative mutants of *Streptococcus pneumoniae*

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The capsular polysaccharide (CPS) is the most important identified virulence factor of *Streptococcus pneumoniae*, a human pathogen of the upper respiratory tract. One limitation in studies of *S. pneumoniae* surface virulence factors is the lack of a reliable procedure for isolation of capsule-negative mutants of clinical strains. This paper presents an approach, based on the immobilization of pneumococci in semi-liquid (0.04 % agar) medium, to easily distinguish and select for non-capsulated mutants. A clinical *S. pneumoniae* type 37 strain was used as a model to show that CPS production results in bacterial immobilization in semi-liquid agar medium and restricts cell sedimentation. Descendants of CPS− mutants sedimented faster under these conditions and therefore could be separated from immobilized parental cells. The CPS− phenotype of the obtained mutants was confirmed by both immunoagglutination and immunostaining experiments using specific type 37 capsular antibodies. Complementation of immobilization with the cloned *tts* gene, encoding type 37 CPS synthase, confirmed that faster sedimentation of mutants was specifically due to loss of the capsule. DNA sequence determination of three independent mutants revealed a point mutation, a 46 nt deletion and a heptanucleotide duplication in the *tts* gene. Immobilization of strains producing other CPSs (type 2, 3 and 6) also resulted in the appearance of CPS− mutants, thus showing that immobilization-based isolation is not restricted to type 37 pneumococci. Bacterial growth in semi-liquid medium proved to be a useful model system to identify the genetic consequences of immobilization. The results indicate that immobilization due to CPS may impose selective pressure against capsule production and thus contribute to capsule plasticity.

**INTRODUCTION**

*Streptococcus pneumoniae* (pneumococcus) is the major cause of community-acquired pneumonia. It also causes meningitis, otitis and septicaemia, with a high incidence of morbidity and mortality throughout the world. The capsular polysaccharide (CPS), which protects pneumococci against phagocytosis during infection, has been identified as the principal virulence factor (Griffith, 1928). Nevertheless, CPS production reduces pneumococcal adhesive capacity, restricts cell passage across the blood–brain barrier (Adamou et al., 1998; Ring et al., 1998) and decreases transformation efficiency (Yother et al., 1986). These effects are probably due to the physical barrier created by CPS between bacterial cells and host receptors or environmental signals.

*S. pneumoniae* polysaccharides have been classified into at least 90 different capsular types according to their structure and immunological properties (Henrichsen, 1995). It is believed that this diversity arose through horizontal transfer of capsular genes from unknown sources (Muñoz et al., 1997, 1998) or by ‘capsular switching’, mediated by recombinational replacement within capsular biosynthesis operons belonging to different types and their flanking regions (Caimano et al., 1998; Coffey et al., 1998, 1999).

In most cases studied so far, capsule formation is encoded by the *cps* (cap) gene cluster. It comprises several genes, and is located in the pneumococcal chromosome between the *dexB* and *allA* genes, which are not involved in CPS synthesis (Garcia et al., 2000). Sequencing of *cps/cap* clusters of the 90 known pneumococcal types is in progress, and will provide new data confirming the involvement of these genes in each CPS biosynthetic process (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS). *S. pneumoniae* type 37 capsule formation constitutes a remarkable exception as the *cap* locus in these isolates is inactive and synthesis of CPS is assured by a single gene, *tts*, located apart from the *cps* locus on the genome (Llull et al., 1999). Type 37
is the only homopolysaccharide described so far among pneumococcal CPSs, and is composed of sophorosyl units (\(\beta\-D\-Glc-(1 \- 2)\-\beta\-D\-Glc\)) interlinked through \(\beta\-(1 \- 3)\) bonds (Adeyeye et al., 1988). It was shown that a mutation in \(tts\) is sufficient to cause a CPS\(^+\) phenotype (Lull et al., 2000). The capacity of a single gene to direct capsule production presents a convenient opportunity to map CPS\(^+\) mutations directly by DNA sequencing.

The experimental use of unencapsulated pneumococcal mutants may be valuable because of their reduced virulence, the exposure of other surface components and their increased transformability. Isolation of such mutants from clinical isolates is limited by the tedious genetic manipulations involved and/or the need to introduce genetic resistance markers by transformation (Pearce et al., 2002; Sung et al., 2001; Trzciński et al., 2003). Furthermore, these treatments could change microbial physiology or have undesirable polar effects. We have used type 37 pneumococci to develop a novel approach for isolation of spontaneous capsule-negative mutants, which emerged from encapsulated parental strains that were immobilized in semi-liquid medium.

**METHODS**

**Growth conditions and bacterial strains.** *S. pneumoniae* was routinely grown in Todd–Hewitt broth supplemented with 0.5% yeast extract (THY medium; Difco, Becton, Dickinson). Semi-liquid medium was prepared by adding 0.04% agar (Difco, Becton, Dickinson) in liquid THY medium as described previously (Mercier et al., 2002).

Pneumococcal strains used in this study were kindly provided by E. García (CSIC, Madrid, Spain) and are listed in Table 1. For construction of PN9, the non-encapsulated laboratory strain M24 (García et al., 1993) was transformed as described previously (Barany et al., 1988) with chromosomal DNA of the type 3 strain 406/90, and a mucous transformant was directly selected on blood agar plates (1% THY agar containing 5% defibrinated horse blood) according to its colony morphology. Type 3 CPS production was verified by immunoglutationin (see below) of transformant PN9 using specific type 3 antcapsular serum (Statens Seruminstitut, Denmark).

Table 1. *S. pneumoniae* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSISP37/2</td>
<td>37</td>
<td>Clinical isolate, Statens Seruminstitut collection (Denmark)</td>
</tr>
<tr>
<td>SSISP6A1/1</td>
<td>6A</td>
<td>Clinical isolate, Statens Seruminstitut collection (Denmark)</td>
</tr>
<tr>
<td>D39</td>
<td>2</td>
<td>Clinical isolate, obtained from E. García (Avery et al., 1944)</td>
</tr>
<tr>
<td>406/90</td>
<td>3</td>
<td>Clinical isolate, obtained from E. García (García et al., 1993)</td>
</tr>
<tr>
<td>NR37-1</td>
<td>CPS(^+)</td>
<td>Derivative of type 37 strain 1235/89, obtained from E. García (Lull et al., 2000)</td>
</tr>
<tr>
<td>M24</td>
<td>CPS(^+)</td>
<td>Late descendant of D39, obtained from E. García (García et al., 1993)</td>
</tr>
<tr>
<td>DN2</td>
<td>37</td>
<td>Derivative of M24, obtained from E. García (Lull et al., 1999)</td>
</tr>
<tr>
<td>PN9</td>
<td>3</td>
<td>Type 3 derivative of M24, this work</td>
</tr>
<tr>
<td>VES2585</td>
<td>CPS(^+)</td>
<td>G to A transition in (tts) gene, this work</td>
</tr>
<tr>
<td>VES2586</td>
<td>CPS(^+)</td>
<td>46 nt deletion in (tts) gene, this work</td>
</tr>
<tr>
<td>VES2587</td>
<td>CPS(^+)</td>
<td>7 nt duplication in (tts) gene, this work</td>
</tr>
<tr>
<td>VES2591</td>
<td>37</td>
<td>VES2585/pDLP49 ((tts^+)), this work</td>
</tr>
<tr>
<td>VES2592</td>
<td>37</td>
<td>VES2586/pDLP49 ((tts^-)), this work</td>
</tr>
<tr>
<td>VES2593</td>
<td>37</td>
<td>VES2587/pDLP49 ((tts^-)), this work</td>
</tr>
</tbody>
</table>

Complementation of mutants VES2585–VES2587 by pDLP49, carrying an intact copy of the \(tts\) gene under the control of its own promoter (Lull et al., 2001), was performed by a similar transformation method, but adding competence-stimulating peptide 2 (CSP-2) (Pozzi et al., 1996) to the transformation mix at a final concentration of 100 ng ml\(^{-1}\). CSP-2 was used instead of CSP-1 after empirical confirmation of its ability to induce competence in type 37 derivatives. Transformants (VES2591–VES2593, respectively) were selected as lincomycin-resistant (1 \(\mu\)g ml\(^{-1}\) clones on blood agar plates.

**Production of pneumococcal CPS-specific antisera.** Pneumococcal capsule-specific antisera were obtained from the Statens Seruminstitut (Denmark). We previously observed that type 37 CPS derivatives, like NR37-1, showed residual activity against commercial type 37 serum in certain culture conditions (unpublished observations). These derivatives are considered non-producers of type 37 CPS; however, they do not react with a serum obtained with the laboratory rough variants (Ravin, 1959). This was unexpected. Rough strains normally react with this type of antibody, as the absence of CPS allows access to cell-surface antigens. Absence of reaction with such antibodies suggests that type 37 CPS\(^+\) strains may express an unknown surface substance that blocks access to antibodies. This substance may also be present on the surface of type 37 capsulated isolates. We considered that commercial type 37 serum, prepared using clinical type 37 isolates, may also contain antibodies against this unknown substance, which would react with mutants lacking type 37 CPS.

To obtain antibodies specific to type 37 capsulated pneumococci, we immunized rabbits with the type 37 laboratory transformant DN2 (Lull et al., 1999), following the procedure described by Lund & Henrichsen (1978). Non-capsular antibodies were eliminated by suspending cells of parental non-encapsulated strain M24 in the type 37 serum and incubating overnight at 4°C with gentle shaking. Serum obtained using DN2 was highly specific for type 37 CPS as it only reacted with type 37 clinical strains (SSISP37/2) and laboratory type 37 transformants (DN2) but not with non-encapsulated derivatives NR37-1 or M24.
Immunostaining. An overnight culture was centrifuged and washed twice with PBS (130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7-2). Cells were fixed with paraformaldehyde (Amann et al., 1990), washed twice and resuspended in PBS. Cells were stored in PBS/ethanol (1:1, v/v) at −20°C for up to 1 week. Fixed PBS-suspended cells were incubated with 1000-fold diluted rabbit anti-CPS antibodies for 1 h, washed twice in PBS and incubated for 1 h with 1000-fold diluted goat anti-rabbit Alexa Fluor 555 coupled IgG antibodies (Molecular Probes). After antibody treatment, cells were washed twice in PBS and spread on gelatin-coated glass slides, air-dried and rinsed with water. For gelatin coating, clean slides were immersed in a solution of 10% KOH in 95% ethanol for 1 h, air-dried, dipped in a hot (70°C) 0.075% gelatin (Merck) solution with 0.01% chromium potassium sulphate dodecahydrate (Merck) and air-dried. Slides with the cells were covered with mounting solution (Citifluor) containing 2-5 µg/ml 4’,6-diamidino-2-phenylindole (DAPI; Sigma). Images were taken with an epifluorescence microscope (×60 objective; Nikon) equipped with an image analysis system (Visiolab 1000; Biocom).

Immunooagglutination. Half volume of specific capsular serum was added to pneumococcal cells previously washed once and suspended in PBS. The mixture was incubated at 4°C for 2–3 h and the agglutination reaction was directly observed using a phase contrast microscope.

DNA techniques, nucleotide sequencing and data analysis. Isolation of chromosomal and plasmid DNA from pneumococcal strains was performed as described previously (Muñoz et al., 1997). The pneumococcal tts gene was PCR-amplified using 5’-TGAA-TGAATCAGCTAGGCTACC-3’ (forward) and 5’-TTGAAGCTGG-TTGCTTGTAGG-3’ (reverse) primers. Reactions for DNA sequence determination were performed for both strands according to the protocol of the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences Europe), using a MegaBACE 1000 automated capillary sequencer (Molecular Dynamics). The previously reported sequence of tts was used as a reference to map mutations (EMBL/GenBank/DDBJ accession number AJ131985).

RESULTS

Growth of pneumococci in semi-liquid medium and isolation of fast-sedimenting mutants

Pneumococcal strain SSISP37/2 (Table 1), producing type 37 CPS, was chosen as a model to select CPS− derivatives. Its capsule is encoded by a single tts gene; therefore, mutations resulting in capsule-defective mutants are readily identified by DNA sequencing. We used type 37 non-encapsulated derivative NR37-1 (Lull et al., 2000) as a CPS− control strain. A stationary-phase culture of strain SSISP37/2 was diluted and 10–50 cells were inoculated into flasks containing THY semi-liquid medium (0.04% agar). Under these conditions, type 37 CPS-producing SSISP37/2 grew as round ‘puffy’ colonies (Fig. 1a). We consider that the CPS of SSISP37/2 causes cells to become entrapped in the agar matrix and therefore immobilized in semi-liquid medium. In contrast, the CPS− NR37-1 strain sedimented and formed streaked colonies (Fig. 1c). When strain SSISP37/2 was grown for 48 h, we observed the appearance of ‘roots’, presumably corresponding to faster-sedimenting mutants (Fig. 1b). Bacteria isolated from these roots (strains VES2585–VES2587, Table 1) sedimented in semi-liquid medium like the CPS− strain NR37-1 (Fig. 1d).

To estimate the mutation frequency, we recovered several round colonies in semi-liquid medium for cell count determination and found that one colony comprised about 104 cells. The majority of such colonies produced faster-sedimenting derivatives. A minimum estimate for mutation frequency is therefore around 10−7. However, as autolysis may occur inside the colony, such values should be taken as approximate.

Transformation of fast-sedimenting mutants VES2585–VES2587 with pDLP49, encoding tts (Lull et al., 2001), gave rise to strains VES2591–VES2593, respectively (Table 1). The presence of pDLP49 restored the wild-type sedimentation pattern (round colonies, Fig. 1e), indicating that a mutation in the capsular tts locus may alone be responsible for the faster-sedimenting phenotype. Moreover, we observed that colonies of plasmid-carrying strains (Fig. 1e) were slightly more round than those of the initial clinical isolate SSISP37/2 (Fig. 1a). We attribute this phenotype to increased tts gene expression in strains carrying the multicopy plasmid.

Immunostaining and immunooagglutination with type 37 CPS-specific antibodies

We used specific DN2 type 37 serum (see Methods) to compare its reactivity with wild-type (SSISP37/2) and faster-sedimenting derivatives VES2585–VES2587. In the immunostaining test, the parental SSISP37/2 strain showed a CPS+ red-staining phenotype (Fig. 2a), as expected for a type 37 clinical strain. In contrast, the control CPS− strain NR37-1 and the VES2585–VES2587 mutants did not give any signal under the same conditions, thus confirming the CPS− phenotype of the isolated mutants (Fig. 2a). The results of the immunooagglutination assays were consistent and confirmed that the fast-sedimenting mutants displayed a CPS− phenotype (Fig. 2b).

Mapping of mutations

DNA sequence determination of the tts gene of the obtained isolates revealed a G to A transition at position 2901 in the sequence of VES2585, leading to a change of Gly to Asp in the Tts type 37 polysaccharide synthase. Similarly, we found a 46 nt deletion (position 3122–3167, strain VES2586) and a duplication of 7 nt (TTAAATT at position 3266) which resulted in a 2 aa insertion as well as a reading frame shift sequence of VES2585, leading to a change of Gly to Asp in the Tts type 37 polysaccharide synthase. Similarly, we found a 46 nt deletion (position 3122–3167, strain VES2586) and a duplication of 7 nt (TTAAATT at position 3266) which resulted in a 2 aa insertion as well as a reading frame shift.

Isolation of mutants from other capsular types

To extend the validity of the method to other capsular types of S. pneumoniae, we isolated CPS− mutants from strains...
Fig. 1. Growth of *S. pneumoniae* strains and their CPS⁻ derivatives in semi-liquid medium. In all cases 0·04% agar was added in THY broth except for the type 6A strain (0·08%). Growth time is indicated on the pictures. First row: type 37 clinical isolate SSISP37/2 strain grown for 18 (a) and 72 (b) h and NR37-1 (c), a spontaneous non-encapsulated mutant of a type 37 strain. Second row: VES2585 (d), a spontaneous faster-sedimenting derivative of SSISP37/2 (two other derivatives, VES2586 and VES2587 showed the same growth pattern; not shown); strain VES2591 (e), a VES2585 derivative transformed with pDLP49 encoding type 37 CPS production (transformants of VES2586 and VES2587 with pDLP49 showed the same growth pattern; not shown); and PN9 (f), the type 3 laboratory transformant. Third row: clinical isolates belonging to capsular types 6A (SSISP6A/1; g), 3 (406/90; h) and 2 (D39; i). Note that CPS⁻ mutants appear as tails hanging down from round colonies.

Fig. 2. (a) Immunostaining of S37 (SSISP37/2) strain and its CPS⁻ derivatives NR37-1 and VES2585 using DN2 type 37-specific serum. Immunostaining of strains VES2586 and VES2587 was the same as that observed for VES2585. (b) Immunoagglutination reactions of pneumococcal strains using anti-DN2 type 37-specific serum. Immunoagglutination was observed after 2 h incubation of cells mixed with the specific serum.
We have also shown that spontaneous CPS\textsuperscript{−} mutants appearing in an immobilized pneumococcal culture may be easily isolated in semi-liquid medium, since they sediment faster and may thus escape from the colony of parental cells (Fig. 1b, f–i).

Mutations were identified in the \textit{tts} gene encoding the type 37 polysaccharide synthase. We noted that strains carrying \textit{tts} on multicopy plasmid pDLP49 (VES2591–VES2593) formed somewhat rounder colonies (Fig. 1e) than the wild-type S37 strain, in which \textit{tts} was present as a single copy on the chromosome (Fig. 1a). More capsule production in the plasmid-carrying strain is likely to account for this immobilization effect.

The versatility of this method was confirmed using other isolates belonging to types 2, 3 and 6A (Fig. 1f–i, Table 1). Our results demonstrate that the use of semi-liquid medium for detection and isolation of CPS\textsuperscript{−} derivatives is not restricted to type 37 pneumococci. Nevertheless, a slight variation of the method (i.e., increasing the agar concentration) was necessary to select mutants from the type 6A strain.

The ability to select CPS\textsuperscript{−} mutants from strains producing the same type 3 capsule in different genetic backgrounds (e.g., PN9 and 406/90), or from strains producing different capsule types within the same genetic background (D39, type 2 and PN9, type 3), suggests that the capsule plays a major role in immobilization of pneumococci in semi-liquid medium.

Screening approaches have been used in the past to isolate CPS\textsuperscript{−} mutants. For example, type 3 or 37 pneumococcal mutants are easily distinguishable on blood agar plates by smooth or rough colony morphology. However, obtaining mutants by such an approach is not always successful. It requires repeated subculture and observation of a large number of colonies and therefore is not a straightforward screening method. Moreover, obtaining CPS\textsuperscript{−} mutants for other less mucous serotypes is hardly possible by simple subculture. Isolation of CPS\textsuperscript{−} mutants may be facilitated by addition of anti-pneumococcal antibodies and exploiting the changes in bacterial sedimentation in liquid medium due to immunoagglutination (Avery et al., 1944). CPS\textsuperscript{−} strain variants can also be identified through their increased surface hydrophobicity (Granlund-Estedt et al., 1993). Finally, efficient enrichment procedures based on buoyant density-gradient centrifugation (Sellin et al., 1995) or use of Sorbarod biofilms (Waite et al., 2001, 2003) have greatly

**DISCUSSION**

We have developed a method for the identification and isolation of pneumococcal CPS\textsuperscript{−} mutants based on immobilization of bacteria in an extracellular matrix of 0·04% agar. We previously showed that, in a similar semi-liquid medium, non-encapsulated bacteria such as lactococci grow as streaked colonies (Mercier et al., 2002). Here, we have shown that, in similar conditions, encapsulated pneumococcal strains behave differently: they do not sediment, and form round colonies. We attribute the diminished sedimentation of CPS-producing pneumococci to a physical entrapment of bacteria by long CPS strands within the agar matrix. Confirmation of this reasoning comes from the observation that CPS\textsuperscript{−} derivatives of a type 37 \textit{S. pneumoniae} clinical isolate sedimented faster in semi-liquid medium and formed streaked colonies (Fig. 1c, d).

Laboratory type 3 transformant PN9 originates from the M24 strain, a late descendant of the type 2 strain D39. It was constructed to produce a type 3 capsule by transformation with chromosomal DNA from the type 3 strain 406/90 (see Methods). Isolation of faster-sedimenting, non-capsulated mutants from both PN9 and 406/90 shows that immobilization of bacteria in an extracellular matrix of semi-liquid medium depends mostly on capsule formation, as we can assume that they both produce the same CPS, despite their different genetic backgrounds.
facilitated isolation of spontaneous capsule-negative mutants. However, while these methods allow separation of a mixture of different phenotypic CPS– forms, they may not all correspond to mutants. In the system described here, round CPS+ colonies give rise to CPS– roots. Roots arising from separate colonies would necessarily originate from independent mutations. Therefore the method presented here greatly facilitates isolation of independent CPS– mutants.

The use of a dilute agar medium for differentiation of phenotypic characteristics of bacteria is not new. It was previously employed in studies of staphylococci (Finkelstein & Sulkin, 1957) and streptococci (Narikawa et al., 1995). However, in those publications, the appearance of faster-sedimenting forms (‘tails’) was not attributed to the emergence of mutants. To our knowledge the method presented here is the first application of immobilization in agar for isolation of capsule-negative mutants.

The capsule is a key virulence factor in S. pneumoniae, since non-capsulated derivatives have been shown to be practically avirulent (Griffith, 1928). On the other hand, the capsule decreases adherence to host cells (Adamou et al., 1998) and trafficking across the blood–brain barrier seems to be easier for non-capsulated pneumococcal derivatives (Ring et al., 1998). Capsule phase variation seems to be a necessary mechanism for pneumococcal survival and mobility in the host. An interesting mechanism of capsule phase variation has been described for types 3, 8 and 37, where spontaneous sequence duplications within their capsular loci switched capsule production off, whereas reversion restored capsule production (Waite et al., 2001, 2003). Remarkably, duplications causing capsule loss were isolated from immobilized bacteria on Sorbarod biofilms. We noted that a similar heptanucleotide duplication in strain VES2587 causing loss of capsule production was also isolated under immobilization conditions (Fig. 3). Moreover, the duplication occurred at the site that already carries duplication of the same, albeit degenerated, heptanucleotide (TTACATT TTAAATT), which could suggest the existence of a duplication-based capsule switch mechanism in this pneumococcal isolate.

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