Spontaneous sequence duplications within capsule genes cap8E and tts control phase variation in Streptococcus pneumoniae serotypes 8 and 37

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Capsule phase variants were isolated from serotype 8 and serotype 37 pneumococcal sorbarods. Sequence duplications within the essential capsule genes – cap8E (type 8) and tts (type 37) – were found to introduce frameshifts and generate acapsular phenotypes. Capsular revertants possessed wild-type cap8E and tts genes, indicating the precise excision of these duplications. Reversion frequencies (OFF–ON) fit a linear relationship between log(frequency of reversion) and log(length of duplication), previously found for serotype three pneumococci [Waite, R. D., Struthers, J. K. & Dowson, C. G. (2001). Mol Microbiol 42, 1223–1232]. This study provides evidence that capsule phase variation can occur in pneumococcal serotypes with either simple (one to three genes) or complex capsule-encoding loci (12 genes). Given the key role of CapE (the first monosaccharide transferase) in other clinically important pneumococci, such as serotypes 14 and 19F with complex capsular loci, the observed duplication within cap8E suggests that capsule phase variation could be controlled by tandem sequence duplication in cap8E homologues in other pneumococcal serotypes that construct their capsules through polymerization of lipid-linked intermediates.

INTRODUCTION

The worldwide increase in antibiotic resistance (Butler et al., 1998) and suboptimal efficacy of existing capsular vaccines (Musher, 1992) has resulted in Streptococcus pneumoniae (the pneumococcus) remaining a major cause of human mortality and morbidity. The pneumococcus is the cause of serious diseases such as pneumonia, bacteraemia, sep席is and meningitis, and less-severe infections such as otitis media and sinusitis (Paton et al., 1993). The groups most at risk from pneumococcal disease are young children, the elderly and the immunocompromised, such as AIDS patients (World Health Organization, 1999). Asymptomatic carriage, with bacteria adhering to the throat or nasopharynx, maintains a large reservoir of pneumococci for human infection (Appelbaum et al., 1996; Austrian, 1986; Sung et al., 1995).

There are currently 90 recorded antigenically distinct capsular serotypes of pneumococci (Henrichsen, 1995). These are composed of repeating polysaccharide units (van Dam et al., 1990) and help confer resistance to complement-mediated opsonophagocytosis (Moxon & Kroll, 1990); hence, they play a key role in survival during systemic infection in vivo and in animal models (Watson & Musher, 1990). Recently, it has been reported that the presence of pneumococcal capsule is also important for murine carriage (Magee & Yother, 2001). Although the majority of pneumococci from clinical isolates are capsulate, a previous population genetic study of pneumococcal carriage identified two pneumococcal isolates from pernasal swabs of children and one from a pernasal swab of a female human immunodeficiency virus-positive patient that were non-serotypable (Muller-Graf et al., 1999). In addition, acapsulate pneumococci have been associated with outbreaks of conjunctivitis (Ertugrul et al., 1997).

There are reports that acapsular pneumococci are more efficient at adhering to bronchial epithelial cells than their capsular parents (Adamou et al., 1998), and that the presence of a polysaccharide capsule significantly reduces the ability of pneumococci to adhere to, and penetrate, a human lung alveolar carcinoma (type II pneumocyte) cell line (Talbot et al., 1996) and brain microvascular endothelial cells (Ring et al., 1998). It is calculated that the polysaccharide capsule inhibits adherence and invasion of eukaryotic cells by up to 200-fold in vitro (Ring & Tuomanen, 2000; Ring et al., 1998).
The ability to ‘flip’ between capsular and acapsular phenotypes, and hence exploit the benefits of each phenotype, as found for Neisseria meningitidis (Hammerschmidt et al., 1996a, b), might aid pneumococci in the progression of invasive disease beyond carriage. Translucent opacity phase variants, which have reduced levels of capsule, and arise by a mechanism different from that described here (Saluja & Weiser, 1995), are already known to adhere more effectively to epithelial cells than their more capsular opaque phase variants.

The genetics of pneumococcal capsule biosynthesis has only recently started to become unravelled, and sequence data are available for a subset of the 90 serotypes (Dillard et al., 1995; Iannelli et al., 1999; Jiang et al., 2001; Kolkman et al., 1997a, b; Llull et al., 1998, 1999; Morona et al., 1997a, b, 1999; Munoz et al., 1997, 1999; Ramirez & Tomasz, 1998). Apart from two notable exceptions (serotypes 3 and 37) the majority of capsule loci are very similar in structure: a central region of serotype-specific genes (of varying number) is preceded by four genes thought to be involved in capsule regulation and export (Guidolin et al., 1994; Jiang et al., 2001; Morona et al., 2000) (Fig. 1). Except for the single gene responsible for biosynthesis of the serotype 37 capsule (tts), the genes responsible for capsule synthesis in all serotypes sequenced so far are located between the dexB and aliA genes (for examples, see Fig. 1). The capsule of serotype 8 pneumococci shares characteristics typical of the majority of serotypes; the capsule locus contains 12 genes (cap8ABCDEFGHIJKL) (Fig. 1), and polymerization of lipid-linked intermediates is thought to be involved in capsule construction (Munoz et al., 1999). The serotype 3 and 37 capsules, however, are relatively simple in structure (repeating units of cellobiuronic acid and sophorosyl, respectively) and are constructed through processive transferase activity (Arrecubieta et al., 1996; Llull et al., 2001).

This study describes capsule phase variants of pneumococcal serotypes 8 and 37 generated in sorbarods, and shows that the molecular mechanisms of phase variation are analogous to those previously found in serotype 3 pneumococci – spontaneous sequence duplication switching the capsule off

![Fig. 1. Organization of capsular loci of serotypes 3, 8, 19F and 37 (not to scale). Arrows indicate functional genes. Patterned blocks indicate truncated non-functional genes. Patterned arrows indicate homologues. Black arrows indicate genes not involved in capsule biosynthesis. Clear arrows indicate serotype-specific genes. Diamond-patterned arrows indicate rhamnose biosynthesis genes. Genes 8E* and tts1 from phase-variable strains 8 and 37 contain black blocks, indicating sequence duplications and functional inactivation.](image-url)
and excision of the duplication re-enabling capsule production (Waite et al., 2001). Furthermore, the frequency of capsule phase variation for duplications within type 8 and 37 phase variants fits the same linear relationship between log(frequency of reversion) and log(length of duplication) demonstrated previously for serotype 3 pneumococci (Waite et al., 2001).

**METHODS**

### Bacterial strains, media and sorbarod culture

*S. pneumoniae* strains 8/555 (serotype 8) and 37/40 (serotype 37) were human blood and human nasopharyngeal isolates, respectively (Table 1). Pneumococci were routinely cultured on brain–heart infusion (BHI; Difco) agar plates supplemented with 4% sheep blood (BHI blood) and incubated at 37 °C in a CO₂/air (5:95, v/v) atmosphere. Sorbarods were set up as described previously (Waite et al., 2001) except that approximately 2 × 10⁸ c.f.u. was used to inoculate sorbarod filters, which were incubated under atmospheric conditions at 34 °C to mimic nasopharyngeal carriage (Keck et al., 2000). Examination of sorbarod cultures by light- and electron microscopy has revealed microcolonies of bacteria scattered throughout the cellulose fibres of the sorbarod (not presented).

Effluent from sorbarod cultures was sampled after 5 or 6 days for serotype 37 and serotype 8, respectively. Effluent samples were diluted in PBS and incubated at 37 °C in a CO₂/air (5:95, v/v) atmosphere on BHI blood plates. Well-isolated, acapsular colonies were restreaked onto BHI blood plates. Those that produced capsular colonies on subculture were identified as having a reversible phenotype. The frequency of reversion was determined by picking single, well-isolated, acapsular colonies with the underlying agar, serially diluting in PBS and plating in triplicate onto BHI blood agar to determine the total number of c.f.u. per acapsular colony and the proportion that reverted to wild-type.

The presence of the type 8 and type 37 capsule was determined using Danish pneumococcal typing antisera (Statens Serum-Institut, Copenhagen, Denmark) by the Quellung reaction.

### Analysis of capsule phase variants

For serotype 8 strain 8/555, PCR was used to amplify the cap8B, cap8C, cap8D, cap8E and cap8L genes of the wild-type organism and capsule phase variants (for primer sequences, see Table 2). For serotype 37 strain 37/40, PCR was used to amplify the tts gene from the wild-type organism and capsule phase variants (for primer sequences, see Table 2). DNA for PCR reactions was obtained by picking individual colonies into dH₂O and heating at 99 °C for 10 min. PCR amplification was carried out under standard conditions, and each reaction was carried out in a final volume of 50 μl: 20 mM Tris/Cl (pH 8.4) and 50 mM KCl, 3 mM MgCl₂, 0·2 mM dNTPs, 0·6 μg primer and 1 U Taq DNA polymerase (Gibco-BRL). Capsule gene PCR products from capsular wild-type (phase-ON) colonies, acapsular (phase-OFF) colonies and revertant capsular (phase-ON) colonies, were purified through Qiagen columns, sequenced using dyeoxy-terminators and fluorescent dyes and run on a Beckman CEQ 2000 sequencer.

PCR amplification and sequencing of trpB/A was as described previously (Waite et al., 2001).

### RESULTS

#### Batch culture, sorbarod culture and identification of capsule phase variants

Both serotype 8 and serotype 37 pneumococci form mucoid colonies (≈1–1·5 mm diameter) on BHI blood agar. When batch cultures were analysed, stable acapsular colonies were isolated from serotype 37 strain 37/40 on each occasion (0·014–0·18% of viable count), which is consistent with other published results for the type 37 pneumococcus (Llull et al., 2000). Serotype 3 and 8 pneumococci only rarely formed acapsular colonies in batch culture.

When serotype 8 strain 8/555 and serotype 37 strain 37/40 were grown for 5 or 6 days as sorbarod cultures they both generated small acapsular colonies at high frequency, i.e. 2·4 and 80%, respectively. A proportion of these acapsular colonies (3·3%, serotype 8 strain; 1·9%, serotype 37 strain) were found to be capsule phase variable as they could revert to capsular phenotype (phase-ON). Phase-OFF and phase-ON phenotypes were confirmed using the Quellung reaction.

It has been shown that trpB/A genes exhibit substantial allelic variation (Muller-Graf et al., 1999). These genes were sequenced from wild-type organisms and their capsule phase-variable derivatives to show that these mutants had arisen from the organisms used to inoculate sorbarod filters and were not contaminants. As expected, both serotype 8 and serotype 37 capsule phase-variable mutants had trpB/A sequences that were identical to those of their parent

<table>
<thead>
<tr>
<th>Strain or mutant</th>
<th>Relevant genotype</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. pneumoniae strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM108</td>
<td>Serotype 3 wild-type</td>
<td>Waite et al. (2001)</td>
</tr>
<tr>
<td>600142</td>
<td>Serotype 3 wild-type</td>
<td>Waite et al. (2001); Whatmore et al. (1999)</td>
</tr>
<tr>
<td>0100993</td>
<td>Serotype 3 wild-type</td>
<td>Throup et al. (2000); Waite et al. (2001)</td>
</tr>
<tr>
<td>8/555</td>
<td>Serotype 8 wild-type</td>
<td>This study</td>
</tr>
<tr>
<td>37/40</td>
<td>Serotype 37 wild-type</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Sorbarod-derived mutants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/555 Sorbarod 223</td>
<td>223 bp cap8E tandem duplication. Capsule phase variant</td>
<td>This study</td>
</tr>
<tr>
<td>37/40 Sorbarod 22</td>
<td>22 bp tts tandem duplication. Capsule phase variant</td>
<td>This study</td>
</tr>
</tbody>
</table>
organisms (data not shown). Each parent strain had a different trpB/A allele (data not shown).

Molecular analysis of serotype 8 capsule phase variation

To identify DNA rearrangements responsible for the observed phase variation, PCR primers were designed to amplify the serotype 8 capsule genes cap8B, cap8C and cap8D (homologous to cpsBCD, which have been found to be essential for capsule production; Morona et al., 2000) from phase-OFF colonies obtained from strain 8/555 capsule phase-variable mutants. PCR primers were also designed to amplify cap8L, which encodes a putative UDP-glucose dehydrogenase with 57% identity to cap3A (Munoz et al., 1999), internal fragments of which have previously been shown to undergo spontaneous duplication resulting in type capsule phase variation (Waite et al., 2001). No regions of DNA duplication were detected within cap8B, cap8C, cap8D or cap8L (data not shown).

CapE plays a central role in capsule biosynthesis for all serotypes examined so far (except serotypes 3 and 37) by transferring a monosaccharide to the lipid carrier in the first step of capsule construction (Kolkman et al., 1996, 1998). The serotype 8 capE homologue (cap8E) is 84% similar to the biochemically characterized Cap14E and >90% identical to cps2E, cps19E, cps23E and cps33FE (Munoz et al., 1999). Examination of cap8E in serotype 8 capsule phase variants revealed that cap8E PCR products from phase-OFF mutants were clearly larger than the PCR product from the parent strain (Figs 1 and 2). Sequencing of cap8E from one capsule phase-OFF variant confirmed the presence of a perfect 223 bp tandem duplication within cap8E (Table 3) which introduced a premature stop codon downstream of the duplicated sequence. As expected from previous work (Waite et al., 2001), phase-ON revertant cap8E PCR products were of identical size and sequence to the parent strain (Fig. 2), indicating that phase variation within serotype 8 isolates is controlled through the same novel mechanism of sequence duplication and excision as that found for serotype 3 phase variation (Waite et al., 2001).

All three type 8 capsule phase-OFF variants examined from a single sorbarod possessed identical RFLP patterns upon restriction analysis of the cap8E PCR product. It is therefore assumed that all three variants were clonally derived from the same progenitor within the sorbarod. Previously, different duplications have been identified from a single sorbarod (Waite et al., 2001).

Table 2. Primers used to PCR-amplify and sequence capsule genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Primer position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpsAup</td>
<td>ATCTTTAAGTACAATATCCT</td>
<td>2294–2313</td>
</tr>
<tr>
<td>cpsAdown</td>
<td>TCAATAGCAGCATAGTAAT</td>
<td>3111–3128</td>
</tr>
<tr>
<td>Abfor1</td>
<td>CAATGGCAGATGGTGGAAATAATC</td>
<td>3003–3026</td>
</tr>
<tr>
<td>Abrev1</td>
<td>GGACGCGCTATCTACATCAA</td>
<td>3663–3683</td>
</tr>
<tr>
<td>cpsBup</td>
<td>CATATCGTTTTTGATGTTAGA</td>
<td>3654–3673</td>
</tr>
<tr>
<td>cpsBdown</td>
<td>ATTGTGCGATGTCATCTTG</td>
<td>4224–4241</td>
</tr>
<tr>
<td>cap8Cfor</td>
<td>GATGAAAGAACAAAACGATAGA</td>
<td>4375–4398</td>
</tr>
<tr>
<td>cap8Crev</td>
<td>GCGATACGACTGCTTCCCT</td>
<td>4772–4791</td>
</tr>
<tr>
<td>cap8Dfor</td>
<td>AGTATTTTCTATACCTCTTGTG</td>
<td>5185–5206</td>
</tr>
<tr>
<td>cap8Drev</td>
<td>ATAATAGCGGCGCATCAAAGGACA</td>
<td>5548–5571</td>
</tr>
<tr>
<td>cap8Efor</td>
<td>AAAAGGGATATTGGATGAACCTTG</td>
<td>5960–5983</td>
</tr>
<tr>
<td>8E seq REV †</td>
<td>ACTGAGAGACAAGCTTCACATAT</td>
<td>6398–6421</td>
</tr>
<tr>
<td>cap8Erev</td>
<td>TCATATCTCCCTTTCGGATTAT</td>
<td>6878–6901</td>
</tr>
<tr>
<td>cap8Lfor</td>
<td>GATAAATATCAAGAAAGTCCAC</td>
<td>12714–12737</td>
</tr>
<tr>
<td>cap8Linvf</td>
<td>TTAGTCCTGAAATTTTTGCGTGAGT</td>
<td>13019–13042</td>
</tr>
<tr>
<td>cap8Linvr</td>
<td>CATTTAAAAACATATTGGGGCTGAAT</td>
<td>13523–13546</td>
</tr>
<tr>
<td>cap8Linvr</td>
<td>AGACGCTCTAATAAAACTAAA</td>
<td>14041–14061</td>
</tr>
<tr>
<td>tssfor1</td>
<td>ACCATGGGCTCCCTTTTACAGA</td>
<td>2054–2077</td>
</tr>
<tr>
<td>tssfor2</td>
<td>GACAGGGATAGATTATTCCTCA</td>
<td>2698–2721</td>
</tr>
<tr>
<td>tssfor3</td>
<td>CTAAACATAAAAATGGGACAGGAAAG</td>
<td>3149–3171</td>
</tr>
<tr>
<td>tssrev1</td>
<td>CCATACCTTCATACATAACTAAG</td>
<td>3734–3758</td>
</tr>
<tr>
<td>tssrev2</td>
<td>CATATTCCCTGCGGTCATTTAG</td>
<td>3089–3111</td>
</tr>
<tr>
<td>tssrev3</td>
<td>TGGGTGCTCACTAAGAAAAAC</td>
<td>3469–3487</td>
</tr>
</tbody>
</table>

*Primer positions correspond to the published serotype 8 capsule locus sequence (accession no. AJ239004) or the published tts sequence (accession no. AJ131985).
†Sequencing primer.
A study by Llull et al. (1999) has shown that a single gene (\textit{tts}) is responsible for synthesis of the serotype 37 capsule. Primers were designed to amplify three different regions of this gene. Using primers \textit{tts}for3 and \textit{tts}rev3, the phase-OFF phenotype was found to contain a perfect 22 bp tandem duplication within the \textit{tts} gene (Figs. 1 and 3, Table 3), resulting in the insertion of a premature stop codon downstream of the duplicated sequence. The phase-ON revertant \textit{tts} PCR product was of identical size to the parent strain PCR product (Fig. 3), and sequencing revealed a wild-type \textit{tts} sequence.

**Frequency of capsule phase variation (OFF–ON) is related to size of duplication**

Analysis of the mean frequency of reversion of both serotype 8 and serotype 37 capsule phase variants revealed that both mutants appeared to adhere to the significant linear relationship between the log(frequency of reversion) and log(length of duplication) previously found with serotype 3 capsule phase variants (Waite \textit{et al.}, 2001) (Fig. 4).

**DISCUSSION**

It has previously been proved that the mechanism of phase variation (OFF–ON) of type 3 capsule formation in sorbarods directly involves the sequential duplication and deletion of apparently random fragments of ORF within \textit{cap3A} (Waite \textit{et al.}, 2001).

The additional finding that such tandem duplications are also directly involved in capsule phase variation in pneumococcal serotypes 8 and 37 (Fig. 1, Table 3) provides evidence that this mechanism is not exclusive to serotype 3 pneumococci. It appears that tandem duplication regulated capsule phase variation can occur in pneumococci regardless of the mechanism of capsule construction. In serotypes 3 and 37 (the two serotypes with the simplest capsular loci structures; Fig. 1), capsule is synthesized by a processive transferase (Arrecubieta \textit{et al.}, 1996; Llull \textit{et al.}, 2001) whilst the capsules of the other sequenced serotypes are thought to be synthesized via lipid-linked repeat unit intermediates. The transferase central to the synthesis of the serotype 37 capsule is encoded by the \textit{tts} gene. The finding that a tandem duplication in \textit{tts} is responsible for the phase-OFF phenotype in serotype 37 capsule phase

### Table 3. Perfect tandem duplications found in serotype 8 and serotype 37 capsule phase-variable pneumococci

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Position of target for duplication</th>
<th>Size of duplication (bp)</th>
<th>Duplicated sequence and flanking sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>555</td>
<td>8</td>
<td>6019–6241†</td>
<td>223</td>
<td>gcacctGGGATTAGTATTTCTAATTTTTTCTTTAGAGGAATGTTTA GTATTTTCCAGAGGAGGATTTTACTCTTCTCACAATATACATGC TCTCTTATGCTATGTGCTAACAATCTTATTTACAGTGTTATTTGA AAGCGGGCATTACCCACACTTTAAGGAAAGTAAGAGGATTCTCC TACCATTACGCAACTCTCTGCTGGTGGAAAGGTGTGGATAGATT AATTGAAATCAAAGGAGTTATTTCTAATTTTTTCTTTAGAGG GGTATTTGATTTTCCAGAGGAGGATTTTACTCTTCTCACA TTACATGCTCTTATGCTATGTGCTAACAATCTTATTTACAGT GGTATTTGAAAGGGCCATTACCCACACTTTAAGGAAAGTAAGAGG AGATTCTCCTAATACAAGCAGCTCTGCTGGTGGAAAGGTGTG TGGATAGATTACCAATCTA\textit{g}</td>
</tr>
<tr>
<td>40</td>
<td>37</td>
<td>3401–3422‡</td>
<td>22</td>
<td>attTGTTTTGATTTTCTTTAGAGGATTTTTCACAGT</td>
</tr>
</tbody>
</table>

*Target for duplication is in bold capitals, and the resulting tandem duplication is underlined. Nucleotides flanking the target region and tandem duplication are shown in lower case type.

†Nucleotide numbers correspond to the published serotype 8 capsule locus sequence (accession no. AJ239004).

‡Nucleotide numbers correspond to the published \textit{tts} gene sequence (accession no. AJ131985).
Independent values plotted for reversion frequencies for each size of duplication were found in the gene encoding ribosomal protein L22 (Musher et al., 2002). An 18 bp tandem repeat resulting in the duplication of 6 aa adjacent to the macrolide-binding site was found in the ribosomal protein L22 of a serotype 3 macrolide-resistant isolate responsible for the mortality of an individual. Sputum samples taken prior to antimicrobial therapy contained the same organism [shown by BOX-PCR (Koeth et al., 1995) and ribotype analysis], but the isolate was sensitive to macrolide antimicrobials, and no tandem duplication was found in the gene encoding ribosomal protein L22 (Musher et al., 2002). Other determinants of capsule phase variation is in agreement with the acapsular phenotype of a serotype 37 mutant with an insertion-inactivated tts (Llull et al., 1999).

In capsules that are synthesized through polymerization of lipid-linked intermediates, the synthesis of repeating units starts with the CapE-mediated transfer of the first monosaccharide to a lipid carrier molecule. Although capE (cpsE) plays in the capsule biosynthetic pathway. As capE appears to play a very prominent role in capsule synthesis, it is conceivable that capsule phase variation controlled through tandem duplications in capE (cpsE) occurs in other serotypes. It is also interesting that phase variation is controlled in a gene found midway through the serotype 8 capsule locus (Fig. 1), as it is known that insertional inactivation of the three genes (capBCD) upstream of capE in serotypes 14 and 19F could also induce an acapsular phenotype (Guidolin et al., 1994; Kolkman et al., 1997a, b; Morona et al., 2000). Our observation is a further illustration of the key role that CapE plays in the capsule biosynthetic pathway.

Although the group of genes present in each pneumococcal capsule locus differs, there is generally a common theme to their organization: a serotype-specific region preceded directly by capE (the first monosaccharide transferase), and four genes (capABCD) upstream of this thought to be involved in capsule regulation and export (Guidolin et al., 1994; Jiang et al., 2001). The amount of pneumococcal surface polysaccharide can be fine-tuned through opacity phase variation (Kim & Weiser, 1998) and by the interactions of CapB, CapC and CapD, where the level of capsule polysaccharide synthesis production is inversely proportional to the cellular level of tyrosine-phosphorylated CapD (Morona et al., 2000). It is therefore interesting that the serotype 8 pneumococcus, which has a capsule locus structure similar to the majority of serotypes, can also undergo capsule phase variation in which the switch is apparently all or nothing. It is possible that different mechanisms of capsule regulation take place in vivo depending on the environment in which the pneumococcus finds itself. It is not yet clear why or how the high cell-density environment within sorbarod cultures drives the formation of acapsular pneumococci – this may involve signalling systems or physiological stress and is currently under investigation.

Pneumococcal tandem duplication-mediated antibiotic resistance has recently been demonstrated in a clinical setting (Musher et al., 2002). An 18 bp tandem repeat resulting in the duplication of 6 aa adjacent to the macrolide-binding site was found in the ribosomal protein L22 of a serotype 3 macrolide-resistant isolate responsible for the mortality of an individual. Sputum samples taken prior to antimicrobial therapy contained the same organism [shown by BOX-PCR (Koeth et al., 1995) and ribotype analysis], but the isolate was sensitive to macrolide antimicrobials, and no tandem duplication was found in the gene encoding ribosomal protein L22 (Musher et al., 2002). Other determinants of variation is in agreement with the acapsular phenotype of a serotype 37 mutant with an insertion-inactivated tts (Llull et al., 1999).

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In capsules that are synthesized through polymerization of lipid-linked intermediates, the synthesis of repeating units starts with the CapE-mediated transfer of the first monosaccharide to a lipid carrier molecule. Although capE (cpsE) plays in the capsule biosynthetic pathway. As capE appears to play a very prominent role in capsule synthesis, it is conceivable that capsule phase variation controlled through tandem duplications in capE (cpsE) occurs in other serotypes. It is also interesting that phase variation is controlled in a gene found midway through the serotype 8 capsule locus (Fig. 1), as it is known that insertional inactivation of the three genes (capBCD) upstream of capE in serotypes 14 and 19F could also induce an acapsular phenotype (Guidolin et al., 1994; Kolkman et al., 1997a, b; Morona et al., 2000). Our observation is a further illustration of the key role that CapE plays in the capsule biosynthetic pathway.

Although the group of genes present in each pneumococcal capsule locus differs, there is generally a common theme to their organization: a serotype-specific region preceded directly by capE (the first monosaccharide transferase), and four genes (capABCD) upstream of this thought to be involved in capsule regulation and export (Guidolin et al., 1994; Jiang et al., 2001). The amount of pneumococcal surface polysaccharide can be fine-tuned through opacity phase variation (Kim & Weiser, 1998) and by the interactions of CapB, CapC and CapD, where the level of capsule polysaccharide synthesis production is inversely proportional to the cellular level of tyrosine-phosphorylated CapD (Morona et al., 2000). It is therefore interesting that the serotype 8 pneumococcus, which has a capsule locus structure similar to the majority of serotypes, can also undergo capsule phase variation in which the switch is apparently all or nothing. It is possible that different mechanisms of capsule regulation take place in vivo depending on the environment in which the pneumococcus finds itself. It is not yet clear why or how the high cell-density environment within sorbarod cultures drives the formation of acapsular pneumococci – this may involve signalling systems or physiological stress and is currently under investigation.
macrolide resistance, such as $erm(B)$ or $mef(A)$, were not found in the resistant isolate.

Now that there is clear evidence that the pneumococcus can utilize tandem sequence duplication to evade antimicrobial therapy in vivo, it remains to be addressed whether tandem duplication-mediated capsule phase variation is used by this organism (or by other organisms) whilst causing disease, whether other virulence determinants are also regulated in this way, and what the environmental conditions or signals are that promote these events.

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