In vitro expression of the first capsule gene of *Streptococcus pneumoniae*, *cpsA*, is associated with serotype-specific colonization prevalence and invasiveness

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The polysaccharide capsule protects *Streptococcus pneumoniae* from phagocytosis during invasive infection, but inhibits adherence. Serotypes vary in their tendency to colonize the nasopharynx or cause invasive infection, and differences in capsule expression may play a role. Expression of the first gene of the capsule operon, *cpsA*, during *in vitro* growth of 43 clinical isolates representing 14 common pneumococcal serotypes was compared using quantitative RT-PCR. Serotypes associated with invasive infection (1, 4, 5, 7F, 8 and 14) expressed an average of twofold ($P=0.0003$) more *cpsA* than serotypes associated with nasopharyngeal colonization (6A, 6B, 9V, 15, 18C, 19F, 23F and 33). There was no difference in *cpsA* expression in response to growth under environmental oxygen or anaerobic conditions between the invasive and colonizing transparent strains tested: oxygen concentration did not affect *cpsA* expression in either the invasive or the colonizing transparent strains. Expression of *cpsA* at OD₆₀₀ 0.6 tended to be greater in strains with a longer lag phase during *in vitro* growth ($P=0.07$). Therefore, *cpsA* expression under ambient oxygen concentrations correlates with serotype-specific invasiveness and is inversely associated with the prevalence of serotype-specific carriage.

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INTRODUCTION

Streptococcus pneumoniae bacteria live mostly in the nasopharynx of young children without causing harm, but when they do invade they cause serious diseases such as pneumonia, sepsis and meningitis. The antigenic and biochemical properties of the polysaccharide capsule are used to characterize *Streptococcus pneumoniae* strains into over 90 distinct serotypes. Some serotypes such as 1, 4, 5, 7F and 14 have been associated with invasive disease, whilst others such as 6A, 6B, 15, 18C, 19F and 23F are more efficient at colonizing the nasopharynx (Brueggemann *et al.*, 2004; Hanage *et al.*, 2005; Kronenberg *et al.*, 2006; Sandgren *et al.*, 2004). However, when the latter do invade they cause more severe disease than serotypes which are commonly associated with invasive disease (Sjostrom *et al.*, 2006). Bättig *et al.* (2006) have recently shown a correlation between the lag phase during *in vitro* growth and

serotype-specific invasiveness. Understanding the characteristics of different serotypes is of great interest in an era in which serotype selection is influenced by vaccination.

The polysaccharide capsule is a major virulence factor of *S. pneumoniae*. It protects the bacteria from phagocytosis after invasion. However, expression of a capsule reduces bacterial attachment to respiratory epithelial cells and may therefore hamper colonization (Adamou *et al.*, 1998; Cundell *et al.*, 1995; Weiser *et al.*, 1994). Hammerschmidt *et al.* (2005) have shown by electron microscopy that bacteria in intimate contact with epithelial cells have a thinner capsule layer, i.e. they may down-regulate capsule expression in order to enhance adherence. It has been suggested that, at least in serotype 3, polysaccharide chain length can be modulated by sugar concentration in the environment (Ventura *et al.*, 2006).

The polysaccharide capsule of most serotypes of *S. pneumoniae* is encoded by a gene cluster located between *dexB* and *aliA*. Capsule operons of different serotypes show a similar structure with some conservation, particularly within the first four genes, downstream of which are the serotype-specific genes (Garcia *et al.*, 1999; Jiang *et al.*, 2001; Bentley *et al.*, 2006). The first gene, *cpsA*, is the most

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Abbreviations: Ct, crossing threshold; OR, odds ratio; UPGMA, unweighted pair group method with arithmetic mean.

conserved and may have a role in regulation of capsule expression (Guidolin *et al.*, 1994).

Expression studies of *cpsA* have shown conflicting results. Transcription of *cpsA* has been shown by RT-PCR to be upregulated fourfold in bacteria recovered 24 h after intraperitoneal infection compared to expression *in vitro* (Ogunniyi *et al.*, 2002). In contrast, a microarray analysis found no increase in *cpsA* expression in bacteria from the blood of mice compared to bacteria grown *in vitro* (Orihuela *et al.*, 2004), and no difference in *cpsA* expression was found among bacteria isolated from the nasopharynx, lungs and blood of mice infected intranasally (LeMessurier *et al.*, 2006).

Here, we aimed to compare *cpsA* expression *in vitro* in clinical isolates representing pneumococcal serotypes associated with invasiveness and serotypes associated with nasopharyngeal colonization.

METHODS

Bacterial strains. Clinical isolates of *S. pneumoniae* were randomly selected from two nationwide surveillance programmes that collected nasopharyngeal and invasive isolates (Mühlemann *et al.*, 2003; Hathaway *et al.*, 2004; Kronenberg *et al.*, 2006). Both surveillance programmes started in 1998 and are ongoing, with short interruptions between January 2000 and March 2002. Capsular serotypes were determined by the Quellung reaction, as described previously (Mühlemann *et al.*, 2003; Hathaway *et al.*, 2004) or the 'agglutination reaction' (Statens Serum Institut, Copenhagen, Denmark). A total of 43 encapsulated *S. pneumoniae* isolates representing 14 serotypes (1, 4, 5, 6A, 6B, 7F, 8, 9V, 14, 15, 18C, 19F, 23F and 33) were used. For each serotype two to six different isolates were included. Isolates from sterile body sites and from the nasopharynx were chosen.

Bacterial culture. Bacteria were stored at -80°C using Protect bacterial preservers (Technical Service Consultants). Bacteria were grown on Columbia sheep blood agar (CSBA) plates at 37°C in a 5% CO_2 atmosphere. An overnight culture was prepared with three to 10 colonies in 5 ml brain heart infusion (BHI; Becton Dickinson) containing 5% fetal calf serum (FCS; Biochrom). A 100 μl sample from the overnight culture was subcultured at 37°C in 5 ml BHI with 5% FCS and grown to OD_{600} 0.5–0.7 (measured using a Perkin Elmer Lambda 2 spectrophotometer with cuvettes of 1 cm path length). After transferring 200 μl of this culture into a tube containing 10 ml BHI, the bacteria were cultured to OD_{600} ~0.6, and were therefore at the end of exponential growth, unless otherwise stated. When anaerobic conditions were required, they were achieved using AnaeroGen sachets (Oxoid) according to the manufacturer's instructions, by incubating within a 37°C room the bacterial cultures in tubes with loosened lids in sealed containers that contained an AnaeroGen sachet. For ambient oxygen conditions, the bacterial cultures in tubes with loosened lids were also incubated in the 37°C room, but without the sealed container or AnaeroGen sachet. No cultures were shaken. In order to determine whether the strains were of an opaque or transparent phenotype, they were plated onto tryptic soy plates containing 1% agar onto which 5000 U catalase (Sigma) had been spread, and viewed by stereomicroscopy using oblique transmitted illumination, as described elsewhere (Weiser *et al.*, 1994).

PFGE. DNA was isolated as described previously (Stutzmann Meier *et al.*, 2003) and PFGE typing was done on all isolates, as described elsewhere (Léchoy *et al.*, 2001), by use of *Sma*I for restriction digestion

of chromosomal DNA. PFGE patterns were analysed with Bio-numerics software (version 3.0, Applied Maths). Patterns were clustered by the unweighted pair group method with arithmetic mean (UPGMA) and a dendrogram was generated from a similarity matrix calculated using the Dice similarity coefficient with an optimization of 1.0% and a tolerance of 1.5%.

Epidemiological data on invasive potential and carriage prevalence of pneumococcal serotypes. Serotypes were grouped as 'invasive' or 'colonizers' based on previous studies with serotype 14 as a fixed reference. This serotype and any with a higher odds ratio (OR) were considered invasive and all others as colonizing. ORs for the invasive potential of individual serotypes were kindly provided by Dr A. Brueggemann, University of Oxford (Brueggemann *et al.*, 2004), or obtained from our local analysis (Kronenberg *et al.*, 2006).

RNA isolation. When the bacteria reached OD_{600} 0.6, twice the culture volume of RNeasy Protect (Qiagen) was added to stop further transcription and prevent RNA degradation. After vortexing and a 5 min incubation at room temperature, the bacteria were pelleted by centrifugation for 10 min at 5000 g. The pellet was resuspended in 200 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) containing 600 μg lysozyme. After 10 min at room temperature with repeated vortexing, 700 μl RLT buffer (Qiagen RNeasy Mini kit) containing β -2-mercaptoethanol was added, and the tubes were vortexed. The mixture was transferred to a 1.5 ml tube containing 0.05 g 100 μm acid-washed glass beads (Sigma) and vibrated for 10 min at half-maximum speed using a Mickle Vibratory Tissue Disintegrator (Mickle Laboratory Engineering). The mixture was then centrifuged and RNA extracted from the supernatant using a Qiagen RNeasy Mini kit according to the manufacturer's instructions. The RNA recovered was treated with Dnase I (Stratagene Europe), according to the manufacturer's instructions, to remove any contaminating DNA.

Quantification of gene expression. A 1 μg volume of total RNA of each sample was reverse-transcribed to cDNA using Superscript II (Amersham) and random hexamer primers, according to the supplier's protocol. Quantification of gene expression was achieved by real-time RT-PCR using TaqMan primers and probes created by the Assay-by-Design Service of Applied Biosystems, based on the most conserved regions of the first gene of the capsule operon in different serotypes of *S. pneumoniae*. The 16S rRNA gene was used as an endogenous control. The primer sequences were: *cpsA* forward primer, 5'-CTCTTTGCAGTACAGCAGTTTGTG-3'; reverse primer, 5'-CTATCTGCTAAAACAGCGACACTGA-3'; probe, 6-carboxy-fluorescein (6-FAM)-ACTGACCAATCGTTTAAATG-minor groove binder (MGB); 16S forward primer, 5'-GACGATACATAGCCGACCTGAGA-3'; reverse primer, 5'-GTAGGAGTCTGGGCCGTGTCT-3'; probe, 6-FAM-CCAGTGTGGCCGATC-MGB. The cDNA was diluted 25-fold in the assay and a reverse-transcription-negative control was performed for every sample. Real-time RT-PCR was performed in 96-well plates using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and the relative gene expression for the different serotypes was calculated from the crossing threshold (Ct) value according to the manufacturer's protocol ($2^{-\Delta\Delta\text{Ct}}$) after normalization using the 16S rRNA endogenous control.

Quantification of capsule. The amount of capsule was determined using the Stains-all assay (Sigma) for detecting acidic polysaccharides (Hammerschmidt *et al.*, 2005). The bacteria were cultured in BHI to OD_{600} 0.6 under aerobic or anaerobic conditions (see above), then 5 ml was centrifuged for 10 min at 5000 g, washed with PBS and resuspended in 0.5 ml 0.85% NaCl. A 10 μl volume was removed to make dilutions in PBS for plating out to quantify the number of bacteria. To the remaining bacterial suspension, 2 ml of a solution containing 20 mg 1-ethyl-2(3-(1-ethylnaphthho-(1,2-*d*)thiazolin-2-ylidene)-2-methylpropenyl)naphthho-(1,2-*d*)thiazolium bromide

(Stains-all) and 60 µl glacial acetic acid in 100 ml 50 % formamide was added, and the OD₆₄₀ determined: 0.5 ml NaCl with 2 ml Stains-all solution was used as a blank. The values for 1×10^7 c.f.u. were calculated from the colony counts of the bacteria plated out.

Statistical analysis. Statistical analyses such as ANOVA were performed using StatView version 5.0 (SAS Institute). The GraphPad Prism software (version 4.01, GraphPad Software) was used to calculate linear regression. A value of $P \leq 0.05$, in a two-tailed test, was considered significant.

RESULTS

Invasive serotypes express more *cpsA* than colonizing serotypes *in vitro*

For each of 43 isolates, representing 14 serotypes, PFGE was performed; Fig. 1 shows that each serotype was represented by isolates with at least two different patterns, demonstrating heterogeneity within the bacterial population used. All strains were predominantly of a transparent phenotype (between 61 and 97 % of colonies). For each isolate, *cpsA* expression was determined by real-time RT-PCR in cultures grown in BHI to OD₆₀₀ 0.6 under ambient oxygen conditions. A serotype 6B isolate was found to have the lowest *cpsA* expression and was given an arbitrary value of 1, and *cpsA* expression values for all other strains were expressed as a multiple of the value for this isolate in order to compare *cpsA* expression levels between isolates. Pneumococcal serotypes have been shown to differ in their propensity to be carried in the nasopharynx or to be associated with invasive disease. Expression of *cpsA* was plotted against serotype-specific carriage prevalence values from an international study (Brueggemann *et al.*, 2004) and a local (Swiss) study (Kronenberg *et al.*, 2006). The serotype-specific carriage prevalence correlated inversely with *cpsA* expression using values from Brueggemann *et al.* (2004) ($P=0.005$, Fig. 2a) or Kronenberg *et al.* (2006) ($P=0.03$, Fig. 2b). The likelihood of a serotype or group being associated with invasive disease rather than with colonization of the nasopharynx can be expressed as an OR (Brueggemann *et al.*, 2004). Brueggemann *et al.* (2004) used serotype 14 as a reference group and considered serotypes with ORs greater than that of serotype 14 to be invaders and those with a lower OR to be colonizers. We applied this method of division into invaders and colonizers to our data according to the ORs of Brueggemann *et al.* (2004) (invaders, serogroups 1, 5, 7 and 14; colonizers, serogroups 4, 6A, 6B, 8, 9, 15, 18, 19, 23 and 33) or according to the ORs of Kronenberg *et al.* (2006) (invaders, serotypes 1, 4, 5, 7F, 8 and 14; colonizers, serotypes 6A, 6B, 9V, 15, 18C, 19F, 23F and 33). Invader serotypes and groups expressed on average 1.6-fold more *cpsA* than colonizers using the ORs from Brueggemann *et al.* (2004) ($P=0.02$, Fig. 2c) or 2.1-fold using those from Kronenberg *et al.* (2006) ($P=0.0003$, Fig. 2d). No significant difference in mean *cpsA* expression was detected between isolates collected from a sterile site (mean 4.78, SD 3.84) and those collected from the nasopharynx (mean 4.82, SD 2.79;

Fig. 1. PFGE results for all pneumococcal isolates used in this study. Chromosomal DNA was digested with *Sma*I. PFGE patterns were analysed with Bionumerics software, version 3.0, and the patterns clustered by UPGMA. The dendrogram was generated from a similarity matrix calculated using the Dice similarity coefficient with an optimization of 1.0% and a tolerance of 1.5%. An isolate name beginning with 'B' indicates isolation in the context of invasive disease, whereas a number alone indicates isolation from the nasopharynx.

$P=0.96$). This was also true when mean *cpsA* expression was compared between isolates collected from a sterile site and those collected from the nasopharynx within groups of invader serotypes and groups (mean 6.3, SD 4.46, vs mean 7.22, SD 2.51; $P=0.61$) and colonizer serotypes and groups (mean 2.70, SD 0.83, vs mean 3.29, SD 2.17; $P=0.31$). The number of isolates was, however, too small to allow for a comparison of *cpsA* expression between different clones belonging to the same serotype or group.

Fig. 2. Expression of *cpsA* in common pneumococcal serotypes. Expression of *cpsA* was assessed by real-time RT-PCR on RNA from clinical isolates cultured to OD₆₀₀ 0.6 in BHI under ambient oxygen conditions. Expression of *cpsA* is displayed as the value for each isolate relative to that of the isolate with the lowest expression, after normalization using 16S rRNA. Relative *cpsA* expression was inversely related to the carriage prevalence of (a) Brueggemann *et al.* (2004), $P=0.005$, and (b) Kronenberg *et al.* (2006), $P=0.03$ (linear regression calculated using GraphPad Prism software, version 4.01). Expression of *cpsA* was greater in invasive serotypes than colonizers, grouped according to (c) the ORs of Brueggemann *et al.* (2004), $P=0.02$, or (d) Kronenberg *et al.* (2006), $P=0.0003$. ANOVA was calculated from the mean *cpsA* expression for each serotype using StatView software, version 5.0. Error bars show SEM.

Effect of anaerobic conditions on *cpsA* expression

To determine whether *cpsA* expression was dependent on oxygen tension, expression was measured in five isolates of five different serotypes (1, 6A, 9V, 15 and 19F) under ambient oxygen and anaerobic conditions in BHI. Fig. 3 shows the association between higher *cpsA* expression under ambient oxygen conditions in serotypes with higher ORs (serotypes 1 and 9V) and a lower expression level for the colonizer serotypes 6A, 15 and 19F. Anaerobic conditions

Fig. 3. Expression of *cpsA* under anaerobic and aerobic conditions. Expression was determined for five isolates representing five different serotypes grown in BHI under ambient oxygen (hatched bars) or anaerobic (grey bars) conditions (achieved using an AnaeroGen sachet in a sealed container). Results are displayed as fold-difference relative to the lowest value for isolate 6A under aerobic conditions and are the summary of five independent experiments. ANOVA was calculated using StatView software. Error bars show SEM.

did not cause a significant difference in *cpsA* expression in any of the five serotypes tested.

The amount of acidic capsule polysaccharide produced does not depend on oxygen tension

The Stains-all assay is only suitable for measuring the amount of capsule for those serotypes which have acidic polysaccharide capsules, and so was used to determine the amount of capsule for the serotype 1 and 19F isolates cultured in BHI under ambient oxygen or anaerobic conditions to OD₆₀₀ 0.6. For both serotypes there was no significant change in the amount of capsule under anaerobic conditions. (OD₆₄₀ for serotype 1 under aerobic vs anaerobic conditions: mean 0.173, SD 0.168, vs mean 0.200, SD 0.217; $P=0.71$. OD₆₄₀ for serotype 19F under aerobic vs anaerobic conditions: mean 0.061, SD 0.038, vs mean 0.096, SD 0.042; $P=0.34$).

Serotypes with a longer lag phase of *in vitro* growth tend to have higher *cpsA* expression

Expression of *cpsA* was assessed by real-time RT-PCR on RNA from clinical isolates cultured to OD₆₀₀ 0.6 in BHI and correlated with the data of Böttig *et al.* (2006), who measured the lag phase when the bacteria were cultured in BHI. Fig. 4 shows that there was a trend towards greater *cpsA* expression in strains with a longer lag phase ($P=0.07$).

DISCUSSION

Several studies have reported an association between *S. pneumoniae* capsular serotype and the likelihood of isolation from a sterile anatomical site (blood, lungs or central nervous system) or from the nasopharynx

Fig. 4. Expression of *cpsA* and *in vitro* growth. Expression was assessed by real-time RT-PCR of RNA from clinical isolates cultured to OD₆₀₀ 0.6 in BHI. log₁₀(relative *cpsA* expression) was plotted against the length of lag phase during *in vitro* growth in BHI (Bättig *et al.*, 2006). There was a trend towards higher *cpsA* expression in strains with a longer lag phase ($P=0.07$).

(Sandgren *et al.*, 2004; Brueggemann *et al.*, 2004; Hanage *et al.*, 2005; Kronenberg *et al.*, 2006). The mechanism leading to this association is not understood.

The polysaccharide capsule protects against phagocytosis but hampers adherence to respiratory cells. Regulation of the amount of capsule produced depending on the anatomical niche might therefore be advantageous (Hammerschmidt *et al.*, 2005). Control of capsule expression is not well understood, except for the case of switching off capsule expression entirely by spontaneous sequence duplications or mutations in capsule genes (Waite *et al.*, 2001, 2003; Arrecubieta *et al.*, 1994), or apparent replacement of capsule genes with novel *aliB*-like ORFs (Hathaway *et al.*, 2004). However, in strains with a complete capsule operon, a role for tyrosine phosphorylation of CpsD, the product of the fourth capsule operon gene, in regulation of capsular polysaccharide production has been proposed (Bender *et al.*, 2003). In this study the role of transcription of the capsule operon was investigated. Since the capsule operon appears to be arranged as a single transcriptional unit (Morona *et al.*, 1997; Munoz *et al.*, 1997), transcription of the first gene of the capsule operon, *cpsA*, was measured. This study was limited to *in vitro* *cpsA* expression with quantification of capsule polysaccharide for only two isolates. We show that under *in vitro* conditions serotypes associated with invasive disease (expressed as the likelihood of isolation in the context of an invasive infection rather than from the nasopharynx) have on average higher *cpsA* expression than serotypes with high colonization prevalence. No difference was found in *cpsA* expression between isolates collected from a sterile site and those collected from the nasopharynx. This appears to indicate that the site of isolation does not play a role in *cpsA* expression. However, it must be borne in mind that the

bacteria have undergone growth *in vitro* before RNA isolation, which may have eliminated any differences in expression that were present *in vivo*.

The association between *cpsA* expression and the invasiveness of serotypes observed in this study was stronger when the OR data from a study of local Swiss epidemiology (Kronenberg *et al.*, 2006) were used rather than OR data from a previous study which represents the epidemiology in several other countries (Brueggemann *et al.*, 2004). This may reflect clonal differences within serotypes between geographical regions (Sandgren *et al.*, 2004; Hanage *et al.*, 2005). For example, clone-specific differences for invasiveness within serotype 1 have been observed (Brueggemann & Spratt, 2003). In the present study, using a clonally mixed strain collection, there was some variation in *cpsA* expression within serotypes between isolates with different PFGE patterns. However, to study this in detail, many isolates representing several clones of the same serotype would be needed to determine with certainty the effect of the genetic background of the isolates on *cpsA* expression.

In the current context of vaccine selection pressure it is especially important to identify serotypes with high invasive potential. We aimed to discover the differences between invasive and non-invasive serotypes, and suggest that one difference is their level of expression of the capsule gene *cpsA*. Although the obvious consequence of this might be a difference in the amount of capsule, it might also be that the capsule genes have some other role, such as in metabolism. As described recently by Bättig *et al.* (2006), the capsule genes also appear to affect growth *in vitro*, with invasive serotypes having a longer lag phase. In this study we show that the length of lag phase tends also to correlate with relative *cpsA* expression at OD₆₀₀ 0.6. If this translates into difficulty in growth in the nasopharynx, the bacteria could be upregulating *cpsA* expression as a response to stress in order to synthesize products essential for survival and growth, which may be unconnected with capsule production. However, care must be taken in extrapolating results of *cpsA* expression at one point in the growth curve to the length of the lag phase, and so further studies are required to clarify this relationship.

In this study with transparent clinical isolates the level of ambient oxygen had no effect on *cpsA* expression in either invasive or non-invasive serotypes. However, after invasion there is a predominance of the opaque phenotype (Weiser *et al.*, 2001) and anaerobic conditions cause an increase in the amount of capsule in opaque variants. As stated by Weiser *et al.* (2001), the bacteria would be exposed to the highest ambient oxygen levels when on the airway surface, but the oxygen tension encountered would be much lower during pneumonia, in blood or in the middle ear. It would be interesting to determine in further studies whether in opaque clinical isolates there is a change in the transcription of *cpsA* under anaerobic conditions and whether this differs between serotypes with high and low invasive potential.

In conclusion, *in vitro* expression of the first gene of the pneumococcal capsule operon, *cpsA*, under ambient oxygen concentrations correlates with the invasiveness of the serotype and inversely with the carriage prevalence of the serotype. No change in *cpsA* expression in response to a change in environmental oxygen concentration was observed in the transparent clinical isolates tested.

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