The genes encoding virulence-associated proteins and the capsule of *Streptococcus pneumoniae* are upregulated and differentially expressed *in vivo*

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The polysaccharide capsule of *Streptococcus pneumoniae* and several well-characterized virulence proteins are known to contribute to the pathogenesis of pneumococcal disease. However, there is a paucity of data on the expression of their respective genes *in vivo*. In this study, the relative abundance of the mRNA transcripts of the genes encoding pneumolysin (*ply*), pneumococcal surface protein A (*pspA*), pneumococcal surface antigen A (*psaA*) and choline-binding protein A (*cbpA*), and of the first gene of the capsular polysaccharide biosynthesis locus (*cps2A*), was measured in virulent type 2 pneumococci harvested from the blood of BALB/c mice at 12 h and 24 h following intraperitoneal infection. The mRNA levels were then compared, using relative quantitative RT-PCR, with those present in organisms grown in serum broth. The expression of *ply* was upregulated threefold at 12 h, and 10-fold at 24 h post-infection; the expression of *pspA* and *psaA* was upregulated threefold and fivefold, respectively, at 12 h post-infection. Interestingly, the expression of *pspA* was 36-fold higher at 24 h post-infection whereas the expression of *cps2A* was upregulated approximately fourfold at 12 and 24 h post-infection. However, *cbpA* mRNA levels remained comparable *in vivo* and *in vitro*. When organisms were grown in whole blood or THY broth, the relative expression of these genes in the two growth media also differed markedly. This work provides direct molecular evidence that known virulence-associated genes of *S. pneumoniae* are differentially expressed *in vivo*. Data on the relative expression of these genes in different growth media also suggests that the regulation of expression of these genes is highly complex and multifactorial.

Keywords: pneumococcus, pneumolysin, surface proteins, mRNA, quantitative RT-PCR

INTRODUCTION

Streptococcus pneumoniae is a very important human pathogen, causing a spectrum of diseases including pneumonia, meningitis, bacteraemia and otitis media (Musher, 1992; Tuomanen *et al.*, 1995). The incidence of pneumococcal disease is particularly high in children under the age of 5 years, the elderly and immunocompromised individuals (Garenne *et al.*, 1992; World Health Organization, 1997). *S. pneumoniae* is conservatively estimated to cause over 1 million deaths in children under 5 years of age in developing countries each year

(World Health Organization, 1997). Furthermore, the alarming rate at which this organism is acquiring resistance to multiple antimicrobials has exacerbated the high morbidity and mortality resulting from pneumococcal disease (Whitney *et al.*, 2000).

The polysaccharide (PS) capsule is strongly anti-phagocytic and is a *sine qua non* of pneumococcal virulence (Austrian, 1981). There are 90 recognized PS serotypes; synthesis of the serotype-specific capsular PS is encoded by distinct clusters of up to 20 tightly linked genes transcribed as single operons (Paton & Morona, 2000). Antibodies to the PS are highly protective, and the global impact of pneumococcal disease has led to the development of polyvalent PS and PS–protein conjugate

Abbreviation: PS, polysaccharide.

vaccine formulations. Although appreciable levels of success have been achieved with these vaccination strategies, the problems of serotype specificity of protection, geographical and temporal variations in serotype distribution, cost of vaccine formulations, and the possibility of nasopharyngeal replacement of vaccine serotypes by non-vaccine serotypes in vaccinated individuals still exist (Obaro, 2000). For this reason, we and others have been investigating the capacity of pneumococcal virulence proteins, administered either singly or in combination, to elicit non-serotype-dependent protection against pneumococcal disease (Alexander *et al.*, 1994; Briles *et al.*, 2000a, b; Ogunniyi *et al.*, 2000).

The pneumococcal virulence proteins which have received the greatest attention to date are the thiol-activated toxin pneumolysin (Ply) (for reviews see Boulnois *et al.*, 1991; Boulnois, 1992; Paton, 1996; Paton *et al.*, 1993), two choline-binding surface proteins called pneumococcal surface protein A (PspA) (Yother & Briles, 1992) and choline-binding protein A (CbpA) (also referred to as PspC or SpsA) (Brooks-Walter *et al.*, 1999; Hammerschmidt *et al.*, 1997; Rosenow *et al.*, 1997), and a metal-binding lipoprotein called pneumococcal surface antigen A (PsaA) (Berry & Paton, 1996; Dintilhac *et al.*, 1997; Paton, 1998). These proteins possess a range of biological activities, indicating that they act at different stages of the pathogenic process. Ply, for instance, has both direct cytotoxic and complement activation properties, mediated by different domains within the toxin (Boulnois *et al.*, 1991). Apart from directly damaging respiratory epithelium (Feldman *et al.*, 1990), the cytotoxic property accounts for inhibition of specific and non-specific immune responses (Ferrante *et al.*, 1984; Paton & Ferrante, 1983), as well as stimulation of the release of inflammatory cytokines from host cells (Houldsworth *et al.*, 1994). Direct activation of the classical complement pathway is the result of binding of Ply to the Fc region of IgG, which also contributes to inflammation and depletes serum opsonic activity (Mitchell *et al.*, 1991; Paton *et al.*, 1984). On the other hand, PspA interferes with complement activation and slows the clearance of pneumococci from the blood of infected mice (McDaniel *et al.*, 1987; Tu *et al.*, 1999). Hammerschmidt *et al.* (1999) have shown that PspA binds lactoferrin, and so it may also function by scavenging iron in the nasopharynx. CbpA is structurally related to PspA, and mediates adherence to cytokine-activated lung cells, as well as playing a major role in colonization of the nasopharynx in an infant rat model (Rosenow *et al.*, 1997). It has also been shown to specifically bind the secretory component of human secretory immunoglobulin A (Hammerschmidt *et al.*, 2000). PsaA forms part of an ABC-type manganese permease complex (Dintilhac *et al.*, 1997) and mutations in *psaA* have pleiotropic effects on various pneumococcal functions, including adherence, autolysis and virulence (Berry & Paton, 1996; Claverys *et al.*, 1999; Novak *et al.*, 1998).

Mutagenesis studies have shown that Ply, PspA, CbpA and PsaA contribute to virulence in a variety of animal

models of pneumococcal disease (Berry *et al.*, 1989, 1999; Berry & Paton, 1996; McDaniel *et al.*, 1987; Rosenow *et al.*, 1997). Moreover, mutagenesis of certain combinations of virulence factor genes results in additive attenuation, implying that their products function independently in virulence (Berry & Paton, 2000). However, it is not currently known whether any of these genes are coordinately regulated, or whether they are specifically up-regulated *in vivo*. Moreover, evidence for *in vivo* expression is largely confined to indirect serological studies (Lankinen *et al.*, 1999; Orihuela *et al.*, 2000; Rapola *et al.*, 2000; Virolainen *et al.*, 2000). In this study, we provide direct molecular evidence for *in vivo* expression of important pneumococcal virulence factors. The levels of mRNA transcript for each gene have been measured using relative quantitative RT-PCR analysis of total bacterial RNA isolated from the blood of mice at various times after intraperitoneal infection. This has permitted what is to our knowledge the first comparison of the relative kinetics of *in vivo* expression of proven pneumococcal virulence factors.

METHODS

Bacterial strain and growth conditions. The bacterial strain used in this study was D39, a virulent encapsulated type 2 *S. pneumoniae* strain which has been described previously (Avery *et al.*, 1944). For isolation of *in vitro*-derived total RNA, and infection of mice, bacteria grown overnight on blood agar were inoculated into serum broth (meat extract broth supplemented with 10% horse serum) and then grown statically for 3 h at 37 °C. For extraction of RNA, cells were harvested by centrifugation at 15 500 g for 5 min. For infection of mice, the density of the serum broth bacterial culture was adjusted to OD₆₀₀ 0.08, which corresponds to approximately 1×10^8 c.f.u. ml⁻¹. The bacteria were then diluted further to the required c.f.u. in serum broth. As an alternative source of *in vitro*-derived RNA, pneumococci were subcultured from blood agar into Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) and incubated statically at 37 °C to OD₆₀₀ 0.3. An aliquot (1.5 ml) of this culture was then centrifuged at 15 500 g for 5 min to pellet the bacteria. In another experiment, bacteria grown overnight on blood agar were subcultured into fresh whole blood obtained from naïve mice and incubated for 3 h at 37 °C.

Infection of mice and harvesting of bacteria for RNA isolation. Two groups of twenty-four 6–8-week-old male BALB/c mice were infected intraperitoneally with approximately 1×10^3 c.f.u. of D39. Mice were exsanguinated after 12 h or 24 h by cardiac puncture under deep anaesthesia, and blood obtained from each group of mice was pooled into sterile heparinized tubes on ice. The bacteria were harvested from the blood by initial centrifugation at 825 g at 4 °C for 5 min to pellet erythrocytes and leucocytes. The resulting supernatant, consisting of a mixture of plasma, bacteria and thrombocytes, was then centrifuged at 15 500 g at 4 °C for 5 min to pellet the bacterial cells. The supernatant was discarded and RNA was extracted from the bacterial pellet. The same procedure was used to harvest bacteria grown in fresh whole blood obtained from naïve mice.

RNA isolation. Total RNA was extracted from *in vitro*- and *in vivo*-harvested bacteria by a modification of the method of Mortier-Barrière *et al.* (1998). The bacterial pellet was resuspended completely in 300 µl prewarmed (65 °C) acid-

Table 1. Oligonucleotide primers

Primer	Sequence (5'→3')	Gene sequence reference*
<i>ply</i> (forward)	GATGGCAAATAAAGCAGTAAATGACT	Walker <i>et al.</i> (1987)
<i>ply</i> (reverse)	TGATGCCACTTAGCCAACAAATCG	
<i>pspA</i> (forward)	CAGAAGAATCTCCCGTAGCCAGT	Yother & Briles (1992)
<i>pspA</i> (reverse)	CTGGTGCTTTTTGTTTAGCTTCTTCTG	
<i>psaA</i> (forward)	GGTACATTACTCGTTCTCTTCTTTCT	Berry & Paton (1996)
<i>psaA</i> (reverse)	GTGTGGGTCTTCTTTTCCTTTTTTC	
<i>cbpA</i> (forward)	GAAAACGAAGGAAGTACCCAAGCA	Rosenow <i>et al.</i> (1997)
<i>cbpA</i> (reverse)	CTTTAACTTTCACATCGAACTCAGC	
<i>cps2A</i> (forward)	AGTGGTAACTGCGTTAGTCC	Iannelli <i>et al.</i> (1999)
<i>cps2A</i> (reverse)	CTGCCAAGTAAGACGAACTC	
16S rRNA (forward)	GGTGAGTAACGCGTAGGTAA	Bentley <i>et al.</i> (1991)
16S rRNA (reverse)	ACGATCCGAAAACCTTCTTC	

* References are for both forward and reverse primer in each pair.

phenol and incubated for 5 min at 65 °C. To this, 300 µl prewarmed NAES buffer (50 mM sodium acetate pH 5.1, 10 mM EDTA, 1% SDS) was added, and the mixture was incubated for another 5 min at 65 °C, with intermittent mixing. The mixture was cooled on ice for 1 min and the phases were separated by centrifugation at 15 500 g for 1 min. The aqueous phase was re-extracted twice with acid-phenol followed by two further extractions with chloroform. Sodium acetate was added to the resulting supernatant to a final concentration of 300 mM and RNA was then precipitated with 2 vols ethanol at -20 °C overnight. The resulting RNA pellet was washed briefly in 70% ethanol and then resuspended in 50 µl nuclease-free water. Recombinant RNasin ribonuclease inhibitor (Promega N251A) was added to a final concentration of 1 U µl⁻¹ and the RNA was then treated with 0.5 U µl⁻¹ RQ1 RNase-free DNase (Promega M610A) at 37 °C for 30 min. The RNA preparation was then treated with RQ1 DNase stop buffer (Promega M198A) to inactivate the DNase. The purity of the RNA preparation was confirmed by subjecting it to one-step RT-PCR with or without reverse transcriptase, using gene-specific primers (see Table 1). An aliquot of the total RNA sample was also electrophoresed on a 1% TAE-agarose gel to check for integrity, and then stored at -70 °C until required.

RNA quantitation. Levels of mRNA for *ply*, *pspA*, *psaA*, *cbpA* and *cps2A* were determined by one-step relative quantitative RT-PCR using the Promega Access RT-PCR system (Promega Biotech, cat. no. A1250). The specific primers used for the various RT-PCR assays are listed in Table 1. As an internal control, primers specific for the 16S rRNA were used. Each primer was used at a final concentration of 3.3 µM, except for 16S rRNA primers, which were used at a final concentration of 0.04 µM. This was necessary because of the high abundance of 16S rRNA in total RNA extracts. At the lower primer concentration, RT-PCR product intensity was linearly related to RNA concentration. To eliminate the possibility of interference between gene-specific and internal control primers, separate RT-PCR reactions were set up (on ice) from a master mix differing only in the constituent primers. The mix was then aliquoted into tubes held on ice and containing the respective upstream and downstream primers, and thoroughly mixed by gentle vortexing. Each mix was finally aliquoted into thin-walled 0.5 ml reaction tubes and placed in a thermal cycler. The RT-PCR cycling conditions comprised one cycle at

48 °C for 45 min (for first-strand cDNA synthesis) one cycle at 94 °C for 2 min (for AMV reverse transcriptase inactivation and RNA/cDNA/primer denaturation) followed by 30 cycles of PCR amplification comprising denaturation (94 °C for 30 s), primer annealing (60 °C for 30 s), and extension (68 °C for 45 s).

The linear range of amplification for each RNA preparation was determined separately under these conditions by removing replicate tubes from the thermal cycler at cycle 10 and at every subsequent fourth cycle. The reaction products were resolved by electrophoresis on a 2% TAE-agarose gel (Agarose LE, Roche Molecular Biochemicals) and stained with Sybr Gold (Molecular Probes). They were then scanned at a resolution of 50 µm at medium sample intensity on a Bio-Rad FX Molecular Imager connected to an external laser and quantified using Quantity One software. The cycle number was plotted against the log of signal intensity to determine the linear range of amplification for each mRNA species. Amplicon intensity in a given RNA extract was determined at a cycle number within the linear range for each species. Between RNA extracts, intensities of individual mRNA species were corrected with reference to that obtained for the internal 16S rRNA control.

RESULTS

Infection of mice and total RNA isolation

The intraperitoneal LD₅₀ of the virulent type 2 pneumococcus used in this study (strain D39) for BALB/c mice has been determined previously to be approximately 10² c.f.u., with death occurring approximately 3 days post-infection. At higher doses, death occurs much sooner (e.g. less than 12 h after infection with 10⁷ c.f.u.). Typically, the bacterial load reaches approximately 10⁹–10¹⁰ c.f.u. ml⁻¹ of blood just before death (Benton *et al.*, 1995; Berry *et al.*, 1999). In this study, the number of bacteria in whole blood at the time of collection was very similar in each case, reaching approximately 1.0–2.0 × 10⁹ c.f.u. ml⁻¹, with approximately 5 × 10⁸ c.f.u. ml⁻¹ recovered from plasma after the initial low-speed centrifugation to remove erythrocytes, etc. Bacteria were recovered from plasma by high-speed

Fig. 1. Agarose gel analyses of virulence gene RT-PCR products from RNA extracted from *S. pneumoniae* D39 obtained from *in vitro* (serum broth) culture and from the blood of infected mice at 12 h and at 24 h post-infection. Aliquots of RT-PCR products taken after 10, 14, 18, 22, 26, and 30 cycles (lanes 1–6, respectively), are shown for *ply*, *pspA*, *psaA*, *cbpA*, *cps2A*, and 16S rRNA as internal control. Samples were electrophoresed on a 2% agarose gel in TAE buffer and then stained with Sybr Gold.

centrifugation, with a final yield of approximately 10^8 c.f.u. from each ml. Total RNA was then extracted from these bacteria as described in Methods, as well as from bacteria grown to mid-exponential phase in serum broth.

Quantitative comparison of virulence factor mRNA levels

Relative quantitative RT-PCR was employed to compare the abundance of *cps2A* (the first gene in the type 2 PS biosynthesis locus), *ply*, *pspA*, *cbpA* and *psaA* mRNAs in total RNA extracts from pneumococci grown in serum broth, or isolated from the blood of mice 12 or 24 h post-infection (see Methods). This involved quantitation of RT-PCR products after 10, 14, 18, 22, 26, and 30 cycles, and for each assay, determining the period during which the rate of RT-PCR product accumulation was directly related to RNA concentration. By way of illustration, agarose gel analyses for the virulence gene and 16S rRNA RT-PCR products from serum broth, and 12 h and 24 h post-infection RNA preparations are shown in Fig. 1. mRNA levels were then expressed relative to that of 16S rRNA; 16S rRNA was selected as an internal control because rRNAs are known to be maintained at a constant level. The various primer pairs for the virulence genes and rRNA were designed to amplify cDNA fragments of comparable size to ensure similar amplification kinetics. This precludes performing RT-PCR for virulence gene transcripts and rRNA in the same tube. However, reaction tubes were prepared from aliquots of the same master mix to which the different primer pairs were added last. They were then amplified in the same PCR run and electrophoresed on the same gel.

Fig. 2. Relative mRNA concentrations of *ply*, *pspA*, *psaA*, *cbpA* and *cps2A* *in vitro*, and at the indicated times after intraperitoneal infection of mice. Between RNA extracts, intensities of individual mRNA species were corrected with reference to that obtained for the internal 16S rRNA control. Data points represent means $\pm$ SE of RT-PCR reactions performed on RNA extracts from bacteria harvested from pooled blood of groups of eight mice from three independent experiments.

The results obtained in the RT-PCR assays using *in vitro*-derived total RNA as template indicated that the mRNA of *cps2A* was the most abundant of the species being studied, followed by the mRNAs of *psaA*, *cbpA*, *ply* and *pspA*, in that order (Fig. 2). Similarly, the mRNA of *cps2A* was the most abundant of all the virulence genes in *in vivo*-derived total RNA at each of

Table 2. Relative *in vivo* expression of pneumococcal virulence genes

Gene	Fold increase relative to <i>in vitro</i> level at indicated times (significance relative to <i>in vitro</i> level)*	
	12 h	24 h
<i>ply</i>	2.8 (NS)	10.3 ($P < 0.05$)
<i>pspA</i>	3.1 (NS)	36.4 ($P < 0.005$)
<i>psaA</i>	4.9 ($P = 0.025$)	6.2 ($P < 0.025$)
<i>cbpA</i>	1.4 (NS)	1.1 (NS)
<i>cps2A</i>	3.6 ($P < 0.01$)	4.2 ($P < 0.05$)

* Determined using Student's *t*-test (one-tailed); NS, not significant.

Fig. 3. *In vitro* expression of *ply*, *pspA*, *psaA*, *cbpA* and *cps2A* in different culture media. Data points represent means $\pm$ SE of RT-PCR reactions performed on RNA extracts from bacteria harvested from each culture medium from three independent experiments.

the indicated times post-infection (Fig. 2). However, the mRNA levels of *pspA* and *ply* were higher than that of *cbpA* at 24 h post-infection. These results were not due to differences in the efficiency of the various amplification reactions, because each primer pair was designed to operate at identical annealing temperatures, and to direct amplification of a similar size fragment. Moreover, no differences in PCR product intensity were observed when *S. pneumoniae* D39 chromosomal DNA was used as template (result not shown). Differences were also seen in the concentration of each mRNA species in RNA extracts from *in vitro* versus *in vivo* cultures. The mRNA levels for *ply*, *pspA*, *psaA* and *cps2A* were elevated in bacteria harvested from the blood of mice 12 h and 24 h after intraperitoneal infection, compared with bacteria grown in serum broth. Upregulation was particularly pronounced for *pspA*; its relative mRNA concentration was 36-fold higher in blood at 24 h post-infection compared with that in serum broth (Table 2). However, *in vivo* expression of *cbpA* was similar to that seen *in vitro*. At 12 h, upregulation was statistically significant for *psaA*

and *cps2A*, while at 24 h, all genes were significantly upregulated except *cbpA* (Table 2).

Comparison of virulence factor mRNA levels in different growth media

The relative expression of the various virulence genes by bacteria grown in serum broth, fresh mouse blood and THY broth was then determined. For each of these culture conditions, bacteria were in mid-exponential phase of growth at the time of harvesting. In general, the expression of the virulence genes was lowest in bacteria grown in THY broth (Fig. 3). The expression of *psaA*, *ply*, and *pspA* was highest in bacteria grown in fresh blood whereas the levels of *cbpA* and *cps2A* mRNAs were highest in serum broth cultures.

DISCUSSION

The continuing high global morbidity and mortality caused by *S. pneumoniae*, in spite of the availability of antibiotics, and the problems identified with existing vaccination strategies, have prompted extensive research on the pathogenesis of pneumococcal disease. The importance of the PS capsule in virulence has been recognized for more than 70 years (Austrian, 1981), and more recent studies have resulted in the identification and characterization of numerous putative virulence proteins (Paton *et al.*, 1993, 1997). Roles for these proteins in pathogenesis have been proposed on the basis of mutagenesis studies in animal models (Berry & Paton, 1996, 2000; Brooks-Walter *et al.*, 1999), and several have been shown to be protective immunogens (Alexander *et al.*, 1994; Briles *et al.*, 2000a, b; Brooks-Walter *et al.*, 1999). Indeed, vaccines based on combinations of pneumococcal proteins may provide effective, non-serotype-dependent protection against this organism (Briles *et al.*, 2000a, b; Ogunniyi *et al.*, 2000). However, pathogenesis of pneumococcal disease is a complex process and involves a series of interactions between pathogen and host. During the course of an infection, the pneumococcus must adapt to a range of environments (e.g. the nasopharyngeal mucosa, lung mucosa, blood, cerebrospinal fluid, etc.) and optimal survival in any one niche may require expression of a distinct subset of the organism's armoury of potential virulence factors. However, direct molecular evidence for alteration in virulence gene expression *in vivo* has hitherto been lacking.

In the present study, we have measured and compared the levels of mRNA for four well-characterized virulence protein genes (*ply*, *pspA*, *psaA* and *cbpA*), as well as the first gene in the type 2 PS biosynthesis operon (*cps2A*) in pneumococci grown in broth culture or isolated from the blood of mice at different times after intraperitoneal infection. This was achieved using relative quantitative RT-PCR, with 16S rRNA as an internal standard. Twelve hours after intraperitoneal infection, the mRNA species of *ply*, *pspA* and *psaA* were up-regulated, unequivocally demonstrating an altered pattern of gene

expression *in vivo* vs *in vitro*. However, the pattern was not constant for the virulence genes during the course of infection. At 24 h, expression of *ply* and, significantly, *pspA*, was again increased, but expression of the other virulence factors was not greatly dissimilar to 12 h levels. These findings clearly show that these virulence genes are not coordinately expressed, under the control of a single global regulatory element.

Interpretation of the significance of differences in virulence-associated gene expression between *in vitro* and *in vivo* conditions is complicated, particularly since marked variation in gene expression between cells grown *in vitro* in THY versus serum broth were observed in the present study. Expression levels in the latter medium were used as a baseline for comparison with expression in blood *in vivo*, which seems appropriate given that virulence gene expression in pneumococci grown in serum broth was similar to that observed for bacteria grown in whole mouse blood *in vitro*. Thus, up-regulation of virulence gene expression in pneumococci in the blood of infected mice appears to be largely attributable to host factors as opposed to the nutritional parameters of blood. While the possibility that initial intraperitoneal growth may have triggered some of the changes in expression observed in mouse blood *in vivo*, the levels of *ply* and *pspA* mRNA are markedly greater in blood at 24 h compared with blood at 12 h post-infection.

Given what is known of the functions of the various virulence factors, it is perhaps not surprising that their genes are upregulated *in vivo*, particularly during the early stages of infection. None of these functions would be expected to confer any advantage on the pneumococcus during *in vitro* culture. *Ply* is known to be important for preventing the establishment of early non-specific host immune responses capable of limiting net exponential growth of pneumococci in the blood (Benton *et al.*, 1995). Thus, high initial levels of expression of *ply* may be critical. *PsaA* is a metal-binding lipoprotein with specificity for Mn^{2+} and possibly also Zn^{2+} (Dintilhac *et al.*, 1997; Lawrence *et al.*, 1998) and so sustained expression of *psaA* *in vivo* is consistent with an ongoing need to scavenge these metal ions from the blood.

The pattern of *pspA* expression was an interesting finding of this study. It was the least abundant mRNA species *in vitro*, but was up-regulated threefold at 12 h after intraperitoneal infection. One of the known functions of *PspA* is inhibition of complement-mediated clearance of pneumococci from the blood, by interference with deposition of C3b on the bacterial surface (Tu *et al.*, 1999). This property would be expected to be advantageous throughout the infectious process. Indeed, levels of *PspA* are reported to be higher in opaque variants of pneumococci, a phenotype associated with systemic virulence (Kim & Weiser, 1998). Remarkably, *pspA* mRNA levels increased approximately 36-fold at 24 h post-infection relative to levels detected in serum broth. Conversely, *cbpA* mRNA levels were similar to *in*

vitro levels at both 12 h and 24 h post-infection. *CbpA*'s primary function is believed to be that of an adhesin with an important role in nasopharyngeal colonization, and effects of mutagenesis of *cbpA* on systemic virulence were reported to be minimal (Gosink *et al.*, 2000; Ring *et al.*, 1998; Rosenow *et al.*, 1997). *CbpA* has recently been shown to interact with the human polymeric immunoglobulin receptor, thereby facilitating invasion of the mucosa (Zhang *et al.*, 2000). However, we have shown that a *S. pneumoniae cbpA/ply* double mutant has massively attenuated systemic virulence compared with either *cbpA* or *ply* single mutants (Berry & Paton, 2000). These latter findings are consistent with a role for *CbpA* in the post-adherence/invasion stages of the pathogenic process. Moreover, *CbpA* has also recently been shown to be capable of binding to C3 (Smith & Hostetter, 2000), and Brooks-Walter *et al.* (1999) have suggested that *CbpA* and *PspA* could perform analogous functions *in vivo*. This is supported by their observation that mutagenesis of *pspA* has a much lesser impact on systemic virulence in *S. pneumoniae* strains which contain *cbpA* than in those which lack it, implying a degree of functional complementation. Thus, the poor expression of *cbpA* observed in the present study could be offset by the marked increase in expression of *pspA*.

The precise manner in which the PS contributes to virulence is not fully understood, although it is known to have strong anti-phagocytic properties in non-immune hosts (Paton & Morona, 2000). The capsular PS of the majority of serotypes is highly charged at physiological pH and this may directly interfere with interactions with phagocytes. Pneumococcal cell wall teichoic acid is capable of activating the alternative complement pathway. In addition, antibodies to this and other cell surface constituents (e.g. surface proteins), which are found in most adult human sera, may result in activation of the classical complement pathway, as does interaction of the teichoic acid with C-reactive protein. However, the PS forms an inert shield, which appears to prevent interaction of either the Fc region of IgG or iC3b fixed to deeper cell surface structures from interacting with receptors on phagocytic cells (Musher, 1992). Thus, in systemic challenge models such as that used in the present study, maximal expression of PS might be expected to be beneficial to the pneumococcus at all stages of the infection. However, studies in nasopharyngeal colonization models suggest that high encapsulation might interfere with the interaction between other surface molecules (e.g. *PspA* and *CbpA*) and the host epithelium (Kim & Weiser, 1998; Talbot *et al.*, 1996).

We believe this to be the first study in which pneumococcal virulence gene mRNAs have been directly quantified from bacteria present in the blood of infected mice. The only previous study addressing *in vivo* pneumococcal virulence gene expression examined mRNA levels in type 3 pneumococci grown in sealed dialysis bags implanted in the murine peritoneal cavity (Orihuela *et al.*, 2000). However, this is a suboptimal *in vivo* surrogate, because it prevents interactions between pathogen and host cells, or host molecules larger than

the dialysis bag pore size (25 kDa cutoff). Using Northern blotting, Orihuela *et al.* (2000) detected a 2.8-fold stimulation in *ply* expression at 8 h, compared with bacteria grown *in vitro*, but intraperitoneal up-regulation of *pspA* expression was not detected. Expression of a gene from the type 3 PS biosynthesis locus (*cap3A*) was also increased 2.2-fold, although we observed slightly higher levels in the present study for *cps2A*. We have now demonstrated that *cps2A* and three of the key virulence protein genes, *ply*, *pspA* and *psaA*, are up-regulated *in vivo*. Moreover, there are marked differences in the patterns of expression of the various genes at different times post-infection, although little is known of the mechanism whereby this occurs. Jakubovics *et al.* (2000) have recently described a *Streptococcus gordonii* gene *scaR*, which encodes a metalloregressor-like protein that regulates the expression of a homologue of PsaA (the Sca Mn²⁺ permease). A homologue of this gene, designated *psaR*, is known to be present in the genome of *S. pneumoniae*, but to date a role in regulation of the *psa* operon has not been demonstrated. Establishing that certain key virulence genes respond to host signals is a major step forward; identifying these as markers will assist in studies of the molecular genetics of virulence gene regulation in this important human pathogen. However, the finding that the relative expression of these virulence genes differed between organisms grown *in vitro* in serum broth, and those grown *in vivo*, or in different *in vitro* media (whole blood or THY broth) suggests that the expression of these genes is highly complex and multifactorial.

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