Differential expression of key pneumococcal virulence genes *in vivo*

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Few studies have examined *in vivo* virulence gene expression in *Streptococcus pneumoniae*. In this study, expression of key pneumococcal virulence genes *cbpA*, *pspA*, *ply*, *psaA*, *cps2A*, *piaA*, *nanA* and *spxB* in the nasopharynx, lungs and bloodstream of mice was investigated, following intranasal challenge with the serotype 2 strain D39. Bacterial RNA was extracted, linearly amplified and assayed by real-time RT-PCR. At 72 h, *cbpA* mRNA was present at higher levels in the nasopharynx and lungs than in the blood. At this time-point, the mRNAs for *PspA* and *PiaA* were most abundant in the nasopharynx, whereas no significant difference in gene expression between niches was observed for *ply*, *psaA* and *cps2A*. Both *nanA* and *spxB* mRNAs were present in higher amounts in the nasopharynx than in the lungs or blood. These findings illustrate the dynamic nature of pneumococcal virulence gene expression *in vivo*.

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a globally significant pathogen, responsible for invasive diseases such as pneumonia, bacteraemia and meningitis (Paton, 1998). The pneumococcus asymptomatically colonizes the nasopharynx, and such carriage is considered essential for subsequent development of disease in susceptible individuals (particularly infants, the elderly, and the immunocompromised). Disease commonly occurs following the aspiration of bacteria from the nasopharynx into the lungs, followed by colonization of the pulmonary epithelium and subsequent development of pneumonia. From this niche, the pneumococcus can invade the bloodstream and cause sepsis. Alternatively, bacteraemia can occur, following direct translocation from the nasopharyngeal epithelium into underlying tissues. The gene regulatory mechanisms involved in transition between, and survival in, these distinct host niches are poorly understood. This has primarily been due to technical difficulties in harvesting sufficient quantities of pneumococci from an animal model to perform accurate and quantitative RNA assays, particularly from niches such as the nasopharynx, in which bacteria exist asymptomatically and in low numbers.

Several studies have been conducted in recent years comparing *in vivo* and *in vitro* pneumococcal gene expression. Orihuela *et al.* (2000) used Northern blotting to assess virulence gene mRNA levels in type 3 pneumococci grown in sealed dialysis bags implanted in the murine peritoneal cavity. In another study, differences in gene expression between virulent type 2 pneumococci harvested from the blood of mice infected intraperitoneally and those grown in serum broth were examined using semi-quantitative

RT-PCR (Ogunniyi *et al.*, 2002). More recently, *in vivo* studies have used microarray technology to quantitate *S. pneumoniae* transcript abundance in the blood of infected mice and the cerebrospinal fluid of infected rabbits (Orihuela *et al.*, 2004b).

The present work is the first to evaluate *in vivo* changes in pneumococcal gene expression during the natural progression of disease from colonization of the nasopharynx to invasion of the lungs and blood. We examined the mRNA expression of genes encoding eight virulence factors considered important in promoting carriage or disease: the choline-binding proteins *cbpA* and *pspA*, the pneumococcal cytotoxin pneumolysin (*ply*), the first gene of the capsular polysaccharide biosynthesis locus (*cps2A*), one of the three known pneumococcal neuraminidases (*nanA*), ion transporters (*psaA* and *piaA*), and pyruvate oxidase (*spxB*).

METHODS

Challenge of mice and harvesting of pneumococci. Outbred 5- to 6-week-old female CD-1 mice (obtained from the Institute of Medical and Veterinary Science, Adelaide, South Australia) were intranasally infected with the type 2 pneumococcal strain D39 (Avery *et al.*, 1944). Two separate experiments were performed, as follows. Before infection, bacteria were grown statically at 37 °C in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY broth) to OD₆₀₀ 0.25 (equivalent to approximately 1×10^8 c.f.u. ml⁻¹). Mice were anaesthetized by intraperitoneal injection of Nembutal (pentobarbitone sodium, Rhone-Merieux) at a dose of 66 micrograms per gram body-weight, and challenged intranasally with approximately 10^7 bacteria suspended in PBS. The challenge dose was confirmed by serial dilution and plating of the inocula on blood agar. Previously, we had established that mice infected intranasally with $1-2 \times 10^7$ c.f.u. of D39 usually succumb rapidly to the infection >72 h after challenge (unpublished results).

Therefore, 72 h was chosen as the end-point of infection in this work, in order to maximize the number of pneumococci recovered from the tissues. At this time-point, eight mice were sacrificed by CO₂ asphyxiation, and nasopharyngeal washes, and lung and blood samples were collected using an adaptation of the protocol described by Wu *et al.* (1997). Briefly, the nasopharynx of each mouse was washed with 1 ml 0.5% trypsin solution in PBS, and the washings were placed on ice until further use. Before perfusion, a sample of blood was recovered from the heart and placed on ice until further use. Mice were subsequently perfused with sterile PBS to remove blood-borne bacteria from the lungs. Lungs were removed and homogenized on ice in 2 ml sterile PBS using a tissue homogenizer (Cat X120, Germany). To separate pneumococci from host cells, lung homogenates and blood samples were centrifuged at 855 g for 6 min at 4 °C, as described previously (Ogunniyi *et al.*, 2002). Nasopharyngeal washes, and lung and blood supernatants were subsequently centrifuged at 15 500 g for 2 min at 4 °C, and the bacterial pellet was stored at -80 °C until further processing. Prior to pelleting harvested bacteria, 40 µl was removed, serially diluted in PBS and plated onto blood agar in order to enumerate pneumococci present in the sample, and to determine the presence, if any, of contaminating microflora. Blood plates were incubated at 37 °C in 95% air/5% CO₂ overnight.

Extraction of RNA from bacteria. RNA was isolated from bacterial pellets with acid-phenol/chloroform/isoamyl alcohol (125:24:1, pH 4.5; Ambion, catalogue no. 9722) essentially as described previously (Ogunniyi *et al.*, 2002). The extract was then precipitated at -80 °C overnight in the presence of 40 ng glycogen µl⁻¹ (Sigma 1767). Subsequently, the preparation was treated with 10 U RNase-free DNase (Roche) at 37 °C for 30 min in the presence of 1 U µl⁻¹ recombinant RNasin ribonuclease inhibitor (Promega N251A), after which RQ1 DNase stop buffer (Promega M198A) was added to inactivate the DNase. The purity of the RNA preparation was confirmed by one-step RT-PCR with or without reverse transcriptase, using 16S rRNA-specific primers, and the products were visualized after electrophoresis on a 2% TBE/agarose gel. In all cases, a PCR product was only seen in the presence of reverse transcriptase. RNA samples from a specific niche from four to five mice were pooled, based on the number of c.f.u. recovered (extracts of samples with low bacterial counts were not included) and also on the absence of contaminating bacteria, and then purified further using a Qiagen RNeasy minikit. RNA obtained from lung homogenates was further enriched for prokaryotic RNA using the MICROBEnrich kit (Ambion). The amount of RNA recovered following purification/enrichment was determined by OD_{260/280} measurements.

Linear amplification of mRNA. Previously, *in vivo* RNA studies for bacteria such as *S. pneumoniae* have been restricted by the amount of bacteria harvested from the animal, and therefore the yield of RNA obtained. This problem was circumvented by using a novel, advanced, RNA linear amplification kit, SenseAMP Plus (Genisphere), which employs terminal transferase to synthesize a poly-T tail onto prokaryotic cDNA. Linear amplification is then driven by a T7 phage promoter, which is incorporated at the end of a synthetic poly-A primer. A second round of amplification was performed for all nasopharyngeal samples in order to obtain sufficient quantities of RNA for analysis by real-time RT-PCR. To determine the integrity of the second-round amplification, primary and secondary amplified RNA samples were compared using real-time RT-PCR. Analysis of the data obtained indicated a high correlation coefficient, and no significant difference was obtained between the amplifications, when subjected to Student's *t* test (data not shown).

RESULTS

Until recently, evidence for expression of virulence factors of pneumococci has been largely limited to indirect serological studies. In this work, we examined *in vivo* changes in pneumococcal gene expression during the natural progression of disease from colonization of the nasopharynx to systemic disease in outbred CD-1 mice. Three niches were examined in this study: the nasopharynx, the lungs and the blood. At 72 h post-infection, the mean c.f.u. of pneumococci harvested from the nasopharynx was approximately 6×10^4 per mouse; in the lungs, the mean c.f.u. was 1×10^6 , whereas approximately 2×10^7 c.f.u. ml⁻¹ bacteria was recovered from the blood. These values were consistent between the two experiments.

Differential expression of pneumococcal virulence genes *in vivo*

We examined the differential expression of the characterized virulence genes *cbpA*, *pspA*, *ply*, *psaA*, *cps2A*, *piaA*, *nanA* and *spxB*. The abundance of each mRNA present in amplified RNA recovered from nasopharyngeal, lung and blood-borne pneumococci was quantified by real-time RT-PCR. Gene-specific LUX fluorogenic primer sets labelled with JOE were designed using the Invitrogen primer designer software, employing primers specific for 16S rRNA as an internal control (Table 1). Primer-pair efficiency was analysed using varying concentrations of *in vitro*-derived template, and the CT values obtained corresponded to the expected relative concentrations of template. The optimal concentrations of primers used in these studies were determined empirically in accordance with the manufacturer's instructions. For relative quantification, total RNA for 16S rRNA quantification was diluted 1/400 before real-time RT-PCR, whereas for mRNA quantification, total RNA was tested neat. Real-time RT-PCR was performed on a Rotorgene RG-2000 thermocycler (Corbett Research, Mortlake, NSW, Australia) using the Superscript III One-Step RT-PCR system (Invitrogen), according to the manufacturer's instructions. Quantitative fold differences for each transcript were determined using the $2^{-\Delta\Delta CT}$ method described by Livak & Schmittgen (2001). Amplification data for each gene were then compared to that obtained for the 16S rRNA control, and presented as a relative fold increase/decrease between niches (Table 2). Data for each independent experiment were analysed relative to the level of the respective transcript in *S. pneumoniae* D39 grown in THY broth to OD₆₀₀ 0.25 (Fig. 1).

The trends in gene expression for *cbpA*, *pspA*, *cps2A*, *piaA*, *ply*, *nanA* and *spxB* were consistent at 72 h post-challenge between the two replicate experiments. The *cbpA* mRNA was detected at the highest levels in bacteria recovered from the nasopharynx and lungs, and at lower levels in bacteria from the blood. Similarly, the level of *pspA* mRNA was highest in bacteria harvested from the nasopharynx and blood, with lower levels detected from pneumococci recovered from the lungs. In contrast, *cps2A* was expressed at a relatively

Table 1. Oligonucleotides used in this study

Primer	Sequence (5'→3')	R6 annotation*
16S F (JOE)	CAACATCACCGCATAAGAGTGGATG(JOE)TG	sprr01
16S R	CAACGCAGGTCCATCTGGTA	
<i>cbpA</i> F (JOE)	GATCGTTTGGTTGGGTAGTTACGACGA(JOE)C	spr1995
<i>cbpA</i> R	GCAGAAGCTAAGAAGAAGGTTGAA	
<i>pspA</i> F (JOE)	CTAGCGAACTGAGGAGAAAGCCGCGC(JOE)AG	spr0121
<i>pspA</i> R	GGCTAGATACGCTTGTGAACTGC	
<i>ply</i> F (JOE)	CAACGAAGGATAGAGGCGACTGTCTG(JOE)TG	spr1739
<i>ply</i> R	GAAAGAAAGAAGCGGAGCTTG	
<i>psaA</i> F	GAACCACTTCCTGAAGACGTTAAG	spr1494
<i>psaA</i> R (JOE)	GAAACACAATCATTGCCACCTGT(JOE)TC	
<i>cps2A</i> F (JOE)	CAACTCGCGGGCATTATGGAG(JOE)TG	spr0314
<i>cps2A</i> R	GAAGTGAAGTTCAATCGCACATAG	
<i>piaA</i> F (JOE)	CAGCACAAGCAAATTACGGTGTAAAGTGC(JOE)G	spr0934
<i>piaA</i> R	TCGTCAAATAGGTTAGCTTTACCA	
<i>nanA</i> F (JOE)	GACGTTGAGCAGTGGTATTTGGAACG(JOE)C	spr1536
<i>nanA</i> R	CCGAAAGTTGAGTTTCATTTGC	
<i>spxB</i> F (JOE)	CACCGGCGACTCACTTGATTAACGG(JOE)G	spr0642
<i>spxB</i> R	TTAACTGGACGTGATCCAAGG	

*Primer sequences derived from the *S. pneumoniae* R6 genome (Hoskins *et al.*, 2001), as deposited in the TIGR Comprehensive Microbial Resource.

constant level in the nasopharynx, lungs and blood. The levels of both *nanA* and *spxB* transcripts were higher in bacteria recovered from the nasopharynx than the lungs or blood. However, *psaA* did not show a consistent difference in expression between niches. The combined data from both *in vivo* experiments and two *in vitro* samples are also depicted as adjusted mRNA concentration, by comparing the CT values for each gene relative to that of 16S rRNA in the same sample (Fig. 2). The results indicate that the mRNA of *spxB* was the most abundant *in vitro*, followed by that of *ply* and *cps2A*, with *nanA* mRNA being the least abundant. In the nasopharynx, the mRNA of *ply*, followed

by that of *cps2A*, *pspA* and *psaA*, was highly abundant relative to 16S mRNA, whereas *nanA* mRNA was the least abundant. In the lungs, however, the mRNA of *cps2A* was the most abundant, followed by that of *ply* and *psaA*. Again, *nanA* mRNA was the least abundant. In the blood, the mRNAs of *cps2A*, *ply*, *pspA*, *psaA* and *piaA* were present in high quantities, *cbpA* mRNA was present at a lower level, while *nanA* mRNA was the least abundant.

DISCUSSION

Whilst for most individuals *S. pneumoniae* is carried asymptotically in the nasopharynx, in susceptible hosts the pathogen can become invasive and cause diseases such as pneumonia, sepsis, meningitis and otitis media. The mechanisms of regulation of key virulence genes required for the survival and growth of the pneumococcus in the different niches that it occupies are poorly understood. Research in this area has been limited due to the inherent difficulties of isolating sufficient quantities of pure and intact bacterial RNA from infected host tissues. Recently, we developed an assay for relative quantitative RT-PCR analysis of total bacterial RNA isolated from the blood of mice at various times after intraperitoneal infection, and the levels of mRNA transcript for several virulence genes were measured. This permitted the first comparison of the relative kinetics of *in vivo* expression of proven pneumococcal virulence factors (Ogunniyi *et al.*, 2002). As an extension of that work, we have now employed a novel, advanced, linear amplification procedure to amplify the low-yield RNA obtained from our *in vivo* model. This has enabled us to perform real-time

Table 2. Differential expression of virulence genes in various niches

Values shown are fold increase in mRNA concentration (mRNA concn_{niche 1}/mRNA concn_{niche 2}). Exp, experiment.

Gene	Nose/lungs		Nose/blood		Lungs/blood	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
<i>cbpA</i>	1.2	3.7	2.2	6.5	1.9	1.7
<i>pspA</i>	6.5	3.8	2.0	1.5	0.3	0.4
<i>ply</i>	1.3	2.8	1.8	1.8	1.4	0.7
<i>psaA</i>	2.5	0.9	2.4	0.9	1.0	1.0
<i>cps2A</i>	0.7	0.6	1.0	1.2	1.4	1.6
<i>piaA</i>	3.1	3.1	2.3	1.8	0.7	0.6
<i>nanA</i>	22.8	15.2	56.6	9.6	2.5	0.6
<i>spxB</i>	4.3	5.6	5.5	4.1	1.3	0.7

Fig. 1. Virulence gene mRNA concentrations in various *in vivo* niches, relative to concentrations *in vitro*. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA control. Quantitative fold differences for each transcript were determined using the $2^{-\Delta\Delta CT}$ method described by Livak & Schmittgen (2001). Data are means $\pm$ SD of triplicate reactions for each gene transcript. Data for two replicate experiments are presented separately.

RT-PCR on the samples recovered from the nasopharynx, lung and bloodstream of infected mice, thereby providing an insight into the behaviour of the pneumococcus at the transcriptional level in these distinct host niches.

In this study, we examined several pneumococcal virulence genes previously identified as being important in colonization and/or invasive disease. There was differential expression of *cbpA*, *pspA*, *piaA*, *nanA* and *spxB* in the various

Fig. 2. Virulence gene mRNA concentrations relative to those of 16S rRNA. The adjusted mRNA concentration for the duplicate *in vivo* experiments and the *in vitro* standard was calculated as $2^{-\Delta CT}$ for each virulence gene, where ΔCT represents the CT value of the gene subtracted from the CT value of the 16S rRNA control, as determined by real-time RT-PCR (Livak & Schmittgen, 2001), corrected for the difference in RNA template dilution. Adjusted values for each experiment were combined and graphed. Data are means $\pm$ SD for the combined experiments.

niches when compared to *in vitro* levels. However, the expression of *ply*, *psaA* and *cps2A* *in vivo* did not appear to change significantly from the expression observed when grown in THY. CbpA, a highly conserved choline-binding protein, is thought to be important in mediating adherence to both the nasopharyngeal epithelium and activated lung epithelial and endothelial cells (Rosenow *et al.*, 1997). In addition, *in vitro* interactions have been reported between CbpA and the human polymeric immunoglobulin receptor (hpIgR) present on nasopharyngeal epithelium (Zhang *et al.*, 2000). A deficiency in CbpA has been shown to reduce the ability of mutant pneumococci to invade nasopharyngeal cells by over 90 % compared to the parent strain, suggesting that pneumococci gain access to the interior of the cell by subversion of hpIgR. Data from the current study indicate that *cbpA* mRNA is present at elevated levels in the nasopharynx and lungs compared to the bloodstream, consistent with the earlier findings. This finding is also consistent with a recent study (Orihuela *et al.*, 2004a) that demonstrated that CbpA is not required for the entry of pneumococci into the bloodstream from the lungs, nor for survival in the blood. On another note, CbpA has also been shown to interact with factor H and C3 (Dave *et al.*, 2001; Janulczyk *et al.*, 2000), and to be important in a mouse sepsis model (Iannelli *et al.*, 2004), suggesting a dual role for CbpA in colonization (adherence) and in systemic disease. This might explain why in this study, the expression of *cbpA* was also significantly upregulated in the lungs and blood, compared to expression *in vitro*.

Iron is essential for pneumococcal growth and survival, as it is for other pathogenic bacteria. PspA, another choline-binding protein, has been shown to bind lactoferrin, an iron-sequestering glycoprotein, at respiratory mucosal sites. The binding of lactoferrin to pneumococci is believed to provide a way for the bacteria to interfere with host immune functions or to aid in the acquisition of iron at the site of infection (Hammerschmidt *et al.*, 1999; Håkansson *et al.*, 2001; Ren *et al.*, 2003; Shaper *et al.*, 2004). However, pneumococci producing a truncated form of PspA incapable of binding lactoferrin have been shown to successfully colonize the human nasopharynx (McCool *et al.*, 2002). Therefore, the relative importance of lactoferrin binding by pneumococci is not certain. The results of our *in vivo* experiments suggest enhanced levels of *pspA* in the nasopharynx compared to the lungs, indicating that PspA is important for colonization of the nasopharynx. This study also demonstrated a higher level of *pspA* transcript in bacteria recovered from the blood of infected mice than from the lungs, consistent with the second function of PspA in preventing complement-mediated opsonization of blood-borne pneumococci (Tu *et al.*, 1999; Ren *et al.*, 2003). Similar results have been obtained by other groups using microarray analysis, which has shown that *pspA* transcription appears to be upregulated both in the blood and in Detroit cells (Orihuela *et al.*, 2004b).

Another protein considered to be important in iron

acquisition is PiaA, which is encoded on an iron-uptake ABC-transporter complex locus (Brown *et al.*, 2001). Although the contribution of PiaA to pneumococcal virulence is not certain, immunization with PiaA has been shown to provide a similar degree of protection to PdB (pneumolysin toxoid) against systemic challenge with *S. pneumoniae*, and is therefore considered a potential vaccine candidate (Brown *et al.*, 2001). We have shown that *piaA* mRNA is present at a higher level in the nasopharynx than in the other niches at 72 h following challenge. The level was approximately threefold greater in the nasopharynx than in the lungs, and approximately twofold greater in the nasopharynx than in the blood.

The expression of *psaA* was consistently high, but not significantly different among the niches examined in this study. PsaA is a metal-binding lipoprotein with specificity for Mn^{2+} , and possibly also for Zn^{2+} (Dintilhac *et al.*, 1997; Lawrence *et al.*, 1998). It has also been shown that mutations in *psaA* have pleiotropic effects on various pneumococcal functions, including resistance to oxidative stress, adherence and virulence (Berry & Paton, 1996; Claverys *et al.*, 1999; Novak *et al.*, 1998; Tseng *et al.*, 2002; McAllister *et al.*, 2004). In addition, immunization with PsaA has been found to confer significant levels of protection against nasopharyngeal carriage (Briles, 2000a, b; Palaniappan *et al.*, 2005). The sustained expression of *psaA* *in vivo* is therefore consistent with an ongoing need to scavenge metal ions from the host, an essential requirement for virulence of pneumococci. This result is also in agreement with microarray data obtained by Orihuela *et al.* (2004b), which demonstrates that PsaA is upregulated to a similar extent in the blood of infected mice and in cultured Detroit-562 nasopharyngeal cells, compared to growth in C + Y media.

Pneumolysin is an important virulence factor, contributing to multiple stages of the pathogenic process, and it is also involved in eliciting an immune response from the host. The cytotoxic characteristic of pneumolysin facilitates progression of disease by inhibiting ciliary beating in the human respiratory epithelium, thus augmenting the migration of bacteria to the lungs (Boulnois *et al.*, 1991). It also acts by disrupting tight junctions between epithelial cells, which may provide an alternative pathway by which the pneumococcus infiltrates the bloodstream (Steinfort *et al.*, 1989). Additionally, numerous studies have indicated that pneumolysin is important in the development of sepsis, with mutants lacking pneumolysin showing reduced replication and survival in the bloodstream of infected animals (Benton *et al.*, 1995; Berry *et al.*, 1999). The current study showed a higher level of *ply* mRNA in the nasopharynx than the blood, which was consistent between the two experiments conducted. This observation was interesting considering the importance of pneumolysin in sepsis, perhaps indicating that pneumolysin production is regulated at a post-transcriptional level, as suggested by Kwon *et al.* (2003). Alternatively, the production of pneumolysin may be regulated independently of environmental stimulus, with

the niche influencing the release of the toxin rather than its transcription or translation.

Immunoelectron microscopy has been used elsewhere to show that transparent pneumococci, the phenotype commonly recovered from the lungs and nasopharynx, possess a significantly lower content of capsular polysaccharide than opaque bacteria, which predominate in the bloodstream (Kim *et al.*, 1999). It was interesting to observe that there did not appear to be significant regulation of expression of the capsule locus, as determined by examining the relative levels of *cps2A* mRNA, suggesting that the pneumococcal capsule may be regulated at a post-transcriptional level. These results support previous work published from our laboratory that suggest that the level of encapsulation is determined by autophosphorylation of CpsD, thereby allowing rapid assembly when the pathogen is exposed to a new environment, such as the bloodstream, in which the anti-phagocytic properties of the capsule are highly desirable (Morona *et al.*, 2000).

The expression of *nanA* was significantly elevated in the nasopharynx of infected mice compared to the other niches examined. NanA has been shown to have a role in facilitating bacterial adherence by removing terminal sialic acid residues from glycoconjugates. Additionally, NanA is able to desialylate the cell surface of *Neisseria meningitidis* and *Haemophilus influenzae* (Ram *et al.*, 1998; Shakhnovich *et al.*, 2002), as well as antibacterial components of human airway secretions (King *et al.*, 2004), potentially reducing pneumococcal clearance whilst promoting the clearance of competing bacteria. Our results provide further support for an important role for NanA in colonization of the nasopharynx by pneumococci.

Pyruvate oxidase (SpxB) is involved in the production of H₂O₂, which is proposed to have multiple roles in pathogenesis. H₂O₂ has been implicated in promoting carriage, as it has antimicrobial effects against competing bacteria *in vitro*, and has previously been shown to be expressed at higher levels in an oxygen- and CO₂-rich environment (Pericone *et al.*, 2000). In addition, the presence of H₂O₂ slows ciliary beating, thereby promoting pneumococcal progression to the lungs. This work indicated elevated levels of *spxB* in pneumococci harvested from the nasopharynx compared to samples recovered from the lungs and bloodstream, which is consistent with its putative roles in carriage and transition to the lungs.

In conclusion, we demonstrate the differential expression of several characterized pneumococcal virulence factors between colonizing and invading pneumococci during progression of disease. This is a new area of *in vivo* RNA research, not only in the pneumococcal field, but also in bacterial pathogenesis for many commensal organisms. The relative expression of these genes was found to support their putative roles in pathogenesis, and suggests which proteins might be more appropriate as vaccine antigens against nasal carriage or invasive pneumococcal disease.

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