Chapter

Streptococcus pneumoniae- Inotrope Interactions: a Contributory Factor in the Development of Pneumococcal Pneumonia?

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Abstract

Host signals have a major impact on the bacterial phenotype. One of them is endogenously produced catecholamine stress hormones, also used therapeutically as inotropes. Recent work form our laboratories have found that stress hormones can markedly increase bacterial growth and virulence. In this report we show that *Streptococcus pneumoniae*, a major cause of community acquired and nosocomial pneumonia, is highly inotrope responsive; therapeutic levels of norepinephrine stimulated pneumococcal growth which mechanistically we showed involved binding of serum transferrin and inotrope-mediated uptake of transferrin iron. Inotrope exposure also markedly altered expression of genes involved in metabolism and virulence. Analysis of the response of *pspC* and *pspA* mutants to the stress hormone showed them to have a central involvement in the catecholamine response mechanism. Collectively, our evidence suggests that catecholamines may act as signals for the pneumococcal transition from colonization to invasion mode, which is key to its capacity to cause life-threatening pneumonia, septicaemia and meningitis.

Introduction

Streptococcus pneumoniae is a major cause of otitis media, bacterial meningitis, septicaemia and community and hospital acquired pneumonia [1]. As well as being a potentially deadly pathogen, the pneumococcus often resides in the human nasopharynx without causing harm, a situation known as carriage. Therefore two fundamental but so far unanswered questions arise from consideration of pneumococcal carriage and the range of diseases it causes. Which host signals trigger transition of the pneumococcus to a pathogenic state, and how does the bacterium sense, process and respond to these signals during the infection in order to modulate its virulence in different tissue sites?

Increasing numbers of studies of infectious bacteria are suggesting that the neuroendocrine (stress hormone) status of a host may

determine the outcome of an infection [2]. The recognition that stress hormone release leads to increased risk of infection has come from the finding that stress –associated chemicals negatively modulate immune function [3], and to their impact on the growth and virulence of bacteria [2] Catecholamines have been shown to augment the growth of species including *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Campylobacter jejuni* and *Bordetella bronchiseptica* [4]. The growth stimulating effect of catecholamines has been largely related to the catechol-containing moiety forming a complex with the iron within transferrin (Tf) or lactoferrin (Lf) which weakens Fe binding and so enables bacteria to acquire the normally inaccessible complexed-Fe [5]. Catecholamines have also been shown to directly modulate bacterial virulence. For instance, norepinephrine (NE) stimulated the inflammatory and secretory responses caused by *E. coli* O157:H7, and augmented the microbe's attachment to intestinal mucosa [6]. NE increased both the cellular cytotoxicity and enterotoxicity of infection caused by *Vibrio parahaemolyticus* and up-regulated the expression of type III secretion system-1 genes [7]. Catecholamine inotropes used in the treatment of acutely ill patients (dopamine and epinephrine) have also been shown to increase staphylococcal and pseudomonad biofilm formation and promote recovery from antibiotic damage [2,8-9].

Most of our knowledge on bacteria-catecholamine interactions originated from the studies of Gram negative gut pathogens, and relatively little is known about the interaction of Gram-positive bacteria with catecholamines. In particular, the infection significance of *S. pneumoniae*-catecholamine interactions is unclear in spite of the demonstration of a significant increase in plasma stress hormone level in patients with pneumococcal pneumonia compared to healthy individuals [10]. Additionally, in an experimental mouse model of pneumococcal pneumonia it was shown that mice pre-exposed to stress were more susceptible to pneumococcal infection [11]. Very recently Marks et al (2013) [12] used a tissue culture biofilm model of infection combined with animal studies to show that treatment of pneumococcal biofilms with a variety of host factors such as ATP, glu-

cose, NE and cell lysates induced bacterial dispersal, and promoted *S. pneumoniae* colonization of normally sterile host tissues. Although this study showed that host chemicals could influence the phenotype of *S. pneumoniae*, the molecular mechanisms by which these behavioural changes were induced were not determined. In this study we show that therapeutic levels of NE can directly affect the growth and virulence of *S. pneumoniae* and identify the genes involved in host signal recognition.

Materials and Methods

Reagents

Human serum transferrin (Tf), ferric nitrate, and the catecholamines norepinephrine were purchased from Sigma Chemical Co. (Poole, Dorset. UK); $^{55}FeCl_3$ (IES, specific activity 5 mCi/mg Fe), 3H-NE (TRK584, l-[7, 8-^{3}H] norepinephrine) were obtained from Amersham Life Sciences, UK.

Bacterial Strains and Growth Conditions

S. pneumoniae type 4 strain TIGR4, and type 2 strain D39 and its isogenic mutants were used in this work. Routinely, pneumococci were grown at 37 °C in microaerophilic conditions either in brain heart infusion broth (BHI), Todd-Hewitt Broth (THB) (Oxoid, Basingstoke, UK) or on Blood Agar Base (Oxoid) supplemented with 5% (v/v) horse blood. Where appropriate the growth medium was supplemented with 100 μg/ml spectinomycin. In addition we used Sicard's defined medium supplemented with 50% (v/v) serum-SAPI (a host-like serum-supplemented minimal medium) in the presence or absence of 10 μM NE.

To prepare passaged D39, mice infected intraperitoneally with 100 μl of overnight grown bacteria in sterile PBS. When the signs of disease were observed, blood was collected by cardiac puncture after deep anaesthesia as described previously [13-14], and 10 ml BHI was inoculated with 50 μl blood. After overnight growth, bacteria were recovered by centrifugation and then the pellet was used to inoculate

10 ml BHI containing 20% (v/v) calf serum (Sigma). When the OD_{500} reached 1.6, growth was ceased and aliquots were kept in -80 °C until required.

Mutant Construction

In vitro mariner mutagenesis was used to introduce mutation to *pspA* as described previously [14-15]. Approximately 2 kb genomic region containing the target gene was amplified with the appropriate primers (Table 1). For transposition reactions 200 ng of PCR fragment was mixed with 200-400 ng of donor mariner plasmid pR412, which contains a spectinomycin resistance cassette, and incubated in the presence of purified *Himar1* transposase, as described previously [14-15]. Gaps in transposition products were repaired with T4 DNA polymerase (New England Biolabs, Ipswick, USA) and subsequently by *E. coli* ligase (New England Biolabs). Repaired transposition products were transformed into *S. pneumoniae* D39 using synthetic competence-inducing peptide [16]. Transformants isolated from selective medium were tested for the presence of mariner mini-transposons through PCR and sequencing [17], and then the purified products were sequenced using MP127 primer. One of the transformants designated as *pspA*$^-$, was selected for further study.

Construction of *pspC* mutant (*pspC*$^-$) was described previously [18]. To construct *pspA* and *pspC* double mutant, the mutated region in the *pspA*- was PCR amplified with pspAF and pspAR primers, and the amplified region was transformed into *pspC*$^-$. The mutation was confirmed as described above. One transformant, designated *pspAC*$^-$, was selected for further study.

Quantitative RT-PCR

The extraction of RNA was done by the Trizol method using mid-log phase cultures as described previously [19]. Before use the RNA was treated with amplification grade DNase I (Qiagen, Crawley, UK) and subsequently purified with an RNeasy Mini Kit (Qiagen). First strand cDNA synthesis was performed on approximately 1 µg DNase-treated total RNA, immediately after isolation, using 200 U of

SuperScript II reverse transcriptase (Invitrogen, Paisley, UK) at 42°C for 55 min, and random hexamers [20]. The transcription level of specific genes was normalised to *gyrB* transcription, amplified in parallel with SPD0709RTF and SP0709RTR primers. To reduce the bias in qRT-PCR we used primer pairs with similar PCR efficiencies. The results were analysed by the comparative C_T method [21].

Pneumococcal Transferrin Binding Assays

To analyse transferrin binding to the pneumococci, overnight cultures grown as described in individual experiments (approximately 10^9 CFU/ml) were harvested by centrifugation at 10,000 *g* for 10 min, washed twice and re-suspended in 1 ml of 100 mM TRIS-SAPI pH7.5.Tf was added at 1 µg/ml; the negative control consisted of addition of an equivalent volume of distilled water. Test and control cultures were incubated at 37 °C for 1 hr, after which the bacteria were centrifuged at 10,000 *g* for 10 min, washed twice in PBS and re-suspended in 100 µl of 100 mM Tris-HCl (pH 6.8) containing 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 0.1% (w/v) bromophenol blue and 100 mM dithiothreitol (DTT). This suspension was heated to 100 °C for 15 min to release bound Tf. The cell free extracts were then centrifuged at 10,000 *g* for 10 min, and the supernatant electrophoresed on 10% SDS-polyacrylamide gels, and electroblotted onto PVDF membranes [22]. Blots were probed with anti-Tf polyclonal antisera and cross-recognition was determined using HRP-conjugated secondary antibodies and enhanced chemiluminescence as described previously [22].

Pneumococcal Transferrin Iron Uptake

To test the ability of *S. pneumoniae* to acquire iron from Tf, 5 ml of sterile SAPI medium [22] buffered with 100 mM Tris-HCl, pH 7.5 containing filter-sterilized ^{55}Fe-Tf (2 x 10^5 cpm ml^{-1}), prepared as in [22] was supplemented with 10 µM NE or an equivalent volume of water (control). Washed cultures were added at 1 x 10^7 CFU/ml and incubated at 37°C in a 5% CO_2 atmosphere for 24 hr. For analysis of catecholamine internalisation, cultures were similarly grown but sup-

plemented with $1x10^5$ cpm per ml of ^{3}H-norepinephrine (control), with and without 50 µM norepinephrine. Cultures were harvested by centrifugation at 10,000 *g*for 10 min, washed in PBS and assayed for cell numbers and for radiolabel incorporation, using scintillation counting as described previously[8,22].

Biofilm Formation

Stress hormone effects on biofilm formation was analysed microscopically and using the crystal violet attachment assay [23].Bacteria were cultured statically as described in individual experiments in 150 µl volumes in triplicate in 96 well plates. After incubation, non-attached bacteria and culture supernatants were removed and the wells washed 3 times with PBS. The wells were then are dried in hot air cabinet set at 50ºC. After drying, crystal violet (0.2% v/v) was added for 15 minutes. Then, the wells washed 3 times with PBS, tapped to remove residual liquid, and the plate left on the bench upside down until dried completely. A mixture of 80% ethanol and 20% acetone was then added to wells, and measurement of attachment at 595 nm recorded.

Quellung Reaction and Microscopy

Polysaccharide capsule was visualized by microscopic examination of pneumococci after treatment with type-specific antibody (Statens Serum Institut, Caopenhagen) as described previously [24]. Briefly, a loop-full of overnight culture, grown with or without NE, was smeared onto a slide and air-dried. This was then covered with a coverslip containing 10 µl of 1% (w/v) methylene blue and 10 µl type specific anti-capsular antibody (Statens Serum Institute, Copenhagen, Denmark). The slide was examined by X1000 oil immersion microscopy.

Statistics

Growth analyses were performed in triplicate and all experiments were performed on at least 3 separate occasions; unless stated

otherwise, numerical data shown are expressed as mean +/- SD.Where appropriate, statistical analysis was first performed using one-way ANOVA, and if significant, an unpaired t-test. Statistical significance was indicated by a *P* value of less than 0•05.

Results

Norepinephrine Stimulates S. Pneumoniae Growth and Viability

Wildtype D39, routinely propagated *in vitro* or its mouse passaged stock, and TIGR4 strain were inoculated into medium containing 50% Sicard and 50% serum-SAPI (a host-like serum-supplemented minimal medium) in order to simulate *in vivo* conditions [25]. A time course of growth in the presence and absence of the catecholamine is shown in Figure 1, which reveals that the cultures grew significantly better ($P<0.01$) when NE was present, indicating that the pneumococcal strains are all stress hormone responsive. The passaged D39 showed overall greater growth levels (Figure 1C) relative to non-passaged bacteria with or without NE. Also, the decline in stationary phase optical density observed in the inotrope supplemented culture of the non-passaged D39 was not present in the NE-stimulated passaged strain. This suggests that during its time within a mammalian host, the bacteria had retained memory of an inotrope encounter.

The effect of NE on cell morphology was analyzed using the cultures obtained from late exponential phase. Figure 1D is a light microscopy image of a quellung reaction assay which shows that for D39 relative to the control, NE had no obvious effect on capsule formation, but reduce the cellular debris probably due to increased cell viability. What is also apparent in Figure 1D is the presence of NE increased clumping (cell-cell association) of the bacteria, which is an important stage in bacterial biofilm formation. Very similar results were also found for strain TIGR4 (data not shown).

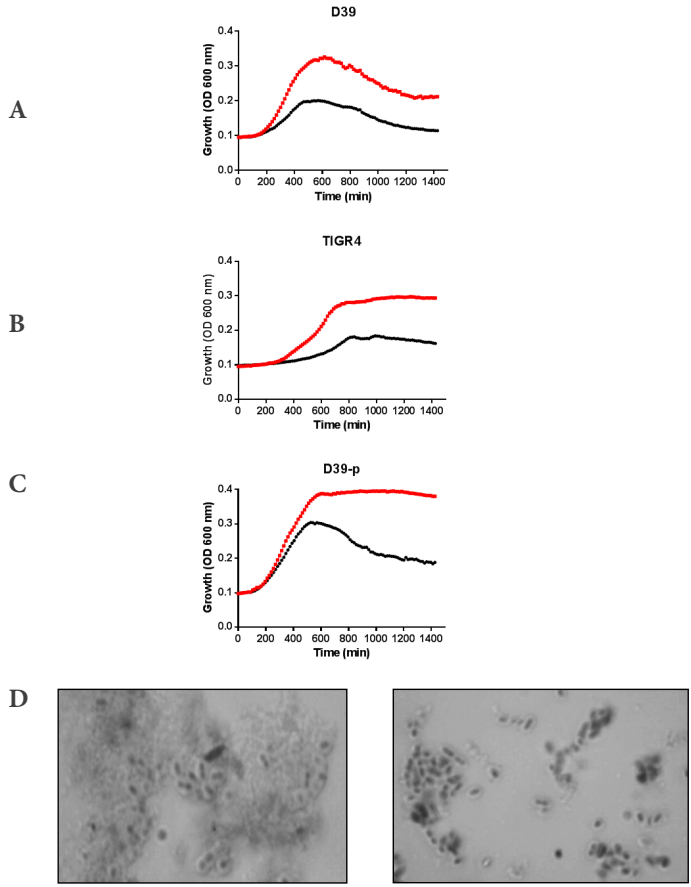


Figure :1 NE stimulates pneumococcal growth and viability
Panels A-C shows the time course of growth of S. pneumoniae strain D39, TIGR4, and passaged D39 (D39-p) in serum-based media, with and without the addition of10 mM NE. Panel D is a microscope image showing how NE increased clumping (cell-cell association) of D39 relative to the control, and also increased viability (shown by reduction in cellular lysis debris). Similar results were also seen with TIGR4 (data not shown). For all experiments, n=3.

pspa and *pspc* are Involved in Pneumococcal Catecholamine Growth Induction and Inotrope Uptake

We have shown that catecholamines can stimulate bacterial growth by enabling access to the iron within host Fe binding proteins, such as transferrin [5,22,26]. Inotropes achieve this by virtue of having a catechol moiety, which can both bind and reduce ferric iron. This reduction to Fe (II) weakens the iron binding affinity of the transferrin, allowing bacteria to then uptake the released iron by either ferric or ferrous uptake systems [5].Previous work has shown that *S. pneumoniae* can utilize ferric and ferrous iron salts, and host Fe sources such as haemoglobin, and haemin, but to a lesser extent the iron within transferrin [27-29]. In terms of the growth stimulation seen in Figure 1, Figure 2A reveals that transferrin is involved as it shows that during growth in serum-based medium *S. pneumoniae* binds the host protein in what appears to be a growth-phase independent manner. Figure 2B shows that incubation of the bacteria with Tf containing radiolabelled Fe (^{55}Fe-Tf) and NE allowed the pneumococcus to obtain normally sequestered host Fe in higher amounts if a stress hormone was also present. Mechanistically, this suggests that Fe delivery from Tf explains the growth induction in serum-medium demonstrated by NE in Figure 1.

The question that arose as to how NE facilitated iron uptake into the pneumococcus, and so the role of key pneumococcal surface proteins, PspA and PspC, in the catecholamine growth induction mechanism was investigated. This was because previous studies had demonstrated the involvement of these proteins in pneumococcal virulence, such as attachment, prevention of complement deposition and factor H recognition [18,30-31] . In addition PspA was shown to be important for binding to lactoferrin [32]. Hence, we constructed *pspA*, *pspC*, and *pspA* and *pspC* double mutants in the D39 pneumococcal strain and analysed their response to NE. Figure 3A-3C show that in marked contrast to wildtype D39 (Figure 2A), the *pspA* and *pspC* mutants, singly or in combination, showed no significant growth

induction by the catecholamine. To understand what the mechanism of this lack of response might be, we also compared the ability of the *psp* mutants with wildtype D39 to acquire Fe from ^{55}Fe-labelled transferrin in the presence and absence of NE. Figure 3D-3F shows that in the absence of the catecholamine the *psp* mutants were able to uptake some ^{55}Fe-iron, but unlike the parent strain (Figure 2A), were unable to utilise the NE to obtain higher levels of the Tf-complexed ^{55}Fe, with the effect most strikingly seen in the double *psp* mutant which was overall severely compromised in its ability to obtain Fe from transferrin (Figure 3F).

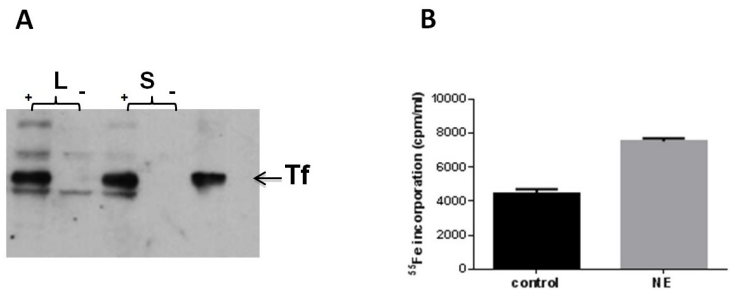


Figure :2 Mechanism of norepinephrine growth induction
Panels A and B show that growth induction in serum-medium by NE involves transferring binding. Panel A) is a western blot of Tf binding to D39 cultures (+) compared with non-Tf supplemented control cultures (-); the influence of growth phase on Tf binding is also shown: log-phase (L), stationary phase (S). Panel B showsthat uptake of Tf-complexed Fe (in the form of ^{55}Fe) is increased in the presence of NE; *, $P<0.05$. Values shown in panel B are means of triplicate counts; n=3.

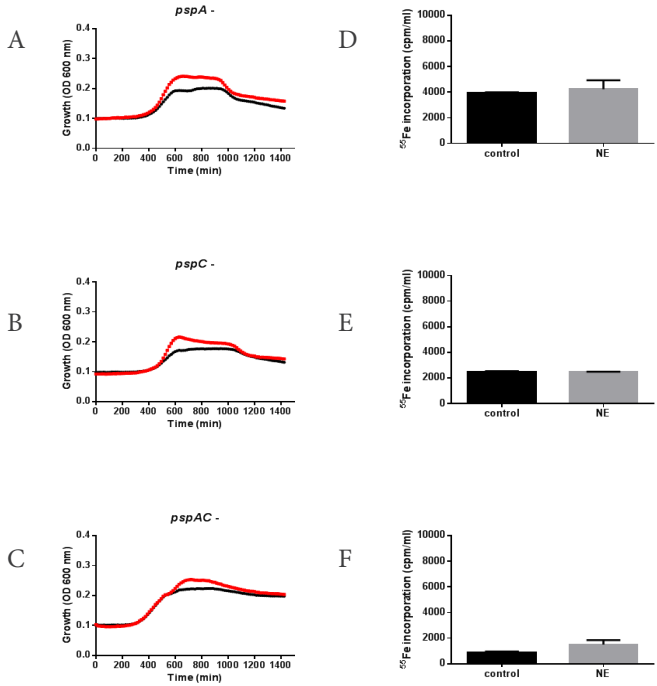


Figure :3 Role of the PspA and PspC in pneumococcal catecholamine growth induction Panels A-C show the time course of growth of wildtype D39 and the *pspA*, *pspC* and pspAC mutants in serum-based media +/-10 mM NE. Panels D-F shows the pspA (pspA$^-$), *pspC*(*pspC*$^-$) and *pspAC* (*pspAC*$^-$)mutant uptake of Tf-complexed Fe (in the form of ^{55}Fe) in the presence and absence of NE; note that wildtype D39 uptake of Tf-Fe in the presence of NE is shown in Figure 2B.Values shown in panels D-F are means of triplicate counts; for all experiments, n=3.

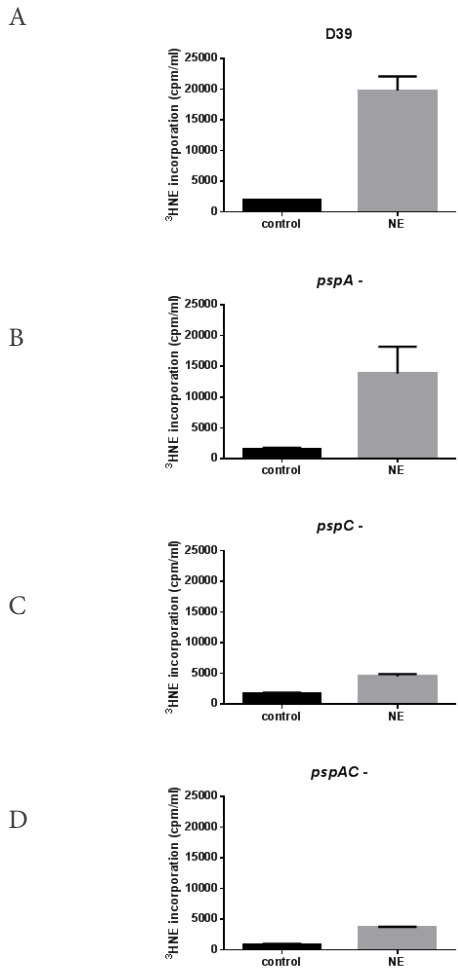


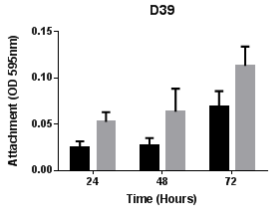
Figure :4 PspA and PspC are involved in norepinephrine uptake
Panels A-D show 3H-NE uptake by wildtype D39 and *pspA*, *pspC* and *pspAC* mutants after 24 hrs incubation in serum-medium as described in Methods in the presence (grey) and absence of NE (black) ; *, P<0.05. Values shown in panels D-F are means of triplicate counts; for all experiments, n=3.

We also investigated if the NE was internalised by the pneumococcus during the catecholamine growth induction process, and if the *pspA* and *pspC* were also involved. Figure 4 compares the uptake of radiolabelled ^{3}H-NE by wildtype D39 with the single and double *pspA* and *pspC* mutants, grown in serum-medium in the presence and absence of added NE. For both wildtype and the *pspA* mutant, the presence of unlabelled NE stimulated uptake of the ^{3}H-NE (Figures 4A and 4B). It can be seen that compared with wildtype D39, mutation of *pspA* had a moderate effect on ^{3}H-NE internalisation, causing an average reduction in uptake of around 25 %. In contrast, inactivation of *pspC* resulted in ^{3}H-NE internalisation levels of >75% less than that of wildtype. The *pspA* and *pspC* double mutant showed similar uptake levels to the single *pspC* mutant.The data in Figures 2, 3 and 4 collectively show that the PspA and PspC are integral elements in the mechanism by which NE induces *S. pneumoniae* growth in serum based medium.

Norepinephrine Stimulates *S. Pneumoniae* Biofilm Formation

The ability of infectious bacteria to attach to surfaces, self-associate and form a biofilm is an aspect of virulence that is particularly important in the development of respiratory infections. It was shown in Figure 1D that NE-treated D39 displayed greater cell-cell clumping, which suggests that as had occurred with another respiratory pathogen (*P. aeruginosa* [8]), the inotrope was also stimulating *S. pneumoniae* biofilm formation. We investigated the effects of NE exposure on D39 attachment, the first step in formation of a biofilm. Figure 5 shows over the course of a 3 day incubation in serum-medium, NE consistently enhanced the attachment of the wildtype D39 ($p<0.05$) (Figure 5A). In the case of the *pspA* and *pspC* mutants (Figures 5B-C), NE appeared to have little effect on attachment, while the *pspAC* (Figure 5D) mutant was generally defective in biofilm initiation ($p<0.05$). This data suggests that *pspA* and *pspC* are also involved in NE mediated pneumococcal biofilm formation.

A



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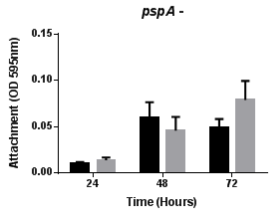

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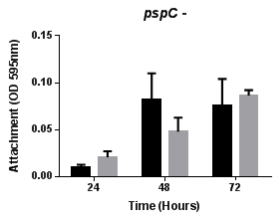

D

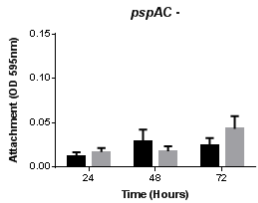

Figure : 5 effects of norepinephrine on pneumococcal biofilm formation
Panels A-D show initial attachment of wildtype D39 and pspA, pspC and pspAC mutants after 24, 48 and 72 hrs incubation in serum-medium. Attachment was measured as described in Materials and Methods. Key: black bar (control); grey bar (NE); *, P<0.05. Values shown in panels D-F are means of triplicate counts; for all experiments, n=3.

Norepinephrine Modulates Pneumococcal Metabolism and Enhances Virulence Gene Expression

We also examined the expression of 15 functionally diverse genes in order to identify genes involved in stress hormone responsiveness of *S. pneumoniae.* The expression of 9 genes manifested greater than two-fold change (Table 1), the commonly agreed level of significance, although sometimes differences of less than two-fold are known to be biologically important. The genes with altered expression included *comX*, a response regulator for genetic competence which is also involved in biofilm formation [33] consistent with our finding that NE increases pneumococcal attachment. Also showing up regulation was the SPD_0939: Rgg family transcriptional regulator, the members of which are involved in oxidative stress response, biofilm formation, quorum sensing and virulence [34-35]. Among two iron transporters, *piuA* and *piA*, a significant up-regulation of *piuA*, but not *piaA*, was detected. In addition, differential expression of genes involved in polysaccharide degradation and sugar utilisation were observed (N-acetyl hexosaminidase, β-galactosidase galactokinase and pyruvate oxidase) [36]. This is significant as *in vivo*, the concentration of free sugars are known to be low in the respiratory tract, forcing the pneumococcus to rely on host glycoproteins such as mucin, and the carbohydrates bound by host proteins such as Tf to satisfy essential carbon needs [37]. Others and we have demonstrated previously that glycosidases, such as neuraminidases and galactosidases are highly important for pneumococcal colonization and invasion [20,38].

Table 1: Oligonucleotide primers used in this study.

Primer I[D]1	Primer Sequence (5'-3')	Target gene in D39
SPD0014RTF	GAAGGCATGCTCTGCTTACA	*comX*
SPD0014RTR	CGCTTCTGACTTTCCTGCTT	
SPD0063RTF	ATCCCAATCATCGGTGGTA	*strH*
SPD0063RTR	CGGTTCAGGTCTTTTGGTA	
SPD0065RTF	GGACCTCTTTGTAACAGGAA	*bga3*
SPD0065RTR	CATCTGCCAATTCCTTAGGA	
SPD0126F	GGAATGAAGGAAGATGATGC	*pspA*
SPD0126R	GCCATCTACAGTTGTGTTG	
SPDRT0144F	GGCGAGAAAGCTTAAGCAGA	*rgg*
SPDRT0144R	TTGTGCCCAAACTCATCAAA	
SPD0344RTF	GCAGAAAAATTGAGCCGAAC	*ritR*
SPD0344RTR	CGAAATACGCGCTACCAGAT	
SPD0420RTF	TGGTGTTTACGCACGTCTTG	*pflB*
SPD0420RTR	CATCAACCCCGTAAAGGTCAC	
SPD0709RTF	TCGTGTGGCTGCCAAGCGTG	*gyrB*
SPD0709RTR	GGCTGATCCACCAGCTGAGTC	
SPD0722RTF	CGTCACCTTCACATGACACC	*spxB*
SPD0722RTR	CATGTTGAATGCTCCGTCAC	
SPD0915RTF	TTTTGCAAAGGAAATTTCTGC	*piaA*
SPD0915RTR	GCAACGGCACCATTTTTAAT	
SPD0939RTF	CAAAATTGAAAAATGGGGCTA	*rgg/mutR*
SPD0939RTR	GCAAGCTGAGAGACAATCTGC	
SPD1463RTF	ACTCATTGTAACCAGCGAAGGAGCA	*psaA*
SPD1463RTR	CCCAGATGTAGGCACTTGGAACACC	
SPD1464RTF	AGAATTGGCTGGACTGGACAA	*tpxD*
SPD1464RTR	CACCGCACCAACGTTTTTG	
SPD1499RTF	GGAGTGAGCCAATTTTTGC	*nanB*
SPD1499RTR	GCAGGCATAACATCAGCT	
SPD1504RTF	AGCAACCTCTGGCAAATGAA	*nanA*
SPD1504RTR	ATAGTAATCTCTTGGAATT	
SPD1634F	TCTCGGTGCTCGTATGACAG	*galK*
SPD1634R	CACCTGCAACTTCAGCGATA	
SPD1652RTF	CTTTGGTGCCAAATCTCGTT	*piuA*
SPD1652RTR	GCAAGGGTACGGTTGATGAC	
MP 127	CCGGGGACTTATCAGCCAACC	*pR412* specific
MP 128	TACTAGCGACGCCATCTATGTG	

1Primers indicated as 'F' or 'R' tag used for amplification of gene targets for mutational work, while 'RTF' or 'RTR' primers utilised for gene expression analysis.

Table 2: S. pneumoniae gene expression in the presence of norepinephrine.

Transcriptional regulators	**Fold change**
***comX* (SPD_0014)**	5.33 (0.15)
***rgg* (SPD_0144)**	1.72 (0.12)
***ritR* (SPD_0344)**	0.77 (0.04)
***rgg*/*mutR* (SPD_0939)**	28.4 (2.2)
Sugar hydrolases	
***strH* (SPD_0063)**	6.56 (0.30)
***bga3* (SPD_0065)**	21.4 (2.0)
***nanB* (SPD_1499)**	22.39 (1.10)
***nanA* (SPD_1504)**	10.77 (0.89)
Cation metabolism	
***piaA* (SPD0915)**	0.73 (0.02)
***piuA* (SPD_1652)**	6.08 (0.20)
***psaA* (SPD_1463)**	1.22 (0.04)
Sugar metabolism	
***pflB* (SPD_0420)**	0.59 (0.03)
***spxB* (SPD_0722)**	0.16 (0.07)
***galK* (SPD_1634)**	25.36 (2.39)
Oxidative stress response	
***tpxD* (SPD_1464)**	0.66 (0.07)

The relative expression was calculated from 3 independent experiments and standard deviation is indicated in parenthesis. The expression of target genes normalised to the housekeeping gene gyrB

Discussion

Nasopharyngeal tissue colonization is the first step of invasive pneumococcal diseases [1]. However, it is not known what triggers the transition from colonization to invasiveness. Our on-going work on pneumococcal biology indicates that environmental factors, such as changing oxygen concentration, differences in metal and sugar composition of tissues, have a fundamental impact on pneumococcal virulence [39-40]. However, although these environmental factors are important, they do not explain fully the sudden change from colonization to invasiveness. Therefore, other host factors, such as stress hormones, must be important for pneumococcal invasiveness [2]. The reason for this assumption stems from the rapid change in the concentration of stress hormones due to physical and emotional stress, from stress hormones' adverse effect on immune system function [3], and from microbial ability to recognize and process stress hormone signals [2].

In this study we showed that *S. pneumoniae* responds to therapeutic levels of inotropes with increased growth and virulence, which could have a major impact on the progression of pneumococcal infection or transmission to new hosts. Many predisposing factors for pneumococcal diseases including emotional and cold stress, and overcrowding are known to increase stress hormone levels. In addition, catecholamine inotropes are administered up to 50% of patients in intensive care unit (ICU) [41], and up to 56% of patients with pneumococcal pneumonia are admitted to ICU [10]. Hence, in addition to endogenously produced stress hormones, pneumococci are exposed to externally applied catecholamine inotropes. Growth stimulation of *S. pneumoniae* came about due to the inotrope providing essential Fe for growth from the host iron binding protein transferrin, which was bound by the bacteria during growth. Interestingly, the supposedly simple in function PspA and PspC surface proteins were found to play a major role for NE mediated growth and biofilm formation. When

the genes for PspA and PspC were mutated, the ability of *S. pneumoniae* to utilize the additional Fe provided from transferrin by the catecholamine was blocked. The uptake of the radiolabelled NE was similarly reduced. In terms of inotrope internalization, the addition of NE appeared to stimulate its own uptake, suggesting up-regulation by the NE of a catecholamine transport system.Effects of the *pspA* and *pspC* mutations were found to be even more widely acting when biofilm induction by NE seen in wildtype D39 was essentially abolished in the *pspA* and *pspC* mutants.

PspA and PspC are choline-binding pneumococcal surface proteins. These two proteins have been shown to play a pivotal role in pneumococcal virulence by inhibiting complement-mediated opsonization [42-43], by preventing lactoferrin mediated killing [44], and by facilitating the microbe's attachment to the nasopharyngeal and lung epithelia and the brain microvascular endothelium [18,30-32]. PspA is also known to bind to lactoferrin [32]. In addition, these two proteins have been shown to elicit protective antibody response against invasive pneumococcal infection, hence they are promising vaccine candidates [30]. Although their contribution to *S. pneumoniae*-host interaction has been well studied, very little is known about their role in pneumococcal physiology. Previously, using recombinant PspA and a strain mutated in *pspA*, it was shown that PspA, but not PspC, is responsible for pneumococcal binding to human lactoferrin, which was suggested to be important to overcome the iron limitation at mucosal surfaces [42-43]. Contrary to previous reports [32,45], in this study we consistently, with both lag phase and stationary phase cultures, demonstrated that *S. pneumoniae* can bind to transferrin, and acquire iron from this glycoprotein in the presence of NE. The reason for this discrepancy could be due to different culture conditions, and detection technology used for transferrin binding. For example, unlike Hakansson *et al*., (2001) [45] we used serum based medium to prepare pneumococcal cultures, which can affect the synthesis of proteins involved in binding to transferrin. Currently, the mechanism of

PspA and PspC mediated pneumococcal response to NE is not clear but based on available data we speculate that these surface proteins are responsible for either recognition and/or uptake of NE since the mutation of either gene resulted in reduction in NE uptake.

A recent study by Marks et al. (2013) [12] showed that NE treatment of biofilms formed *in vitro*, and in vivo in nasopharynx leads to dispersion of *S. pneumoniae*, and the dispersed cells display distinct phenotypic traits that are different from those of both biofilm and broth-grown, planktonic bacteria. The dispersed pneumococci were shown to have differential virulence gene expression, and had a significantly increased ability to disseminate and cause infection in the middle ear, lungs, and bloodstream. Our results are consistent with Marks *et al*., (2013) [12] in that the pneumococcus responds to NE, and the treatment with NE leads to differential gene expression. On the other hand, contrary to the Marks *et al*. study, who used biotic surfaces to determine NE's role in pneumococcal dispersion from biofilms, our results show that in host like media the catecholamine aggregates the pneumococci and promotes biofilm formation on abiotic surfaces. The reason for this seeming discrepancy can be due to methodological differences and also be attributed to NE's dual function in biofilm formation. In other words, NE can initially promote bacterial biofilm formation and after a certain stage in the infection process, depending also the microbial growth phase, may also promote dispersion of the pneumococci. Therefore, further work is required to test this hypothesis.

In this study NE mediated iron uptake has been identified to be responsible for the observed growth effect of NE in serum based media. However, our gene expression analysis shows that NE has much wider effect on pneumococcal physiology and virulence gene expression. For example, the expression of genes coding for glycosidases (*nanA, nanB, bgaC* and *strH*), which are responsible for deglycosylation of host glycans and play important role in pneumococcal colonization and invasiveness [36-37], were significantly upregulated in the

presence of NE. Moreover, differential expression of genes involved in transcriptional regulation (SPD_0939), competence development (*comX*), galactose metabolism (*galK*), and iron transport (*piuA*) was also detected, indicating the comprehensive effect of NE on pneumococcal metabolism. Currently it is not known how the pneumococcus detects and processes stress hormone signals nor whether the pneumococcus would respond differentially to different stress hormones as was demonstrated in *Mycoplasma hypopneumoniae* [46], hence we plan to investigate underlying genetic mechanisms for detection and processing of NE signals.

Bacteria have evolved mechanisms to sense the changes in the stress hormone levels by receptors, which are specific and can differentiate different stress hormones [47]. Using α and β receptor antagonists, we showed the presence of putative adrenergic and dopaminergic receptors in three Gram-negative bacteria: *Escherichia coli, Salmonella enterica* and *Yersinia enterocolitica* [48]. Our results demonstrated that catecholamine- induced growth in these bacteria could be blocked by catecholamine α-receptor antagonists, but not by the antagonists for β adrenergic receptors. But, so far, no comprehensive study has been conducted to investigate proteins responsible for stress hormone recognition in Gram positive bacteria. Identification of such receptors enhances our understanding of *S. pneumoniae*-host interactions and can offer alternative therapeutic options against the pneumococcal diseases.

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