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Differential gene expression in *Streptococcus pneumoniae* in response to various iron sources

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ABSTRACT

Iron is a critical co-factor for several enzymes and is known to regulate gene expression in many pathogens. *Streptococcus pneumoniae* (pneumococcus) normally colonizes the upper respiratory mucosa, which is an iron-restricted environment. In contrast, during bacteremia available iron from heme and non-heme proteins potentially increases. In iron-depleted medium pneumococcal strain TIGR4 showed reduced growth, however, addition of several physiological iron sources restored growth. Gene expression of selected known and putative pneumococcal virulence factors was analyzed by quantitative RT-PCR in response to iron sources in vitro and during colonization, pneumonia, and bacteremia in a mouse model. Change in mRNA levels relative to transcription in iron-depleted medium was reported. In presence of iron sources, transcription of *cps4A*, *zmpA*, *pavA*, hemolysin and a putative exfoliative toxin was significantly increased, but *nanB* was suppressed. Hemoglobin at physiological concentration repressed *ply* and *pspA* expression. Ferritin, an acute phase protein, increased expression of an iron ABC transporter and repressed expression of a bacterial non-heme iron-containing ferritin. Transcription of *cps4A*, *nanB*, hemolysin, and a putative exfoliative toxin were significantly up-regulated during pneumonia and bacteremia, while mRNA of *pavA* and non-heme ferritin were expressed at higher levels during pneumonia and carriage. An iron ABC transporter was most up-regulated during bacteremia, while *pspA* and *ply* were expressed only in pneumonia. Transcription of *zmpA* was elevated during both pneumonia and bacteremia. These findings suggest that a subset of virulence genes in pneumococci is differentially regulated in response to the quantity and form of iron sources available in a host.

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1. Introduction

Streptococcus pneumoniae (pneumococcus) is a major cause of community-acquired pneumonia, meningitis, septicemia, otitis media and community-acquired pneumonia throughout the world, causing significant mortality and morbidity [17,23]. The pathogenesis of pneumococcal disease is poorly understood. The ability to bind, invade, and damage host tissue is attributed to virulence factors such as capsule, pneumolysin, adhesins, IgA protease, and various surface proteins [4,7,9,14,22,41,43]. The differential expression of virulence factors during carriage and invasive disease is only beginning to be studied. Pneumococcus colonizes the upper respiratory tract of humans and can be isolated from the nasopharynx of up to one-fourth of healthy adults and 80% of children less than five years of age [6]. Carriage can subsequently lead to disease in susceptible individuals (particularly infants, the elderly, and the immunocompromised). Infection primarily results from aspiration of nasopharyngeal bacteria into the lungs. Sepsis develops in about 10–15% of cases of pneumococcal pneumonia. It is presumed that bacterial surface components play a major pathogenic role early in infection by inhibiting complement deposition and preventing phagocytosis [7,43], however, the sequential events that allow progression of the infection are still unclear.

Iron is an absolute requirement for nearly all cells, since it is important in many biological processes. Iron has limited solubility in aqueous solutions and is associated with proteins or functional groups in vivo. In vertebrates, extracellular iron is bound to carrier proteins such as transferrin, lactoferrin, haptoglobin, and hemopexin. Intracellular iron is primarily bound to heme-containing proteins, ferritin, and iron–sulfur proteins [5].

The ability to acquire iron under physiological conditions has been associated with virulence in a variety of bacterial pathogens [16,29,30]. Bacteria have acquired highly efficient iron acquisition

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systems to scavenge iron during growth in their hosts. In certain cases, this involves the secretion and internalization of various types of extracellular ferric chelators called siderophores. The most common types include hydroxamates and catecholate compounds [5]. Ferrous iron can be directly imported by the G protein-like transporter, FeoB in *Escherichia coli*. Some pathogens have membrane transporters for protein–iron complexes and can utilize these directly as iron sources. Bacterial iron storage proteins such as bacterioferritin serve as intracellular iron reserves when external supplies are restricted.

The first published pneumococcal genome sequence revealed the presence of putative iron ABC transporters, however, no genes encoding siderophore biosynthetic enzymes or transferrin receptors have been annotated [39]. Pneumococci have been reported to utilize ferric and ferrous iron salts, hemoglobin, and hemin as iron sources [8,36]. The choline-binding surface protein PspA binds human lactoferrin, however, lactoferrin does not support the growth of pneumococci in iron-deficient medium [8,19,38]. Pneumococci can bind hemin, and a putative hemin-binding protein has been identified [38]. Although pneumococci do not secrete any detectable siderophores, a ferric hydroxamate transport system has been detected that can transport ferrichrome and ferrioxamine B siderophores as iron sources [35].

Pneumococci require iron and must acquire it from the host in the form of iron–protein complexes. Mucosal surfaces are functionally devoid of free iron as a result of chelation by iron-binding proteins [45]. In blood, heme iron is the predominant source of iron. Hemoglobin is released by lysis of erythrocytes and plasma ferritin levels increase during acute inflammatory responses. The transition from an iron-restricted environment to one which potentially contains more iron in the form of different protein complexes may signal differential expression of pneumococcal genes, including some virulence factors.

Several studies comparing pneumococcal gene expression in vivo and in vitro have been reported. Differential expression of certain virulence genes in pneumococci has been reported by northern blotting, semi-quantitative RT-PCR, and microarrays [26–28]. In this study utilization of physiological iron sources to support growth of pneumococci was assessed. We evaluated expression of a select group of known and putative virulence genes in response to potential iron sources during nasopharyngeal colonization, lung infection, and bacteremia. We also examined the expression of genes after exposure of pneumococci to CSF.

2. Results

2.1. Growth in chemically defined medium

We initially examined growth kinetics of a type 4 pneumococcus to determine if growth can occur in physiologically relevant levels of iron-containing proteins. Growth of pneumococcal cells was observed in CDM, CDMct and CDMct supplemented with various iron sources that bacteria may encounter in a host. Pneumococci grown in CDM started exponential growth at about 9 hrs. Time to reach logarithmic phase growth in CDMct was delayed and cells grew to lower density before reaching death phase with no preceding stationary phase. Cells grown in CDMct supplemented with either 6 μM ferrous sulfate or ferric chloride had a longer lag-phase but the cells eventually grew to similar density as in CDM before reaching the stationary phase (Fig. 1A). Cells grown with hemin, ferrioxamine, ferritin and hemoglobin started exponential growth at times between that in CDM and CDMct with iron salts (Fig. 1B). However, the cells eventually grew to similar density as that observed in CDM before reaching the stationary phase.

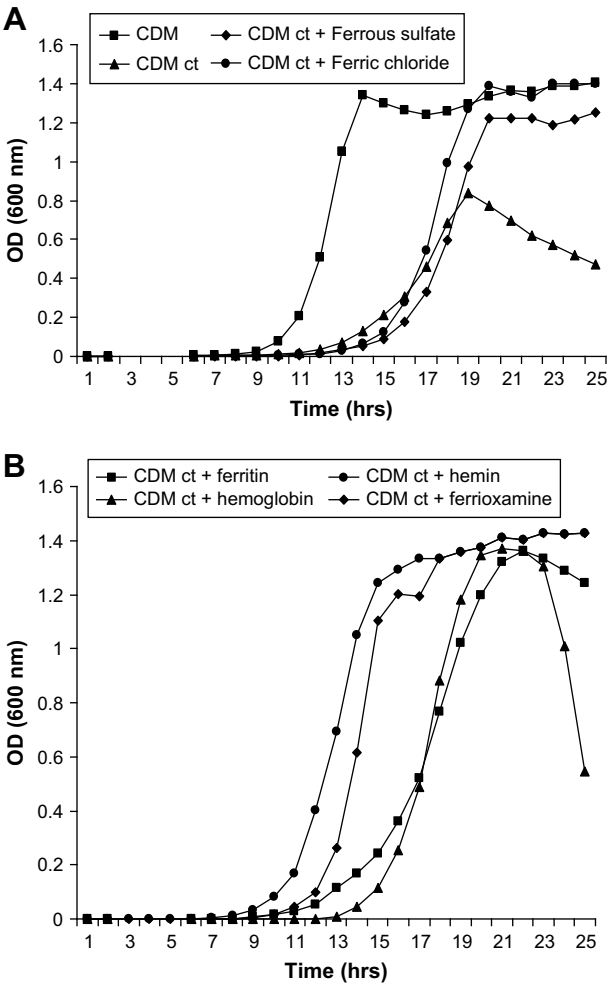

Fig. 1. Growth of pneumococcal strain TIGR4 in presence of several iron sources. A – CDM, CDMct, CDMct + ferrous sulfate, CDMct + ferric chloride. B – CDMct + ferritin, CDMct + hemoglobin, CDMct + hemin, and CDMct + ferrioxamine E.

Notably, cells grown in presence of hemoglobin had a short stationary phase as compared to growth in other iron sources before culture density started to decline (Fig. 1B). The results shown are the average of two independent experiments done in triplicate.

2.2. Cell morphology

Pneumococcal cells had similar exponential growth phase slopes with various iron substrates, although their lag-phase was variable. As cell morphology can affect optical density we examined the morphology of the growing cells. When grown in CDM, cells appear as diplococci or short chains (Fig. 2A). Pneumococci grown in CDMct (in absence of iron) formed long chains in which rapidly dividing cells failed to separate into characteristic diplococcal forms or short chains (Fig. 2B). Pneumococcal cells grown in CDMct with 75 μg/ml of hemoglobin appear mostly as aggregates or clusters, occasionally as diplococci, and also appear to produce an extracellular matrix material. This extracellular matrix appears as a fine reticular network. The cells, whether in aggregates, clusters or small chains, appeared to be associated with this matrix material (Fig. 2C & D). Pneumococci grown in CDMct supplemented with other iron sources and with 30 μg/ml of hemoglobin exhibited similar morphology to cells grown in CDM (not shown).

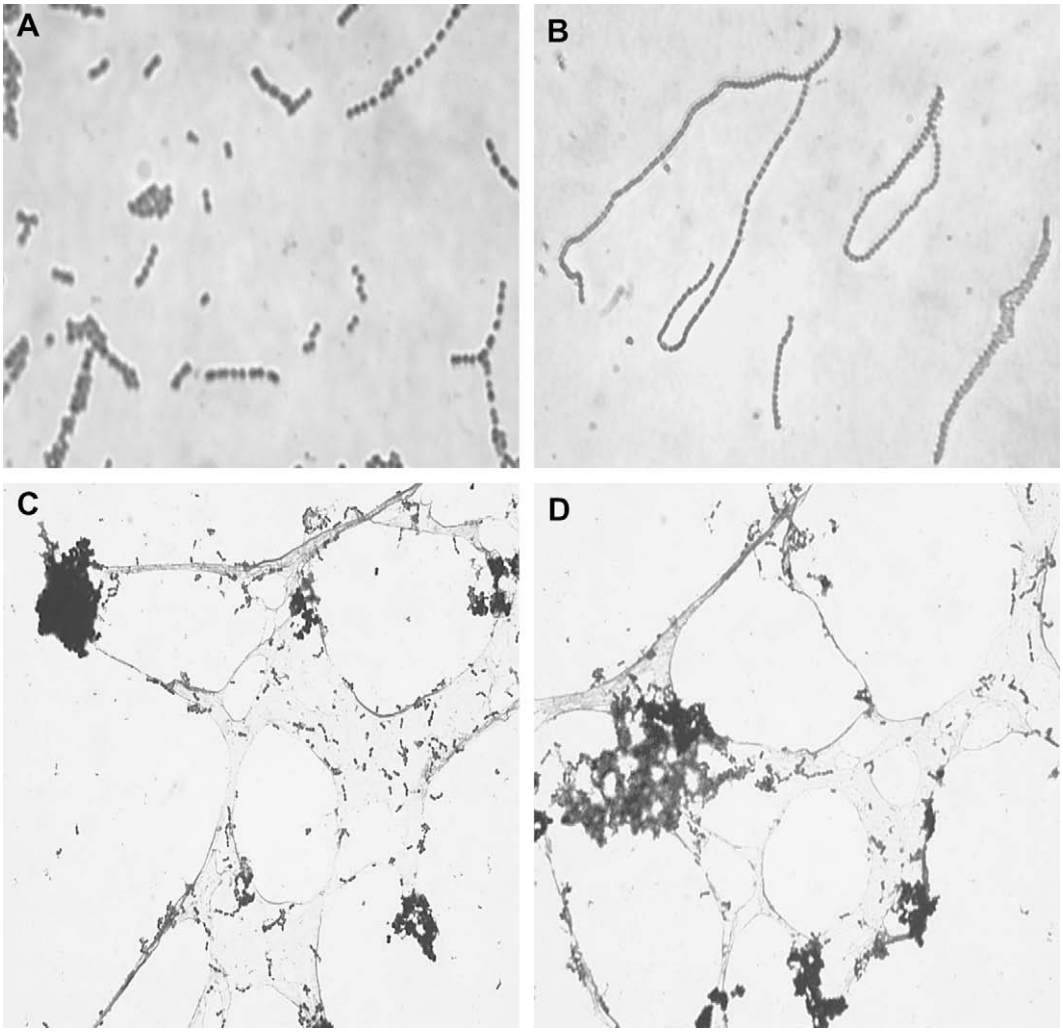

Fig. 2. Gram stain comparing morphology of TIGR4 cells grown in CDM (A), CDMct (B), and CDMct supplemented with 75 μg/ml of hemoglobin (C, D).

3. Gene expression in vitro

We analyzed gene expression of selected known and putative pneumococcal virulence factors (Table 1) in response to various iron sources potentially available in vivo. Quantitative RT-PCR (qRT-PCR) was performed using gene specific primers (Table 1). The mRNA levels of pneumococcal cells grown in CDMct were compared to cells grown in CDMct supplemented with 6 μM hemin, 1 μg/ml ferritin, and either 30 μg/ml or 75 μg/ml of hemoglobin. The data shown is the average of two different biological samples. The results indicate that, under in vitro growth conditions the transcription of *cps4A*, *zmpA*, *pavA*, a gene encoding hemolysin, and a gene encoding a putative exfoliative toxin was up-regulated with ferritin, hemin, and hemoglobin. *pspA* was down-regulated with 30 μg/ml of hemoglobin but was up-regulated with other iron sources. Interestingly, no significant change for *ply* was observed with ferritin, hemin or 75 μg/ml of hemoglobin but with 30 μg/ml of hemoglobin *ply* transcription was significantly reduced. A neuraminidase gene, *nanB*, was down-regulated during growth in all iron sources compared with growth in CDMct (Fig. 3A). A gene encoding a component of an iron ABC transporter was up-regulated only with ferritin and was not affected by growth in other iron sources. A non-heme iron-containing ferritin-like protein in TIGR4 was down-regulated with ferritin but was up-regulated with other iron sources (Fig. 3B).

3.1. Gene expression in vivo

In this study, we also examined in vivo expression of the same pneumococcal genes during the natural progression of disease from nasopharyngeal colonization to systemic disease in a murine model. CBA/N mice express the X-linked immunodeficiency gene *xid* and are unable to produce antibodies against polysaccharide capsule, but can mount immune responses to protein antigens. Quantitative RT-PCR was performed to measure mRNA levels from pneumococcal cells grown in CDMct and compared to transcripts from cells collected during nasopharyngeal colonization, pneumonia, and bacteremia. A two-fold or greater change in mRNA levels relative to growth in CDMct was considered significant. Each experiment was performed in triplicates and the data in the figure are a composite of three biological samples. Uninfected control mice did not amplify any of the gene products.

The results suggest that transcription of *cps4A*, *nanB*, *pavA*, a gene encoding hemolysin, a gene encoding a putative exfoliative toxin, a gene encoding a component of an iron ABC transporter, and a non-heme iron-containing ferritin protein in TIGR4 was up-regulated both during colonization and bacteremia (Fig. 4A & B). Surprisingly, neither *pspA* nor *ply* had any significant change under the same conditions. However, *zmpA* was up-regulated during bacteremia but remained unchanged during colonization (Fig. 4A). In pneumonia studies we found that, expression of all genes was

Table 1
Characterized and putative pneumococcal virulence genes examined in this study.

Genes/TIGR Locus name[a]	Function	Primer Sequence
16S rRNA	House keeping gene	5′-CTGCGTTGTATTAGCTAGTTGGTG-3′ 5′-TCCGTCCATTGCCGAAGATTC-3′
cps4A (SP0346)	Biosynthesis of capsule polysaccharides	5′-TCAATCAGTGTCGCTGTTTTA-3′ 5′-TTCATTATTAGTCCCAGTCGG-3′
pspA (SP0117)	Binds complement and lactoferrin	5′-TGCTACTGCTCAACATCAAG-3′ 5′-ATAACTTCTGTGCCATCATC-3′
Exfoliative toxin (SP0410)	Toxin production (putative)	5′-ACGGTTCTCTATGTGGGG-3′ 5′-ACTCAAGGTCGCATAGGC-3′
ply (SP1923)	Pneumolysin	5′-CTACCCGATGAGTTTGTTGTT-3′ 5′-TCCAGGATAGAGGCGACT-3′
Hemolysin (SP1466)	Cytolysin (putative)	5′-AGCCTTGCTCTTTACCTGATT-3′ 5′-GAGACTCCAGAAAACGGGTGT-3′
pavA (SP0966)	Pathogenesis and cell adhesion	5′-GGTCGCATCCAGAAAATC-3′ 5′-AGAAAGGAGCAGGCGATG-3′
nanB (SP1687)	Neuraminidase B	5′-GGTGAGATAGGTGTAGAAATAAGA-3′ 5′-ATTTTCAACTGCCTGTCCTTT-3′
zmpA (SP1154)	Zinc metalloproteinase	5′-ACCAATGTTCTCAGCGATGTT-3′ 5′-CTTGCTGCTGAATGTGTCCTT-3′
Fe ABC transporter (SP1872)	Binding and transport of iron compounds	5′-GCTGAAACACCTGCTGCT-3′ 5′-TGATTCAAGTCCGCCTCC-3′
Non-heme iron-containing ferritin (SP1572)	Intracellular iron storage protein	5′-GATGAAGAAGGTGACGATGTG-3′ 5′-TTGTCCAAGTTCGGCTGC-3′

[a] Sequences from The Institute for Genomic Research (http://cmr.jcvi.org/tigr-scripts/CMR/genome).

up-regulated (Fig. 4B), except *pspA* whose expression was unchanged (Fig. 4A).

3.2. Gene expression during CSF exposure

The expression profile of the same genes (Table 1) in CDM (~22 μM iron) [42], and CSF (~2.2 μM iron) [18] was compared. The transcription levels of cells grown in CDMct were compared to levels in CDM and cells incubated in CSF. The results indicate that expression of *cps4A*, *pspA*, *pavA*, a gene encoding hemolysin and a non-heme iron-containing ferritin-like protein was up-regulated and the expression of *ply*, *zmpA*, *nanB,* and a gene encoding a component of an iron ABC transporter was reduced in presence of ionized iron (CDM) (Fig. 5A, B). A gene encoding a putative exfoliative toxin did not have any significant change in the same condition. On the other hand, mRNA levels of *cps4A*, *pavA*, *nanB*, *zmpA* and a gene encoding hemolysin were not affected by exposure to CSF (Fig. 5A, B). A gene encoding a putative exfoliative toxin, a non-heme iron-containing ferritin-like protein and *ply* were down-regulated in CSF. Of all genes studied a component of an iron ABC transporter was the only gene up-regulated in CSF (Fig. 5B).

4. Discussion

Concentration and form of iron vary at different anatomical locations in a host and it is possible that bacterial pathogens sense these differences and regulate gene expression in response to iron sources. One such pathogen is pneumococcus which is well-adapted to colonize the mucosal surfaces of the nasopharynx. It can also invade normally sterile sites such as the lower respiratory tract, blood, and subarachnoid space. Iron bound to heme groups is predominant in blood, while trace amounts of both heme and non-heme iron is found on epithelial /mucosal surfaces. The total complement of mechanisms used by pneumococci to sense and acquire iron in a human host is not fully defined.

In the growth experiments we observed that pneumococcus utilized iron salts as well as iron proteins. Ferritin at 5 μg/ml did not support the growth of a serotype 3 pneumococcus [8], therefore, we used a lower concentration for growth experiment because of the possible inhibitory effect of a higher concentration. Pneumococcus does not produce any known siderophores, but we wanted to know if the bacteria could utilize iron from saturated siderophores. Free hemoglobin at a concentration of 30 μg/ml is within the normal adult physiologic range [2]. For the gene expression studies we used physiological hemoglobin concentration and 75 μg/ml, which may occur during bacteremia and intravascular hemolysis. The transient exposure of bacteria to hemoglobin may provide a source of iron, by a mechanism as yet unidentified. Pneumococci have been shown to contain hemin-binding polypeptide and therefore we examined gene expression in presence of hemin [37].

In iron-restricted medium the lag-phase of TIGR4 cells was prolonged and cells grew to a lower density as compared with other media containing iron, possibly because cells became depleted of intracellular iron. Time in lag-phase for TIGR4 cells in media supplemented with either ferrous or ferric iron was slightly prolonged, possibly because of the removal of other trace cations during chelation, or may be due to the slight variations in solubility of supplemented cations. Hemoglobin contains four heme groups and is a highly complex protein, whereas hemin has one heme moiety complexed with a chlorine atom and may be more efficiently utilized for growth than hemoglobin. Cells growing with hemoglobin had a short stationary phase before lysis occurred as compared with other iron sources. The reason for this is not known but hemoglobin appears to somehow facilitate density-dependent autolysis. Although, pneumococus can utilize multiple iron sources in vitro, the molecular mechanisms of iron acquisition are unknown. Pneumococci appear to lack heme oxygenase, heme / hemoglobin receptors, and cell-bound reductase or protease [39]. However, it is one among the large list of pathogenic bacteria which utilize complex heme iron compound [10,12,21,24,31,32,34].

Pneumococcus produces H_2O_2 that is thought to help establish colonization of nasopharynx by reducing competition, yet also has cytotoxic effect on epithelial cells [13,31]. Epithelial cell turnover may make intracellular iron-containing proteins such as ferritin available to pneumococci. Certain bacteria have the ability to utilize xenosiderophores [5] and some bacteria such as *Hemophilus influenzae* produce siderophores but not use them [34]. In this study we demonstrated that pneumococci can utilize ferritin and a hydroxamate siderophore as iron sources. The use of ferritin as a source of iron is uncommon in human bacterial pathogens. It is possible that pneumococci utilize ferritin, and/or xenosiderophores during nasopharyngeal colonization. These iron compounds may be derived from epithelial cell turnover and produced by other commensal species. The results of our in vitro investigations suggest that several sources of iron are potentially available for pneumococci growth, which confirm and expand a previous report on iron utilization in pneumococcus [36].

Pneumococcal cells grow as long filamentous chains in iron-restricted medium, suggesting an inhibition of daughter cell separation. This observation for pneumococci is similar to that noted in iron-starved *E. coli* [20]. In presence of supra-physiological concentration of hemoglobin, pneumococcal cells grow as aggregates and also secreted an apparent extracellular matrix material to which cells are attached. Heme iron is associated with virulence in many pathogens, although the mechanism is unclear. Extracellular polymer and cell aggregates are suggestive of biofilm formation. Cellular aggregates and cluster formation in pneumococci have been associated with biofilm formation [3,25].

During bacteremia pneumococci multiply rapidly in blood. Bacterial proteases, hemolysins and pneumolysin may make heme

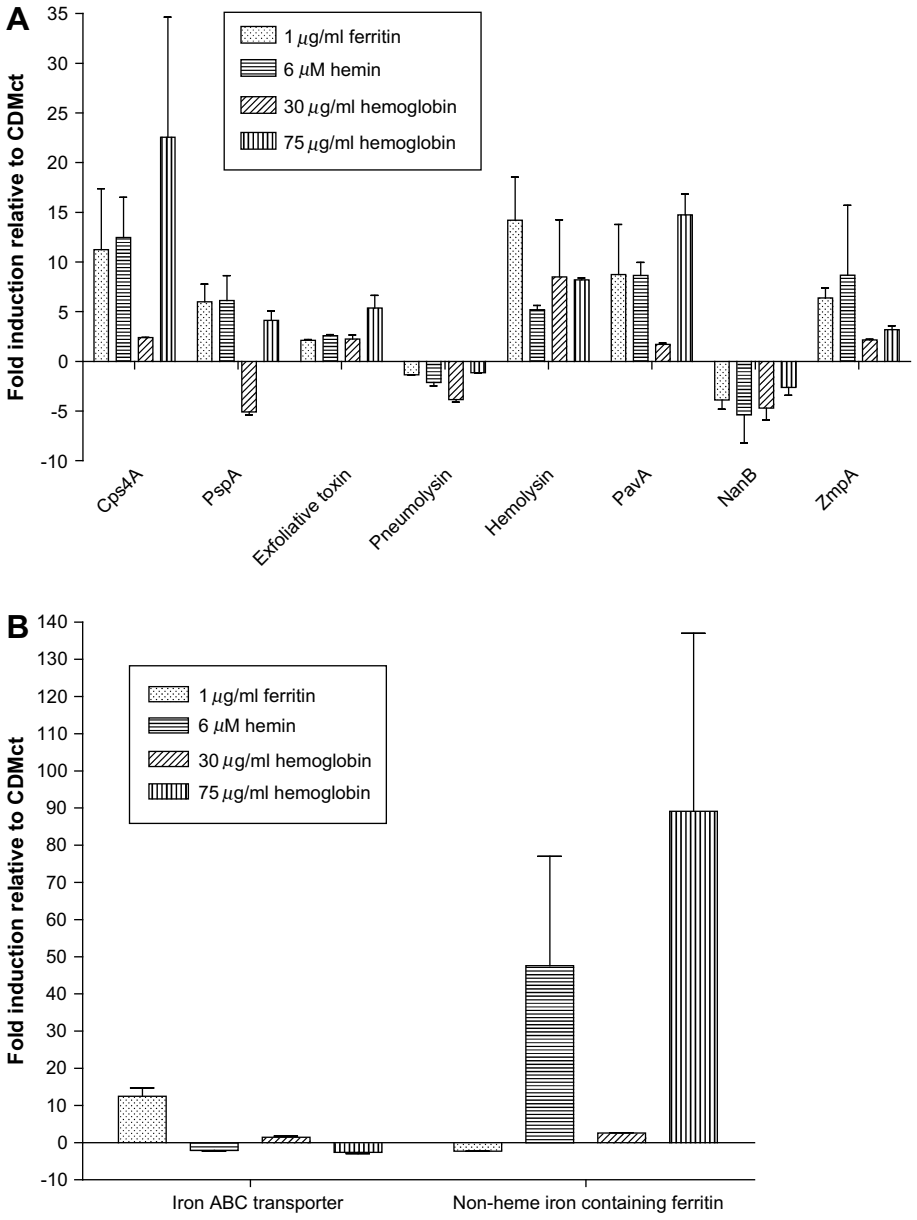

Fig. 3. Gene expression in TIGR4 cells in presence of various iron sources in vitro. A – Characterized and putative virulence genes of pneumococcus. B – Iron associated proteins in pneumococcus.

iron available from erythrocytes [15]. Ferritin, an acute phase protein, also increases in blood during inflammation. During the course of infection, the translocation of pneumococcus from the nasopharynx to a relatively iron rich environment (blood), may potentially increase expression of certain virulence factors that may provide a survival advantage. In this study, we analyzed and compared the levels of expression of several well-characterized and putative virulence genes in pneumococci grown in presence of iron-containing compounds which are likely to be encountered in a host. The transcription of *cps4A*, *zmpA*, a putative hemolysin, *pavA*, and a putative exfoliative toxin were highly regulated by various heme and non-heme iron sources, whereas regulation of *pspA*, an iron ABC transporter, and a non-heme ferritin were varied by the iron sources. Notably, transcription of *ply* and *nanB* was suppressed with all iron sources.

Pneumolysin, *zmpA*, and *pspA* are not up-regulated during colonization. This is in contrast to what is seen in *ply*, *pspA*, and *zmpA* in vitro with various iron sources. Regulation of these genes during colonization in a mouse model is probably not by iron-containing proteins tested here. In our studies with a mouse model of pneumonia, we observed that transcription of all the tested genes were affected, except that the *pspA* transcription remained unchanged. Histopathology of mouse lungs infected with TIGR4 shows significant alveolar hemorrhage, red hepatization, and hemosiderin formation early in infection (picture not shown), suggesting that increased amounts of heme and non-heme iron sources available in lungs during acute inflammation may represent a component of environmental signal for virulence gene expression. Although some iron-containing compounds affect *pspA* transcription in vitro the expression of this gene did not appreciably change in our mouse pneumonia studies.

During growth in blood, bacteria potentially encounter increased amounts of heme as well as non-heme iron. Increased RBC lysis releases heme iron and acute inflammation is associated

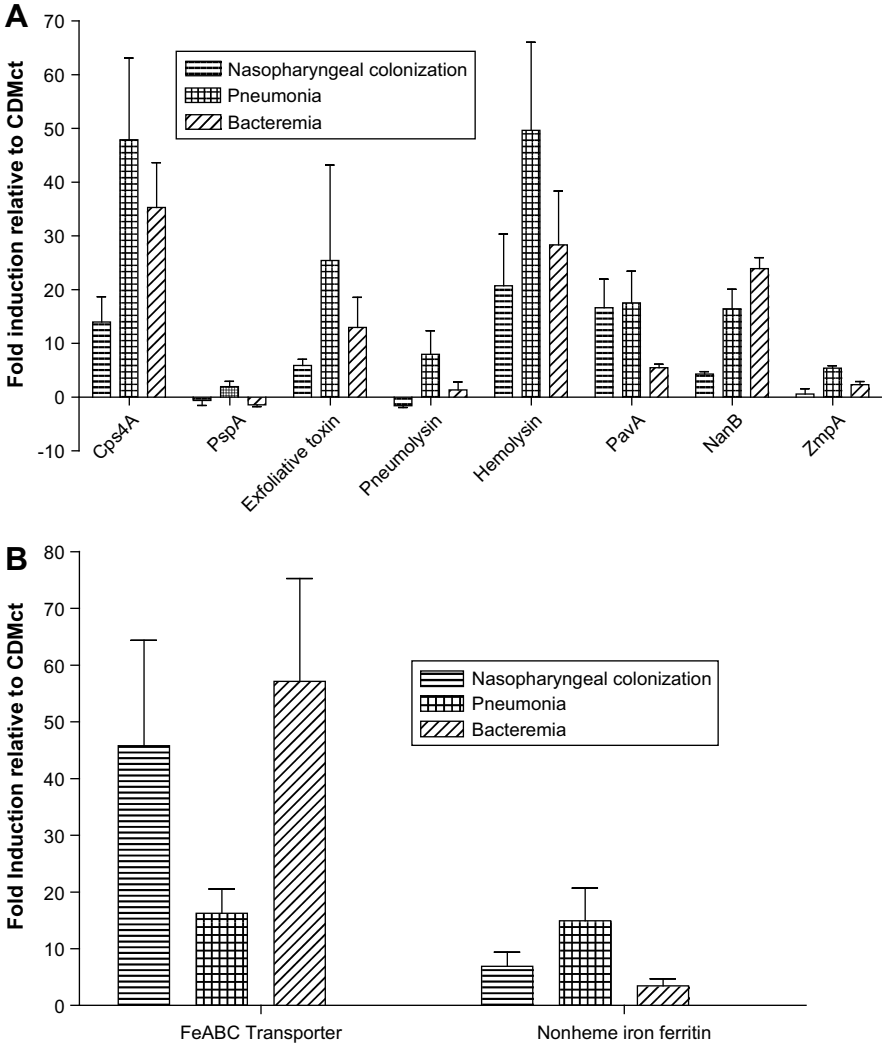

Fig. 4. Gene expression in TIGR4 cells during nasopharyngeal colonization, pneumonia, and bacteremia infection in a murine model. A – Characterized and putative virulence genes of pneumococcus. B – Iron associated proteins in pneumococcus.

with elevated level of ferritin. Possibly, high amounts of ferritin during inflammation negatively regulate non-heme iron-containing ferritin in pneumococci during bacteremia. This would parallel the in vitro down-regulation of the gene in presence of ferritin. Alternatively, *ply* and *pspA*, two well-characterized virulence-associated genes, remained unchanged in vivo. Although, *nanB* was found to be up-regulated in all in vivo models, expression of this gene appears to be regulated by some in vivo signal other than iron, as transcription was reduced with all iron sources tested in vitro.

Cerebrospinal fluid (CSF) is an ultrafiltrate of plasma that has very low iron content physiologically. Low iron content in CSF may be a mechanism of host defense against pathogens invading the central nervous system. Our data indicate that the transcription of almost all genes were either down-regulated or unchanged when bacterial cells were exposed to CSF. Interestingly, only the iron ABC transporter gene demonstrated increased transcription in CSF. Up-regulation of the iron ABC transporter in CSF suggests that the gene responds to low iron in the environment by increased transcription. These results support the hypothesis that certain virulence genes are regulated by iron availability.

In this study, we examined the regulation of several known and putative pneumococcal virulence genes. Our results indicate that pneumococcus can utilize various iron-containing compounds for growth and multiplication. A selected subset of potentially important virulence factors is differentially regulated in pneumococcus in response to various iron sources in vitro. It is possible that these genes are regulated in vivo by the quantity and form of available iron. This is the first report suggesting regulation of capsule expression in response to iron sources. Gene expression is the result of the sum of all environmental signals which are sensed by bacterial cells; however, iron, as a critical co-factor for many metabolic functions likely has significant effects on global gene expression in bacteria which live in iron-restricted environments.

5. Methods

5.1. Bacterial strains and media

S. pneumoniae TIGR4, a capsular type 4 strain was used in all studies. This strain is highly virulent in mouse models of infection and its genome has been sequenced [1,39]. Bacterial cells were grown in THY broth, (Todd Hewitt broth supplemented with 0.5% yeast extract; Difco Laboratories, Detroit, MI USA) to mid to late logarithmic phase (O.D. 0.8–1.0), at 37 °C and were collected by centrifugation. The cells were washed twice in sterile phosphate-buffered saline (PBS, pH 7.0) at 4 °C and stored at −80 °C in PBS

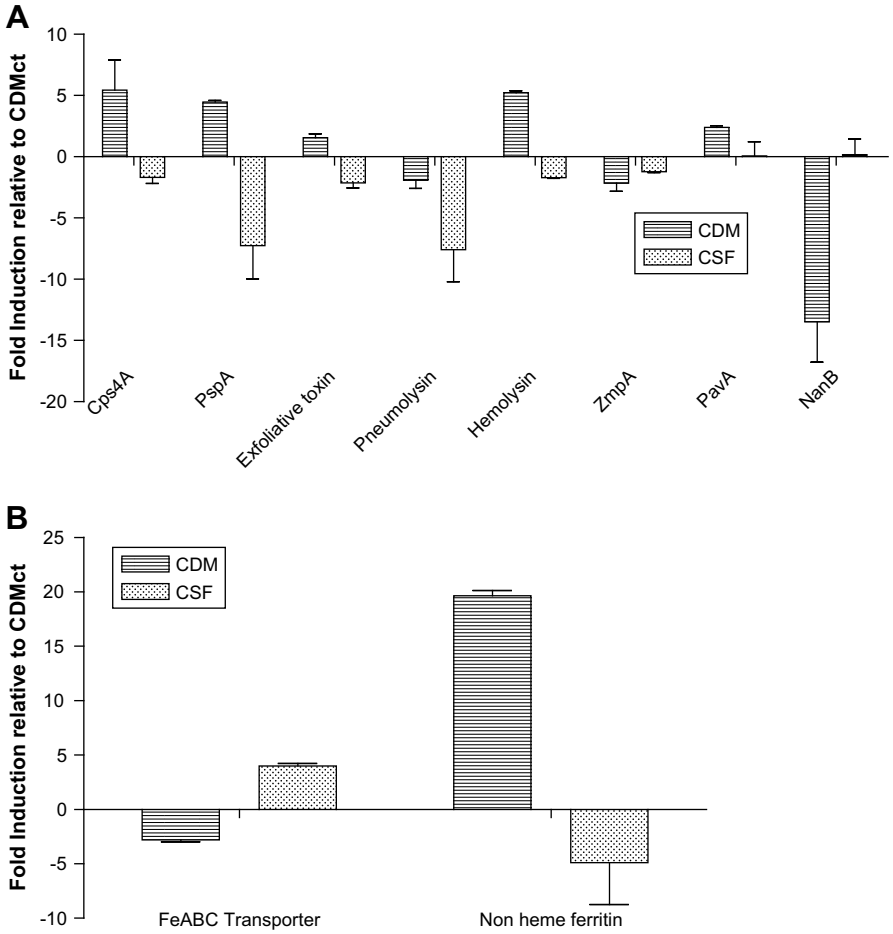

Fig. 5. Gene expression in TIGR4 cells after exposure to CSF for 6 hrs. A – Characterized and putative virulence genes of pneumococcus. B – Iron associated proteins in pneumococcus.

with 20% glycerol. Bacteria from frozen stocks were used to inoculate chemically defined medium (CDM) (JRH Bioscience, Lenexa, KS USA) [42]. The CDM was supplemented with 0.1% choline, 0.25% sodium bicarbonate and 0.073% cysteine for every 1 liter of stock. Iron-depleted CDM was prepared by treatment with chelex-100 (Bio-Rad, Hercules, CA USA) at 3% w/v for 20 hrs. Chelex-treated CDM was supplemented with $MnSO_4$, $MgSO_4$ and $CaCl_2$ salts (to a final concentration same as that in CDM) and was designated CDMct. CDM and CDMct media were sterilized using a 0.22 μm filter and stored at 4 °C.

5.2. Growth conditions

Disposable polystyrene tubes were used for all cultures. Bacterial stocks were initially grown in CDM at 37 °C to mid-log-phase (0.4–0.6 O.D.) and cells were harvested by centrifugation, washed twice with sterile PBS and then used as inoculum for growth studies. Approximately 4×10^5 CFU were added at time zero and was confirmed by serial dilution and plate counting. Bacteria were grown at 37 °C in CDM or CDMct supplemented with the following compounds: 6 μg/ml of either $FeSO_4$ or $FeCl_3$, 30 μg/ml human hemoglobin, 6 μM of hemin [36], 2.5 μg/ml of ferritin [44], 2 μM of ferrioxamine E [11] (Sigma, St. Louis, MO USA). Bacterial growth was monitored for 24 hrs by measuring absorbance at 600 nm. All cultures were briefly vortexed to thoroughly resuspend the cells before removing aliquots for spectrophotometry. After growth kinetics experiments were completed terminal subcultures were performed by plating on to 3% sheep blood agar plates (BAP) to assure purity and identity of the cultures. Bacteria were grown in CDM, CDMct, and CDMct supplemented with iron compounds at 37 °C until mid-logarithmic phase growth. Cells were collected by centrifugation and bacterial pellets were stored at −80 °C until further processing.

5.3. RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNA was purified with GenElute ™ Total RNA Purification Kit, (Sigma, St. Louis, MO USA) according to the manufacturer's instructions. Contaminating genomic DNA was removed using DNase I. Purified RNA was dissolved in nuclease-free water and stored at −80 °C. Purity and integrity of the isolated RNA was confirmed by measuring 260/280 ratio with ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE USA) and by electrophoresis on denaturing agarose gels. Presence of contaminating DNA was assessed by PCR using 16S rRNA - specific primers, and the products were visualized after electrophoresis on a 1% Tris-borate EDTA (TBE) agarose gel. All RNA samples used for qRT-PCR did not generate amplicons with this assay. One microgram of RNA was used as the template for reverse transcription with iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA USA). After reverse transcription, cDNA (1:100 dilution) was amplified by quantitative PCR with gene specific primers (Table 1) using iQ™ SYBR Green Supermix and MyiQ™ Single Color Real Time PCR Detection System (Bio-Rad, Hercules, CA USA) according to the manufacturer's

instruction. Assays were conducted in triplicate using 96-well plate in 25 μl reaction volumes as previously described [40]. Relative gene expression was analyzed using PFAFFL method [33] and fold change normalized to16S rRNA was reported.

5.4. Cell morphology

Bacteria were grown in CDM, CDMct, and CDMct supplemented with various iron sources to mid-logarithmic phase and were Gram stained. Cell morphology at 1000X magnification was observed using Axiovert S 100, inverted light microscope (Carl Zeiss Micro Imaging Inc, Thornwood, NY USA).

5.5. Nasopharyngeal colonization

Bacteria from frozen stock cultures were used for in vivo experiments. CBA/N mice (5–6 wks old) were used for all infection studies. All procedures performed were in accordance with the Institutional Animal Care and Use Committee guidelines. The external nares of each mouse was cleaned with 70% alcohol to remove any contaminant flora before intranasal challenge and also prior to collection of nasal washes after infection. For all experimental procedures mice were anesthetized with isoflurane. A bacterial suspension was administered into the nares of anesthetized mice (20 μl per mouse). The inoculum ($\sim 10^6$ CFU) was confirmed by serial dilution and plate counting. All animal models of infection used this mode of delivery. The same protocol was used for the control mice except that these animals received sterile PBS only. Three groups each with 5 mice were used separately for infection and control experiments. Two days after intranasal challenge mice were sacrificed by CO_2 asphyxiation and nasopharyngeal washes were collected using a modified version of the protocol described previously [46]. Briefly, the thoracic cavity was cut open and an incision was made in the anterior region of trachea, into which 200 μl of PBS was injected and collected from the nares into a sterile tube. The nasal washes were pooled for RNA extraction. An aliquot of this pooled wash was streaked on a BAP and incubated overnight at 37 °C to determine the presence of any contaminating flora and to confirm the purity of pneumococci in the wash. The remaining nasal wash was immediately centrifuged at 10,000 × *g* for 10 min at 4 °C and the bacterial pellet was stored at −80 °C until further processing. Before performing the nasal wash, a sample of blood was recovered from all mice by retro-orbital bleeding and streaked on BAP to assure that mice were not bacteremic. The same procedure was followed on the control mice to collect the nasal wash and detect the presence, if any, of contaminating nasal microflora.

5.6. Pneumonia

On day 4 following infection mice were euthanized and the lungs were aseptically removed and homogenized in sterile bag containing 1 ml of PBS. All lung homogenates were pooled into sterile tubes for each group. An aliquot of the homogenate was streaked on a BAP to determine the presence of any contaminating microflora and to confirm the purity of pneumococci in the sample. The remaining lung homogenate was centrifuged at 1000 × *g* for 25 s to remove particulates and unhomogenized debris, as described previously [26]. The supernatant was subsequently centrifuged at 10,000 × *g* for 10 min at 4 °C and the bacterial pellet was stored at −80 °C until further processing. Control animals were treated in an identical manner to infected animals.

5.7. Bacteremia

A protocol described previously [46] was used with slight modification. On day 5 after infection blood was collected by retro-orbital puncture and pooled in a sterile heparinized tube on ice for each group. Before collecting blood the surface of the eye was wiped with 70% alcohol to remove any contaminant bacteria. An aliquot of the blood sample was streaked on a BAP to determine the presence of any contaminating microflora and to confirm the presence of pneumococci in the blood. The remaining blood was centrifuged at 1000 × *g* for 25 s to separate plasma from red cells and leukocytes, as described previously [26]. The plasma was subsequently centrifuged at 10 000 × *g* for 5 min at 4 °C and the bacterial pellet was stored at −80 °C until further processing.

5.8. CSF exposure

Cells were grown in CDM at 37 °C until early log-phase (O.D. ∼0.3), a cell pellet was obtained by centrifugation and washed with sterile PBS. The cells were then resuspended in 1 ml of normal human cerebrospinal fluid (CSF) and incubated at 37 °C for 6 hours. After incubation cell pellets were obtained by centrifugation and were stored at −80 °C for RNA extraction. Prior to centrifugation, an aliquot from the tubes was streaked on BAP to assure purity and identity of the cultures.

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