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Role of polyamine transport in *Streptococcus pneumoniae* response to physiological stress and murine septicemia

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ABSTRACT

Streptococcus pneumoniae has a potential ABC-type transporter (Pot) for extracellular polyamines. Polyamine transport protein D (PotD) is a membrane-associated, surface protein that putatively binds polyamines such as putrescine and spermidine. In this study we used quantitative PCR (qPCR) to analyze *potD* mRNA expression under physiologically relevant stress conditions in vitro, during in vivo infection, and in the presence of polyamines and choline. Expression of *potD* mRNA was elevated 2- and 4-fold when cells were grown at either 34 or 42 °C, respectively, in a choline restricted environment. Expression increased by 5- and 11-fold in response to oxidative stress in either low or high choline environments, respectively. Putrescine led to an increase in *potD* mRNA transcription, while choline and spermidine resulted in decreased gene expression. Transcription of *potD* in pneumococci harvested from blood of systemically infected mice was 43-fold higher compared to in vitro transcription levels. Flow cytometry analysis using PotD antiserum confirmed increased PotD expression on the pneumococcal surface. These results indicate that polyamines and polyamine transport systems potentially play an important role in *Streptococcus pneumoniae* pathogenesis, and may be important for bacterial response to temperature shock, oxidative stress, choline limitation and in vivo growth.

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1. Introduction

Polyamines such as putrescine, spermidine and spermine are polycationic molecules at physiological pH and are ubiquitous in cells [1,2]. Polyamines exist primarily as complexes with RNA molecules in prokaryotes and have pleiotropic effects on translation [3,4]. In *Escherichia coli* polyamines modulate gene expression and membrane permeability, as well to protect cells from oxidative, nitrosative and acid stress [5–9]. Polyamine uptake, synthesis and degradation in bacteria are a coordinated process, and intracellular polyamine levels are stringently regulated [10,11]. Most prokaryotes have a de novo synthesis pathway in which polyamines are synthesized via enzymatic modification of precursor amino acids [2,12]. In addition, almost all bacteria have polyamine transport systems, which allow them to utilize extracellular polyamines from their environment. *E. coli* has a four-gene polyamine uptake ABC transport operon identified as polyamine transport operon (*pot*) that has been well characterized. PotD is the periplasmic substrate-binding protein that binds putrescine and spermidine and facilitates their uptake via transmembrane proteins PotB and PotC, while cytosolic PotA is an ATPase hydrolyses that supplies energy for this process [13].

Polyamines and polyamine transport systems have also been implicated in virulence of human bacterial pathogens [2]. In *Pseudomonas aeruginosa* polyamines increase susceptibility to antibiotics [14]. *Yersinia pestis* mutants that are unable to synthesize polyamines fail to produce biofilm [15]. Additionally, genes encoding proteins with homology to polyamine transporters have been associated with biofilm formation in *Pseudomonas putida* [16] and *Vibrio cholerae* [17]. Putrescine has been shown to act as an extracellular signal required for swarming in *Proteus mirabilis* [18] and restore virulence gene expression in *Shigella flexneri* mutants which are unable to synthesize modified nucleosides necessary for tRNA synthesis [19].

Streptococcus pneumoniae (pneumococcus) is a gram positive encapsulated human pathogen that colonizes the nasopharynx. From the upper respiratory tract it can enter the lungs and cause pneumonia, bacteremia and meningitis. Pneumococcal infections lead to more than a million deaths worldwide, especially in young children, the elderly, and the immunocompromised [20–22].

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Antibiotic resistance in pneumococci has become common and has added complexity to treatment [23]. A four-gene *pot* operon homologous to the *E. coli* PotABCD transport system has been described in *S. pneumoniae* [24]. PotD in *S. pneumoniae* is a surface-exposed and a membrane-associated protein expressed by diverse capsular serotypes and is antigenically conserved [25]. Pneumococcal cells with a deletion in *potD* are attenuated in a murine septicemia model and immunization with PotD reduces mortality in mice with systemic pneumococcal infections [26,27]. These observations indicate that polyamine transporter systems may play an important role in pneumococcal pathogenesis and immunity.

Pneumococci require choline for optimal cell growth and infection. Choline is covalently attached to the teichoic acids where it anchors choline-binding proteins that are important virulence factors [28]. Pneumococci may encounter choline limitation in various host imposed microenvironments in vivo, and mutants deficient in choline biosynthesis and transport are attenuated in various animal models of infections [29,30]. Pneumococci can substitute choline with either polyamines or ethanolamine under laboratory conditions which then get incorporated in the bacterial cell wall [24,31]. Thus, small molecule metabolites which are similar in charge and structure may potentially serve as choline analogues in choline restricted growth environments during infection. Additionally, environmental polyamine acquisition may play an important role in pneumococcal response to temperature, oxidative and pH stress in manner similar to other bacterial pathogens [2].

In this study we have examined the differential expression of *S. pneumoniae* PotD in response to stress conditions, which are likely to be encountered by bacteria in various host environments and during murine septicemia.

2. Results and discussions

2.1. *PotD is expressed in multiple* S. pneumoniae *capsular serotypes implicated in clinical disease*

Relative conservation and distribution of PotD among multiple pneumococcal serotypes was evaluated by sequence analysis, immunoblotting and flow cytometry. All completely sequenced pneumococcal genomes (TIGR4, D39, R6 and Hungary19A-6) have genes coding for the *pot* operon. Additionally, analyses of 19 pneumococcal isolates, which are currently being sequenced (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) also have genes homologous to the *potABCD* system. Thus, polyamine transport genes seem to be well conserved in all sequenced pneumococcal isolates. We evaluated expression of PotD in different *S. pneumoniae* capsular serotypes (one non-pathogenic, unencapsulated isolate was also included) implicated in pneumococcal disease and colonization. PotD expression was tested by immunoblotting with polyclonal PotD antiserum to probe of whole cell lysates of all isolates grown under laboratory conditions with minimum passages (<4) after isolation. Since only the assessment of either presence or absence of PotD in various serotypes was being made, equal volumes of whole cell lysates were analyzed without adjusting for protein concentrations. A single band corresponding to the predicted molecular weight of PotD was seen in all isolates (Fig. 1). Finally, flow cytometry was used to evaluate expression of PotD molecules on cell surfaces of all isolates. The mean fluorescence intensity of cells stained with PotD antiserum was significantly higher than control cells stained with secondary antibody alone (data not shown). These results indicate that PotD is relatively well conserved among multiple capsular serotypes common in human infections. Pneumococcal serotype 2 isolate D39 was chosen for all subsequent qPCR and flow cytometry experiments because it replicates to high levels in blood in a mouse bacteremia model [32].

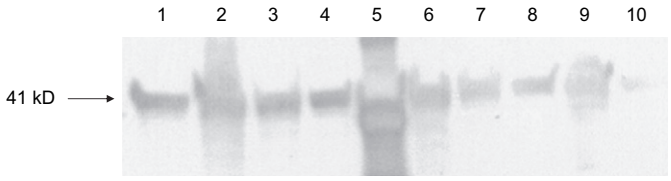

Fig. 1. Detection of PotD in different *S. pneumoniae* capsular serotypes by immunoblotting. Whole cell lysates of exponentially growing pneumococci were separated by SDS-PAGE and probed with PotD antiserum. Lane number and strain (serotype): 1 – WU2 (3), 2 – TIGR4 (4), 3 – R36A (unencapsulated), 4 – MW (9), 5 – recombinant PotD, 6 – JW48 (23), 7 – EF3030 (19), 8 – DV4834 (14), 9 – D39 (2), 10 – BC51 (6).

2.2. *Effect of choline and polyamine concentrations on* potD *expression*

Transcription of *potD* increased by 7.5-fold in low choline conditions compared to that in a high choline environment (Fig. 3); while a 2.5-fold reduction in gene expression was observed in high choline conditions compared to expression in low choline environment (Fig. 2). This suggests that polyamine transport may be regulated in response to choline concentrations during pneumococcal growth in vivo. Putrescine and spermidine, as well as the amino alcohol choline, are small hydrocarbon molecules with positively charged quaternary nitrogen's and similar molecular weights. Putrescine and ethanolamine have previously been shown to substitute for choline during pneumococcal growth and are associated with the bacterial cell wall [24,33]. Bacterial ABC transporter substrate-binding proteins like PotD often bind multiple ligands, which are structurally related compounds [34]. Increase in *potD* transcription in response to choline deprivation may be a result of PotD being involved in binding and transport of choline. Alternatively, PotD upregulation in response to low choline may represent increased use of putrescine for cell wall synthesis. It has been shown that *licD2* (a choline transporter) is upregulated during choline starvation [35]. These results suggest that pneumococci may have multiple mechanisms to overcome choline limitation during growth in specific host microenvironments.

Addition of spermidine to a low choline medium, resulted in 2.5-fold down regulation in *potD* expression while putrescine led to a 3-fold increase in *potD* induction as compared to expression levels

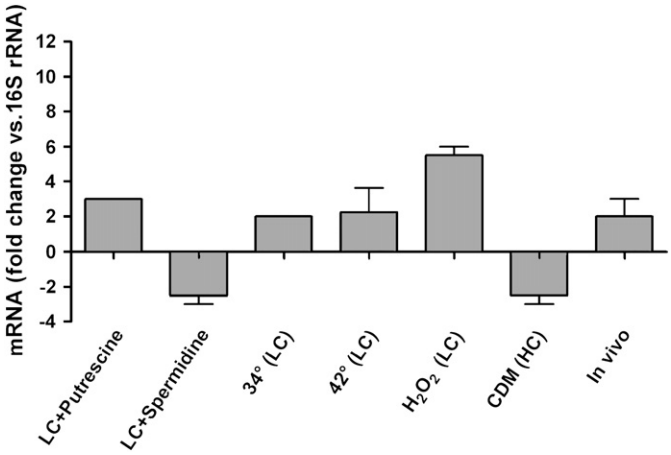

Fig. 2. Quantitative PCR analysis of *potD* expression in *Streptococcus pneumoniae* D39 cells grown in CDM supplemented to 6 μg/ml (low choline/LC) and harvested at OD_{600} 0.5. Transcription of *potD* in various growth conditions compared with *potD* transcription in CDM + LC alone at 37 °C. In vivo bar represents *potD* expression in cells harvested from blood of mice systemically infected with D39 compared to expression levels in CDM + LC at 37 °C expression levels. Bars represent the means of three experiments with error bars representing standard errors of the means.

in a low choline environment without spermidine or putrescine (Fig. 2). Spermidine is a negative regulator of the *pot* operon in *E. coli* and reduction in pneumococcal *potD* transcription in response to spermidine was not unexpected [36]. Since putrescine is structurally related to choline it is possible that, during conditions of choline starvation, putrescine uptake via PotD could potentially serve to substitute for pneumococcal choline in teichoic acids. Another possibility is that this response is independent of the presence of putrescine and is solely a result of choline starvation as observed in the previous experiment. However, further experimentation is required to confirm these observations.

2.3. *Expression of* potD *in response to oxidative stress*

Aerobic respiration by bacteria generates reactive oxygen species, which can react with many cellular components, including DNA, RNA, proteins, and lipids, and can result in decreased growth rates, mutagenesis, and cell death. Respiratory burst by phagocytes generates reactive oxygen species leading to microbial cell death. *S. pneumoniae* does not produce catalase, yet it can survive exposure of upto 3 mM hydrogen peroxide (H_2O_2) generated during aerobic growth in high oxygen environments such as respiratory mucosal surfaces. The enzyme pyruvate oxidase (SpxB), which is responsible for endogenous H_2O_2 production in pneumococci, has also been shown to confer resistance to hydrogen peroxide [37]. PsaA, a lipoprotein component of an Mn^{+2} ABC transport operon, also seems to play a protective role against oxidative stress in pneumococci [38]. Polyamines have been implicated in protection from toxic effects of reactive oxygen species by either scavenging free radicals, binding and protecting nucleic acids from oxidative damage, and inducing operons that encode enzymes capable of inactivating hydrogen peroxide [2,39–42]. *E. coli* cells deficient in polyamine synthesis are killed by concentrations of H_2O_2 that are non-toxic to wild-type cells [7].

In addition to *spxB*, polyamine transport systems like the *pot* operon may play an important role in protecting *S. pneumoniae* from oxidative stress. D39 cells in mid-log phase of growth were exposed to a previously reported sub-lethal concentration of 3 mM H_2O_2 for 1 h at 37 °C [38]. Oxidative stress increased *potD* transcription 11-fold (Fig. 3) in medium containing high choline levels and by 5.5-fold in a low choline medium (Fig. 2), compared to control cells. Increased PotD production during an oxidative stress response, may lead to transport of additional extracellular polyamines into the cells and protect nucleic acids from reactive oxygen species. Thus, polyamines may allow *S. pneumoniae* to resist the oxidative stress response imposed by phagocytic cells and also may be an additional mechanism to survive endogenous hydrogen peroxide production.

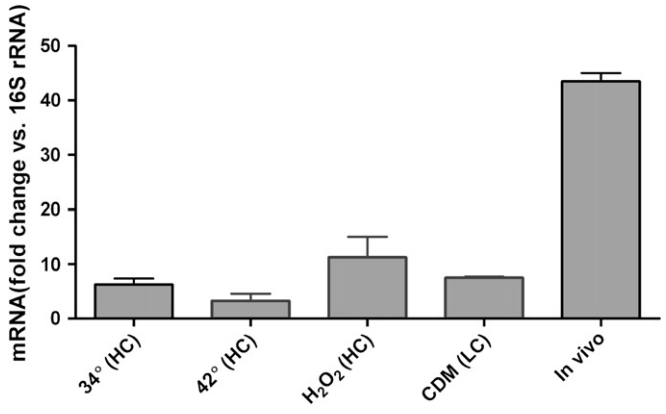

Fig. 3. Quantitative PCR analysis of *potD* expression in *Streptococcus pneumoniae* D39 cells grown in CDM supplemented to 20 μg/ml choline (high choline/HC) and harvested at OD_{600} 0.5. Transcription of *potD* in various growth conditions compared with *potD* transcription in CDM + HC alone at 37 °C. In vivo bar represents *potD* expression in cells harvested from blood of mice systemically infected with D39 compared to expression levels in CDM + HC at 37 °C expression levels. Bars represent the means of three experiments with error bars representing standard errors of the means.

2.4. *Effect of low and high temperatures on* potD *expression*

The global response of pneumococci during growth at low or high temperatures is not well characterized. In *E. coli*, addition of exogenous putrescine allows more efficient protein synthesis in polyamine auxotrophic mutants exposed to temperature stress [43]. In these experiments we characterized pneumococcal *potD* expression in response to different temperatures.

The normal environmental niche for *S. pneumoniae* is the nasopahrynx; where the ambient temperature is 2–3 °C lower than core body temperature of 37 °C. When exposed to 34 °C in both low and high choline conditions, *potD* transcription increased by 2- and 5-fold compared to expression at 37 °C (Figs. 2 and 3). Thus, polyamine acquisition may be a part of the cellular physiological response that facilitates pneumococcal nasopharyngeal colonization. Additionally, fever is a common sequela of almost all invasive pneumococcal infections, which subjects bacterial cells to temperatures above 37 °C. Exposure of bacterial cells to 42 °C for 60 min increased *potD* expression by 2- and 3-fold in low and high choline conditions respectively, as compared to gene expression at 37 °C (Figs. 2 and 3). Taken together, these data signify that polyamines and polyamine transport systems may play important physiological roles in *S. pneumoniae* during temperature stress, possibly by their pleiotropic effects on nucleic acid stability, transcription and translation.

2.5. *Expression of* potD *during murine septicemia*

The first evidence of a role for PotD during infection was a signature tagged mutagenesis analysis of *S. pneumoniae*, which led to identification of an attenuated clone with a mutation in *potD* [44]. *S. pneumoniae* PotD mutants have significantly increased LD_{50} compared to wild-type cells in murine bacteremia, emphasizing the importance of polyamines for pneumococcal virulence and pathogenesis [26]. Results from these in vitro experiments suggested that polyamine transport might be important for pneumococcal survival and multiplication during infection. We evaluated *potD* transcription levels in vivo during systemic murine infection. Transcription of *potD* mRNA from bacteria harvested from blood of bacteremic mice was significantly increased compared with cells grown in vitro in either low (Fig. 2) or high choline environments (Fig. 3). These results suggest that polyamine acquisition may play an important role during infection and need to be further evaluated to better understand pneumococcal virulence and pathogenesis.

2.6. *Flow cytometry analysis of pneumococci*

Flow cytometric analyses were performed to confirm that an increase in *potD* mRNA transcription does, in fact, result in enhanced protein expression on the bacterial cell surface. Mean fluorescence intensity of pneumococci exposed to different temperature shifts and H_2O_2 was significantly higher than control cells (Table 1). These results indicate that increased transcription of *potD* results in increased protein expression on the bacterial cell surface during environmental stress and that polyamine acquisition may contribute to a pneumococcal stress response. Similarly, growth in a medium containing low choline and putrescine resulted in approximately 2-fold increase in mean fluorescence intensity compared to cells grown in low choline alone, in agreement with transcription data (Table 1). However, no significant change in PotD

Table 1
Flow cytometry and quantitative PCR analyses of *S. pneumoniae* PotD expression in D39 during growth in indicated conditions

Growth conditions		*potD* mRNA fold-change	Mean fluorescence intensity ± SEM
Presence of choline/polyamines	Low choline	1.00 ± 0.0	21.03 ± 1.59
	High choline	1.00 ± 0.0	22.94 ± 1.15
	Low choline + putrescine	3.00 ± 0.2	49.14 ± 2.59
	Control		2.11 ± 0.41
Physiological stress	Low choline: 34 °C	2.00 ± 0.0	51.81 ± 4.38
	Low choline: 42 °C	2.24 ± 1.37	45.49 ± 2.91
	Low choline: H_2O_2	5.50 ± 0.5	37.55 ± 3.50
	High choline: 34 °C	6.25 ± 1.10	53.01 ± 6.59
	High choline: 42 °C	3.25 ± 1.31	59.55 ± 12.20
	High choline: H_2O_2	11.25 ± 3.77	38.67 ± 7.36
In vivo	Bacteremic mice	43.50 ± 1.5	94.00 ± 2.00

Control represents D39 cells stained with secondary antibody alone. Numbers represent results from three independent experiments ± standard errors of means (SEM).

expression was seen on comparison of mean fluorescence intensities of cells grown in high choline medium compared with those grown in low choline conditions. The reasons for this are unknown, but may be related to up or downregulated expression of other proteins during growth in other stress conditions, which may affect PotD expression.

Mean fluorescence intensity of D39 harvested from mice increased approximately 9-fold compared with in vitro cultured bacteria, suggesting that increased *potD* transcription in vivo leads to increase in protein expression (Table 1 and Fig. 4) and that polyamine acquisition may play an important role in pneumococcal pathogenesis. Overall, pneumococci express more PotD during in vivo growth and in response to certain in vitro stressors, suggesting that polyamine transport maybe an important defense and/or survival mechanism during *S. pneumoniae* infection.

Polyamines play pleiotropic roles during bacterial stress responses in many different pathogens; however, few insights are available concerning the exact molecular mechanisms of polyamine action. Recent reports indicate that polyamine-mediated protection against stress in bacteria might involve direct binding of polyamines and nucleic acids, scavenging of the reactive oxygen species or differential expression of protective enzymes like superoxide dismutase [2]. All of these mechanisms most likely contribute to variable degrees, with the relative contribution depending on the

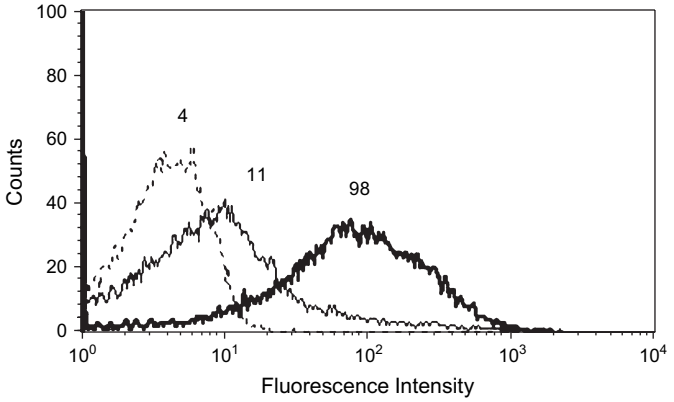

Fig. 4. Representative flow cytometric analysis of PotD expression on *Streptococcus pneumoniae* D39 cells collected from the blood of systemically infected mice. Dashed line-D39 cells stained with the secondary antibody alone (negative control); thin line-D39 cells grown in CDM; thick line-D39 cells collected from infected mice. Data are shown as fluorescence intensity of the total cell population, and the geometric mean of each peak is indicated.

metabolic state of the cell, growth medium or the type of stress. Although polyamines are ubiquitous in all cells and are crucial to normal cellular growth, control of physiological concentrations of extracellular polyamines in mammals is poorly understood. Polyamine transport may be modulated in pneumococci in different microenvironments in the host to supplement intracellular polyamine levels produced by de novo synthesis. In addition, environmental polyamine uptake may be a bacterial response to oxidative and temperature stress from host immune responses. *S. pneumoniae* subjected to a choline deficient environment is known to upregulate expression of a choline transporter [35]. Our results indicate that increase in extracellular choline levels results in reduction in *potD* transcription and vice versa. Because of structural similarity between polyamines and choline it is possible that PotD may bind and transport choline into pneumococcal cells, in choline limited microenvironments. Alternatively, polyamine transporters like PotD may function to increase intracellular polyamines, which may substitute for choline during teichoic acid synthesis during choline limitation [24].

Overall, our data suggests that transcription and expression of PotD maybe significantly upregulated in various host microenvironments where pneumococci are subjected to temperature shifts, nutrient limitation, and toxic metabolic products as well as during infection in an animal model. Acquisition of environmental polyamines may play an important role in disease and further characterization of the role of polyamines in pneumococcal physiology will lead to a better understanding of pneumococcal pathogenesis.

3. Materials and methods

3.1. Bacterial strains, media and growth conditions

All qPCR, flow cytometry and in vivo experiments were performed with mouse-virulent *pneumococcal* serotype 2 strain D39, whose genome has been recently sequenced [45]. Approximately 4×10^3 CFU of D39 from frozen stocks were used to inoculate chemically-defined medium (CDM) (JRH Bioscience, Lenexa, KS, USA), which has been previously described [46]. CDM was supplemented with filter-sterilized choline chloride at 6 μg/ml (low choline, LC) or 20 μg/ml (high choline, HC) [35].

All cultures were grown in disposable polystyrene tubes and incubated in 5% CO_2 at 37 °C. Bacteria were grown in both low and high choline conditions until mid-log phase (OD_{600} 0.5), then exposed to various alterations in growth conditions, including exposure to either 34 °C or 42 °C for 60 min or 3 mM hydrogen peroxide (H_2O_2) for 60 min. After exposure to various environmental shifts, cells were collected by centrifugation and the pellets were stored at −80 °C. To analyze the effect of PotD ligands, putrescine and spermidine on gene expression, bacteria were grown in CDM supplemented with 50 μg/ml of putrescine or spermidine, harvested when cells reached mid-log phase, and stored at −80 °C. D39 cells grown in LC or HC conditions and harvested at mid-log phase served as controls. Terminal subcultures, serial dilutions and plate counts on 5% sheep blood agar plates were performed with all cultures to confirm the purity and viability of the harvested cells before qPCR or flow cytometry analysis.

3.2. Immunoblot analysis

Distribution and conservation of PotD among different pneumococcal capsular serotypes was evaluated by immunoblot analysis. Cultures were grown in Todd–Hewitt broth supplemented with 0.5% yeast extract at 37 °C until mid-log phase and harvested by centrifugation. Samples represent equal volumes of whole cell lysates of various pneumococcal serotypes separated by SDS-PAGE with 3–12% gels, electroblotted onto PVDF membranes, and probed

with specific antiserum as previously described (Shah, 2006 #9; Shah, 2006 #10).

3.3. RNA purification and reverse transcription

RNA was purified from frozen bacterial pellets using Qiagen RNeasy kit (Qiagen, Valencia, CA, USA) by following the manufacturer's protocol. Isolated RNA was treated with DNase to remove genomic DNA contamination, and purity was checked, by performing PCR using primers specific for 16S rRNA in the presence or absence of reverse transcriptase. RNA concentration was quantified spectrophotometrically, and quality was determined by $A_{260/A280}$ and agarose gel electrophoresis; purified RNA was stored in nuclease free water at −80 °C. One microgram of total RNA from each sample was used for cDNA synthesis using the iScript Reverse Transcriptase kit (Biorad Laboratories, Hercules, CA, USA) according to manufacturer's instructions.

3.4. Harvesting pneumococci from mice

Animal studies were approved by the local animal care and use committee. Six to 8 week old CBA/CAHN-XID/J (CBA/N) mice (Jackson Laboratories, Bar Harbor, ME, USA) were infected intravenously with 2×10^4 CFU of D39. Blood was collected by retro-orbital puncture 24–48 h following infection. Bacteria were harvested from mouse blood as previously described [47] and stored at −80 °.

3.5. Quantitative PCR

Gene-specific oligonucleotide primers were designed using the Beacon Designer 2.1 software program (Premier Biosoft International, Palo Alto, CA, USA) and purchased from Operon Biotechnology (Huntsville, AL, USA). Primers used to amplify a 117 base pair internal fragment of *potD* were: *potD* forward – 5′-AGTTGGAAGAGACAGTGGATAAGC-3′ and *potD* reverse – 5′-AGAAGGTCACGCCGATTGC-3′. 16S rRNA served as a relatively invariant housekeeping control gene and was amplified using the following primers: *16S rRNA* forward – 5′-CTGCGTTGTATTAGCTAGTTGGTG-3′ and 16S rRNA reverse – 5′-TCCGTCCATTGCCGAAGATTC-3′. Amplification efficiency of both *potD* and 16S rRNA cDNA was evaluated by using serial dilutions of the template DNA, ranging from 10^1 to 10^4 target copies per reaction. Quantitative PCR experiments to measure expression of *potD* mRNA levels were performed using iQ SYBR Green Supermix Kit (Biorad Laboratories, Hercules, CA, USA) as previously described [48]. Briefly, the optimized real time PCR reaction mix contained 0.5 μM each of forward and reverse primers, and the SYBR Green Supermix reagent. For each specific reaction mixture, 1 μl of cDNA for amplification of *potD* and 16S rRNA was added to a 24 μl master mix. Real-time data were obtained during the extension phase, and threshold cycle values were obtained at the log phase of each gene amplification. Gene expression results were analyzed by using the PFaffl method of real time PCR analysis [49]. Results are expressed as fold changes and first normalized against 16S rRNA expression levels within the same sample before being compared between different growth conditions. The experiments were repeated two times with each quantitative PCR being done in triplicate. Bars represent the means of three experiments with error bars representing standard errors of the means.

3.6. Flow cytometric analysis of bacteria harvested from mice

For flow cytometry analysis, bacteria collected from bacteremic mice as well as all pneumococci exposed to various stress conditions were processed as previously described [25]. Briefly, cells were incubated with 100 μl PotD rabbit antiserum, washed with phosphate buffered saline (PBS) and then incubated with biotinylated goat anti-rabbit IgG antibody (Southern Biotech, Birmingham, AL, USA). Finally, cells were suspended in streptavidin conjugated to Alexafluor 488 (Southern Biotech, Birmingham, AL, USA) and analyzed with FACScan cytometer (Beckton Dickinson, Franklin lakes, NJ, USA).

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