Polyamine Transport Is Required for Stress Responses and Capsule Production in Streptococcus Pneumoniae

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Research Article

Keywords: Streptococcus pneumoniae, polyamine transporter, oxidative stress, nitrosative stress, transcriptome, metabolome

Posted Date: February 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-192362/v1

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Abstract

Infections due to *Streptococcus pneumoniae*, a commensal in the nasopharynx, still claim a significant number of lives worldwide. Genetic plasticity, antibiotic resistance, limited serotype coverage of the available polysaccharide-based conjugate vaccines confounds therapeutic interventions. Pathogenic systems that allow successful adaption and persistence in the host could be potential innovative targets for mediations. Polyamines are ubiquitous polycationic molecules and regulate many cellular processes.

We previously reported that deletion of *potABCD*, an operon that encodes a putrescine/spermidine transporter (Δ*potABCD*), resulted in an un-encapsulated attenuated phenotype. Here we characterize the transcriptome, metabolome, and stress responses of *S. pneumoniae* that is dependent on the polyamine transporter. Expression of genes involved in oxidative stress responses and the central metabolism was reduced while that of genes involved in the Leloir, tagatose, and pentose phosphate pathways was increased in Δ*potABCD*. Downregulation of genes of the central metabolism will reduce production of precursors of capsule polysaccharides. Metabolomics results show reduced glutathione and pyruvate levels in the mutant. We also show that the *potABCD* operon protects pneumococci against hydrogen peroxide and nitrosative stress. These results show the importance of the *potABCD* operon and polyamine transport in pneumococcal physiology and fitness that represents a novel target for therapeutic interventions.

Introduction

Despite years of intensive research, infections due to *Streptococcus pneumoniae* (pneumococcus) still claim countless lives across the entire globe [1]. Pneumococci account for up to 15% of pneumonia cases in the USA and 27% worldwide [2]. Following colonization of the nasopharynx, pneumococci can translocate to sterile sites and cause infections such as community-acquired pneumonia, meningitis, septicemia and otitis media [3]. Well-coordinated metabolic networks for efficient exploitation of the host micro-nutrients and immune response evasion strategies are crucial for pneumococcal pathogenesis. Serotype diversity, limited serotype coverage of the available vaccines, serotype replacement, and increase in multidrug-resistant strains confound intervention strategies to limit the spread of pneumococci [4–6]. A better understanding of pneumococcal physiology and survival mechanisms in the host can identify novel therapeutic targets.

Polyamines are polycationic molecules that interact with RNA, DNA, and phospholipids and modulate cellular processes such as cell division, transcription, and translation [7, 8]. Putrescine, spermidine and cadaverine are the principal bacterial polyamines and their intracellular concentrations are tightly regulated by transport, biosynthesis, and degradation [8]. In pathogenic bacteria, polyamines are known to regulate virulence, biofilm formation, stress responses, *in vivo* fitness, and host-pathogen interactions [9]. Therefore, failure to sustain intracellular polyamine levels could alter regulatory homeostasis which could interfere with *in vivo* survival and pathogenesis. Our previous work has shown that the polyamine transport operon *potABCD* is essential for virulence in murine models of pneumococcal infections [10]. We have shown that in a murine model of pneumonia, Δ*potABCD* is more invasive than the wild type.
strain (WT) but is more susceptible to opsonophagocytosis. Uptake of ΔpotABCD by neutrophils does not require antibody opsonization [11]. PotD, either alone or in combination with other proteins, has been shown to elicit protection against pneumococcal colonization, pneumonia, and sepsis in mice [12–14]. We have recently shown that deletion of the potABCD operon resulted in reduced intracellular concentrations of putrescine and spermidine and an un-encapsulated phenotype [10, 15]. Since pneumococcal capsular polysaccharide (CPS) is a determinant of virulence, reduced CPS could explain reported in vivo attenuation of ΔpotABCD. Initial characterization of the ΔpotABCD proteome identified altered expression of over 100 proteins including virulence factors such as pneumolysin [10]. However, the limited proteome coverage did not allow for the identification of specific mechanisms of metabolic regulation that could explain the observed phenotype. To determine polyamine dependent metabolic regulation that is at the intersection of pneumococcal virulence, we compared the ΔpotABCD and WT transcriptome and metabolome using RNA-Seq and metabolomics. Given the role of polyamines in the defense against reactive radicals, and the diversity of stress conditions encountered by pneumococci, reduced intracellular polyamine levels are expected to adversely affect stress responses that are critical for in vivo fitness. We therefore, examined the role of polyamine transport on the susceptibility of pneumococci to oxidative and nitrosative stress. We determined the impact of impaired polyamine transport on the intracellular pH (pH$_i$), GSH/GSSG ratio, and production of Nicotinamide adenine dinucleotide phosphate (NADPH), and hydrogen peroxide (H$_2$O$_2$). Our results show that polyamine transport is required for the regulation of stress responses and central metabolism that adversely affect capsular polysaccharide synthesis in pneumococci and present an attractive target for developing novel therapeutics.

Results

Polyamine transport modulates pneumococcal gene expression.

Comparison of the transcriptome profiles of TIGR4 and ΔpotABCD identified regulatory mechanisms responsive to polyamine transport. We identified a total of 1333 genes whose expression varied significantly in ΔpotABCD. Expression of 651 and 682 genes was downregulated and upregulated in ΔpotABCD, respectively (Tables 1–3 and Supp Table 2). Gene Ontology analysis of the differentially expressed genes (DEGs) identified significant enrichment of five categories which included: phosphotransferase systems (PTS), galactose metabolism, ABC transporters, amino and nucleotide sugars as well as fructose and mannose metabolism. Major biological functions and pathways represented by the DEGs with a fold change of ≥ 2 and metabolites are discussed in the following sections and shown in Tables 1–3 and Table 5, respectively.

Polyamine transporter and pneumococcal stress responses.

Polyamines protect cells against reactive oxygen species (ROS) via regulation of stress response genes or by directly scavenging reactive free radicals [8, 16]. Therefore, deficiency of polyamines could impact the redox status and render ΔpotABCD susceptible to oxidative stress. Downregulation of genes that encode
treR, a scavenger of H₂O₂, molecular chaperones and their regulator, hrcA (Table 3), indicate impaired redox and repair systems which could compromise in vivo fitness of ΔpotABCD [17, 18]. Reduced expression of genes that encode several tRNAs (Supp. Table 2) could represent cellular adaptation during stress to meet the demand for redox homeostasis [19]. Increased expression of some genes that encode regulators implicated in pneumococcal stress responses, the arginine deiminase system (ADS) and glutamine transporters could be in response to oxidative stress in the mutant [20, 21] (Table 1). Elevated glutamine influx and ADS could be in response to meet the increased demand for energy (ATP) and to restore the buffering capacity (ammonia). Increased expression of ABC transporters for the import of iron, manganese, and phosphate (Tables 1) could result in cationic imbalance and negatively impact cellular functions and redox homeostasis [22]. These results show that deficiency of the polyamine transporter impairs pneumococcal stress systems, renders the mutant susceptible to oxidative stress and triggers polyamine independent redox systems to combat the stress.
Table 1
The *potABCD* operon is essential for pneumococcal stress responses.

| Locus tag | Gene   | Fold change $\Delta potABCD/TIGR4$ | Description                                                      |
|-----------|--------|-------------------------------------|-----------------------------------------------------------------|
| SP_1884   | SP_1884| -3.6                                | PTS trehalose transporter subunit IIBC                           |
| SP_1883   | SP_1883| -3.2                                | Trehalose6-phosphate hydrolase                                  |
| SP_1907   | groES  | -3.9                                | Co-chaperone                                                    |
| SP_0519   | dnaJ   | -3.8                                | Molecular chaperone                                             |
| SP_0517   | dnaK   | -4.2                                | Molecular chaperone                                             |
| SP_1906   | groEL  | -3.3                                | Molecular chaperone                                             |
| SP_1870   | SP_1870| 2.7                                 | Iron-compound ABC transporter                                   |
| SP_1871   | SP_1871| 2.9                                 | Iron ABC transporter ATP-binding protein                        |
| SP_1241   | SP_1241| 3.1                                 | Glutamine transport system substrate-binding                    |
| SP_1242   | SP_1242| 3.2                                 | Glutamine transport system ATP-binding protein                  |
| SP_2087   | pstB   | 41.8                                | Phosphate ABC transporter ATP-binding protein                    |
| SP_2085   | pstC   | 37.2                                | Phosphate ABC transporter permease subunit                      |
| SP_2084   | pstS   | 27.2                                | Phosphate ABC transporter substrate-binding                     |
| SP_2086   | pstA   | 40.6                                | Phosphate ABC transporter permease protein                      |
| SP_1650   | psaA   | 3.2                                 | Manganese ABC transporter substrate-binding                     |
| SP_1648   | psaB   | 3.4                                 | Metal ABC transporter ATP-binding protein                       |
| SP_1649   | psaC   | 3.5                                 | Metal ABC transporter permease                                  |
| SP_0502   | glnA   | 4.2                                 | Glutamine synthetase                                            |
| SP_2148   | arcA   | 4.6                                 | Arginine deiminase                                              |
| SP_2151   | arcC   | 3.7                                 | Carbamate kinase                                                |
| SP_0798   | ciaR   | 3.0                                 | DNA-binding response regulator                                   |
| SP_0799   | ciaH   | 3.0                                 | Two-component sensor histidine kinase                           |
| SP_0501   | merR   | 4.0                                 | MerR family transcriptional regulator;                          |
Galactose utilization and the pentose phosphate pathway (PPP).

The signature of oxidative stress was further revealed by metabolic shift towards the PPP in the mutant which is usually in response to oxidative stress to produce NADH/NADPH which are cofactors in antioxidant systems [23].

| Locus tag | Gene | Fold change $\Delta \text{potABCD}/\text{TIGR4}$ | Description |
|-----------|------|-----------------------------------------------|-------------|
| SP_0515   | hrcA | -4.3                                          | Transcriptional regulator |
| SP_2088   | phoU | 47.2                                          | Phosphate uptake regulator |

Table 2
The $\text{potABCD}$ operon promotes galactose utilization and the pentose phosphate pathway.

| Locus tag | Gene | Fold change $\Delta \text{potABCD}/\text{TIGR4}$ | Description |
|-----------|------|-----------------------------------------------|-------------|
| SP_2127   | tktN | 222.7                                         | Transketolase |
| SP_2128   | tktC | 225.5                                         | Transketolase |
| SP_2130   | SP_2130 | 211.5                                      | Ascorbate PTS system EIIB |
| SP_2129   | SP_2129 | 214.7                                      | PTS ascorbate transporter subunit IIC |
| SP_1192   | lacB | 2.8                                           | Galactose6-phosphate isomerase subunit |
| SP_1193   | lacA | 2.8                                           | Galactose6-phosphate isomerase subunit |
| SP_1190   | lacD | 2.8                                           | Tagatose1,6-diphosphate aldolase |
| SP_1191   | lacC | 2.8                                           | Tagatose6-phosphate kinase |
| SP_1186   | lacF-2 | 2.0                                       | Lactose PTS system EIIA component |
| SP_2165   | fucU | 4.5                                           | Fucose isomerase |
| SP_2166   | fucA | 3.8                                           | L-fuculose phosphate aldolase |
| SP_1853   | galK | 6.1                                           | Galactokinase |
| SP_0066   | galM | 2.7                                           | Galactose mutarotase |
| SP_0064   | SP_0064 | 3.4                                      | PTS mannose PTS system EIIA |
| SP_0645   | SP_0645 | 8.8                                      | PTS galactose PTS system EIIA |
| SP_0646   | SP_0646 | 9.0                                      | PTS galactose PTS system EIIB |
| SP_2164   | SP_2164 | 3.7                                      | PTS mannose transporter subunit IIA |
| SP_2161   | SP_2161 | 3.3                                      | PTS mannose transporter subunit IID |
| SP_2162   | SP_2162 | 3.7                                      | PTS mannose PTS system EIIC |
Expression of genes that encode enzymes of the Leloir pathway involved in galactose catabolism and generation of UDP-galactose (UDP-Gal) was upregulated in the mutant (Table 2). Expression of lacF-2 and the lacDCBA operon involved in the import and interconversion of galactose via the tagatose pathway to fructose-6-phosphate and glyceraldehyde-3-phosphate (G3P) was upregulated (Table 2). Upregulated Leloir and tagatose pathways could be in response to high demand for PPP precursors in the mutant. Increased PPP and G3P may contribute to upregulation of tktC and tktN which encode a transketolase that catalyzes the interconversion of sugar-phosphates in the pathway (Table 2). There was an increase in the expression of genes that encode enzymes that degrade fucose (Table 2). Fucose degradation by triosephosphate isomerase yields G3P, an intermediate of glycolysis and PPP [24]. Increased expression of a putative PTS system involved in the import and interconversion of L-ascorbate to xylulose-5-phosphate, an intermediate of PPP (Table 2) further suggests increased activity of the PPP. Genes involved in ascorbate utilization are co-transcribed upstream with a transcriptional regulator (bglG) whose expression was upregulated in ΔpotABCD (Table 2). Shunting carbons to the PPP may be in response to increased demand of NADPH due to oxidative stress in the polyamine transporter-deficient mutant.

**Glycolytic pathway and production of precursors for the pneumococcal capsule.**

During stress, organisms modulate their gene expression to limit energy consuming processes such as CPS synthesis to preserve energy for redox systems. Gene expression profile in ΔpotABCD indicate a limited flow of carbohydrates through the main glycolytic pathway which could be in response to stress. Expression of a sucrose operon regulator and genes involved in sucrose uptake was reduced. Expression of malP, which encodes an enzyme that breaks down glycogen to glucose-1-phosphate and the malXCD operon, that encodes a maltose/maltodextrin transporter, was downregulated (Table 3). Reduced influx of sucrose, maltose, and reduced breakdown of glucose in glycolysis will deplete glycolytic intermediates in ΔpotABCD. UDP-N-acetylglucosamine (UDP-GlcNAc) is a precursor of the three pneumococcal CPS sugar repeat units (UDP-N-acetylmannosamine (UDP-ManNAc), UDP-N-acetylglactosamine (UDP-GalNAc) and UDP-N-acetylfucosamine (UDP-FucNAc). UDP-GlcNAc levels are expected to be low due to the elevated levels of nagA and nagB, involved in its breakdown to fructose-6-phosphate (Table 3) and subsequently reduce the levels of precursors for CPS synthesis. Impaired CPS production was further apparent with the reduced expression of genes involved in the transport of N-acetylglactosamine (Tables 3).
Table 3
The *potABCD* operon modulates central metabolism and production of precursors for the pneumococcal capsule.

| Locus tag | Gene | Fold change $\Delta potABCD/TIGR4$ | Description |
|-----------|------|-----------------------------------|-------------|
| SP_2131   | bglG | 247.3                             | Transcriptional regulator, |
| SP_0100   | padR | 6.8                               | Transcriptional regulator |
| SP_1854   | galR | 3.9                               | LacI family transcriptional regulator |
| SP_2109   | malC | -2.5                              | Maltodextrin ABC transporter permease |
| SP_2110   | malD | -2.4                              | Maltodextrin ABC transporter permease |
| SP_2108   | malX | -2.4                              | Maltose/maltodextrin-binding protein |
| SP_1894   | gtfA | 2.3                               | Sucrose phosphorylase |
| SP_1722   | SP_1722 | -51.0                             | PTS sucrose system EIIBCA or EIIBC |
| SP_0648   | bgaA | 7.0                               | Beta galactosidase |
| SP_1898   | aga  | 2.2                               | Alpha galactosidase |
| SP_1721   | scrK | -10.0                             | Fructokinase |
| SP_1725   | scrR | -9.2                              | LacI family transcriptional regulator, |
| SP_1278   | pyrR | -2.7                              | Bifunctional pyrimidine operon transcriptional regulator |
| SP_1724   | scrB | -10.5                             | Sucrose6-phosphate hydrolase |
| SP_1415   | nagB | 3.2                               | Glucosamine-6-phosphate deaminase |
| SP_2056   | nagA | 2.3                               | N-acetylglucosamine6-phosphate deacetylase |
| SP_0321   | SP_0321 | -2.3                             | PTS N-acetylglactosamine transporter subunit IIA |
| SP_0323   | SP_0323 | -2.6                             | PTS N-acetylglactosamine PTS system EIIB |
| SP_1277   | pyrB | -2.4                              | Aspartate carbamoyltransferase |
| SP_1014   | dapA | -4.3                              | 4-hydroxy-tetrahydrodipicolinate synthase |
| SP_1013   | asd  | -5.5                              | Aspartate-semialdehyde dehydrogenase |

Moreover, the *pyr* operon involved in the *de novo* synthesis of pyrimidine nucleotides and *pyrR*, the regulator of this operon, were repressed (Table 3). Repression of pyrimidine biosynthetic genes will reduce UTP levels, a precursor for UDP required for the activation of UDP-sugars for CPS synthesis [24]. Expression of *asd* and *dap* involved in the synthesis of lysine, a constituent of the pneumococcal peptidoglycan layer (PG), was reduced (Table 3). The overall effect of the above gene expression changes
is reduced carbon flow through the central metabolism possibly due to stress and this will impact the production of precursors for CPS repeat unit sugars and the PG layer in the mutant. To validate RNA-Seq results, we measured the expression of selected genes using qRT-PCR (Table 4). Genes that encode for *tktC*, *tktN*, the ascorbate regulator (*bglG*), and the choline-binding protein *pcpA*, were upregulated, which is consistent with RNA-Seq (Table 4). Although not differentially expressed in the RNA-Seq results, polyamine synthesis genes *speE* and *speA* were upregulated in the qRT-PCR results, suggesting that polyamine synthesis could compensate for the loss of polyamine transport.

**Table 4**

| Locus tag | Gene   | Fold change Δ*potABCD*/TIGR4 | Description                   |
|-----------|--------|------------------------------|-------------------------------|
| SP_0916   | *speA* | 16.9                         | Arginine decarboxylase        |
| SP_0918   | *speE* | 14.4                         | Spermidine synthase           |
| SP_2127   | SP_2127| 20.3                         | Transketolase, C-terminal subunit |
| SP_2128   | SP_2128| 27.7                         | Transketolase, N-terminal subunit |
| SP_2131   | *BglG* | 5.5                          | Transcriptional regulator     |
| SP_2136   | *pcpA* | 7.2                          | Choline binding protein       |

**Redox state and regulation of intracellular pH in** *S. pneumoniae*.

Bacteria adapt to changing microenvironments by modifying metabolite levels to maintain redox homoeostasis. To gain further insight into the role of polyamine transport on the pneumococcal stress signature observed at the transcriptome level, intracellular levels of NADPH, endogenous H$_2$O$_2$, pH$_i$, and GSH/GSSG ratio between WT and Δ*potABCD* were compared. Our results show that deletion of the *potABCD* operon does not impact pH$_i$, as the pH$_i$ of TIGR4 (7.5) and that of the mutant (7.2) were both within physiological range (Fig. 1).

There was no significant difference in the amount of NADPH produced by Δ*potABCD* (3.8 ± 0.4 µg/mg) compared to the WT (4.0 ± 0.6 µg/mg). We observed no significant difference in the amount of H$_2$O$_2$ generated by Δ*potABCD* (1 mM ± 0.05) and WT (1 mM ± 0.01). However, we observed a significant difference between the GSH/GSSG ratio of the WT (1.3 ± 0.1) and Δ*potABCD* (1.7 ± 0.1, $p \leq 0.05$). A high GSH/GSSG ratio indicates increased GSH production, for which one stimulus is oxidative stress. These results show that intracellular levels of NADPH, H$_2$O$_2$, and intracellular pH$_i$ are not dependent on polyamine transport.

**Metabolic profile of polyamine transporter deficient** *S. pneumoniae*.

Our metabolomics results identified significant differences in the levels of several metabolites in response to *potABCD* deletion (Table 5). Levels of N-acetylglucosamine (GlcNAc) and pyruvate were reduced which
could impact CPS production in \( \Delta potABCD \). Increased activity of the PPP was evident by the higher levels of sedoheptulose1, 7-bisphosphate, a precursor for either erythrose4-phosphate or ribose5-phosphate. Increased galactose metabolism was shown by high levels of UDP-glucose, a precursor for glucose1-phosphate, an intermediate of glucose6-phosphate that can be channeled to PPP.

| Locus tag                  | Fold change \( \Delta potABCD/TIGR4 \) | Description                                   |
|----------------------------|------------------------------------------|-----------------------------------------------|
| Trehalose6-phosphate       | -12.1                                    | Starch and sucrose metabolism                 |
| N-Acetylglucosamine        | -2.0                                     | Amino sugar and nucleotide sugar metabolism   |
| Pyruvate                   | -1.7                                     | Pyruvate metabolism                           |
| L-Methionine               | -1.3                                     | Cysteine and methionine metabolism            |
| D-Gluconate                | -1.2                                     | Pentose phosphate pathway                     |
| Glutathione (GSH)          | 2.6                                      | Glutathione metabolism                        |
| UDP-glucose                | 5.7                                      | Galactose metabolism                          |
| Sedoheptulose1,7-bisphosphate | 4.0                                    | Galactose metabolism                          |

The metabolome also showed reduced concentration of trehalose6-phosphate which could impact oxidative stress responses in \( \Delta potABCD \). The concentration of glutathione was higher, which could be in response to oxidative stress in \( \Delta potABCD \). Metabolomics results are concordant with our RNA-Seq results.

**PotABCD is required for combating hydrogen peroxide and nitrosative stress.**

Host defense against bacterial pathogens includes generation of ROS such as superoxide anions \( (O_2^-) \), \( H_2O_2 \) and hydroxyl radicals \( (OH^-) \) as well as reactive nitrogen species (RNS). To further corroborate the stress signature observed at the transcriptome and metabolome levels, we compared the susceptibility of TIGR4 and \( \Delta potABCD \) to unstable oxygen and nitrogen radicals. Our results show that \( \Delta potABCD \) is more susceptible to hydrogen peroxide stress compared to the WT. When cultured in the presence of low concentrations of exogenous hydrogen peroxide \( (0.5, 0.75 \text{ and } 1 \text{ mM}) \), there was no significant effect on the growth of WT and \( \Delta potABCD \) at 15- and 30 min post exposure (data not shown). However, in the presence of 2.5 mM \( H_2O_2 \), viability of \( \Delta potABCD \) was reduced by (54%) relative to WT at 15 min post exposure but was restored by \( \sim 25\% \) in the complement pABG5-\( \Delta potABCD \) strain or with \( \frac{1}{4} \text{MIC (2.5 mM)} \) cadaverine supplementation (Fig. 2). Polyamine supplementation was done on \( \Delta potABCD \) cultured in chemically defined growth medium (CDM) which lacks synthesized polyamines.
Compared to WT, ΔpotABCD was significantly more susceptible to S-nitrosoglutathione (GSNO) that generates RNS. Exposure to GSNO for 60 min at a concentration of 2.5 mM resulted in a significant reduction in the percentage survival of ΔpotABCD that ranged between 32% at 15-min and 73% at 60 min post exposure compared to the WT (0% at 15 min and 10% at 60 min post exposure). There was no significant difference in the survival of the complement pABG5-potABCD strain or supplementation with polyamines in the presence of GSNO (Figure 3). These results suggest that ΔpotABCD is more susceptible to H₂O₂ and nitrosative stress than WT. Data with pABG5-potABCD complement or polyamine supplementation indicates that impact of impaired polyamine transport could be specific to the type of stress.

Discussion

Findings in this study show that deficiency of polyamine transport impairs pneumococcal stress responses and disrupts CPS and PG synthesis (Fig. 4). This study also shows that the potABCD operon is involved in hydrogen peroxide and nitrosative stress responses in pneumococci. Polyamines contribute to cellular homeostasis by scavenging reactive radicals or balance intracellular pH through the consumption of a proton during their synthesis via the decarboxylation of amino acids [25, 26]. Therefore, reduced intracellular polyamine levels reported in Ayoola et al. [15] and decreased expression of some genes involved in redox balance in the current study, could render ΔpotABCD susceptible to stress. In addition, polyamines regulate transcription of several genes including those involved in oxidative stress responses. Downregulation of expression of treR that impact the levels of trehalose a scavenger of ROS and the impaired DNA repair system could make the mutant sensitive to stress. The damage caused by H₂O₂ can be amplified via the Fenton reaction with the generation of hydroxyl radicals, the primary cause of damage to biomolecules such as DNA [27]. Therefore, increased expression of the iron transporter could aggravate the effects of oxidative stress and impair pneumococcal survival in vivo, as it moves through different host niches.

In response to the stress caused by polyamine transport deficiency, ΔpotABCD triggered other known stress response systems to restore the redox status. Acquisition of Pᵢ and manganese is essential for normal growth, virulence, and oxidative stress responses in many pathogenic bacteria [17, 22, 28]. The PPP is often upregulated as a quick cellular response to meet NADPH demands to combat oxidative stress [23]. The ADS produces ammonia (NH₃) that protect cells against acid stress and a molecule of ATP for basal cellular functions during stress [29, 30]. Reduced glutathione and glutathione metabolism play a significant role during oxidative stress in many organisms [31−33]. Therefore, increased expression of genes involved in cation influx, PPP, the ADS, and a high GSH/GSSG ratio could be in response to stress in ΔpotABCD [17, 34]. Upregulation of the ADS could explain the observation that ΔpotABCD can maintain pHᵢ in the physiological range. The polyamine modulon which represent genes whose expression is regulated by polyamines is well described in Escherichia coli and includes genes involved in cell proliferation, biofilm formation, and detoxification of ROS [35]. Our results show that deficiency of the polyamine transporter affected the transcript level of several regulators, and thus
indirectly altering genes controlled by these regulators. Interestingly, we observed that some polyamine transporter responsive genes are also part of the CcpA regulatory network, for example genes involved in stress responses, amino acid synthesis, and carbohydrate metabolism [36, 37]. These results suggest that the intersection of polyamine metabolism and CcpA regulatory network is needed for \textit{in vivo} fitness. These results warrant future studies to identify mechanisms of co-regulation of polyamine metabolism and other transcriptional regulators that impact pneumococcal virulence.

Furthermore, upregulation of the Leloir and the tagatose pathways that produce PPP intermediates, and transketolase, a key enzyme in PPP, further suggest decreased carbon flow through glycolysis, supported by decreased pyruvate levels in \textit{ΔpotABCD}. Decreased glycolysis could result in reduced acetyl-CoA, a precursor for UDP-GlcNAc, and a donor for the three N-acetylated sugars at the intersection of CPS and PG repeat unit biosynthesis [38, 39]. However, upregulation of genes involved in the Leloir pathway in \textit{ΔpotABCD} is contrary to what was observed in \textit{ΔspeA} [15]. Deletion of arginine decarboxylase (\textit{speA}) resulted in downregulation of genes of the Leloir pathway, implicating a role for polyamine synthesis in the regulation of this pathway. Arginine decarboxylase in \textit{ΔpotABCD} could result in the upregulation of the Leloir pathway which warrants further investigation. The PPP generates ribulose5-phosphate which can either be used for nucleotide synthesis or converted to sedoheptulose1, 7-bisphosphate, a precursor for erythrose4-phosphate and G3P. High levels of sedoheptulose1, 7-bisphosphate suggest that \textit{ΔpotABCD} promotes PPP activity possibly to increase production of NADPH levels required in redox systems. Downregulation of the \textit{pyr} operon, responsible for the interconversion of uracil and uridine monophosphate (UMP), could impact UDP production necessary for UDP-sugar repeat unit synthesis and further impair CPS biosynthesis. Results from this study and our previous report [15] demonstrate that polyamine mediated CPS and PG regulation is dependent on both polyamine transport and synthesis. Reduction of CPS precursors could explain the un-encapsulated phenotype and attenuation of \textit{ΔpotABCD} in murine models of colonization, pneumonia, and sepsis [10, 15].

Hydrogen peroxide produced by the pneumococcus is essential for its pathogenesis and protection against other common respiratory tract inhabitants. H$_2$O$_2$ is cytotoxic to host cells, causes apoptosis in respiratory epithelial cells, and promotes colonization of the upper respiratory tract [40]. Therefore, pneumococci must adapt to survive the high levels of H$_2$O$_2$ produced via the pyruvate oxidase system. Despite a high GSH/GSSG ratio, maintenance of NADPH levels and upregulation of several genes involved in oxidative stress responses, \textit{ΔpotABCD} was more susceptible to hydrogen peroxide and nitrosative stress comparable to the WT. The increased susceptibility to H$_2$O$_2$ cannot be attributed to increased intracellular H$_2$O$_2$ levels in \textit{ΔpotABCD} since they were comparable to that produced by WT. These results suggest that polyamines could be essential for the regulation of up/down stream functions of pneumococcal oxidative stress responses which warrants further investigation. These results are consistent with the known roles of polyamines in other bacterial pathogens [8]. Putrescine and spermidine protect cells from ROS by increasing expression of genes that code for free radical scavengers [41, 42]. Polyamine synthesis [43] or transport genes are upregulated [9] in response to H$_2$O$_2$ stress. Cadaverine protects \textit{Salmonella typhimurium} and \textit{E. coli} against nitrosative and acid stress [44,
Pneumococci increase transcription of the substrate binding protein (potD) of the polyamine transporter in response to oxidative and thermal stress and during murine bacteremia [9]. We also reported that speA, a gene that encodes an arginine decarboxylase, regulates pneumococcal nitrosative, \( \text{H}_2\text{O}_2 \) and superoxide stress responses (manuscript under review). These results show that polyamine uptake from the environment may potentially help pneumococci to survive various host microenvironments. However, only supplementation with 2.5 mM cadaverine restored mutant viability in the presence of \( \text{H}_2\text{O}_2 \) but not GSNO. These results indicate that providing the transport function in trans (pABG5-potABCD complement) or by exogenous polyamine supplementation has varying effects on the viability of \( \Delta \text{potABCD} \) that is dependent on the type of stress and the type and concentration of polyamines.

In summary, polyamine transport is essential for pneumococcal stress responses and a dysregulation of these responses impacts the synthesis of CPS and PG. Through horizontal gene transfer, a non-virulent pneumococci can become encapsulated and virulent during coinfections in the host [46] which confounds management strategies. Therefore, gaining insights into virulence mechanisms that are capsule independent but modulate capsule production, like polyamine metabolism, present a novel avenue for exploring new vaccine and therapeutic interventions against this deadly pathogen. Polyamine transport systems are conserved across pneumococcal serotypes and are a promising therapeutic avenue due to their immunogenic potential [12, 14]. Future studies focusing on uncovering the interconnected network of pneumococcal polyamine redox homeostasis, CPS and PG synthesis will contribute towards deconvolution of the complex regulatory networks that impact stress responses and pneumococcal adaptation in vivo.

Materials And Methods

Bacterial strains and growth conditions.

\( \text{S. pneumoniae} \) serotype 4 strain TIGR4 [47], \( \Delta \text{potABCD} \) [11], and the complement strain (pABG5-potABCD) [15] were used in this study. All strains were grown in either chemically defined medium (CDM) [48] or Todd- Hewitt broth supplemented with 0.5% yeast extract (THY) or on 5% sheep blood agar plates (BAP) in 5% CO\(_2\) at 37°C. All assays were performed in triplicate in three independent experiments.

RNA Sequencing.

Total RNA was isolated and purified from mid-log phase cultures (OD\(_{600}\) 0.4) of TIGR4 and \( \Delta \text{potABCD} \) (n = 4) grown in THY (a complete medium that mimics nutrients in the host milieu) using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). RNA quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA-Seq analysis was performed as described earlier [15]. Briefly, libraries for RNA-Seq were prepared with the KAPA RNA Hyper Kit with RiboErase (KAPA Biosystem, Wilmington, MA, USA) with 5 \( \mu \)g RNA as input. The concentration and quality of libraries were determined by the Qubit ds DNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) and Agilent Tapestation (Agilent
Technologies, Santa Clara, CA, USA). Sequencing was done on Illumina Hiseq 3000, the quality of the
data was checked with Illumina SAV and de-multiplexing was performed with Illumina Bcl2fastq2 v 2.17.
Removal of failed reads, mapping of the short sequence reads to the TIGR4 reference genome, and
identification of differentially expressed genes were performed with CLC Genomic Workbench 20.0.3
(Qiagen, Valencia, CA, USA).

Paired end reads of both WT and ΔpotABCD were mapped to the TIGR4 genome using CLC proprietary
read mapper and read counts were estimated by EM estimation algorithm [49] and DEGs were identified
based on the fold change generated by the edgeR algorithm. Changes in gene expression with a fold
change of ± 1.3 at a false discovery rate (FDR) of ≤ 0.05 were considered significant. Functions and
pathways represented by DEGs were identified utilizing multiple bioinformatics resources such as
MetaCyc [50], Gene Ontology [51], KEGG [52], UniProt [53], and STRING [54]. RNA-Seq raw data and
metadata are available at NCBI GEO with the accession number XXXXXXX.

Quantitative real time PCR.

To validate RNA-Seq results, we measured expression of selected genes by quantitative reverse
transcription PCR (qRT-PCR). The primers used for qRT-PCR are listed in supplementary material (Supp.
Table 1). All primers were validated by performing a melt curve analysis with SYBR Green (Thermo Fisher
Scientific Waltham, MA, USA). In brief, total RNA was purified from mid-log phase cultures (OD 600 0.4) of
TIGR4 and ΔpotABCD grown in THY (n = 3). Purified RNA (7.5 ng/reaction) was reverse-transcribed into
cDNA and PCR was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit
(Thermo Fisher Scientific, Waltham, MA, USA) as previously described [55]. Relative quantification of gene
expression was determined by the Stratagene Mx3005P qPCR System (Agilent, Santa Clara, CA, USA).
Expression of selected genes was normalized to the expression of gapDH and fold changes determined
by the comparative C_T method.

Measurement of intracellular pH.

The intracellular pH (pH_i) was determined based on the method described by Clementi et al., [56] with
slight modifications. Briefly, mid-log phase cultures (OD 600 0.4) of TIGR4 and ΔpotABCD grown in THY (n
= 3) were collected by centrifugation, washed, and suspended in phosphate buffered saline (PBS). Cells
(10^8 CFU/mL) were loaded with 5 mM BCECF/AM dye (Millipore-Sigma, St. Louis, MO, USA) and
incubated for 30 min at 30°C in the dark. Cells were then pelleted, washed, and reenergized with 10 mM
glucose in PBS. To obtain the in vivo calibration curve for each strain, 400 µL of energized cells were
pelleted and suspended in potassium buffers ranging from pH 6.5 to 8.0. Nigericin (1 mM) (Thermo
Fisher Scientific, Waltham, MA, USA) was added to the cells (to equilibrate the pH_i of the cells to the pH of
the surrounding buffer) and incubated at 37°C for 5 min. Fluorescence was then measured by a Synergy
H1 plate reader (BioTek, Winooski, VT, USA), and a calibration curve was obtained by plotting fluorescence
against the pH of the buffers. To measure the pH of individual samples, 200 µL (10^8 CFU/mL) of the
loaded and energized cells was added to the wells of a 96-well plate in duplicate and fluorescence
detected using a plate reader for 5 min. 10 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to one well (to serve as control) and the reading taken for another 5 min. CCCP is a protonophore that uncouples proton motive force and causes a rapid decrease in pH\textsubscript{i} (Millipore-Sigma, St. Louis, MO, USA). Nigericin was added to both CCCP-treated control and untreated sample (to a final concentration of 1 mM) to equilibrate the pH\textsubscript{i} to the pH of the buffer and fluorescence was read for an additional 5 min. Fluorescence was calculated and the pH\textsubscript{i} was interpolated from the calibration curve.

**Measurement of intracellular NADPH.**

The intracellular concentration of NADPH was determined using the NADP/NADPH Assay Kit (Abcam, Cambridge, MA, USA). Mid-log phase cultures (n = 3) were harvested at 5,000 × g for 10 min at 4°C, suspended in PBS and transferred to beadbeater tubes (MP Biomedicals, Irvine, CA, USA). Cell suspensions were lysed with a FastPrep-24™ Classic benchtop homogenizer (MP Biomedicals, Irvine, CA, USA) and centrifuged at 6,000 × g for 5 min at 4°C. The cells were processed according to the manufacturer’s instructions. NADPH concentrations were determined with a SpectraMax® M5 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). The concentration of the protein extracts was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and used to normalize NADPH concentrations.

**Measurement of intracellular glutathione.**

The ratios of reduced (GSH) to oxidized (GSSG) intracellular glutathione concentrations were determined using the GSH/GSSG-Glo™ Assay Kit (Promega, Madison, WI, USA). The cells were processed, and protein concentration determined as was done for NADPH quantification above. Luminescence was measured with a Cytation™ 5 cell imaging multi-mode reader (BioTek, Winooski, VT, USA) and used to calculate glutathione concentrations. The concentration of the protein extracts was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and used to normalize glutathione concentrations. GSH/GSSG (reduced/oxidized glutathione) ratios were calculated from the normalized glutathione concentrations according to the manufacturer’s instructions.

**UPLC–HRMS untargeted metabolomics.**

Approximately 10\textsuperscript{9} CFU/mL of cells from mid-log phase cultures of TIGR4 and ΔpotABCD grown in THY (n = 5) were transferred onto a 0.2 µm Whatman polycarbonate membrane by vacuum filtration. The membranes were snap-frozen in liquid nitrogen and stored at −80°C. Metabolites were extracted from bacteria on the membranes with extraction solvent (40:40:20 methanol, acetonitrile, and water with 0.1% formic acid) at 4°C (16). The extracts were transferred to 2.0 mL tubes, centrifuged for 5 min (16,100 × g) at 4°C and the supernatant transferred to new 2.0 mL tubes. Tubes containing ∼1.7 mL of the total supernatant were dried under a stream of N\textsubscript{2} and solid residue was suspended in 300 µL of sterile water and transferred to autosampler vials for mass spectrometric analysis. A 10 µL aliquot was injected through a Synergi 2.5-micron reverse-phase Hydro-RP 100, 100 × 2.00 mM LC column (Phenomenex, Torrance, CA, USA) kept at 25°C. The eluent was introduced into the MS via an electrospay ionization
source conjoined to an Exactive™ Plus Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) through a 0.1 mm internal diameter fused silica capillary tube. The mass spectrometer was run in full scan mode with negative ionization mode with a window from 85 to 1000 \textit{m/z} with a method adapted from [57]. Samples were run with a spray voltage of 3 kV. The nitrogen sheath gas was set to a flow rate of 10 psi with a capillary temperature of 320°C. Automatic gain control target was set to 3e6. The samples were analyzed with a resolution of 140,000 and a scan window of 85–800 \textit{m/z} from 0 to 9 min and 110–1000 \textit{m/z} from 9 to 25 min. Files generated by Xcalibur (RAW) were converted to the open source mzML format [49] via the open source msconvert software as part of the ProteoWizard package [49]. Maven (mzroll) software, Princeton University [58, 59] was used to automatically correct the total ion chromatograms based on the retention times for each sample [58, 59]. Metabolites were manually identified and integrated using known masses (± 5 ppm mass tolerance) and retention times (1 ≤ 1.5 min). Unknown peaks were automatically selected via Maven’s automated peak detection algorithms. A database of 275 metabolites verified using exact \textit{m/z} and known retention times, expanded from the original database [57] was used. The statistical analysis on metabolite peak intensity post CFU normalization was done by MetaboAnalyst 4.0 [60]. Quantile normalization which is highly efficient in normalizing metabolite variations from mass spectrometry [61] was used to normalize the data. Significant differences in metabolite peak intensity between \( \Delta \text{potABCD} \) and TIGR4 were identified by a T-test at an adjusted FDR of ≤ 0.05.

**Hydrogen peroxide production.**

\( \text{H}_2\text{O}_2 \) generated from mid-log phase cultures of TIGR4 and \( \Delta \text{potABCD} \) (n = 3) was compared using a quantitative peroxide assay (Pierce, Thermo Fisher Scientific Waltham, MA, USA). Briefly, 1 mL of bacterial culture (10\(^8\) CFU/mL) grown in THY was centrifuged at 4°C for 2 min at 10,000 x g and the supernatant filtered with a 0.22 µm filter. The concentration of \( \text{H}_2\text{O}_2 \) was measured in the filtrate following the manufacturer’s instructions.

**Hydrogen peroxide survival.**

Mid-log phase cultures of TIGR4, \( \Delta \text{potABCD} \) and the complement pABG5-\( \text{potABCD} \) strain grown in either THY or CDM (\( \text{OD}_{600} 0.4–0.5 \)) were centrifuged at 10,000 x g for 2 min and suspended in PBS. The cells (10\(^8\) CFU/mL) (in 1 mL PBS) were then supplemented with final concentrations of hydrogen peroxide 2.5 mM and incubated at 37°C with 5% \( \text{CO}_2 \) for 15 min. CDM is devoid of polyamines but has the amino acid precursors for polyamine synthesis. To determine the effects of polyamines on pneumococcal \( \text{H}_2\text{O}_2 \) stress, \( \Delta \text{potABCD} \) challenged with 2.5 mM \( \text{H}_2\text{O}_2 \), was supplemented with cadaverine, putrescine or spermidine (½MIC, ¼MIC, ⅛MIC) and incubated for 15 min. MICs for cadaverine, spermidine or putrescine are 10.0, 4.8 and 5.7 mM, respectively. Control reactions had untreated bacteria, and CFUs were determined by serial dilution in PBS and plating on BAP. Results from three independent experiments were expressed as percent survival of treated bacteria relative to the untreated bacteria.

**\( \text{S}^-\text{nitrosoglutathione susceptibility.} \)**
Mid-log phase cultures of TIGR4, ΔpotABCD and the complement pABG5-ΔpotABCD strain grown in CDM were centrifuged at 10,000 x g for 2 min and cells suspended in PBS. The cells (10^7 CFU/mL) in 100 µl were supplemented with a final concentration of 2.5 mM GSNO (Sigma-Aldrich, Israel), a nitric oxide producer, and incubated at 37°C in 5% CO₂ for 60 min. In addition, ΔpotABCD challenged with 2.5 mM GSNO was supplemented with cadaverine, putrescine or spermidine (½MIC, ¼MIC, MIC). Control reactions had untreated bacteria, and CFUs were determined by serial dilution in PBS and plating on BAP every after 15 min. Results from three independent experiments were expressed as percent survival of treated bacteria relative to the untreated bacteria.

**Statistical analysis.**

Significant differences between the susceptibility of TIGR4, ΔpotABCD and the complement pABG5-ΔpotABCD strain to the different stressors, changes in pHᵢ, production of endogenous H₂O₂, levels of NADPH and GSH/GSSG ratio, as well as changes in gene expression measured by qRT-PCR were determined by a Student's T-test at \( p \leq 0.05 \).

**Declarations**

**Authors and contributors.**

B.N. conceived, designed, and supervised the experiments. M.F.N performed the experiments and drafted the manuscript. L.A.S., M.M. and M.B.A. performed the experiments. B.N. and E.S. contributed to the final draft. All authors approved the final version of the manuscript.

**Conflicts of interest.**

The authors declare no conflict of interest in this work.

**Funding information.**

Grant: #P20GM103646 (Center for Biomedical Research Excellence in Pathogen Host Interactions) from the National Institute for General Medical Sciences.

**Acknowledgements.**

This work was supported by grant #P20GM103646 (Center for Biomedical Research Excellence in Pathogen Host Interactions) from the National Institute for General Medical Sciences.

Metabolomics extraction and mass spectrometric analyses were performed at the Biological and Small Molecule Mass Spectrometry Core, University of Tennessee, Knoxville, Knoxville, TN, United States, with the assistance of Dr. Shawn R. Campagna, Dr. Hector F. Castro, and Joshua B. Powers.

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Figures
Intracellular pH of S. pneumoniae TIGR4 and ΔpotABCD. Three replicates of TIGR4 and ΔpotABCD were loaded with 5 mM of pH sensitive fluorescence dye BCECF-AM, washed with PBS, re-energized with 10% glucose, and baseline fluorescence readings were established in the first 5 mins. Controls were supplemented with 10 µM CCCP as a protonophore (triangles), and fluorescence of CCCP-treated controls and untreated samples (squares) was measured for an additional 5 mins. The pHi of untreated TIGR4 (blue squares) and ΔpotABCD (red squares) is 7.5 and 7.2, respectively. Nigericin (20 µM) was added to both treated and untreated samples to dissipate transmembrane gradients over that last 5 mins. Graphs represent the mean of three independent experiments.
Figure 2

Hydrogen peroxide susceptibility of S. pneumoniae TIGR4, ΔpotABCD and pAGB5-potABCD. The graph shows bacterial sensitivity to 2.5 mM H2O2 at 15 min post exposure. Also included is ΔpotABCD supplemented with cadaverine (¼MIC). The results represent an average of three independent experiments. Percentage survival relative to the untreated control is shown as a bar with the standard error of the mean, with ** representing $p \leq 0.01$, determined by a Student's T-test.
Figure 3

S-nitrosoglutathione susceptibility assay of S. pneumoniae TIGR4, ΔpotABCD and pABG5-potABCD. The graph shows bacterial sensitivity to 2.5 mM GSNO at 15-60 min post exposure. The results represent an average of three independent experiments. Percentage survival relative to the untreated control is shown as a bar with the standard error of the mean, with * representing $p \leq 0.01$ based on a Student’s T-test.
Figure 4

Intersection between polyamine metabolism, stress responses, carbohydrate metabolism and CPS/PG production in S. pneumoniae. Multi-step reactions are shown as dotted arrows, (blue represents upregulated while red represents downregulated reactions). Deletion of the polyamine transporter results in reduced intracellular polyamine levels (red oval), impairing the redox system (light green rectangle), rendering the pneumococci susceptible to NO and H2O2 stress (dark green oval). The stressful conditions alter gene regulation (orange rectangle) to limit processes (grey square; glycolysis, pyrimidine, and amino sugar nucleotide synthesis) that yield precursors for CPS and PG synthesis (yellow vertical rectangle). Reduced CPS, PG and protein synthesis could be to save energy for redox homeostasis (dark blue vertical rectangle; ADS, PPP, and GSH metabolism) and cation inflow. Influx of carbohydrates via phosphotransferase systems (PTS) that promote synthesis of glycolysis is inhibited in favor of inflow of carbohydrates that favor the Leloir pathway and tagatose pathways (light blue rectangle) probably to provide PPP intermediates.

Supplementary Files

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