Polyamine biosynthesis and transport mechanisms are crucial for fitness and pathogenesis of *Streptococcus pneumoniae*

Pratik Shah,1 Bindu Nanduri,2 Edwin Swiatlo,3 Yinfa Ma4 and Ken Pendarvis2

1Department of Microbiology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA
2College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA
3Research Service, Veteran Affairs Medical Center, 1500 East Woodrow Wilson Drive, Jackson, MS 39216, USA
4Department of Chemistry, Missouri University of Science and Technology, Rolla, MO 65409, USA

Polyamines such as cadaverine, putrescine and spermidine are polycationic molecules that have pleiotropic effects on cells via their interaction with nucleic acids. *Streptococcus pneumoniae* (the pneumococcus) is a Gram-positive pathogen capable of causing pneumonia, septicemia, otitis media and meningitis. Pneumococci have a polyamine transport operon (*potABCD*) responsible for the binding and transport of putrescine and spermidine, and can synthesize cadaverine and spermidine using their lysine decarboxylase (*cad*) and spermidine synthase (*speE*) enzymes. Previous studies from our laboratory have shown that an increase in PotD expression is seen following exposure to various stresses, while during infection, *potD* inactivation significantly attenuates pneumococcal virulence, and anti-PotD immune responses are protective in mice. In spite of their relative importance, not much is known about the global contribution of polyamine biosynthesis and transport pathways to pneumococcal disease. Mutants deficient in polyamine biosynthesis (∆*speE* or ∆*cad*) or transport genes (∆*potABCD*) were constructed and were found to be attenuated in murine models of pneumococcal colonization and pneumococcal disease. The ∆*speE* mutant was also attenuated during invasive disease, while the ∆*potABCD* and *cad* genes seemed to be dispensable. HPLC analyses showed reduced intracellular polyamine levels in all mutant strains compared with wild-type bacteria. High-throughput proteomic analyses indicated reduced expression of growth, replication and virulence factors in mutant strains. Thus, polyamine biosynthesis and transport mechanisms are intricately linked to the fitness, survival and pathogenesis of the pneumococcus in host microenvironments, and may represent important targets for prophylactic and therapeutic interventions.

INTRODUCTION

*Streptococcus pneumoniae* is a Gram-positive pathogen that asymptomatically colonizes the nasopharynx of humans. However, in some cases it can cause pneumonia, septicemia, otitis media and meningitis (Kadioglu et al., 2008). Two capsular polysaccharide-based vaccines are currently licensed to prevent pneumococcal colonization and disease (Iyer et al., 2005; Iyer & Camilli, 2007). However, these vaccines are ineffective in reducing disease incidence in children and the elderly (Fedson, 1999; Huang et al., 2005). Pneumococci are one of the leading causes of community-acquired pneumonia and otitis media in the USA (Dagan, 2000; File, 2004). Worldwide, the situation is worse, as approximately one million children succumb to pneumococcal disease annually (O’Brien & Nohynek, 2003). The pneumococcus continues to be a serious public health concern, and there is a need for new and improved ways to combat and prevent infections.
Significant advances have been made in the identification and characterization of classical bacterial virulence factors such as toxins, capsule, adhesins, secretory systems and immune evasion. However, the contributions of host and microbial metabolism to the establishment and progression of disease have often been underappreciated. Indeed, bacterial metabolic pathways such as the tricarboxylic acid cycle and gluconeogenesis play an important role in pathogenesis (Alteri et al., 2009). Additionally, the availability of key nutrients in the host also modulates the expression of bacterial phenotypes that may affect disease outcome (Somerville & Proctor, 2009). A large proportion of the pneumococcal genome is devoted to basic metabolic functions (Tettelin et al., 2001). This is of particular importance, as it spends most of its life cycle on nutritionally restricted mucosal surfaces, and the acquisition of scarce but essential nutrients represents a critical cellular function. Indeed, pneumococci possess multiple mechanisms for carbohydrate uptake and metabolism that are intricately linked to their pathogenesis (Iyer et al., 2005; Iyer & Camilli, 2007). Transport and biosynthesis of amino acids, manganese and iron have been shown to be important for pneumococcal pathogenesis (Basavanna et al., 2009; Gupta et al., 2009; Nanduri et al., 2008; Rosch et al., 2009; Yesilkaya et al., 2000). A thorough understanding of pneumococcal metabolism is required for designing effective therapeutic and prophylactic strategies.

Polyamines are small polycationic molecules with hydrocarbon backbones and are positively charged at physiological pH (Shah & Swiatlo, 2008). Intracellular polyamine pools are stringently regulated in all organisms, and polyamines are required for optimal cell growth and division (Shah & Swiatlo, 2008). Most bacteria have de novo biosynthesis pathways and membrane transporters to satisfy cellular polyamine requirements (Tabor & Tabor, 1985). Cadaverine, putrescine and spermidine are the most common and well-characterized bacterial polyamines (Tabor & Tabor, 1985). Recent reports also suggest that norspermidine (a derivative of spermidine with an extra carbon atom) plays important biological roles in Vibrio spp. (Lee et al., 2009). Most research on polyamines in prokaryotes has focused on their effects on transcription and translation by virtue of their interactions with negatively charged nucleic acids. Little is known about the role of polyamines in the physiology and virulence of bacterial pathogens. However, over the last few years reports linking polyamines to cancer, biofilm formation, escape from phagolysosomes, bacteriocin production, toxin activity and stress responses have been published, providing insights about their other important but lesser known functions in bacteria (Shah & Swiatlo, 2008). Functional genomic analyses suggest that pneumococci have a membrane polyamine transporter encoded by potABCD (Sp_1386–1389) similar to the polyamine transport operon in Escherichia coli that binds and transports putrescine and spermidine (Igarashi et al., 2001; Ware et al., 2005). The pneumococcal chromosome also has annotated lysine decarboxylase (cad; Sp_0916) and spermidine synthase (speE; Sp_0918) genes, suggesting that it can synthesize cadaverine and spermidine from precursor amino acids. Signature-tagged mutagenesis screens have identified both the pot operon and lysine decarboxylase to be vital for pneumococcal disease formation in murine models (Hava & Camilli, 2002; Polissi et al., 1998).

Previous studies from our laboratory have shown that inactivation of potD in a mouse-virulent capsular type 3 strain significantly attenuates the progression of disease in systemic and pulmonary murine models, supporting a role for polyamine uptake in pneumococcal pathogenesis (Ware et al., 2006). We have also shown that polyamines may serve as functional analogues for choline molecules during in vitro growth, and a significant increase in PotD expression is seen when pneumococci are exposed to environmental stress and during murine septicaemia (Shah et al., 2008; Ware et al., 2005). Additionally, immunization with the surface-exposed PotD protects mice against colonization and lethal pneumococcal infections (Gupta et al., 2009; Shah et al., 2006; Shah & Swiatlo, 2006). Despite their relative importance, not much information is available about the global contribution of polyamines to pneumococcal disease. We hypothesized that genetic deficiencies in the ability to synthesize or transport polyamines would result in profound effects on pneumococcal colonization, invasive disease and stress responses. The data presented in this study show that polyamine biosynthesis and transport loci are conserved across multiple pneumococcal capsular serotypes. Mutant strains deficient in polyamine biosynthesis and transport genes were significantly attenuated in murine models of pneumococcal colonization, pneumonia and invasive infections. Measurement of intracellular polyamine pools and of survival during oxidative and pH stresses, and large-scale proteomic analyses were also performed with the mutant strains. Our data strongly suggest that polyamines are intricately linked to the in vivo fitness, pathogenesis and virulence factor expression of the pneumococcus, and may represent promising targets for novel prophylactic and therapeutic interventions against this pathogen.

MATERIAL AND METHODS

Bacterial strains and growth conditions. All experiments were performed with S. pneumoniae serotype 4 clinical isolate TIGR4 (Tettelin et al., 2001). Pneumococci were routinely grown in Todd–Hewitt broth with 1% yeast extract (THY) or on blood agar plates (BAP) at 37°C in a 5% CO2 atmosphere. Broth cultures were typically grown to a final OD600 of 0.4. All primers used in this study are listed in Table 1.

PCR analyses. PCR was used to examine the distribution of polyamine biosynthesis and transport genes among selected pneumococcal capsular serotypes. Primers were designed on the basis of 5' and 3' sequences of cad (cadA/cadR), potD (potD/potDR) and speE (speE/speER) genes (Table 1). Bacterial cultures were statically grown in 2 ml THY medium at 37°C in 5% CO2 and harvested during the exponential phase of growth (OD600 0.4) by centrifugation at
Table 1. Sequences of primers used in this study

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</tr>
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*Underlined sequences are complementary to S. pneumoniae TIGR4 chromosomal DNA.

Animal experiments. The institutional animal care and use committee approved all animal studies. All experiments were performed with 8- to 12-week-old CBA/N mice (Jackson Laboratory) (Briës et al., 1982). Pneumococci were grown at 37 °C in 5% CO₂ in THY medium. Cells were harvested by centrifugation during exponential phase, and were resuspended in fresh medium containing 10% (v/v) glycerol and stored at −80 °C. One week prior to infection, stock cultures were thawed, serially diluted in lactated Ringer’s solution (LR) and plated on BAP to determine c.f.u. Approximately 4 × 10⁹ viable cells resuspended in 10 μL LR were used for nasopharyngeal colonization experiments (Briës et al., 2003). Five days post-infection animals were killed and nasal cavities were washed with 1000 μl sterile LR, as previously described (Shah et al., 2009). All collected samples were serially diluted and plated on BAP with 4 μg gentamicin ml⁻¹ (for the wild-type strain) or 4 μg gentamicin ml⁻¹ and 50 μg TP ml⁻¹ (for mutant strains). For pneumonia experiments, approximately 4 × 10⁹ cells were resuspended in 40 μL LR and administered intranasally (i.n.) to anaesthetized mice. Forty-eight hours after infection, mice were euthanized, and lungs were aseptically harvested, homogenized and serially diluted in LR and plated on BAP with either 4 μg gentamicin ml⁻¹ or 4 μg gentamicin ml⁻¹ and 50 μg TP ml⁻¹. Retro-orbital puncture was used to collect blood from all animals in colonization and pneumonia experiments to assess bacteremia. For comparison of in vivo growth and ability to cause septicemia, mice were infected intravenously (i.v.) using approximately 1 × 10⁶ cells. Blood was collected at regular intervals post-infection and was plated on BAP with and without TP to enumerate pneumococci. Infected animals were closely monitored and the survival time was recorded.

In vitro growth assays. S. pneumoniae mutant and wild-type strains were grown at 37 °C in THY broth following inoculation with approximately 10⁵ cells. Exponentially growing cells were diluted in fresh medium to achieve an OD 600 of approximately 0.04 and OD 600 readings were taken every 60 min. For enumeration of c.f.u. during growth, equal numbers of pre-enumerated mutant or wild-type cells were inoculated in THY broth at 37 °C, aliquots were taken at periodic intervals, and serial dilutions and plate counts were performed.

Determination of polyamine concentrations. Bacterial cells in exponential phase growth were harvested by centrifugation and washed three times in a glucose citrate buffer [100 mM sodium citrate (pH 5.5), 2% glucose] followed by sterile PBS. Cells were resuspended in 5% HClO₄, vigorously vortexed, and centrifuged. The bacterial cell extract solution was adjusted with 8.0 M NaOH to pH 3–4 to precipitate proteins, and the supernatant was removed after centrifugation at 10 000 g for 15 min at 4 °C. Precipitates were washed twice with 0.1 M HClO₄. Polyamines were derivatized with fluorescein-5-isothiocyanate and quantified by the capillary electrophoresis method (Du et al., 2004).

Exposure to oxidative and acid stress. To determine the sensitivity of mutant and wild-type strains to superoxide, exponentially growing cultures in THY broth were treated with 50 mM paraquat, a generator of intracellular superoxide (Hassett et al., 1987). Untreated cultures were used as negative controls. At defined time points, samples were removed from treated and untreated cultures, serially diluted in sterile LR, and plated on BAP to obtain c.f.u. The assay was performed in triplicate. For acid-stress experiments, mutant and wild-type bacteria were inoculated...
in THY broth (pH 7.15) and incubated at 37 °C until all cultures reached OD₅₆₀ = 0.4. Cells were then collected by centrifugation, washed in LR and resuspended in sterile THY broth (pH 5.5). Cells were incubated for 3 h at 37 °C. For each strain, c.f.u. were determined pre- and post-exposure.

Proteomic analyses

Cell lysis using pressure cycling technology (PCT). Bacterial cells were processed using a ProtonSolve-SB kit (Pressure Biosciences) followed by lysis by PCT using a Barocycler NEP2017 pressure cycling instrument (Pressure Biosciences). In PCT, samples are subjected to alternating cycles of ambient and high pressure, up to 35,000 p.s.i. (241.500 kPa), resulting in cell lysis (Smejkal et al., 2006).

Trypsin digestion and tandem MS analysis. Proteomic analyses were carried out with proteins isolated from independent triplicate cultures of wild-type and ApotABCD and speE mutant strains. Proteins were trypsin-digested and desalted as previously described (Nanduri et al., 2008). Briefly, approximately 20 μg protein was reduced with 5 mM dithiothreitol at 65 °C for 5 min and alkylated with 10 mM iodoacetamide at 37 °C for 30 min. Trypsin digestion was carried out using molecular biology grade porcine trypsin (2 μg; 37 °C; 16 h; 50:1 ratio of protein:trypsin; Promega). Tryptic peptides were desalted using a peptide macrotrap (Michrom BioResources), eluted in 0.1% trifluoroacetic acid, 95% acetonitrile (ACN) solution, vacuum-dried, and resuspended in 20 μl 0.1% formic acid for 2D liquid chromatography-electrospray ionization tandem MS (2D LC/ESI MS/MS). Liquid chromatography (LC) analysis was accomplished by reverse-phase LC coupled directly in-line with an ESI ion trap mass spectrometer (LCQ Deca XP Plus, ThermoElectron). Tryptic peptides were loaded onto a BioBasic C18 reversed-phase column (Thermo 72105-100266) that was equilibrated for 20 min with 5% ACN, 0.1% formic acid. The HPLC flow rate was set at 500 nl min⁻¹ and all solvents contained 0.1% formic acid. Peptide separation was achieved with an ACN gradient: 5–25% ACN in 450 min, followed by 25–50% in 130 min, followed by a 20 min wash with 95% ACN and equilibration with 5% ACN for 55 min. Data were collected for 655 min over the duration of the HPLC run using repetitive MS scans immediately followed by three MS/MS scans of the three most intense MS peaks. Dynamic exclusion was enabled with a duration of 2 min with a repeat count of two.

Protein identification. Mass spectra and tandem mass spectra were searched against an in silico trypsin-digested protein database for S. pneumoniae TIGR4 downloaded from the National Center for Biotechnology Information (NCBI). All searches were done using TurboSEQUEST (Bioworks Browser 3.2, ThermoElectron) (Eng et al., 1994). Cysteine carboxymethylmethylation and methionine single and double oxidation were included in the search criteria. Decoy searches from a reversed version of the S. pneumoniae TIGR4 protein database were derived using the reverse database function in Bioworks 3.2. The reversed database was in silico trypsin-digested and used for searches with tandem mass spectra as described above. The probability for peptide identification was estimated using a method described for Sequest data analysis and was set at P ≤ 0.05 (Qian et al., 2005). Probabilities of protein identifications being incorrect were calculated using published methods (Lopez-Ferrer et al., 2004; MacCoss et al., 2002). Differential protein expression analysis based on ∑Xcorr was carried out using ProtQuant (Bridges et al., 2007). When comparing two datasets, ProtQuant utilizes tandem mass spectra present at Xcorr values below the user-defined threshold for peptide identification to fill in the missing Xcorr values in a dataset, thus improving the specificity (i.e. decreasing type I errors), provided at least three peptides are identified in the corresponding dataset at a user-defined identification threshold. ProtQuant generated an ANOVA-based P value for significant changes in protein expression. The P values were corrected for multiple testing using the Benjamini–Hochberg method (Benjamini & Hochberg, 1995), and proteins with an adjusted P value of ≤ 0.05 were considered to be significantly differentially expressed. We calculated fold-changes based on ∑Xcorr for proteins that had a significant change in expression using a published method (Old et al., 2005). For protein identifications where ∑Xcorr = 0 in a dataset, this method accounts for the discontinuity in identification by including a correction factor. We used a correction factor of 0.5 (Nanduri et al., 2008) for reporting the log₂ ratio of protein abundance.

Statistical analyses. All pneumococcal c.f.u. are reported as log₁₀ values where indicated. Statistical analysis was performed using the GraphPad software program (GraphPad Software). Bacterial counts obtained from the nasopharynx, lungs and blood were compared using Mann–Whitney two-sample rank tests. Data on survival of mice were analysed by using the Kaplan–Meier graph and log rank test. P values less than 0.05 were considered to represent significant differences between groups.

RESULTS

Distribution of pneumococcal polyamine biosynthesis and transport genes

Sequence alignment and analyses showed that the pneumococcal Cad protein is similar to other bacterial pyridoxal-dependent decarboxylases. It has a conserved lysine residue that can bind pyridoxal phosphate and decarboxylates lysine to cadaverine (Fig. 1). The amino acid sequence of pneumococcal spermidine synthase has the characteristic glycine-rich aminopropyltransferase motif that catalyses the production of spermidine from putrescine and decarboxylated L-adenosylmethionine (SAM) (Fig. 1). Similarly, S. pneumoniae PotD has a bacterial spermidine/putrescine-binding motif and is similar to other polyamine ABC transporters (Fig. 1). PCR analyses showed that cad, speE and potD genes were present in 12 different S. pneumoniae clinical isolates (Table 2). Additionally, BLAST searches using cad, potABCD and speE sequences as templates showed that these genes were conserved, with more than 99% identity in the genomes of all sequenced pneumococcal isolates (http://strepneumo-sybil.igs.umaryland.edu/).

The polyamine biosynthesis genes cad and speE, and potABCD contribute to pneumococcal nasopharyngeal colonization

In E. coli, the lysine decarboxylase gene is co-transcribed with a membrane transporter cadB involved in lysine–cadaverine exchange and pH regulation (Sokswatmaekhin et al., 2004). The pneumococcal lysine decarboxylase locus annotated as cad is transcribed independently, and this is suggested by the presence of a 5’ promoter and 3’ stem–loop transcriptional terminator element and by transcriptional analyses of wild-type TIGR4 cells grown under laboratory conditions. MEGABLAST searches using the cadB sequence from E. coli confirmed the absence of a homologous membrane transporter in all sequenced pneumococcal genomes (results not shown). Inter-
Interestingly, sequence analyses also showed that a cadB homologous sequence is absent in other Gram-positive pathogens. These findings suggest that Gram-positive bacteria that do not have lysine–cadaverine antiporters may use cadaverine for novel intracellular functions in addition to pH regulation. The pneumococcal cad mutant was significantly attenuated in nasopharyngeal colonization compared with the wild-type strain (Fig. 2a). It was also outcompeted by the isogenic parent during nasopharyngeal colonization in CI experiments (Fig. 2b).

Although the pneumococcal polyamine transporter has been implicated in both pneumonia and murine septicemia, its role in nasopharyngeal colonization was unknown (Polissi et al., 1998; Ware et al., 2006). Similar to the cad deletion strain, a potABCD mutant was significantly attenuated in a murine respiratory tract colonization model (Fig. 2a). CI experiments with the mutant strain also showed that the ΔpotABCD mutant was outcompeted by wild-type TIGR4 during colonization of respiratory mucosal surfaces (Fig. 2b).

Spermidine biosynthesis in Gram-negative bacteria is usually carried out by the spe operon, consisting of the speD and speE genes (Xie et al., 1989). The speD locus seems to be absent in all sequenced pneumococcal isolates.

Fig. 1. Sequence alignments of a representative set of polyamine biosynthesis and transport domains. Alignment of the conserved lysine decarboxylase, spermidine synthase and the spermidine-binding domains of PotD from S. pneumoniae TIGR4 and other human bacterial pathogens. The boxed sequences in the alignment represent signature motifs in each domain representing either polyamine biosynthesis or transport functions. Asterisk, identical amino acid residues in all sequences; colon, highly conserved amino acids; period, similar amino acids; blank, dissimilar amino acids or gaps in sequences.
Additionally, similar to cadB, the speD locus is also absent in a large subset of Gram-positive pathogenic bacteria (data not shown). Thus, lysine–cadaverine antiporters and SAM decarboxylases may be non-essential for some Gram-positive bacteria, and other compensatory or novel pathways may account for polyamine biosynthesis. To elucidate the role of spermidine in pneumococcal colonization, an in-frame speE deletion mutant was constructed. Similar to cad and potABCD mutants, the speE deletion strain was less fit to colonize the nasopharynx, either alone or in competition with the isogenic parent strain (Fig. 2a, b).

Polyamine biosynthesis and transport loci play an essential role in pneumococcal pneumonia

Deletion of cad or the polyamine transporter resulted in severe attenuation of the mutants in a pneumonia model, either alone or in competition with the wild-type strain (Fig. 3). It was also shown for the first time, to our knowledge, that the biosynthesis of spermidine is also a key determinant in pneumococcal pneumonia, as the speE deletion mutant was significantly outcompeted by the wild-type parent (Fig. 3).

Role of pneumococcal polyamine biosynthesis and transport during invasive infection

To elucidate the in vivo growth kinetics of mutant strains, an i.v. model of murine bacteraemia was used. Fewer mutant strains were recovered from infected mice, suggesting that they could be cleared more rapidly compared with wild-type TIGR4 (Fig. 4a). The mean time to death of mice infected with the potABCD and speE mutants (P<0.05) was longer compared with the wild-type parent (Fig. 4b). The cad locus, on the other hand, did not confer a significant survival or growth advantage (Fig. 4a, b).

In vitro growth

Both the cad and the speE mutant grew similarly to the wild-type TIGR4 in vitro (Fig. 5a). The potABCD mutant initially grew more slowly but eventually attained OD<sub>600</sub> values similar to those of the wild-type parent (Fig. 5a). Provision of excess polyamines (0.5 μM) in the growth medium did not affect the growth of the potABCD strain (data not shown). Colony counts of all mutant and wild-type cells during growth in THY also showed that all strains had identical growth kinetics in vitro either alone (Fig. 5b) or in competition with the wild-type TIGR4 (data not shown).

Table 2. Conservation of cad, potD and speE genes among different S. pneumoniae capsular serotypes implicated in disease

<table>
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*Nasopharynx. †Not available.

Fig. 2. Lysine decarboxylase, speE and the potABCD operon contribute to pneumococcal nasopharyngeal colonization. Inactivation of cad, potABCD or speE results in significant attenuation of the ability of the pneumococcus to colonize the murine respiratory tract following infection either alone (a) or in competition with the wild-type parent (b). Data represent log<sub>10</sub>(c.f.u.) of either the mutant or the wild-type strain (a), or the CI ratios (mutant : wild-type) recovered from the nasopharynx (b) of infected mice (n=6 in each group) 5 days post-infection. The dashed line in (a) represents the lower limit of bacterial detection for the colonization experiment.
Polyamine measurement

Intracellular polyamine pools of mutant strains were measured and compared with that of the wild-type TIGR4. Similar depletion in cadaverine, putrescine and spermidine levels was seen in all mutant strains (Table 3). Spermidine was the most abundant polyamine in the pneumococcus, followed by cadaverine and putrescine. Indeed, genes responsible for putrescine biosynthesis (speABC) are absent in the TIGR4 chromosome, suggesting that most of its intracellular putrescine is acquired from the environment via the polyamine transporter or synthesized by as yet unknown pathways.

Susceptibility to oxidative and pH stress

Polyamines have been implicated in oxidative stress responses in many bacterial species (Chattopadhyay et al., 2003; Ha et al., 1998; Jung & Kim, 2003a, b; Khan et al., 1992). It has previously been shown that environmental polyamine acquisition might play a role in oxidative stress responses, as expression of PotD significantly increases when pneumococci are exposed to sublethal H₂O₂ levels and temperature stress (Shah et al., 2008). Mutants and wild-type cells were exposed to an oxidizing stress-inducing agent (paraquat) and survival was enumerated by plate counts. No significant differences in survival rate were observed between mutant and wild-type cells following paraquat exposure at all time points (Fig. 6b). We further tested our mutant strains by using different concentrations of paraquat and taking earlier time points but saw no difference in survival rates (data not shown). Similarly, when rapidly dividing mutant or wild-type cells were transiently exposed to a low-pH environment, no significant differences in survival were observed (Fig. 6a).

Proteomic analyses

2D LC ESI MS/MS analyses were used to identify proteins that are uniquely or differentially expressed in the ΔpotABCD and ΔspeE mutant strains compared with the wild-type TIGR4, as these mutants showed severe attenuation during colonization, pneumonia and invasive infection. Proteomic analyses with the ΔpotABCD strain and wild-type TIGR4 identified 92 proteins that were detected only in wild-type, while 79 proteins were exclusive to the ΔpotABCD mutant strain and 359 proteins were common to both datasets (Supplementary Table S1).
Differential expression analysis based on $\sum X_{corr}$ showed a significant increase in the expression of approximately 35 proteins, while 76 proteins were downregulated in the $\Delta potABCD$ mutant strain compared with wild-type TIGR4 (Supplementary Table S2). While 16 proteins showed a significant increase in expression, 23 proteins were downregulated in the $\Delta speE$ mutant strain and wild-type TIGR4 also showed that 124 proteins were unique to the wild-type strain, 96 were detected in the $\Delta speE$ strain alone, and 535 were common to both datasets (Supplementary Table S4). Differentially expressed proteins in mutant strains included known virulence factors, such as capsular polysaccharide, pneumolysin, zinc metalloprotease, amino acid transporters, and several growth and replication factors, signifying the involvement of polyamines in regulating pneumococcal physiology and pathogenesis. We are currently characterizing pneumococcal transcriptional responses to various polyamines to corroborate the reduced expression of key virulence factors.

DISCUSSION

Bacterial pathogens face diverse environmental stresses during growth in a host and have consequently made multiple adaptations to survive and multiply under difficult conditions. Recently, a growing body of literature has alluded to the role of basic metabolites and carbon metabolic pathways in the overall growth, fitness and virulence of bacterial pathogens (Barelle et al., 2006; Munoz-Elia & McKinney, 2005; Naderer et al., 2006; Shelburne et al., 2008; Tchawa Yimga et al., 2006). In this study a similar degree of attenuation was seen for both transport and biosynthesis mutants during nasopharyngeal colonization, suggesting that genetic deficiencies affecting the availability of certain polyamines are equally detrimental to successful mucosal colonization. These data are the first demonstration, to our knowledge, that polyamines may be important for the growth and fitness of the pneumococcus on nutritionally restricted mucosal surfaces.

Pneumococcal pneumonia requires prolonged bacterial persistence in the lungs of infected individuals, and most patients succumb to the subsequent massive influx of polymorphonuclear neutrophils and lobar pneumonia in the absence of bacteraemia (McCullers & Tuomanen, 2001). The metabolic requirements governing $S. pneumo- niae$ persistence during pulmonary infection are crucial for understanding pneumococcal disease. Severe attenuation in a murine model of pneumococcal pneumonia was observed for $potABCD$ and cad mutant strains in competition with wild-type TIGR4. Additionally, spermidine biosynthesis seems to be equally important during pneumococcal pneumonia. These data strongly suggest that the biosynthesis and acquisition of polyamines are crucial for pneumococcal fitness and disease during lower respiratory tract infections, and represent novel targets for prophylactic or therapeutic interventions.

### Table 3. Polyamine concentrations of wild-type TIGR4 and the $\Delta cad$, $\Delta potABCD$ and $\Delta speE$ strains grown in THY

Values represent mean results from three independent experiments ± SEM.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polyamine</th>
<th>Concentration (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Cadaverine</td>
<td>9.17 ± 1.20</td>
</tr>
<tr>
<td></td>
<td>Putrescine</td>
<td>4.14 ± 2.03</td>
</tr>
<tr>
<td></td>
<td>Spermidine</td>
<td>14.22 ± 1.21</td>
</tr>
<tr>
<td>$\Delta cad$</td>
<td>Cadaverine</td>
<td>5.59 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>Putrescine</td>
<td>1.64 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>Spermidine</td>
<td>9.60 ± 0.29</td>
</tr>
<tr>
<td>$\Delta potABCD$</td>
<td>Cadaverine</td>
<td>3.29 ± 1.77</td>
</tr>
<tr>
<td></td>
<td>Putrescine</td>
<td>1.99 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>Spermidine</td>
<td>11.29 ± 0.30</td>
</tr>
<tr>
<td>$\Delta speE$</td>
<td>Cadaverine</td>
<td>5.95 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>Putrescine</td>
<td>1.74 ± 0.91</td>
</tr>
<tr>
<td></td>
<td>Spermidine</td>
<td>9.12 ± 1.15</td>
</tr>
</tbody>
</table>
In septicaemia experiments the time to death of mice infected with either the potABCD or the cad mutant strain was almost identical to that for wild-type TIGR4, suggesting that these loci are dispensable for the pneumococcus during invasive infections. However, mice infected with the ΔspeE strain had a median time to death of 144 h compared with 54 h for animals infected with the wild-type parent. These data suggest that the biosynthesis and intracellular availability of spermidine play a role in pneumococcal pathogenesis during invasive infections. The functions of the cad and potABCD loci may be compensated for by genetic redundancy or other unknown compensatory mechanisms, and hence do not affect disease outcome. It is also possible that spermidine is the key polyamine required by pneumococci during murine septicaemia, and that cad (deficient in cadaverine) and potABCD (deficient in putrescine) mutants synthesize excess spermidine to compensate for the deficiency of other polyamines. However, the infection of mice with potABCD or cad mutant strains grown in media with excess spermidine did not result in increased virulence (data not shown), suggesting as yet unknown functions of spermidine during infection. These data suggest that deficiencies in cadaverine biosynthesis and putrescine or spermidine transport have marginal or no effects on pneumococcal growth and virulence during invasive infections. Spermidine biosynthesis, however, may confer certain survival and fitness advantages upon pneumococci and play an adjunctive role during septicaemia.

Overall, the in vivo data show that deletion of genes involved in polyamine biosynthesis or transport results in severe attenuation during pneumonia formation, followed by upper respiratory tract colonization, and little or no effect during murine septicaemia. Interestingly, the availability of free polyamines is highest in the blood, followed by the nasal mucosa, and is severely limited in pulmonary tissues (Shah & Swiatlo, 2008). At least two different, although not necessarily mutually exclusive, mechanisms may be responsible for polyamine-mediated pneumococcal disease outcomes. (i) Polyamines may be important nutrients that are indispensable for pneumococcal growth,
replication and persistence during colonization and infection. For example, putrescine and spermidine are eventually shuttled into the tricarboxylic acid cycle as carbon and nitrogen sources (Chou et al., 2008). (ii) Deficiencies in polyamines may modulate the expression of key pneumococcal growth and virulence factors that are indispensable for in vivo fitness and infection, and result in an attenuated phenotype. Indeed, polyamines regulate the translation of numerous proteins, many of which are key regulators of growth, replication and virulence (Shah & Swiatlo, 2008; Tabor & Tabor, 1985).

We tested both these hypotheses by measuring intracellular polyamine pools of mutant and wild-type bacteria and in vitro growth kinetics, and by performing large-scale proteomics with the ΔpotABCD and ΔspeE strains, as they showed significant attenuation in all disease models. Capillary electrophoresis analyses showed a reduction in intracellular pools of cadaverine, putrescine and spermidine in all mutant strains compared with the wild-type TIGR4. Depletion of intracellular polyamines, however, does not affect the in vitro growth kinetics of mutant strains, suggesting that polyamines are dispensable for growth under routine laboratory conditions in an enriched medium (THY). There are no well-defined minimal media for S. pneumoniae, partly due to its complex growth requirements. We tested the growth kinetics of mutant strains in a completely defined medium that provides all essential nutrients in measured amounts and again saw no difference in growth rates (data not shown). These results are similar to the observations made by Chattopadhyay and Tabor, which showed the non-essentiality of polyamines for aerobic growth of E. coli (Chattopadhyay et al., 2009). Large-scale proteomic analyses with the potABCD mutant revealed a significant decrease in the expression of oligopeptide and amino acid ABC transporters involved in pathogenesis, as well as several well-characterized virulence factors such as capsular polysaccharide biosynthesis proteins, pneumolysin and pneumococcal surface protein A (Kadioglu et al., 2008). Decreased expression of several proteins involved in growth and replication was also seen in the polyamine transport-deficient strain. Tandem MS analysis with the speE mutant strain also showed a reduction in the expression of important virulence factors such as oligopeptide and amino acid ABC transporters, zinc metalloprotease ZmpB and choline-binding protein PcpA. Similar to the ΔpotABCD strain, a decrease in the expression of several growth and cell division proteins, such as DivIVA, MrcC and FtsX, was also seen in the spermidine synthase mutant strain. Interestingly, the speE mutant strain showed increased lysine decarboxylase expression. It is possible that increased cadaverine production compensates for spermidine deficiency during infection and may be a counteracting mechanism. These results suggest that polyamines regulate the expression of proteins required for pneumococcal replication as well as virulence. The attenuated phenotype of the mutant strains could be a collective outcome of reduced expression of proteins responsible for regulating pneumococcal growth, replication and/or virulence, with the exact contribution of each group of proteins probably dependent on the host micro-environment and the level of polyamines in it.

Polyamines have often been implicated in the transcriptional and translational control of genes and transcripts involved in stress responses. However, all mutants had survival rates similar to that of the wild-type strain following paraquat and low-pH exposure. Also, the pneumococcus might employ polyamine-mediated defence mechanisms to overcome nitrosative stress during infection, as described for E. coli via the merR transcriptional regulator (Bower et al., 2009; Potter et al., 2010). Thus, subtle perturbations in intracellular polyamine levels may not significantly affect pneumococcal stress responses in vitro. However, further evaluation by constructing mutants with additional deletions in polyamine biosynthesis and transport pathways is required to fully comprehend the role of polyamines in S. pneumoniae survival during stress.

Our data strongly suggest that polyamines are one of the key nutrients utilized by the pneumococcus during in vivo growth, and that they regulate the expression of certain virulence factors during infection. These results also establish, for the first time to our knowledge, a link between S. pneumoniae polyamine biosynthesis and transport and colonization and disease outcomes. Similar observations have been made with respect to the roles of sucrose, sialic acid metabolism and manganese transport in pneumococcal pathogenesis (Manco et al., 2006; Rosch et al., 2009; Yesilkaya et al., 2008). It is becoming clear that understanding the metabolic requirements of a pathogen may promote a deeper understanding of the underlying disease aetiology and result in novel prophylactic and/or therapeutic interventions.

REFERENCES


Role of polyamines in pneumococcal disease


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