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

Pratik Shah,[1]† Bindu Nanduri,[2] Edwin Swiatlo,[3] Yinfa Ma[4] and Ken Pendarvis[2]

Correspondence
Pratik Shah
pratik.shah@molbio.mgh.harvard.edu

[1]Department of Microbiology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA

[2]College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA

[3]Research Service, Veteran Affairs Medical Center, 1500 East Woodrow Wilson Drive, Jackson, MS 39216, USA

[4]Department 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, septicaemia, 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 (Δ*speE* or Δ*cad*) or transport genes (Δ*potABCD*) were constructed and were found to be attenuated in murine models of pneumococcal colonization and pneumonia, either alone or in competition with the wild-type strain. The Δ*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.

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INTRODUCTION

Streptococcus pneumoniae is a Gram-positive pathogen that asymptomatically colonizes the nasopharynx of humans. However, in some cases it can cause pneumonia, septicaemia, 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.

†Present address: Department of Molecular Biology, Massachusetts General Hospital and Harvard Medical School, 185 Cambridge Street, Boston, MA 02114, USA.

Abbreviations: ∑Xcorr, sum of cross correlations; 2D LC ESI MS/MS, 2D liquid chromatography-electrospray ionization tandem MS; ACN, acetonitrile, CI, competitive index; LR, lactated Ringer's solution; PCT, pressure cycling technology; SAM, *S*-adenosylmethionine; TP, trimethoprim.

Four supplementary tables, showing the results of tandem MS experiments, are available with the online version of this paper.

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% CO_2 atmosphere. Broth cultures were typically grown to a final OD_{600} 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* (*cad*F/*cad*R), *potD* (*potD*F/*potD*R) and *speE* (*speE*F/*speE*R) genes (Table 1). Bacterial cultures were statically grown in 2 ml THY medium at 37 °C in 5% CO_2, and harvested during the exponential phase of growth (OD_{600} 0.4) by centrifugation at

Table 1. Sequences of primers used in this study

Primer	Sequence* (5′–3′)
*cad*F	CACCTTGAAAGAGTTAGATCA
*cad*R	TTGACTTTTCTTATAGTTT
*potD*F	CACCATGTTAGATAGTAAAATCAAT
*potD*R	CTTCCGATACATTTTAAACTGTA
*speE*F	CACCATGGATTTATGGTTTTCT
*speE*R	TTTTTTTCCTTCCTCTTCTTCT
0916F1	AGCAAATATAAACCCGAGTAAAAA
0916R1	CAGGTACCGCTTGTGACCTGGAACATC
0916F2	CAGAGCTCGTTTCGGTTTGCGATTTT
0916R2	GATCTTCCGTCCCTTGGAG
1389-86F1	AGCCCCGATCGGTTAATCT
1389-86R1	CAGAGCTCAGAAAGTTTGCGGAT
1389-86F2	CAGGTACCACAGGGAAATATAGCGACC
1389-86R2	TATAAAGGTGCCTATCACCCAAT
0918FI	AAACTTTATATCCTTGTTCATGCAG
0918R1	CAGGTACCCTACTGCCAAAGCCCAA
0918F2	CAGAGCTCTTGCCCAAGTTGCTATTTT
0918R2	ACACCTGGGTCAAAACCAGA

*Underlined sequences are complementary to *S. pneumoniae* TIGR4 chromosomal DNA.

~17 000 ***g*** in an Eppendorf 5810 centrifuge, and pneumococcal chromosomal DNA was isolated with a MasterPure Gram-positive DNA purification kit (Epicenter) following the manufacturer's protocol. PCR was performed with GoTaq DNA polymerase (Promega Biotechnology), an equal concentration of chromosomal DNA was added to each mix and reactions were run for 30 cycles (94 °C, 1 min; 52 °C, 0.5 min; 72 °C, 1.5 min). Amplified products were separated on a 1% agarose gel and visualized following ethidium bromide staining.

Construction of Δ*potABCD*, Δ*cad* and Δ*speE* strains. The TIGR4 *potABCD* operon was replaced with a trimethoprim (TP)-resistance cassette, *tmp*^{r}. The *tmp*^{r} cassette was obtained from the *pkoT* plasmid by digestion with *Kpn*I and *Sac*I enzymes (Adrian *et al.*, 2000). Approximately 500 bp DNA fragments flanking the 5′ and 3′ ends of the *pot* operon were PCR-amplified from TIGR4 genomic DNA using primer pairs 1389-86F1/1389-86R1 and 1389-86F2/1389-86R2 (Table 1). One primer in each pair was designed to incorporate either a *Kpn*I (1389-86F2) or *Sac*I site (1389-86R1). The *tmp*^{r} cassette was ligated to the two PCR products flanking the *pot* operon, and the resulting construct was PCR-amplified using primer pair 1389-86F1 and 1389-86R2, and used for transforming *S. pneumoniae* TIGR4 as described by Lau *et al.* (2002). The double recombination event was selected by plating on BAP containing 50 μg TP ml^{-1}. Construction of *cad* and *speE* mutants was done in a similar manner by replacing the *cad* or *speE* genes with the *tmp*^{r} cassette. Briefly, approximately 500 bp fragments 5′ and 3′ of either *cad* (0916F1-0916R1 and 0916F2-0916R2) or *speE* genes (0918F1-0918R1 and 0918F2-0918R2) were PCR-amplified. One primer in each primer pair had either a *Kpn*I (0916R1 and 0918R1) or the *Sac*I site (0916F2 and 0918F2) (Table 1). The *tmp*^{r} cassette with *Kpn*I- and *Sac*I-generated ends was ligated with PCR fragments with complementary ends. PCR amplification was used for amplification of constructs of the correct size. All constructs were used for transformation of TIGR4 and transformants were selected on BAP with 50 μg TP ml^{-1} (Bricker & Camilli, 1999). PCR and DNA sequencing was performed to confirm the deletion of target genes.

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) (Briles *et al.*, 1982). Pneumococci were grown at 37 °C in 5% CO_2 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 4×10^5 viable cells resuspended in 10 μl LR were used for nasopharyngeal colonization experiments (Briles *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 4×10^5 cells were resuspended in 40 μl LR and administered intranasaly (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 bacteraemia. For comparison of *in vivo* growth and ability to cause septicaemia, mice were infected intravenously (i.v.) using approximately 1×10^4 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 vivo* competitive index (CI) experiments for colonization and pneumonia infections were essentially performed as described by Iyer & Camilli (2007). Pre-enumerated stock cultures were thawed, and mutant and wild-type cells were mixed to yield an input ratio of 1 : 1. Plating on media containing TP and regular BAP free of antibiotics was used to distinguish between competing strains. The CI [CI= ($mutant_{output}$/$competitor_{output}$)/($mutant_{input}$/$competitor_{input}$)] was calculated for each animal. A CI of 1 indicates that the mutant and competitor strains colonize to equal levels. A CI <1 indicates that the mutant is outcompeted and has a colonization/lung infection defect.

***In vitro* growth assays.** *S. pneumoniae* mutant and wild-type strains were grown at 37 °C in THY broth following inoculation with approximately 10^6 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% $HClO_4$, 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 two times with 0.1 M $HClO_4$. 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 OD_{600} ~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 ProteoSolve-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 Δ*potABCD* 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 30 °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% triflouroacetic 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 carboxyamidomethylation 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 $P \leqslant 0.05$ (Qian *et al.*, 2005). Probabilities of protein identifications being incorrect were calculated using published methods (López-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 $\leqslant 0.05$ 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 $\log_2$ ratio of protein abundance.

Statistical analyses. All pneumococcal c.f.u. are reported as $\log_{10}$ 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 *S*-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 (Soksawatmaekhin *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-

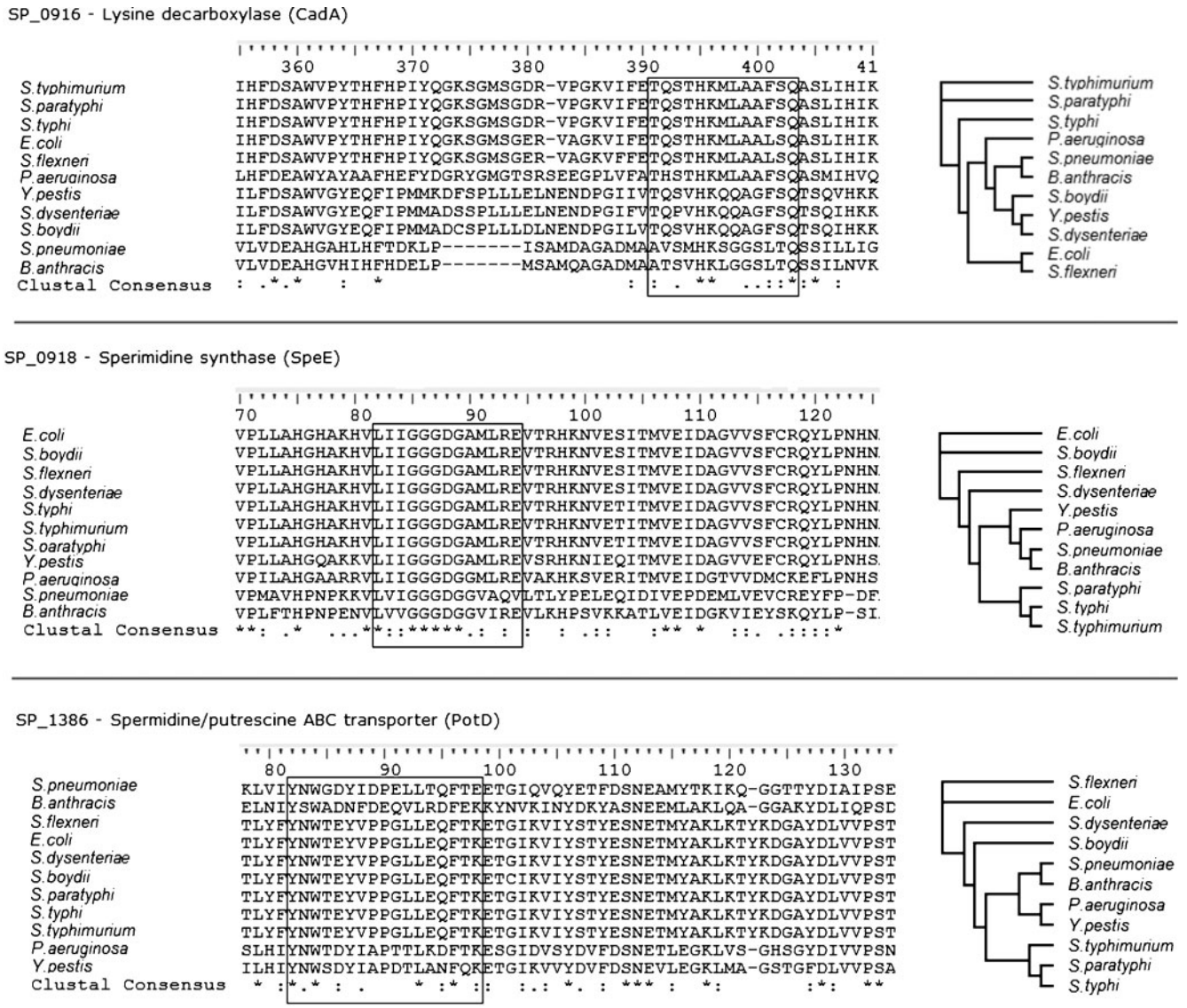

Fig. 1. Sequence alignments of a representative set of polyamine biosynthesis and transport domains. Alignment of the conserved lysine decarboxylase, spermidine synthase and the spermidine-binding domains of PotD from *S. pneumoniae* TIGR4 and other human bacterial pathogens. The boxed sequences in the 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.

estingly, 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 septicaemia, 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 Δ*potABCD* 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.

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

Strain	Serotype	Patient sex	Age (years)	Sample
AW234	3	F	78	Sputum
TIGR4	4	M	30	Blood
AW61	6A/B	M	53	Sputum
AW130	7F	F	57	Sputum
AW230	9V	M	83	Sputum
SP11-BS70	11	Child	11	NP*
AW315	14	F	41	Sputum
AW207	18	M	58	Sputum
G54	19F	NA†	NA†	Sputum
AW66	23F	M	78	Sputum
AW212	33F	F	69	Sputum
AW205	35B	F	60	Sputum

*Nasopharynx.
†Not available.

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 nasophraynx, 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).

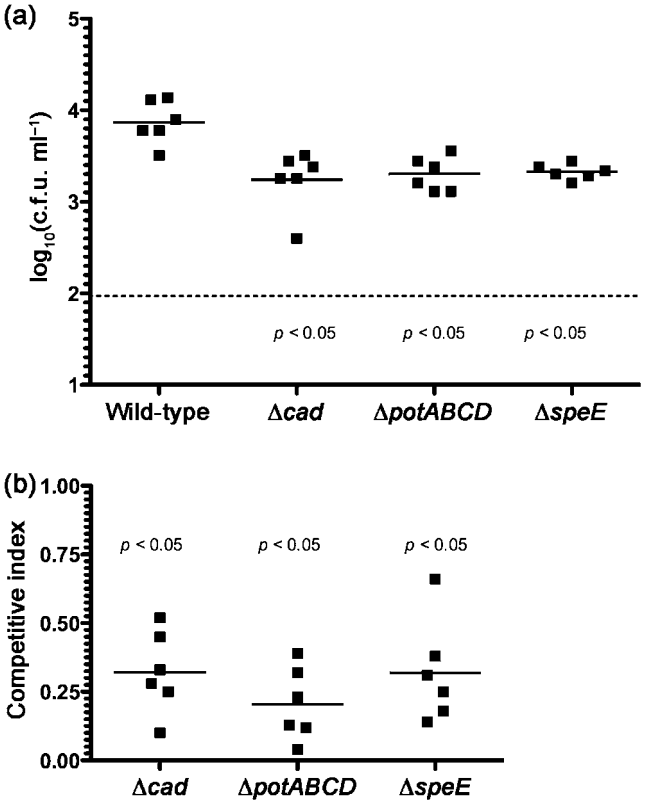

Fig. 2. Lysine decarboxylase, *speE* and the *potABCD* operon contribute to pneumococcal nasopharyngeal colonization. Inactivation of *cad*, *potABCD* or *speE* results in significant attenuation of the ability of the pneumococcus to colonize the murine respiratory tract following infection either alone (a) or in competition with the wild-type parent (b). Data represent $\log_{10}$(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.

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_{600} 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).

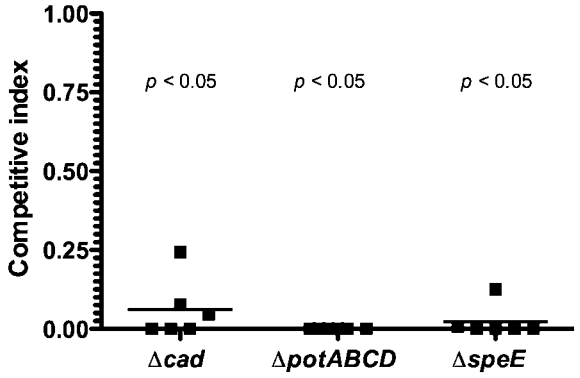

Fig. 3. Polyamine biosynthesis and transport genes are essential for pneumococcal pneumonia formation. Deletion of *cad*, *speE* or the *potABCD* genes results in severe attenuation in the pneumonia model of infection in competition with the wild-type strain. Data represent CI ratios (mutant : wild-type) of the $\log_{10}$(c.f.u.) recovered from lung homogenates of mice (n=6 in each group) 48 h post-infection.

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 H_2O_2 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).

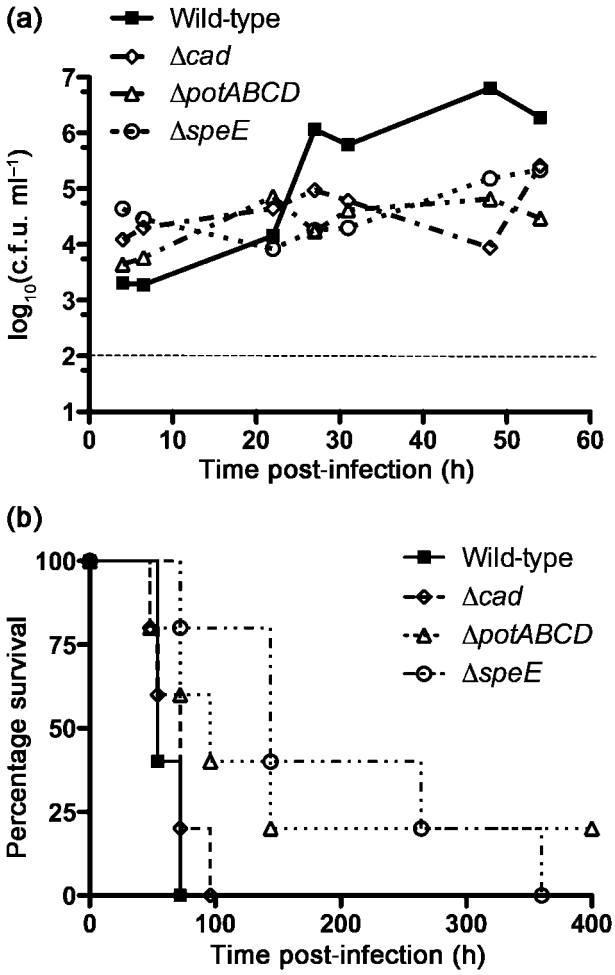

Fig. 4. *S. pneumoniae* polyamine biosynthesis and transport genes affect pneumococcal survival and virulence during septicaemia. (a) Deletions in *cad*, *speE* or the *potABCD* genes affect pneumococcal septicaemia in mice following intravenous challenge with mutant strains. Each data point represents $\log_{10}$(c.f.u.) of either the mutant or the wild-type strain recovered from blood of infected mice at various time points. (b) The median survival of mice intravenously infected with the Δ*speE* (P<0.05) and Δ*potABCD* strains is longer than that of mice infected with wild-type TIGR4. The dashed line in (a) represents the lower limit of bacterial detection for the experiment.

Proteomic analyses

2D LC ESI MS/MS analyses were used to identify proteins that are uniquely or differentially expressed in the Δ*potABCD* and Δ*speE* mutant strains compared with the wild-type TIGR4, as these mutants showed severe attenuation during colonization, pneumonia and invasive infection. Proteomic analyses with the Δ*potABCD* strain and wild-type TIGR4 identified 92 proteins that were detected only in wild-type, while 79 proteins were exclusive to the Δ*potABCD* mutant strain and 359 proteins were common to both datasets (Supplementary Table S1).

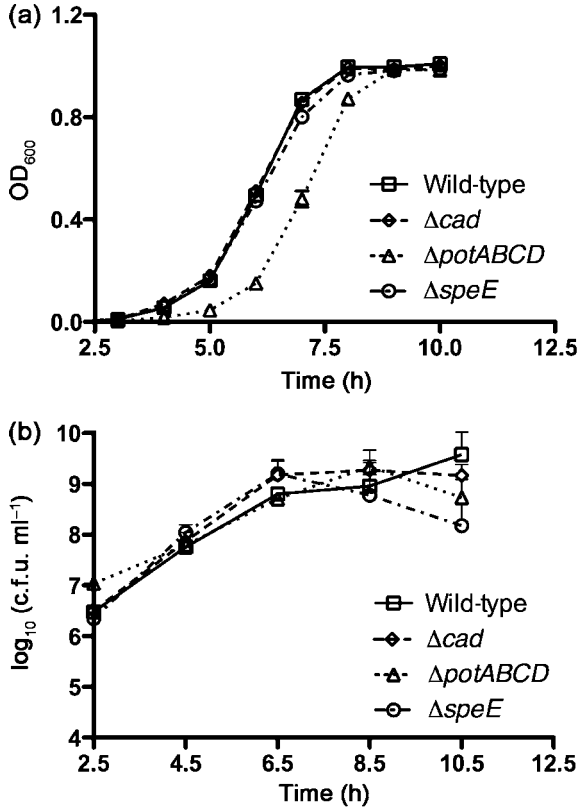

Fig. 5. Growth of wild-type TIGR4 and *cad*, *potABCD* and *speE* deletion strains. (a) OD_{600} measurements and growth curves of wild-type TIGR4 and the *cad*, *potABCD* and *speE* deletion strains in THY medium. (b) Growth rate and c.f.u. of the *cad*, *potABCD* and *speE* deletion strains and wild-type TIGR4 in THY medium. All assays were performed in triplicate and data are presented as mean ± SEM.

Differential expression analysis based on ∑Xcorr showed a significant increase in the expression of approximately 35 proteins, while 76 proteins were downregulated in the Δ*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 Δ*speE* mutant strain compared with wild-type TIGR4 (Supplementary Table S3). Tandem MS analysis with the Δ*speE* mutant strain and wild-type TIGR4 also showed that 124 proteins were unique to the wild-type strain, 96 were detected in the Δ*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.

Table 3. Polyamine concentrations of wild-type TIGR4 and the Δ*cad*, Δ*potABCD* and Δ*speE* strains grown in THY

Values represent mean results from three independent experiments ± SEM.

Strain	Polyamine	Concentration (pM)
Wild-type	Cadaverine	9.17 ± 1.20
	Putrescine	4.14 ± 2.03
	Spermidine	14.22 ± 1.21
Δ*cad*	Cadaverine	5.59 ± 0.19
	Putrescine	1.64 ± 0.40
	Spermidine	9.60 ± 0.29
Δ*potABCD*	Cadaverine	3.29 ± 1.77
	Putrescine	1.99 ± 1.04
	Spermidine	11.29 ± 0.30
Δ*speE*	Cadaverine	5.95 ± 0.92
	Putrescine	1.74 ± 0.91
	Spermidine	9.12 ± 1.15

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; Muñoz-Elías & 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. pneumoniae* 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.

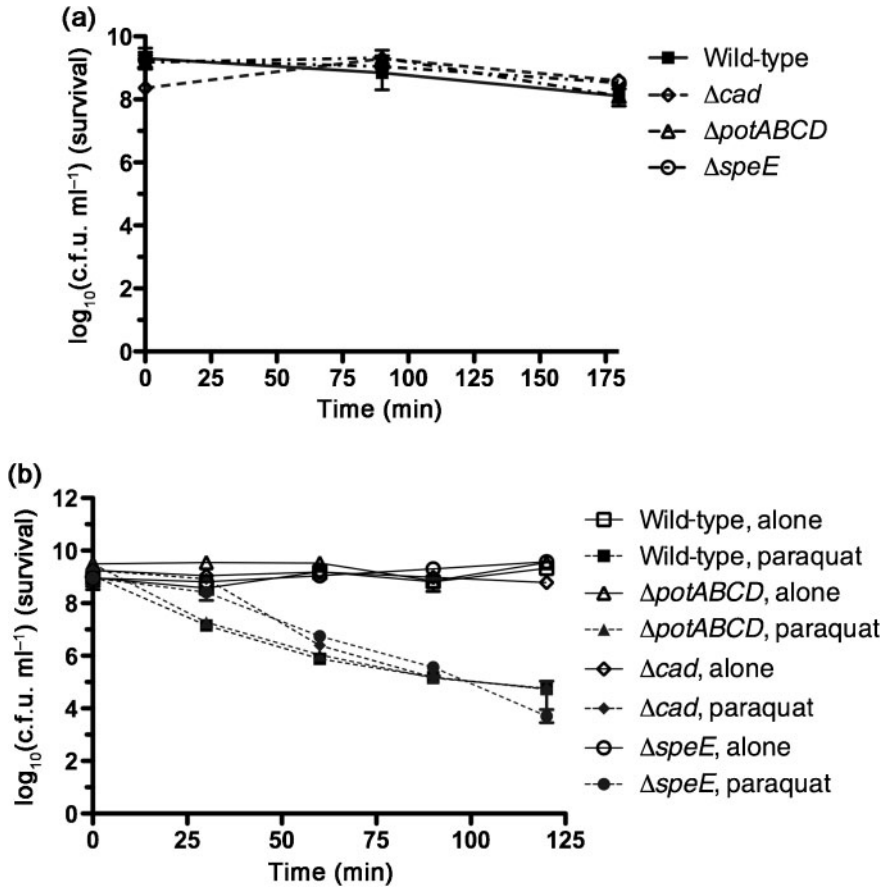

Fig. 6. Stress responses. (a) Wild-type TIGR4 and the *cad*, *potABCD* and *speE* mutant strains were exposed to a low-pH (5.0) environment and post-exposure c.f.u. enumerated. (b) Wild-type TIGR4 and the *cad*, *potABCD* and *speE* mutant strains were incubated with or without 50 mM paraquat, and survival was determined at various time points. All assays were performed in triplicate and data are presented as mean ± SEM.

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 Δ*speE* 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 Δ*potABCD* and Δ*speE* 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 Δ*potABCD* strain, a decrease in the expression of several growth and cell division proteins, such as DivIVA, MreC 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.

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