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Utilization of Putrescine by *Streptococcus pneumoniae* During Growth in Choline-limited Medium

D. Ware[1], J. Watt[2,‡] and E. Swiatlo[1,2*]

[1]*Department of Microbiology, University of Mississippi Medical Center, 2500 N. State Street Jackson, MS 39216, USA*
[2] *Research Service, Veterans Affairs Medical Center, 1500 Woodrow Wilson Drive, Jackson, MS 39216, USA*
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Polyamines such as putrescine are small, ubiquitous polycationic molecules that are required for optimal growth of eukaryotic and prokaryotic cells. These molecules have diverse effects on cell physiology and their intracellular content is regulated by *de novo* synthesis and uptake from the environment. The studies presented here examined the structure of a putative *po*lyamine *t*ransporter (Pot) operon in *Streptococcus pneumoniae* (pneumococcus) and growth of pneumococci in medium containing putrescine substituted for choline. RT-PCR experiments demonstrated that the four genes encoding the Pot system are co-transcribed with *murB*, a gene involved in an intermediary step of peptidoglycan synthesis. Pneumococci grown in chemically-defined media (CDM) containing putrescine without choline enter logarithmic phase growth after 36-48 hs. However, culture density at stationary phase eventually reaches that of choline-containing medium. Cells grown in CDM-putrescine formed abnormally elongated chains in which the daughter cells failed to separate and the choline-binding protein PspA was no longer cell-associated. Experiments with CDM containing radiolabeled putrescine demonstrated that pneumococci concentrate this polyamine in cell walls. These data suggest that pneumococci can replicate without choline if putrescine is available and this polyamine may substitute for aminoalcohols in the cell wall teichoic acids.

Key words: polyamine, putrescine, *Streptococcus pneumoniae*

Polyamines such as spermidine and putrescine are small, polycationic molecules which are present in all prokaryotic and eukaryotic cells. These molecules are necessary for normal cell growth and have been associated with a wide variety of physiological processes, primarily through their interaction with negatively charged nucleic acids and ATP (Igarashi and Kashiwagi, 2000). Polyamines have been shown to modulate virtually all aspects of cellular physiology involving nucleic acids, including DNA synthesis, transcription, and ribosome assembly (Goldemberg and Algranati, 1989). Intracellular polyamines are derived from both *de novo* synthesis from amino acids and uptake from the environment. Cellular levels of polyamines are highly regulated in *Escherichia coli* by transcriptional control of genes encoding a polyamine transporter and substrate inhibition of polyamine biosynthetic enzymes (Kashiwagi and Igarashi, 1988; Antognoni *et al.*, 1999).

Despite the known pleiotropic effects of polyamines on protein synthesis and cell growth in many types of cells, little is known about polyamine physiology in pathogenic bacteria. A four-gene operon encodes an ABC transporter for putrescine and spermidine in *E. coli* and an additional four-gene operon encodes a putrescine-specific transporter (Igarashi and Kashiwagi, 1999). Additionally, *E. coli* expresses a transmembrane protein which functions as a putrescine/ornithine antiporter (Kashiwagi *et al.*, 1992). *E. coli* mutants unable to synthesize polyamines *de novo* grow at a much slower rate compared with wild-type cells (Tabor *et al.*, 1980) and hyperosmotic shock induces polyamine excretion in *E. coli* through an unknown membrane transport mechanism (Schiller *et al.*, 2000). Cadaverine synthesis is induced at low pH in *E. coli* and may confer adaptive acid tolerance by altering the function of outer membrane porins (Samartzidou *et al.*, 2003). Mutant *E. coli* cells which cannot synthesize polyamines are hypersusceptible to the toxic effects of high oxygen concentration (Chattopadhyay *et al.*, 2003).

What is known about the physiology of polyamines in prokaryotes has been derived from *E. coli,* and knowledge of polyamine metabolism in other human pathogens is extrapolated from functional genomic analysis of completed genome projects. Many human-associated bacterial species contain genes for members of a superfamily of amino acid/polyamine/organocation (APC) transporters based on sequence analysis (Jack *et al.*, 2000), but almost

* To whom correspondence should be addressed.
(Tel) 1-601-364-1315; (Fax) 1-601-364-1390
(E-mail) swed@sprintmail.com
‡University of Alabama at Birmingham, Department of Microbiology, Bevill Building, Room 658, Birmingham, AL 35294, USA

nothing is known about their function during in vitro growth or in a human host. It has been shown that supplemental putrescine can restore virulence gene expression in *Shigella flexneri* mutants which are unable to synthesize modified nucleosides necessary for tRNA synthesis (Durand & Bjork, 2003). *Enterococcus faecalis* can transport agmatine by an agmatine-putrescine antiporter for use as an energy source (Driessen *et al.*, 1988). Putrescine and cadaverine have been found in the peptidoglycan in *Veillonella* spp. (Kamio and Nakamura, 1987).

The human pathogen *Streptococcus pneumoniae* (pneumococcus) is generally considered to be the most common bacterial cause of pneumonia and frequently progresses to septicemia and meningitis. The polysaccharide capsule of pneumococci has long been recognized as an important virulence factor of this organism. However, many surface-associated and secreted proteins are being studied for their role in pathogenesis and protective immunity (Briles *et al.*, 1998; Jedrzejas, 2001; Swiatlo, 2003). Pneumococcal genes with homology to a *po*lyamine *t*ransporter (Pot) operon in *E. coli* have been implicated in the pathogenesis of pneumococcal infection in a mouse model of bacteremia and pneumonia (Polissi *et al.*, 1998; Ware and Swiatlo, 2003).

Pneumococci require choline, or a closely related analog ethanolamine, for growth in vitro (Mosser and Tomasz, 1970). The polyamine putrescine and the aminoalcohol choline are both small hydrocarbons molecules with similar molecular weights and quaternary nitrogens with a net positive charge at physiological pH. This structural similarity of putrescine and choline and the requirement for choline, or analogs, for pneumococcal growth suggested that putrescine may be an additional substrate that can substitute for choline. The purpose of this study was to examine the transcriptional organization of the pneumococcal *pot* operon and test for the potential utilization of putrescine during in vitro growth of pneumococci in a choline-limited environment.

Methods

Bacterial strains and Growth Conditions

S. pneumoniae WU2 expresses serotype 3 capsule (Briles *et al.*, 1981) and was used in all experiments. This strain is highly virulent in a mouse model of sepsis (Briles *et al.*, 1992). Bacterial stocks were grown to late exponential phase in Todd Hewitt Yeast Extract (THY) broth at 37°C in 5% CO_2 and cells were collected by centrifugation. The cells were washed twice in sterile phosphate-buffered saline (PBS, pH 7.0) at 4°C and stored at -80°C in PBS with 20% glycerol. Bacterial stocks were quantitated by counting CFUs in serial dilutions plated on THY plates containing 4% sheep erythrocytes (Colorado Serum Co., USA). Bacteria from frozen stocks were used to inoculate chemically-defined medium (CDM) (JRH Bioscience, USA) which has been previously described (van de Rijn and Kessler, 1980). The CDM was supplemented with either filter-sterilized choline chloride (CDM-CC), ethanolamine (CDM-EA), or putrescine (CDM-Pt) and all cultures were performed in disposable polystyrene tubes. Cultures in CDM were incubated in 5% CO_2 at 37°C. Bacterial growth in liquid media was monitored by measuring absorbance of resuspended cells at 600 nm. Before removing aliquots for absorbance measurement all cultures were vortexed vigorously to thoroughly resuspend those cells which grow in long chains that settled to the bottom of culture tubes. Terminal subcultures were done after all experiments by plating bacteria from the liquid media onto blood agar plates and testing for optochin sensitivity to assure purity and identity of the cultures.

RT-PCR

Pneumococcal strain WU2 was grown in THY broth at 37°C in 5% CO_2 until mid-logarithmic phase growth ($O.D._{600} \sim 0.5 - 0.6$). Cells were collected by centrifugation and lysed by adding $1/5^{th}$ the culture volume of 10 mM Tris buffer (pH 6.8) containing 0.1% SDS and 0.05% deoxycholate and incubating for 30 min at room temperature. Total RNA from lysed cells was purified with TRI REAGENT-LS (Molecular Research Center, Inc., USA) according to the manufacturer's instructions. Contaminating DNA was removed using the RNA-Free kit (Ambion, USA). Purified RNA was suspended in DEPC-treated water in RNase free tubes and stored at -80°C. This RNA was used as the template for a one–step RT-PCR procedure with the MasterAmp High Fidelity RT-PCR kit (Epicentre, USA). Oligonucleotide primers specific for the 5'-end of *murB* gene and the 3'- end of *potD* gene were used to first synthesize, and subsequently amplify, a cDNA product from total RNA. The primer sequence specific for the 5'-end of *murB* was: 5'-CGTTCGTGATGGTGGGATTCGTGGA-3' and the 3'-specific primer for *potD* was: 5'-AGCA-xA-GCATGCCTTGATAGAACCGATACATTTTAAACTG-3'. Primers were derived from examining both published pneumococcal genomes, which have identical sequence at the locus which was targeted by the oligonucleotides (Hoskins *et al.*, 2001; Tettelin *et al.*, 2001). Following reverse transcription cDNA was amplified by PCR: denaturation for 1 min at 95°C, annealing at 55°C for 1 min, extension at 72°C for 3 min, with a total of 35 cycles. A final extension was done at 72°C for 5 min after the last cycle.

Immunoblot Analysis

WU2 was grown to mid-log phase and bacterial cells were collected by centrifugation. Culture supernatants were filter-sterilized and concentrated to 1/10 the original culture volume in Amicon Centriplus YM - 30 kD molecular weight cutoff concentrators (Millipore, USA). The cells were washed twice in PBS and serial 10-fold dilutions of cell suspensions were made in PBS. Ten micro-

liters from each dilution was applied to a nitrocellulose membrane, allowed to air dry, washed twice with PBS, and blocked for one hour in PBS containing 1% BSA and 0.5% Tween® 20 (Sigma, USA). Each blot was then incubated with one of the following combinations of antibodies: 1) mouse anti-phosphocholine IgA (TEPC15; Sigma, USA) as the primary antibody followed by alkaline phosphatase (AP)-conjugated goat anti-mouse IgA (Sigma, USA); 2) anti-PspA monoclonal antibody Xi126 followed by AP-conjugated goat anti-mouse IgG (Sigma, USA). The blots were developed using an alkaline phosphatase buffer, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl-phosphate as previously de-scribed (Briles *et al.*, 1996). The color development was observed for 5 min immediately after addition of AP substrates. Further development was prevented by removal of reaction solution and washing the membrane three times in distilled water.

Growth in Radiolabeled Substrates

Frozen stocks of WU2 were used to inoculate 10 ml of CDM containing a mixture of unlabeled choline or putrescine and 50 μCi of either [^{3}H]-choline or [^{3}H]-putrescine (Moravek Biochemicals, USA) to give a final total concentration of 50 μg/ml. The cells were grown to mid-logarithmic phase, collected by centrifugation, and washed in PBS. The cell pellets were resuspended in 1 ml of protoplast buffer (20% sucrose, 5 mM Tris (pH 7.4), 2.5 mM $MgSO_4$, 25 μg/ml of mutanolysin) and the suspension was incubated for 1 h at 37°C. The suspension was centrifuged at 500 × *g* for 10 min and the supernatant was collected as the cell wall fraction. The pellet was washed in 1/5 the original culture volume of protoplast buffer and resuspended in distilled water. An equal volume of chloroform was added and the suspension was vortexed for 1 min to emulsify the layers. The chloroform/cell suspension was centrifuged at 500 × *g* for 10 min and the aqueous layer containing the water-soluble cytoplasmic contents was removed. The remaining suspension was lyophilized in a vacuum centrifuge for 15 min and the pellet, containing membranes and insoluble cytoplasmic contents, was suspended in water. Each fraction was counted in a Packard TriCarb 2200CA liquid scintillation counter. Bacteria incubated in CDM alone or CDM supplemented to a final concentration of 50 μg/ml with either unlabeled choline or putrescine were fractionated in a manner identical to bacteria grown with radiolabeled substrates and used as controls.

Results

The sequenced genomes of pneumococcal strains TIGR4 and R6 have been published and both strains contain four contiguous genes which encode a putative spermidine/putrescine transporter (Hoskins *et al.*, 2001; Tettelin *et al.*, 2001). These gene sequences were submitted to the DNA and protein databases of the National Center for Biotechnology Information for homologous sequences using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). The genes *potABCD* in *E. coli* have homologs in many Gram-positive bacterial pathogens (Table 1). The *potA* gene in pneumococcus is predicted to encode a typical ATP-binding protein containing consensus Walker A and B sites which are considered to define an ATP-binding cassette (Schneider and Hunke, 1998). The proteins encoded by

Table 1. Representative proteins with sequence homology to PotABCD in *S. pneumoniae*

S. pneumoniae protein	Genbank Accession No.	Predicted function	Organism	% Identity/similarity	No. of amino acids compared
PotA	AAAM99992	ATP-binding	*S. agalactiae*	88/94	384
	AAK33980	ATP-binding	*S. pyogenes*	87/93	384
	AAN58676	ATP-binding	*S. mutans*	88/92	384
	AAO82360	ATP-binding	*E. faecium*	72/84	361
	A40840	ATP-binding	*E. coli*	53/68	378
PotB	AAK33981	Transmembrane permease	*S. pyogenes*	75/88	264
	AAN586775	Transmembrane permease	*S. mutans*	74/86	264
	AAN79945	Transmembrane permease	*E. coli*	31/54	287
PotC	AAN58678	Transmembrane permease	*S. mutans*	77/86	258
	AAK33982	Transmembrane permease	*S. pyogenes*	74/85	258
	C40840	Transmembrane permease	*E. coli*	33/60	264
PotD	AAM99989	Polyamine-binding protein	*S. agalactiae*	66/81	357
	AAK33983	Polyamine-binding protein	*S. pyogenes*	64/80	357
	ZP000376	Polyamine-binding protein	*E. faecium*	61/75	357
	AAN79867	Polyamine-binding protein	*E. coli*	41/58	348

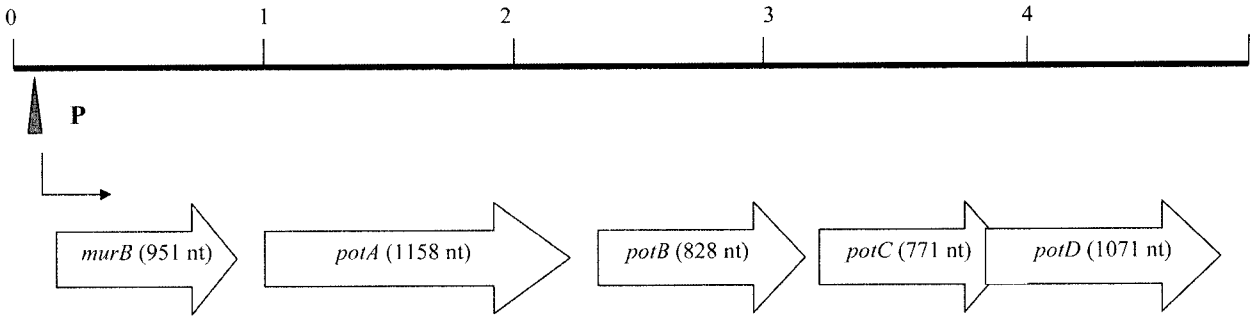

Fig. 1. Organization of the Pot operon in *S. pneumoniae*. The operon encodes a polycistronic mRNA of 4870 bases containing five open reading frames. A potential extended -10 promoter (5'-AATGGTATAAT-3') is located 29 bases 5' to the *murB* start codon. Fourteen bases 3' to the translational stop codon of *potD* is a 12 bp inverted repeat (5'-AAGACGAATAG-3') which may function as a rho-independent transcription terminator. The start codon of *potD* is located within the *potC* ORF, 28 bases from its translational stop site. Length of individual genes is noted next to gene name (nt - nucleotides).

potBC contain six (PotB) and seven (PotC) α-helical hydrophobic domains which are typical of integral membrane proteins. The *potD* gene product has homology to PotD in *E. coli* which has been shown to function as a spermidine- and putrescine-binding protein (Kashiwagi *et al.*, 1993). Although pneumococcal PotD has a typical Gram-positive signal peptide when analyzed by the SignalP algorithm (Neilsen *et al.*, 1997), it contains no motifs characteristic of lipoproteins, sortase-processed proteins, and choline-binding proteins. The extracellular fate of PotD is currently being investigated.

The *pot* genes in pneumococcus are arranged in an operon as shown in Fig. 1. Immediately upstream from the 5' end of *potA* is *murB* which encodes UDP-*N*-acetylenolpyruvoylglucosamine reductase, an enzyme involved in peptidoglycan biosynthesis. There is no obvious promoter sequence within the *pot* operon and immediately upstream of *potA*. The first potential promoter sequence is located 29 bases upstream of the ATG start codon for *murB*. This sequence (Fig. 1) is a putative extended -10 pneumococcal promoter sequence which initiates transcription without -35 promoter sequences (Sabelnikov *et al.*, 1995). Located 14 bases downstream from the termination codon of *potD* is a 12 bp inverted repeat which may function as a factor-independent transcription terminator.

RT-PCR was used to examine the size of transcripts that contain the *potABCD* genes. Reverse transcription was performed with a primer specific for the 3' end of *potD*. PCR was then performed with the same primer and a specific primer for sequence between the -10 promoter and the ATG start codon of *murB*. These primers amplified a product of approximately 4.8 kb, which is the predicted size of *murB* plus *potABCD* (Fig. 2). This result suggests that, under in vitro growth conditions, *murB* is co-transcribed as part of a polycistronic mRNA with *potABCD*. Efforts to determine alternative *potABCD* transcript sizes by northern blotting and hybridization were unsuccessful. It remains possible that the *pot* genes are also transcribed from an alternative, non-consensus promoter which does not include *murB*.

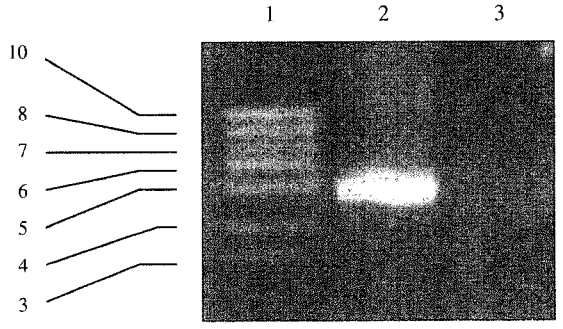

Fig. 2. Agarose gel electrophoresis of RT-PCR of WU2 RNA. Reverse transcription was performed with a *potD*-specific primer, then PCR was performed with a *murB*- and *potD*- specific primers. Lane numbers: 1 - DNA ladders (kilobase pairs); 2 - total RNA from WU2; 3 - PCR of WU2 RNA without reverse transcription (control).

Pneumococci are known to require either choline or ethanolamine for growth in vitro. The similarities in size and net charge between choline and putrescine led to the hypothesis that putrescine can substitute for choline during growth in a choline-limited environment. Choline was replaced with putrescine in a defined medium to test whether pneumococci can use putrescine for growth in the absence of aminoalcohols. The growth kinetics of WU2 in CDM supplemented with choline, ethanolamine, and putrescine are shown in Fig. 3. Growth of pneumococci was observed in putrescine concentrations ranging from 10 - 100 μg/ml (data not shown). The final concentration of putrescine, ethanolamine, and choline was adjusted to 50 μg/ml for all subsequent experiments. Pneumococci grown in choline started exponential growth at about 15 h after inoculation of the medium with 10^5 CFU. Growth in ethanolamine is delayed by about 12 h compared with choline, but cells eventually grew to similar density before reaching stationary phase. Cells grown in putrescine did not start exponential phase growth until at least 36 h after inoculation. However, the rate of cell division during expo-

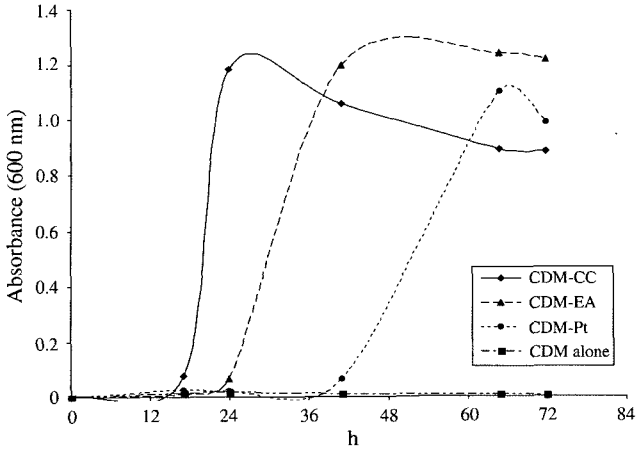

Fig. 3. Growth kinetics of WU2 in chemically defined medium (CDM) supplemented with choline (CC), ethanolamine (EA), or putrescine (Pt) to a final concentration of 50 μg/ml. Results are representative of experiments performed in triplicate.

nential phase was similar to that seen with choline and ethanolamine.

Pneumococcal cells growing without choline formed long chains in which rapidly dividing cells failed to separate into the characteristic diplococcal forms or short chains. Functional autolysin is necessary for peptidoglycan remodeling and cell fission during replication, and the major pneumococcal autolysin responsible for daughter cell separation (LytB) must bind choline for enzymatic activity. Without active autolysin dividing cells remain connected by incompletely processed peptidoglycan. Pneumococcal cells growing in putrescine formed elongated chains resembling cells grown in ethanolamine or mutants which lack a functional autolysin (Fig. 4). When WU2 cells grown in putrescine were washed and inoculated into medium containing choline they reverted back to the more typical morphology of cell pairs or short chains (data not shown).

To track the subcellular location of putrescine during pneumococcal growth cells were grown in CDM containing radiolabeled substrates. Exponentially dividing bacteria were treated with muramidase and detergent to produce cell wall, membrane, and cytoplasmic fractions. Putrescine was found to be concentrated in the cell wall at levels similar to those noted for choline. Putrescine and choline concentrations in the cytoplasm and membrane fractions were at least 1000-fold lower than those in the cell wall (Fig. 5).

If putrescine can associate with teichoic acids, it is important to know if proteins which anchor to the cell surface by binding choline can use putrescine as an alternative ligand. Pneumococcal surface protein A (PspA) is a well-characterized choline-binding protein (CBP) in pneumococci and was used a surrogate for pneumococcal CBPs in WU2. Cells grown in CDM + CC or THY media expressed phosphocholine and PspA on their surface, and no soluble PspA

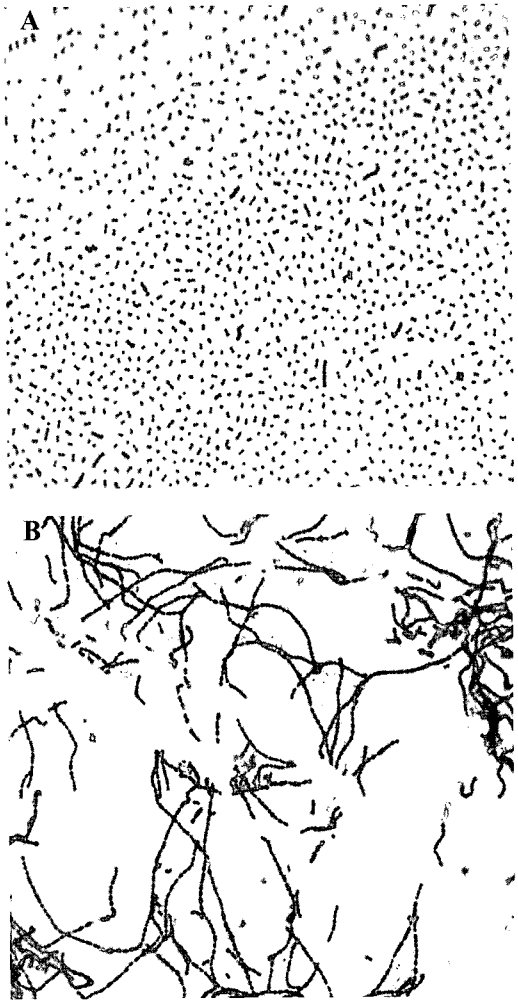

Fig. 4. Gram's stain of WU2 in CDM supplemented with choline (A) and putrescine (B). Pneumococci grown in choline appear as diplococci or short chains. Cells growing in putrescine formed elongated chains in which daughter cells were unable to completely separate.

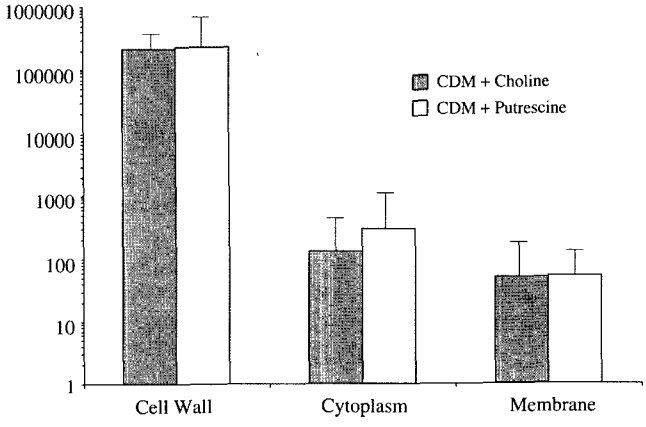

Fig. 5. Sub-cellular localization of choline or putrescine in pneumococcal strain WU2 growing in CDM+[^{3}H]-choline or CDM+[^{3}H]-putrescine. The cells were fractionated into cell walls, water-soluble cytoplasmic contents, and membranes (containing insoluble cytoplasmic contents). Results presented are geometric means of three independent experiments.

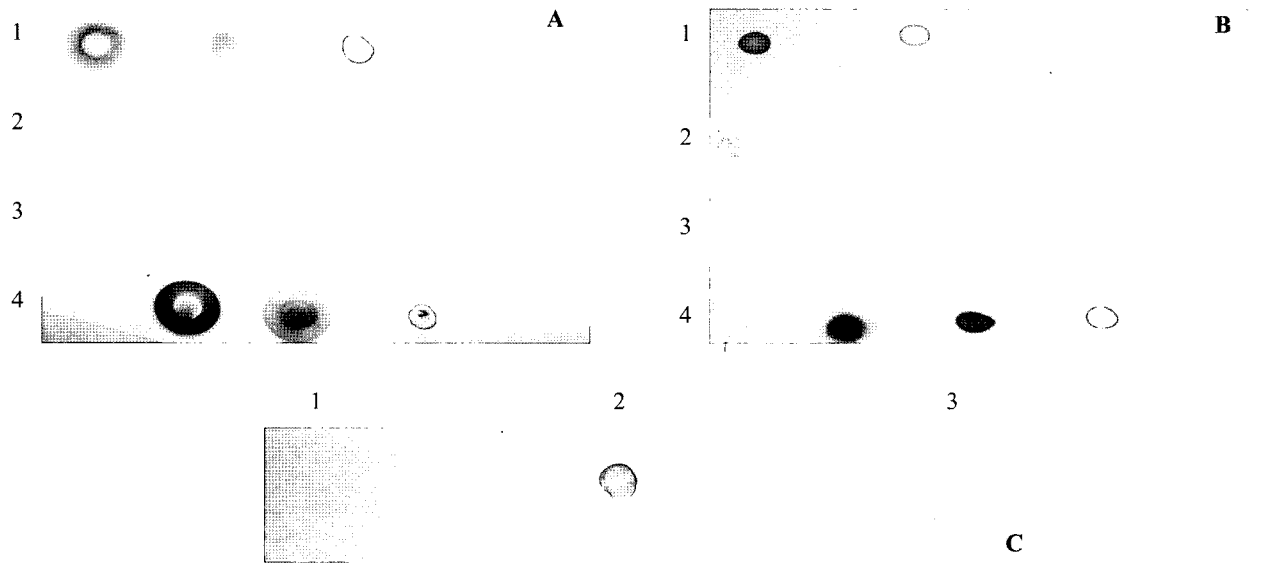

Fig. 6. Immunoblots of cells (A,B) and concentrated culture supernatant (C). Panels A and B: serial 10-fold dilution of WU2 cells growing in: 1 - CDM-CC; 2 - CDM-EA; 3 - CDM-Pt; 4 - THY. The primary antibodies used were TEPC15 (anti-phosphocholine) in panel A and Xi126 (anti-PspA) in panel B. Panel C is concentrated culture medium from WU2 growing in: 1 - CDM-CC; 2 - CDM-Pt; 3 - THY. The primary antibody used was Xi126. For panels A - C appropriate secondary antibody was conjugated to alkaline phosphatase and blots were developed as described in Materials and Methods.

could be detected free in the medium, even after concentration (Fig. 6). In contrast, cells grown in CDM-EA and CDM-PT contain no phosphocholine and no surface-associated PspA. It has been shown previously that pneumococci growing in ethanolamine released PspA and other CBPs into the culture medium (Swiatlo *et al.*, 2002). The same observation was made for cells growing in CDM-Pt, as demonstrated by the detection of soluble PspA in the culture supernatant (Fig. 6C).

Discussion

Polyamines such as putrescine are ubiquitous molecules found in all prokaryotic and eukaryotic cells. These polycations interact extensively with negatively charged nucleic acids and nucleotides and, consequently, have pleiotropic effects on transcription and translation. In pneumococci a putative ABC transporter for polyamines (Pot) has been identified from sequence analysis of a virulent type 4 strain (TIGR4) and of an unencapsulated laboratory strain (R6). Data from RT-PCR experiments now demonstrates the presence of Pot genes in the capsule type 3 strain WU2. A polycistronic mRNA from cells grown *in vitro* includes four genes which comprise the transmembrane Pot complex plus *murB*, a gene involved in peptidoglycan synthesis. This organization of the Pot operon in pneumococci is unusual and is not suggested by sequence analysis of other human bacterial pathogens whose genomes have been completed. Although there are no consensus promoter sequences immediately upstream of *potA*, an as-yet undefined promoter sequence may be functional under growth conditions not replicated in these studies, specifically, growth in a mammalian host. Cell wall synthesis and intracellular polyamines are both required at maximal levels during periods of rapid cell division, which implies that both MurB and the Pot transporter may be upregulated during periods of rapid cell growth. During growth in a choline-limited environment in the presence of polyamines cotranscription of a gene for a cell wall synthesis enzyme with a polyamine transporter may ensure that equimolar amounts of this enzyme are synthesized to maintain some basal level of peptidoglycan synthesis.

Putrescine has a molecular weight and structure similar to choline. For this reason, putrescine was studied as a potential growth substrate in a defined medium. Pneumococci were able to grow in CDM that contains putrescine, although the lag time prior to exponential cell growth is extended when compared with choline or ethanolamine. Once exponential phase growth was reached, however, the slopes of the growth curves for all substrates is similar. This observation suggests that cell doubling times are similar for all substrates during logarithmic phase growth and the difference in growth kinetics, seen in the initial lag phase, may reflect the time necessary to respond to extracellular signals with upregulation of enzymes and transporters which are expressed at low levels during growth in choline or ethanolamine. Cellular morphology of pneumococci growing in putrescine is very similar to the morphology of cells growing in ethanolamine, suggesting that LytA is not functional when pneumococci are growing in putrescine. Currently, choline is the only moiety associated with pneumococcal teichoic acids which

has been shown to be sufficient for activation of LytA enzymatic activity.

Although putrescine is chemically similar to choline and can support growth in the absence of choline, a pneumococcal mutant which can grow without any added choline or ethanolamine has been described (Yother *et al.*, 1998). The generation of this phenotype required extensive adaptation of an unencapsulated strain to decreasing concentrations of ethanolamine. In this study the capsular type 3 strain WU2 was shown to grow directly in putrescine-containing media with no period of adaptation. It is known, though, that polyamines have significant interactions with DNA and RNA and multiple spontaneous mutations or transcriptional/translational errors may be induced or stabilized by polyamines. Spontaneous mutations or mechanistic errors in transcription or translation may be responsible for the observations reported here.

The radiolabeled substrate experiments were designed to follow the cellular fate of putrescine. If putrescine is accumulated in the cytoplasm and not transported to surface structures then trace amounts of label would be seen in cell wall fractions. This was the opposite of what was observed, however, and the data here suggests that this polyamine is concentrated in the pneumococcal cell wall in a manner similar to that of ethanolamine and choline. Whether putrescine is covalently attached to galactosamine in the teichoic acids, in a manner similar to choline, is not known. Alternatively, putrescine may interact with amino acids in the peptide bridges in peptidoglycan. It has been shown that putrescine and cadaverine are covalently attached to glutamic acid residues in the peptidoglycan of Veillonella and Selenomonas species (Kamio *et al.*, 1981; Kamio, 1987). Polyamines are positively charged molecules and may be non-specifically associated with the negatively charged capsule or peptidoglycan by electrostatic interactions. The lowest concentration of putrescine was seen in membrane fractions although lipoteichoic acids, as well as teichoic acids in peptidoglycan, are also substituted with choline. One possible explanation for this may be that putrescine is a poor substrate in LTA synthesis reactions downstream from the point at which teichoic and lipoteichoic acid synthesis pathways diverge.

Putrescine associated with cell wall teichoic acids is not a ligand for the choline-binding domain of PspA. Since all pneumococcal choline-binding proteins contain repeats of a 20 amino acid domain which is necessary for choline-binding, it is likely that those CBPs which are surface-attached through choline binding are not anchored to the cell surface when pneumococci grow in a choline-depleted environment containing putrescine. Some CBPs such as PspA, CbpA/PspC, and PcpA have been associated with virulence and protective immunity, therefore, polyamine metabolism in specific *in vivo* environments may have implications for pathogenesis and host immunity.

Bacterial pathogens translocate across diverse micro-environments and pneumococci in particular have adapted mechanisms to survive and grow in anatomic locations such as the nasopharynx, bronchial epithelium, alveoli, blood, and cerebrospinal fluid. The nutrients and substrates preferentially used in one location may be limited in another. Although the requirement by pneumococci for choline, or the analagous ethanolamine, has been well-established, it is likely that these compounds occur in low concentrations under some circumstances *in vivo*. It has recently been shown that pneumococci upregulate a gene involved in choline attachment to lipoteichoic acid in response to choline starvation (Desai *et al.*, 2003). This finding implies that pneumococci encounter micro-environments with limited choline within the human host. Certainly, upregulated transcription of genes involved in choline metabolism is one response to this condition. Alternatively, or additionally, utilization of choline analogs may have an important role during the colonization or infectious process when choline is limited in the environment. The data presented here suggests that the polyamine putrescine can be considered a choline analog which supports pneumococcal growth *in vitro*, in a manner similar to that of ethanolamine. The role of choline analogs during pneumococcal replication in a host has not been studied but deserves further attention. The transport and metabolism of these compounds may provide novel insights into the pathogenesis of pneumococcal infections.

Acknowledgments

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