Cellular location of polyamine transport protein PotD in *Streptococcus pneumoniae*

Pratik Shah[1], Mary Marquart[1], Lisa R. Quin[1] & Edwin Swiatlo[1,2,3]

[1]Department of Microbiology, University of Mississippi Medical Center, Jackson, MS, USA; [2]Department of Medicine, University of Mississippi Medical Center, Jackson, MS, USA; and [3]Research Service, Veterans Affairs Medical Center, Jackson, MS, USA

Correspondence: Edwin Swiatlo, Research Service (151), Veterans Affairs Medical Center, 1500 Woodrow Wilson Drive, Jackson, MS 39216, USA. Tel.: +601 364 1315; fax: +601 364 1390; e-mail: swed@sprintmail.com

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Abstract

Streptococcus pneumoniae encodes a transporter for polyamines that contributes to virulence in an animal model. The putative polyamine-binding protein, PotD, has an amino-terminal secretory peptide but no other domains known to be involved in anchoring proteins to the surface of Gram-positive bacteria. Cell fractionation and immunoblotting, along with flow cytometry, suggest that PotD is surface-exposed and anchored to the cytoplasmic membrane by a potentially novel mechanism.

Introduction

Polyamines are ubiquitous compounds found in all eukaryotic and prokaryotic cells. These small amine-containing molecules have pleiotropic effects on nucleic acid functions, including DNA and RNA synthesis, as well as mRNA translation (Tabor & Tabor, 1985; Antognoni *et al*., 1999). The human pathogen *Streptococcus pneumoniae* contains a four-gene operon that encodes a putative ABC transporter for polyamines with a high degree of sequence homology to a polyamine transporter in *Escherichia coli* (Ware *et al*., 2005). The polyamine transport protein D (PotD) in pneumococcus contributes to virulence in a murine sepsis model and may be a potential target for antimicrobial therapy or a vaccine candidate (Ware *et al*., 2006). Pneumococcal PotD protein possesses a characteristic Gram-positive signal peptide (Ware *et al*., 2005), suggesting its processing by the general secretory pathway and subsequent extracellular location. However, this protein does not contain any obvious amino acid motifs that would suggest its method of attachment to the cell surface, such as choline-binding domains, LPXTG sequences, or lipoprotein processing sequences (Navarre & Schneewind, 1999). The ultimate location of PotD cannot be readily predicted from amino acid analysis, a characteristic noted for certain subsets of Gram-positive surface proteins (Chhatwal, 2002; Ling *et al*., 2004). This study was designed to examine the surface of intact pneumococcal cells for PotD using antibody binding and to ascertain the subcellular location of this protein.

Materials and methods

The gene for PotD from capsule type 4 strain TIGR4 (Sp1386; http://www.tigr.org) was amplified with *Pfu* polymerase PCR using primers that exclude the coding region for the 31 amino acid peptide at the N-terminus (*potD* forward: 5′-CACCATGTTAGATAGTAAAATCAAT-3′; *potD* reverse: 5′-CTTCCGATACATTTTAAACTGTA-3′). The amplified product was cloned into pET101/D-TOPO, which expresses a 6 × His tag at the C-terminus of the recombinant protein. Recombinant PotD was expressed in *E. coli* host strain BL21Star and purified by affinity chromatography with Ni-DEAE columns according to the manufacturer's instructions (Invitrogen, Carlsbad, CA)

High-titer polyclonal antiserum against recombinant pneumococcal PotD was raised by subcutaneous immunization of 8–10-week-old New Zealand White rabbits at bi-weekly intervals for a total of three immunizations. The antiserum, at 1 : 3000 dilution, recognized both recombinant PotD (which does not contain the 31 amino acid N-terminal leader peptide) and whole-cell lysates of

pneumococcal type 3 strain WU2 by immunoblotting. A single band of 41 kDa for WU2 lysates and 38 kDa for recombinant PotD expressed in *E. coli* were observed (data not shown).

Flow-cytometric analysis was carried out to determine whether PotD is exposed on the surface of intact pneumococcal cells. WU2 and Rx1 cells were grown to $OD_{600\,nm}$ 0.5 in Todd–Hewitt broth containing 0.5% yeast extract (Difco Laboratories, Detroit, MI) and serial dilution plate counts were performed on sheep blood agar plates. Approximately 10^5 CFU were collected by centrifugation, washed twice in phosphate-buffered saline (PBS; pH 7.2), and incubated with 100 μL of PotD rabbit antiserum (1 : 50 dilution in PBS) for 30 min at 37 °C. Bacteria were then washed with PBS and incubated with 30 μg mL^{-1} of biotinylated goat antirabbit IgG antibody (Southern Biotechnology Associates, Birmingham, AL) for 30 min at 37 °C. Cells were washed with PBS and suspended in 1 μg mL^{-1} streptavidin conjugated to AlexaFluor 488 (Molecular Probes) for 30 min at room temperature in the dark. After a final wash in PBS, bacteria were suspended in 2 mL of PBS and analyzed with a FACScan cytometer (Beckton Dickinson). A polyclonal rabbit anti-PspC serum (1 : 100 dilution with PBS) was used as a positive control and cells incubated only with the biotinylated goat antirabbit IgG were used as a negative control.

Pneumococcal cell fractionation was carried out with an equal number of cells of capsular type 3 strain WU2 and the unencapsulated strain Rx1 as described (Vijayakumar & Morrison, 1986). Protein concentrations for the fractions were determined using the Bradford reagent (Pierce, Rockford, IL). The fractions were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblot analysis was performed using 1 : 3000 dilution of the rabbit polyclonal antiserum against PotD using standard protocols (Lefkovits, 1997).

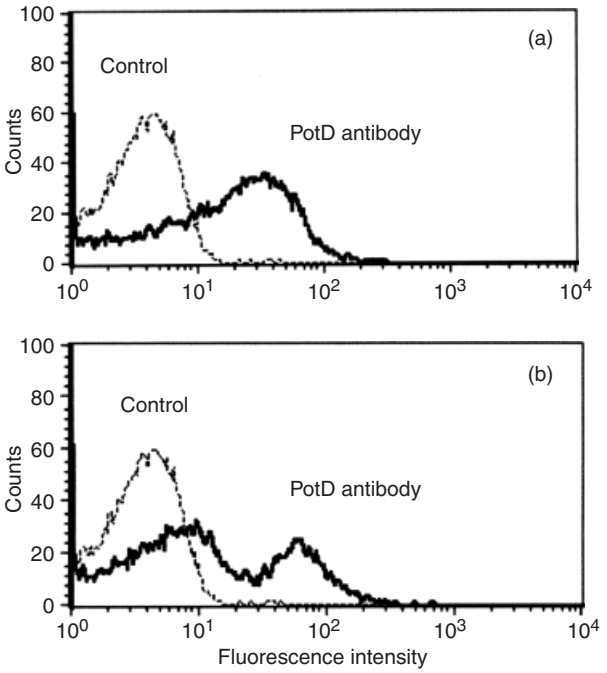

Fig. 1. Flow cytometric measurement of binding of PotD antibodies to the pneumococcal surface. (a) unencapsulated strain Rx1; (b) capsule type 3 strain WU2. Each graph is a representative result of three independent experiments.

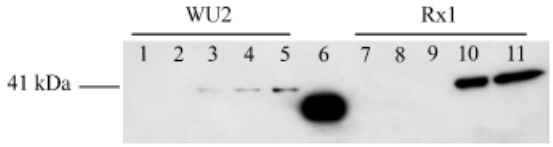

Fig. 2. Immunoblot analysis with subcellular fractions of pneumococcal strains WU2 and Rx1. Lanes: 1, 7, secreted proteins; 2, 8, noncovalently attached surface proteins; 3, 9, cell wall-associated proteins; 4, 10, soluble cytoplasmic contents; 5, 11, membranes, insoluble cytoplasmic contents; 6, recombinant PotD (control).

Results and discussion

Pneumococcal cells bound the PotD antibody when examined by flow cytometry with secondary antibody conjugated to biotin and subsequently incubated with streptavidin-conjugated fluorescent dye (Fig. 1). The mean fluorescence intensity for unencapsulated strain Rx1 was 49 ± 3.1 (mean ± standard error for three independent experiments) compared with a mean of 4 for Rx1 cells incubated with secondary antibody alone (negative control) (Fig. 1a). Capsule type 3 strain WU2 also bound PotD antibody with a mean fluorescence intensity of 60 ± 5.3 (Fig. 1b). WU2 cells from the same culture appear to consist of two populations when examined for Anti-PotD binding by flow cytometry (Fig. 1b). The reason for this remains speculative, but the dichotomy may arise from varying amounts of capsule expressed within a culture growing in liquid medium and affecting access of antibodies to surface-bound PotD.

The subcellular location of PotD was examined by fractionating logarithmically dividing cells and testing the fractions for their reactivity with PotD antibodies (Fig. 2). For both strains Rx1 and WU2, the reactivity was noted primarily in the membrane and cytoplasmic fractions. Finding surface proteins such as PotD in the cytoplasm of rapidly replicating cells is not unexpected, as cells at this stage of growth have high rates of protein synthesis and all proteins, to a greater or lesser degree, will be detected in the cellular compartment where protein synthesis is occurring. In strain WU2, a trace amount of PotD is detected in the cell wall fraction (Fig. 2, lane 3). This may represent a small amount of free PotD that copurifies with the cell wall or, alternatively, small amounts of contaminating membrane

that are bound to peptidoglycan. Lysates of pneumococcal strains representing capsule types 2, 3, 4, 6A, 9, 14, and 23 were immunoblotted with rabbit antiserum and a single protein band of 41 kDa was seen for all strains (data not shown). This suggests that PotD is expressed by diverse capsule types and is relatively antigenically conserved. Further large-scale surveys will need to be performed to support the use of PotD as a vaccine component.

Extracellular proteins of Gram-positive bacteria such as the pneumococcus can be broadly categorized as lipoproteins anchored in the lipid bilayer of the cytoplasmic membrane, choline-binding proteins electrostatically attached to choline residues of the (lipo)teichoic acids, sortase-processed proteins covalently bound to peptidoglycan of the cell wall, and unattached proteins secreted into the extracellular medium (Fischetti *et al*., 1990; Rosenow *et al*., 1997; Navarre & Schneewind, 1999; Swiatlo *et al*., 2002; Kharat & Tomasz, 2003; Ridgen *et al*., 2003). The putative polyamine-binding protein of pneumococcus, PotD, has a typical N-terminal leader sequence but contains no consensus motif that suggests its attachment to the cell surface. This would be an unusual organizational structure for a component of an ABC transport system, where most ligand-binding proteins are anchored to the cell in proximity to their cognate transmembrane channels (Nikaido & Hall, 1998; Schneider & Hunke, 1998).

In this study, it has been shown that pneumococcal PotD is accessible to antibodies at the surface of intact bacteria, in both unencapsulated cells and those expressing the highly hydrated and mucoid capsule type 3. PotD is found primarily in association with cytoplasmic membranes, which suggests that it is a lipoprotein. PotD does not contain an LXXC amino acid motif, which is the most common site of attachment for fatty acids in bacteria, primarily palmitic acid. The lipidation signal of PotD remains undefined, but may potentially reveal a novel mechanism for synthesis of lipoproteins in bacteria. Pneumococcal PotD is presently being studied for its ability to induce protective antibody responses in a mouse model of bacteremia and pneumonia. Because PotD is a surface-exposed protein, it offers potential as a protective immunogen and may be part of an improved, next-generation vaccine.

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