Identification of the Key Protein for Zinc Uptake in Hemophilus influenzae*

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Very little is known about specific mechanisms for zinc accumulation and transport in bacteria. In this study a putative adhesin B in Hemophilus influenzae, the product of gene HI0119, has been identified as a periplasmic zinc-binding protein (PZP1). A pzp1-deficient mutant has been constructed which is defective for growth under aerobic conditions and grows poorly under anaerobic conditions. The growth defect is specifically rescued by supplementing the growth medium with high concentrations of zinc. Subcellular fractionation was used to localize PZP1 to the periplasmic region in a nontypeable H. influenzae strain and in a transfected recombinant Escherichia coli strain (TApzp1). Recombinant PZP1, purified from a periplasmic extract of E. coli strain TApzp1, contained two zinc atoms/protein molecule as determined by neutron activation analysis and atomic absorption spectroscopy. The zinc atoms could be removed by incubation with EDTA, and, by further addition of zinc, a total of five zinc atoms/PZP1 could be bound. Direct binding of $^{65}$Zn to the recombinant protein by Western blot was demonstrated. Taken together, these results provide direct evidence that PZP1 plays a key role in zinc uptake by H. influenzae.

Zinc is essential for all organisms because it plays a critical role in the catalytic activity and/or structural stability of many proteins. More than 300 zinc-dependent enzymes have been identified (1). Several important motifs commonly found in transcriptional regulatory proteins are stabilized by zinc, including the zinc finger, zinc cluster, RING finger, and LIM domain (2). Despite this importance, very little is known about the mechanisms and regulation of zinc transport in bacteria (3). The few studies reported suggest that bacteria appear to possess a specific energy-dependent zinc transport system (4–7). However, studies concerned with the intracellular accumulation of the metal have been confounded by both the nonspecific binding of zinc to the bacterial surface and the rapid exchange of cellular zinc with zinc in the medium (7–9). For unknown reasons, the zinc requirement for bacteria seems to be much lower than that for fungi or other eukaryotic cells (8, 9). The exceedingly small requirements for zinc have frustrated studies in this field. So far, a specific zinc transport mechanism has not been properly demonstrated, and thus there has been no means to study the regulation of the transport of this metal in prokaryotes (3).

Hemophilus influenzae is a commensal of the human upper respiratory tract and can cause both localized and invasive infections in humans (10, 11). Recently, we reported the partial characterization of HI0119, identified from the H. influenzae genomic sequence (13) as a putative adhesin B because of its homology with the adhesin fimA of Streptococcus parasanguis. However, this 37-kDa protein is distinct from fimA because of a central histidine-rich domain, potent celite binding ability, and a COOH-terminal disulfide-bonded domain. Expression of HI0119 is highly conserved in all H. influenzae clinical strains tested. In this study, we demonstrate that this putative adhesin B is in fact, a periplasmic zinc-binding protein (PZP1) which plays a key role in the zinc uptake of H. influenzae. This is the first description of a potential prokaryotic zinc-specific transport protein.

MATERIALS AND METHODS

Clinical strains of H. influenzae were kindly provided by the Department of Microbiology, Hospital for Sick Children. Pfu and Tag polymerase were purchased from Stratagene and Pharmacia Biotech Inc., respectively. Restriction enzymes and buffers used for DNA manipulation were purchased from Pharmacia. DNA and protein standards were purchased from Life Technologies, Inc. and Bio-Rad, respectively. TA cloning kit, pTrc plasmids, and Escherichia coli strains Top10 and INVa were purchased from Invitrogen. SpectroPor dialysis tubing (MWCO 12,000–14,000 kDa) was from Fisher. The tubing was washed extensively with double distilled H$_2$O before use. Goat anti-rabbit horseradish peroxidase conjugate was from Bio-Rad. $^{65}$ZnCl$_2$ was purchased from Amersham. Restriction analysis and plasmid constructions were performed using standard techniques as outlined by Sambrook et al. (14). Amino acid analysis was performed by the Biotechnology Service Center at the University of Toronto.

Bacterial Cultures

Nontypeable H. influenzae strain NTHI6564 was obtained immediately following isolation and stocks stored at −70 °C in glycerol citrate. The strain was plated from frozen stocks onto chocolate agar plates and grown overnight at 37 °C under aerobic conditions or anaerobic conditions using a BBL GasPak Plus generator with catalyst (Baxter HealthCare Corporation, Medford, MA). Each strain was subcultured twice before assay. E. coli was grown in Luria-Bertani or BHI broth or agar, aerobically at 37 °C and supplemented with 100 μg/ml ampicillin where appropriate.

Subcellular Fractions of H. influenzae

Triton X-114 Extraction of Integral Membrane Proteins—Integral membrane proteins of NTHI6564 were extracted with Triton X-114 essentially as described by Swanecutt et al. (15). Briefly, bacterial cultures were grown to an A$_{600}$ of 0.5, harvested by centrifugation at 4 °C, and washed using 1 volume of 200 mM Tris-HCl (pH 8.0). The pellets were suspended in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2% Triton X-114, and 1 mM PMSF. A 1:10 dilution of the suspension to form a 5% Triton X-114 solution was used for all further experiments.


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Triton X-114. After incubation for 4 h at 4 °C, cellular debris was removed by centrifugation. The supernatants were warmed to 37 °C to allow phase separation. After centrifugation at 14,000 × g for 10 min, the aqueous phase was separated from the detergent phase. The detergent phases were washed three times using 20 mM Tris-HCl (pH 8.0), 10 mM EDTA.

**Osmotic Shock Extraction of Periplasmic Proteins**—Proteins in the periplasmic space were obtained by the osmotic shock procedure described by Ames (16). Briefly, bacterial cultures were grown at 37 °C overnight and harvested by centrifugation. The bacterial pellet was gently resuspended in 30 mM Tris-HCl (pH 8.0), 20% sucrose. EDTA was added to a final concentration of 1 mM, and the bacteria were incubated at room temperature for 5–10 min with shaking. The cells were recovered by centrifugation and then resuspended in ice-cold 5 mM MgSO4 with shaking for 10 min at 4 °C. The supernatant, containing periplasmic proteins, was collected as osmotic shock fluid.

**Expression and Purification of PZP1 in E. coli**

PCR amplification of the pzp1 gene and its upstream 500-base pair region was performed as described previously. The primers used in amplification were PZU500, 5'-GACTACGTCATTGGGATGTTGGTCTCAACAG-3' and HIMA2, 5'-GAATTCATATGCTAAACATTCCATGTAGC-3'. The 6-kb amplified product was ligated into a TA cloning vector, pCRII. The resulting plasmid was designated as pTApzp. The orientation of the insert was determined by restriction enzyme digestion; two clones, containing either orientation, were checked to assess whether the native pzp1 promoter has function in E. coli.

The E. coli transformant containing the plasmid pTApzp was grown overnight in 1 liter of BHI medium with ampicillin. The cells were harvested by centrifugation, and the periplasmic proteins were isolated as described above. The concentrated periplasmic extract was dialyzed overnight against 25 mM imidazole-HCl buffer (pH 7.4) and was applied to a column of Polybuffer exchanger 94 (Pharmacia) equilibrated with the same buffer. Elution was carried out with a degassed solution of Polybuffer 74 (Pharmacia) diluted 1:8 with distilled water and adjusted to pH 4.0 with HCl. Fractions were monitored for absorbance at 280 nm, and for pH, and the PZP1-positive fractions (identified by Western blotting) were pooled and lyophilized. To remove ampholytes, the lyophilized material was dissolved in 1 ml of water and applied to a G-75 fine column (1.5 × 80 cm) equilibrated with 50 mM Tris-HCl (pH 7.2). The column was run at 0.5 ml/min, and fractions were monitored at 254 nm.

A standardized stock solution of PZP1 of known concentration was prepared as follows. Purified protein was dialyzed extensively against water, and the protein concentration was determined by amino acid analysis in triplicate. The absorbance at 280 nm was measured for dilutions of stock solution prepared in 20 mM Tris-HCl (pH 7.1), 20 mM NaCl, and from these values the extinction coefficient of PZP1 was calculated to be 36,800 liters mol⁻¹ cm⁻¹.

Metal Composition of Purified PZP1

**Neutron Activation Analysis**—Purified PZP1 with a concentration of 0.56 mg/ml was heat sealed in a clean polycrylyene vial. This was irradiated for 5 min at a neutron flux of 1.0 × 10¹² n cm⁻² s⁻¹ in the SLOWPOKE reactor of the University of Toronto. The irradiated solution was transferred to a clean vial, and after a delay time of 2.5 h to the SLOWPOKE reactor of the University of Toronto. The irradiated solution was transferred to a clean vial, and after a delay time of 2.5 h, the sample was added to a final concentration of 1 mM, and the bacteria were incubated at room temperature for 5–10 min with shaking. The cells were recovered by centrifugation and then resuspended in ice-cold 5 mM MgSO4 with shaking for 10 min at 4 °C. The supernatant, containing periplasmic proteins, was collected as osmotic shock fluid.

**Circular Dichroism Measurements**

CD spectra were recorded between 190 and 250 nm on a Jasco 720A spectropolarimeter using a 1-mm quartz cell at 25 °C. The concentration of PZP1 was 0.56 mg/ml in 20 mM Tris-HCl (pH 7.1), 20 mM NaCl.

**Electrophoresis and Western Blotting**

SDS-PAGE and Western blotting were carried out by the methods of Laemmli (18) and Towbin et al. (19), respectively. For Western blots, a rabbit polyclonal antiserum (1/1,000 dilution) raised against a 6× histidine-tagged PZP1 fusion protein (His-PZP1) was used.

**Construction of the pzp1 Isogenic Mutant**

A 3.5-kb DNA fragment from the NTHI6564 chromosome, including the pzp1 gene and its 1.2-kb upstream and 1.3-kb downstream region, was amplified by PCR as described previously. The primers used for PCR were HZU5, 5'-CGGATCTCTTCTTGAACATGGCTCTCATGCT-3' and HZD3, 5'-GAATTCATATGCTAAACATTCCATGTAGC-3'. The amplified product was cloned into vector pCRII, generating plasmid pTA3.5. The 2.5-kb BamHI-EcoRI fragment from pTA3.5 was subcloned into vector pTrc99A. The resulting plasmid, pTrc2.5, was digested with PstI to remove an 822-base pair internal region of the pzp1 gene and ligated to a 1.2-kb kanamycin-resistant cassette from pUC4K. The resulting plasmid was designated as Ypzp::kan.

The plasmid Ypzp::kan was digested with BamHI and EcoRI, and this digestion mixture was used to transform NTHI6564 cells made competent for transformation with M-IV medium as described previously. The transformants were selected on chocolate agar plates containing 20 µg of kanamycin/ml under both aerobic and anaerobic conditions.

Transfomant transformant colonies were screened by direct amplification of chromosomal DNA from single colonies by PCR. Oligonucleotide primers used in the screening PCR were primers P1, 5'-GAATTCATCAGTAACACC-3', and P2, 5'-TATTTAGCTAAACATTCCATGTAGC-3'.

**Localization of PZP1 in H. influenzae**—We have shown previously that the NH₂-terminal 24-amino acid sequence of PZP1 forms a signal peptide, which suggests that this protein is extracytoplasmic. Because PZP1 was originally purified from a surface protein (water) extract from an NTHI strain, immunofluorescence was used to determine the possible surface localization of this protein. This study revealed that this protein is not on the H. influenzae cell surface (data not shown). Furthermore, Western blotting showed that after overnight growth of H. influenzae in liquid culture, PZP1 is cell-associated and is not secreted into the culture medium (data not shown). Other members of the fimA family possess an NH₂-terminal lipid linkage consensus sequence LXXC (21). Although PZP1 contains no such sequence and does not contain obvious membrane-spanning hydrophobic regions, the possibility of membrane anchorage was investigated.

A characteristic of integral membrane proteins, including outer membrane proteins from Gram-negative bacteria, is their selective partitioning into detergent phases. Therefore, detergent-resistant membranes were added to a final concentration of 1 mM, and the bacteria were incubated in the presence of 1 mM Zn(NO₃)₂ or 5 mM EDTA at 4 °C overnight. The samples were then dialyzed extensively against 20 mM Tris-HCl (pH 7.1), 20 mM NaCl, and from these values the extinction coefficient of PZP1 was calculated to be 36,800 liters mol⁻¹ cm⁻¹.

**RESULTS**

**Localization of PZP1 in H. influenzae**—We have shown previously that the NH₂-terminal 24-amino acid sequence of PZP1 forms a signal peptide, which suggests that this protein is extracytoplasmic. Because PZP1 was originally purified from a surface protein (water) extract from an NTHI strain, immunofluorescence was used to determine the possible surface localization of this protein. This study revealed that this protein is not on the H. influenzae cell surface (data not shown). Furthermore, Western blotting showed that after overnight growth of H. influenzae in liquid culture, PZP1 is cell-associated and is not secreted into the culture medium (data not shown). Other members of the fimA family possess an NH₂-terminal lipid linkage consensus sequence LXXC (21). Although PZP1 contains no such sequence and does not contain obvious membrane-spanning hydrophobic regions, the possibility of membrane anchorage was investigated.

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**Atomic Absorption Analysis**—Aliquots of purified PZP1 were incubated in the presence of 1 mM Zn(NO₃)₂ or 5 mM EDTA at 4 °C overnight. The samples were then dialyzed extensively against 20 mM Tris-HCl (pH 7.1), 20 mM NaCl at 4 °C for 4 days. Zinc content was calculated to be 36,800 liters mol⁻¹ cm⁻¹.
that this protein was localized in the periplasmic space in *H. influenzae*.

**Expression and Purification of PZP1 in E. coli**—We amplified the *pzp1* gene and its upstream 590-base pair region from NTHI6564 chromosomal DNA and cloned this 1.6-kb fragment into the TA cloning vector pCRII to generate plasmids pTApzp1 and pTApzp2. In pTApzp1, the *pzp1* gene is in the same orientation as the *lac* promoter but is inverted with respect to this promoter in plasmid pTApzp2. Recombinant *E. coli* strains harboring either plasmid were found to overexpress this 37-kDa protein at a similar level, suggesting that transcription of this protein is initiated at a *H. influenzae* promoter that is well recognized by *E. coli* RNA polymerase. Recombinant PZP1 was found to localize in the periplasmic space of *E. coli* as it did in *H. influenzae* and constitutes about 35% of the total protein in the periplasmic shock fluid. Recombinant protein was purified from periplasmic extracts by chromatofocusing (Fig. 2A) and gel filtration chromatography. PZP1 thus obtained has been purified to >98% homogeneity as determined by SDS-PAGE with a yield of approximately 7 mg of pure protein from 1 liter of bacterial culture. Recombinant PZP1 was recognized by antisera against fusion protein His-PZP1 on Western blots (Fig. 2B). The observed isoelectric point of this protein, as estimated by chromatofocusing, was 6.4, which is close to the theoretical isoelectric point (pH 6.7) predicted from the amino acid sequence.

Like the native protein, recombinant PZP1 contains a disulfide bond as determined by differential migration by SDS-PAGE under reducing and nonreducing conditions and has potent celite binding capability, which is similar to the native PZP1 (data not shown). Amino acid analysis of purified recombinant PZP1 gave the expected amino acid composition for the mature, processed form of the protein (results not shown).

**PZP1 Is a Zinc-binding Protein**—Our previous data have suggested that PZP1 may be capable of binding metal(s). To

![Fig. 1. Localization of PZP1 in NTHI6564. Triton X-114 extract, periplasmic and whole cell extracts of NTHI6564 were suspended in sample buffer and boiled for 3 min under reducing conditions before loading. Panel A, SDS-PAGE analysis, stained with Coomassie Blue. Panel B, Western blotting with antisera against fusion protein His-PZP1. Lane 1, Triton X-114-insoluble protein of NTHI6564. Lane 2, Triton X-114 detergent fraction. Lane 3, Triton X-114 aqueous fraction. Lane 4, periplasmic proteins of NTHI6564. Lane 5, whole cell extract of NTHI6564. Lanes 1–3 contain extracts from 3 × 10⁸ organisms; lanes 4 and 5, extracts from 10⁶ organisms.

![Fig. 2. Expression and purification of recombinant PZP1. PZP1 was purified from a periplasmic extract of TApzp1 using chromatofocusing and identified by Western blotting with antisera against His-PZP1 or ⁶⁵Zn binding. Panel A, chromatofocusing elution profile showing A₂₈₀ nm (●) and pH (○) of each fraction. The PZP1 peak (identified by Western blotting) is indicated with an arrow. Panels B–D, the periplasmic extracts from *E. coli* strains which contained plasmid pTApzp1 (lane 1) or pCRII (lane 2) and purified PZP1 (lane 3) were analyzed by 12% SDS-PAGE followed by Coomassie Blue staining (panel B), Western blotting with anti-His-PZP1 (panel C), or with ⁶⁵Zn followed by autoradiography (panel D). In ⁶⁵Zn blotting, the filter was equilibrated for 2 h in metal binding buffer (100 mM Tris-HCl (pH 8.0), 50 mM NaCl) and probed with ⁶⁵ZnCl₂ (20 µCi/10 ml) for 1 h. After washing, the filter was exposed at −80 °C to Kodak X-Omat film.**
determine the nature and amount of metals bound to PZP1, the purified protein was analyzed by neutron activation. Neutron activation analysis revealed that PZP1 contained about two zinc atoms/protein molecule but did not contain measurable levels of chromium, nickel, scandium, iron, cobalt, or manganese. The zinc content of purified PZP1 was also confirmed by atomic absorption spectroscopy (Table I).

To assess the zinc binding capacity of PZP1, the purified protein was incubated in the presence of 5 mM EDTA or 1 mM Zn(NO₃)₂ at 4 °C overnight. After exhaustive dialysis, zinc content was determined by atomic absorption spectroscopy. Untreated PZP1 contained 1.58 zinc atoms/protein molecule, whereas EDTA treatment reduced bound zinc to an undetectable level. On incubation with zinc, a total of five zinc atoms/protein molecule could be bound (Table I). In addition, ⁶⁵Zn binding to PZP1 was demonstrated using a ⁶⁵Zn blotting technique (Fig. 2D).

Construction of the pzp1 Negative Mutant—To construct a mutant deficient in the expression of PZP1, we amplified a 3.5-kb DNA fragment from NTHI6564 chromosomal DNA by PCR, including a 1.2-kb upstream and 1.3-kb downstream region of the pzp1 gene and cloned this DNA fragment into vector pCRII. The resulting plasmid was designated as Ypzp1::kan. Restriction endonuclease analysis of Ypzp1::kan confirmed that the kanamycin cassette had been inserted into the expected site of the pzp1 gene. The deleted region was replaced by a 1.2-kb kanamycin resistance (kan*) gene. Panel B, PCR analysis of chromosomal DNA from NTHI6564 wild type (wt) and the pzp1 mutant.

![Fig. 3. Construction of the pzp1-deficient mutant. Panel A, schematic of the H. influenzae chromosomal region encompassing the pzp1 gene. Open reading frames are indicated by boxes. The gene chlN encodes a putative molybdopterin biosynthesis protein. The arrows indicate the direction of transcription. The localization of oligonucleotide primers (P1 and P2) used in screening reactions is shown above. The deleted region was replaced by a 1.2-kb kanamycin resistance (kan*) gene. Panel B, PCR analysis of chromosomal DNA from NTHI6564 wild type (wt) and the pzp1 mutant.](image)

![Fig. 4. SDS-PAGE and Western blotting of wild type (wt) and the pzp1 mutants. The whole cell extracts from NTHI6564 (lane 1) and the two separate pzp1 mutants (c1 and c2) (lanes 2 and 3) were suspended in sample buffer and boiled for 3 min under reducing conditions before loading the gel. Panel A, 12% SDS-PAGE. Panel B, Western blotting with antisera against fusion protein His-PZP1.](image)
mutant under both aerobic and anaerobic conditions was rescued by the addition of zinc (Fig. 5), suggesting that the pzp1 mutant is defective in zinc uptake. To assess the substrate specificity of suppression, 100 μM CaCl₂, MgCl₂, CuCl₂, NiCl₂, Cd(NO₃)₂, MnCl₂, FeCl₃, ZnCl₂, or Zn(NO₃)₂ was added into 20% Levinthal broth. Only ZnCl₂ and Zn(NO₃)₂ rescued the growth defect of the pzp1 mutant under aerobic conditions (Fig. 6A), indicating that suppression of the growth defect is zinc-specific.

The growth characteristics of the pzp1 mutant at different zinc concentrations under aerobic conditions were compared with that of the wild type parent strain (Fig. 6B). In the presence of 5 μM ZnCl₂, the growth defect of the mutant cannot be rescued. At 100 μM ZnCl₂, the growth rate of the mutant was similar to that of the wild type strain, although the cell density at stationary phase was lower than that of the wild type strain (Fig. 6B).

**DISCUSSION**

Despite the rapidly increasing knowledge of zinc function at molecular and cellular levels, a zinc-specific transporter system in bacteria has not been properly demonstrated (3). There are several reasons for this discrepancy. First, extremely low zinc concentrations (0.5–1 μM) are required for optimal bacterial growth. Second, elimination of zinc from medium using solvents or alumina has generally been unsuccessful (8). Finally, the high electrostatic affinity of zinc for anionic sites on the microbial surface is not readily distinguishable from zinc trans-vents or alumina has generally been unsuccessful (8). Finally, the high electrostatic affinity of zinc for anionic sites on the microbial surface is not readily distinguishable from zinc trans-vents or alumina has generally been unsuccessful (8). Finally, the high electrostatic affinity of zinc for anionic sites on the microbial surface is not readily distinguishable from zinc trans-vents or alumina has generally been unsuccessful (8). Finally, the high electrostatic affinity of zinc for anionic sites on the microbial surface is not readily distinguishable from zinc trans-vents or alumina has generally been unsuccessful (8). Finally, the high electrostatic affinity of zinc for anionic sites on the microbial surface is not readily distinguishable from zinc trans-vents or alumina has generally been unsuccessful (8). Finally, the high electrostatic affinity of zinc for anionic sites on the microbial surface is not readily distinguishable from zinc trans-vents or alumina has generally been unsuccessful (8). Finally, the high electrostatic affinity of zinc for anionic sites on the microbial surface is not readily distinguishable from zinc trans.

The ATP-binding cassette system is involved in the transport of a diverse array of macromolecules across the cytoplasmic membranes of bacteria and eukaryotes (23). This system consists of three basic parts: one or two ATPases, one or two integral membrane proteins, and one substrate-specific binding protein. In Gram-negative bacteria the binding protein is soluble and periplasmic, but in Gram-positive bacteria the binding protein is lipid linked to the cytoplasmic membrane. Usually these three components are encoded together in one operon in bacteria (24, 25). PZP1 of *H. influenzae*, a periplasmic zinc-binding protein, is 23.7% identical and 47.8% similar to fimA of *S. parasanguis*. The *S. parasanguis* fimA is a lipoprotein that is involved in adherence of these bacteria to the salivary pellicle of dental surfaces (26–28). DNA sequence data showed that the *S. parasanguis* fimA locus encodes an ATP-binding membrane transport system (29). Interestingly, a fimA isogenic mutant did not display an obvious growth defect in vitro but was found to be less virulent in animal models (28). Our previous studies have suggested that there is functional heterogeneity between PZP1 of *H. influenzae* and fimA of *S. parasanguis*. We speculate that fimA of *S. parasanguis* may be involved in an ATP transport system similar to that proposed for PZP1 but have different substrate binding specificity. Furthermore, a recent BLAST search showed that PZP1 has 49.2% identity and 59.4% similarity to an unidentified protein (YebL) in *E. coli*, a 31.1-kDa protein in the mshB-rvB intergenic region precursor. Compared with the sequence of PZP1, YebL in *E. coli* seems to have a similar domain structure including a central potential metal binding
domain of 21 amino acid residues and two conserved cysteines in the COOH-terminal region which may form a disulfide bond. The YeaL locus also appears to be organized in an operon. YeaL of E. coli may thus have a function similar to that of PZP1 of H. influenzae, which is involved in the transport of zinc.

In general, there is little sequence conservation between the binding proteins for different substrates among ATP-binding protein cassette transport systems (23). However, the pairs of periplasmic binding proteins that interact with a common membrane receptor have extensive homology (23). In the H. influenzae genome, another putative adhesin B (HI0362) has 21% identity to PZP1, and the gene HI0362 locus seems to have a genetic organization similar to that of typical ATP-binding protein cassette systems (13). Even though the pzp1 locus does not appear to be part of an operon as is usually found for comparable transporters, it is possible that PZP1 and the product of the HI0362 gene may interact with the same core transmembrane complex.

Our results demonstrate that the pzp1 mutant cannot grow under aerobic conditions and grows poorly under anaerobic conditions. Only zinc can suppress the growth defects of the pzp1 mutant. This suggests that the metabolic process under aerobic conditions may be more dependent on zinc than that under anaerobic conditions in H. influenzae. Alternatively, there may be an additional, lower affinity zinc transport system operating when H. influenzae grows under anaerobic conditions. Western blotting has shown that the expression level of PZP1 was not found to be decreased during anaerobic growth (results not shown), supporting the former explanation.

PZP1 of H. influenzae contains an unusual histidine-rich domain of about 47 amino acids. This domain is extremely rich in potentially metal-binding amino acids, including 23 histidines, 10 aspartic acids, and 6 glutamic acids. We expect that PZP1 was not found to be decreased during anaerobic growth (results not shown), supporting the former explanation.

Recent studies showed that zinc uptake in yeast Saccharomyces cerevisiae is transporter-mediated by at least two systems, one with high affinity and second with lower affinity. The transporters that are responsible for both uptake systems have been identified because of their significant similarity to IRT1, an Fe(II) transporter gene from the plant Arabidopsis thaliana (30). The zrt1 gene encodes the transporter protein of the high affinity system (31), whereas the zrt2 gene encodes the transporter of the low affinity system (12). Based on protein sequence divergences, ZRT1 and ZRT2 were predicted to be integral membrane proteins containing eight potential transmembrane domains. However, the zrt1/zrt2 double mutant is viable, indicating the existence of additional zinc uptake pathways (12).

In this study, we demonstrate that PZP1 is a highly soluble periplasmic zinc-binding protein. Our results suggest that unlike in yeast, there may be only one zinc uptake system in H. influenzae.

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