Cloning of the gene and characterization of the enzymatic properties of the monomeric alkaline phosphatase (PhoX) from *Pasteurella multocida* strain X-73

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Abstract

We have identified a new *phoX* gene encoding the monomeric alkaline phosphatase from *Pasteurella multocida* X-73. This gene was not found in the published genome sequence of *Pasteurella multocida* pm70. Characterization of the recombinant PhoX of *Pasteurella multocida* X-73 showed that it is a monomeric enzyme, activated by Ca^{2+} and possibly secreted by the Tat pathway. These features distinguish phosphatases of the PhoX family from those of the PhoA family. All proteins of the PhoX family were found to contain a conserved motif that shares significant sequence homology with the calcium-binding site of a phosphotriesterase known as diisopropylfluorophosphatase. Site-directed mutagenesis revealed that D527 of PhoX might be the ligand bound to the catalytic calcium. This is the first report on identification of homologous sequences between PhoX and the phosphotriesterase and on the potential calcium-binding site of PhoX.

Introduction

Pasteurella multocida is an important pathogen of domestic animals and an opportunistic pathogen of humans. It is the causative agent of fowl cholera in domestic birds, hemorrhagic septicemia in cattle, and atrophic rhinitis in pigs (Hunt *et al.*, 2000). Human infections with *Pasteurella multocida* largely arise from the bite of an infected carnivore, but other types of infections are occasionally reported (Hubbert *et al.*, 1970; Frederiksen, 1989).

Alkaline phosphatase is a ubiquitous enzyme produced by organisms ranging from bacteria to mammalian cells. This enzyme releases a free inorganic phosphate from many phosphate-containing compounds and provides bacteria with the inorganic phosphate as a nutrient (Torriani, 1990). *Escherichia coli* has a single gene (*phoA*) for alkaline phosphatase, which is a homo-dimer enzyme containing two Zn^{+2} and one Mg^{+2} per monomer (Bradshaw *et al.*, 1981; Kim & Wyckoff, 1991). PhoA-like alkaline phosphatases are found in many other bacteria and these alkaline phosphatases have functionally important domains similar to those in *E. coli* PhoA (Bradshaw *et al.*, 1981; Kim & Wyckoff, 1991).

Another type of alkaline phosphatase, $PhoA_{VC}$, was first identified in *Vibrio cholerae* (Roy *et al.*, 1982). In contrast to phosphatases of the PhoA family, $PhoA_{VC}$ is a monomeric enzyme that is activated by Ca^{2+} and shares no sequence homology with PhoA. Recent reports indicate, however, that $PhoA_{VC}$ shares sequence homology with a group of proteins known as PhoX (Majumdar *et al.*, 2005). In *Pseudomonas fluorescens*, PhoX was the major enzyme responsible for the phosphatase activity (Monds *et al.*, 2006). Majumdar *et al.* (2005) proposed that $PhoA_{VC}$ from *V. cholerae*, PhoX from *Pseudomonas fluorescens*, together with related homologues in other bacterial species, constitute a new family of alkaline phosphatases. In the current Conserved Domain Database (Marchler-Bauer & Bryant, 2004), the PhoX family includes 180 bacterial and one eukaryotic sequence. The eukaryotic PhoX is a calcium-dependent phosphatase from the multicellular alga, *Volvex carteri* (Hallmann, 1999). Of the 180 bacterial PhoX sequences, only $PhoA_{VC}$ has been enzymatically characterized, and it is unclear whether other PhoX family members share the same set of enzymatic properties. In addition, the tertiary structure of PhoX remains unknown and the amino acid residues involved in the metal

binding and catalytic reactions of PhoX have not been identified.

This paper reports the complete nucleotide sequence of a new PhoX from *Pasteurella multocida* X-73, a highly virulent strain of *Pasteurella multocida* widely used in the challenge test of fowl cholera (Rice *et al.*, 1978). The enzymatic properties of this PhoX are characterized and compared with those of $PhoA_{VC}$. Common enzymatic features shared by PhoX and $PhoA_{VC}$ are reported, and the potential amino acid residue involved in binding of calcium to PhoX is investigated.

Materials and methods

Bacterial strains, DNA extraction, and Southern blot analysis

Pasteurella multocida P-1059 (ATCC 15742) was obtained from the Bioresource Collection and Research Center in Hsinchu, Taiwan. Other strains, including *Pasteurella multocida* X-73 (ATCC 11039) and *Pasteurella multocida* P-1662, were obtained from Dr Chin Chen at the National Animal Research Institute in Taipei, Taiwan. The identities of all strains were confirmed using species-specific and serotype-specific PCR methodology (Kasten *et al.*, 1997; Townsend *et al.*, 2001). All strains were grown at 37 °C in brain heart infusion broth (Difco Laboratories, MI). Bacteria DNA was isolated using the DNeasy tissue kit (Qiagen, Hilden, Germany). Southern blot analysis was conducted as previously described (Wu *et al.*, 2003).

PCR amplification and sequence analysis

Two primers, P1 (+) and P1 (−), were designed using the published genome sequence of *Pasteurella multocida* pm70 (May *et al.*, 2001). Their sequences are as follows: P1 (+): 5′-GAC AAT GAA GCG ATC AAA GA-3′ and P1 (−): 5′- TCC AAT GCA ATT GTT GGC TT-3′. P1 (+) and P1 (−) were able to amplify a 2.4-kb DNA fragment containing the *phoX* gene from *Pasteurella multocida* X-73. The PCR products were purified using a QIAquick gel extraction kit (Qiagen) and sequenced from both directions using a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) in an automatic sequencer (ABI-3730XL DNA Analizer, Applied Biosystems). Sequences were compiled using the SEQMAN program in the LASERGENE package (DNASTAR Inc. Madison, WI). Nucleotide and protein sequences were searched for homology in GenBank using the BLAST program provided by NCBI (Altschul *et al.*, 1990). The nucleotide sequence determined in this study is available in the GenBank under the accession number DQ986289.

Expression and purification of recombinant proteins

Two primers, PhoX (+): 5′-CCA TGG GCA TGA CTA ATT CAG TAC AAG AA-3′ and PhoX (−): 5′-AAG CTT TGA ACT AAT AAC CCC ACC GTC-3′, were used to amplify the DNA fragment containing the entire *phoX* ORF. Another two primers, ΔPhoX (+): 5′-CCA TGG GCA ATC CTT CAC AGC CAC TGC AA-3′ and ΔPhoX (−): 5′ AAG CTT TGA ACT AAT AAC CCC ACC GTC-3′, were used to amplify the *phoX* fragment without the region encoding the signal peptide. These primers contained restriction enzyme (NcoI or HindIII) cutting sites at their 5′-ends (underlined sequences), followed by sequences specific to *phoX*. The PCR products were cloned into the expression vector pET28a as described in the user's manual (Novagen, Inc. Madison, WI). Recombinant plasmids were transformed into *E. coli* strain BL21 (DE3) and recombinant proteins were purified by nickel chromatography as previously described (Chang *et al.*, 2002). In brief, the *E. coli* extract was loaded into a column containing 'His-bind' resin (Novagen). The column was washed with the washing buffer (20 mM pH 7.9 Tris, 30 mM imidazole, 500 mM NaCl) to remove the unbound proteins. The bound protein was eluted with the eluting buffer (20 mM pH 7.9 Tris, 250 mM imidazole, 500 mM NaCl); only the first 3 mL of the elute was collected. Protein concentration was determined using the 'Protein Assay' kit (BIO-RAD, Hercules, CA).

Alkaline phosphatase assay

APase activity was assayed by measuring the release of paranitrophenol from paranitrophenol phosphate (PNPP) (Roy *et al.*, 1982). One unit of enzyme was defined as the amount required to release 1 nmol of paranitrophenol per min at 37 °C.

Gel-filtration chromatography of recombinant PhoX

Purified PhoX was chromatographed on an FPLC Superdex HR200 column (Amersham Pharmacia Biotech, NJ). The column was pre-equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl. The molecular-mass standards and their retention times in the HR200 column were as follows: β-amylase (200 kDa, 16.78 min), albumin (66 kDa, 29.4 min), carbonic anhydrase (29 kDa, 34.14 min), and cytochrome c (12.4 kDa, 36.97 min).

Effect of metal ions and substrate specificity

Apoenzymes were prepared by dialyzing purified PhoX against a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 100 mM NaCl. The resulting apoenzymes were then dialyzed against a buffer containing 20 mM

Tris-HCl (pH 7.5) and 100 mM NaCl, to remove EDTA. Apoenzyme activity was measured in the presence of 2 mM of various divalent metal ions.

Substrate specificity was determined by assaying the ability of the enzymes to release orthophosphate from various organic phosphate esters. The 2 mL reaction mixture contained 1.0 M Tris-HCl buffer (pH 10.0), 2 mM substrate, and 0.1 μg of enzyme. The amount of orthophosphate released was determined using the Saheki method (Saheki *et al.*, 1985).

Site-directed mutagenesis

Four sets of primers were used to create four PhoX mutants. The sequences of these primer sets were as follows: D527E (+): 5′-TTG GTA GTC CTG AAG GGT TAC GTT TTG-3′/D527E (−): 5′-CAA AAC GTA ACC CTT CAG GAC TAC CAA-3′, D527N (+): 5′-TTG GTA GTC CTA ACG GGT TAC GTT TTG-3′/D527N (−): 5′-CAA AAC GTA ACC CGT TAG GAC TAC CAA-3′, D522E (+): 5′-TTA ATG GTG ATG AAT TTG GTA GTC C-3′/D522E (−): 5′-GGA CTA CCA AAT TCA TCA CCA TTA A-3′, and D522N (+): 5′-TTA ATG GTG ATA ATT TTG GTA GTC C-3′/D522N (−): 5′-GGA CTA CCA AAA TTA TCA CCA TTA A-3′. The Quik Change Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate the mutants, and the entire *phoX* coding region was sequenced to verify that the mutation occurred specifically.

Results and discussion

Identification of a new *phoX* gene in *Pasteurella multocida* X-73

We are interested in elucidating the function of the *plpB* gene, which appears to encode the 39 kDa cross-protecting protein of *Pasteurella multocida* (Tabatabai & Zehr, 2004). Sequence analyses of the region downstream of *plpB* showed that a new *phoX* gene exists at the locus between the *plpB* and *glm*S of *Pasteurella multocida* X-73. This *phoX* gene was not found in the published genome sequence of *Pasteurella multocida* pm70 (May *et al.*, 2001). The presence of the *phoX* gene in *Pasteurella multocida* X-73 was revealed by PCR amplification using primers P1 and P2, which annealed to the 3′-ends of the *plpB* and *glmS* genes, respectively (Fig. 1a). According to the published genome sequence of *Pasteurella multocida* pm70, P1 and P2 should amplify a 0.9-kb fragment. As shown in Fig. 1b, the two primers amplified the expected 0.9-kb fragment from all strains of *Pasteurella multocida* that were examined, except the strain X-73, from which the primers amplified a 2.4-kb fragment (Fig. 1b). Sequence analyses showed that the 0.9-kb sequence from different strains of *Pasteurella multocida* shared 78.4–99.3% identity with the sequence from *Pasteurella multocida* pm70

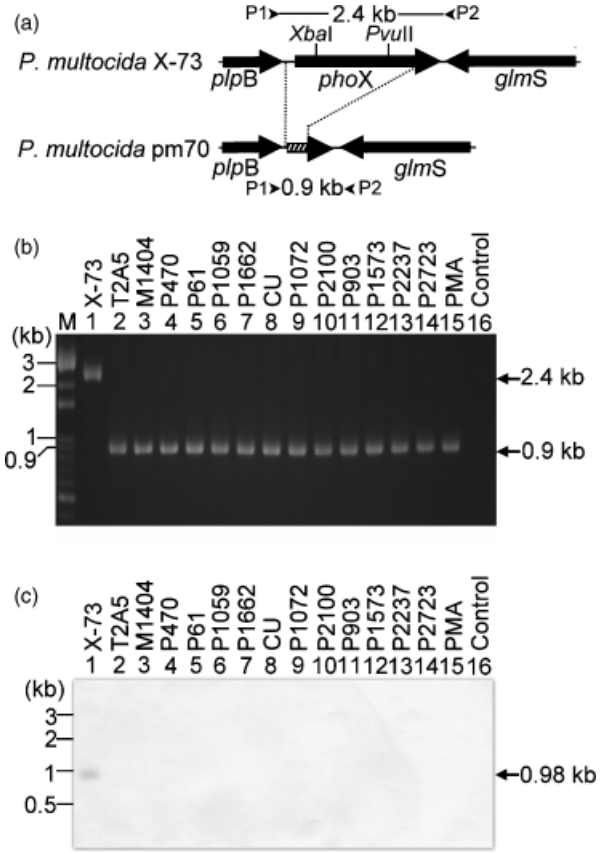

Fig. 1. (a) Diagram of the locus containing *plpB*, *phoX*, and *glmS* of *Pasteurella multocida* strains X-73 and pm70. The location and transcriptional orientation of the genes are shown by filled arrows. The positions where primers P1 and P2 bind are indicted by arrowheads, and the size of each PCR product are also shown. The region containing deletions and mismatches in the 0.9-kb fragment is indicated by a hatched box. (b) Photograph of the ethidium bromide-stained agarose gel showing the PCR products amplified from different strains of *Pasteurella multocida*. The names of each strain are shown on the top of each lane. (c) Southern blot analysis of bacterial DNA digested by XbaI and PvuII. The probed used was the 0.98-kb XbaI–PvuII fragment of *phoX*. The band derived from *phoX* is indicated by an arrow.

(May *et al.*, 2001), and no ORF existed in this 0.9-kb region. In contrast, the 2.4-kb fragment from strain X-73 contained an ORF consisting of 1944 nucleotides that encoded the PhoX protein. When the 2.4- and 0.9-kb sequences were compared, extensive deletions and mismatches were found between nt 56 downstream of the *plpB* termination codon and nt 1683 downstream of the *phoX* initiation codon (Fig. 1a). This result suggests that in strains other than X-73, the promoter and most of the *phoX* coding region were excised from the bacterial genome, while nt 1684–1944 of *phoX* remained. Southern hybridization using the 0.98-kb XbaI–PvuII fragment of *phoX* as the probe confirmed that only strain X-73 contains *phoX* (Fig. 1c).

PhoX from *Pasteurella multocida* X-73 is a new member of the PhoX family, sharing 48.3% amino acid identity with $PhoA_{VC}$ from *V. cholerae* and 46.2% with PhoX from *Pseudomonas fluorescens*. The amino acid sequence of PhoX from *Pasteurella multocida* X-73 has an 56 amino acid signal sequence that was predicted using SIGNALP 3.0 (Bendtsen *et al.*, 2004) (Fig. 2). This signal peptide has a twin arginine motif (SRRKILQ) at residues 28–33 (Fig. 2), suggesting that PhoX from *Pasteurella multocida* X-73 is secreted by the twin arginine translocation (Tat) pathway. In fact, all bacterial PhoX proteins appear to have a signal peptide containing the Tat motif. In general, the Tat pathway is responsible for secreting redox proteins that fold with their redox cofactor before secretion (Berks *et al.*, 2003; Robinson & Bolhuis, 2004). It remains unknown why PhoX, a non-redox enzyme, utilizes this pathway for secretion.

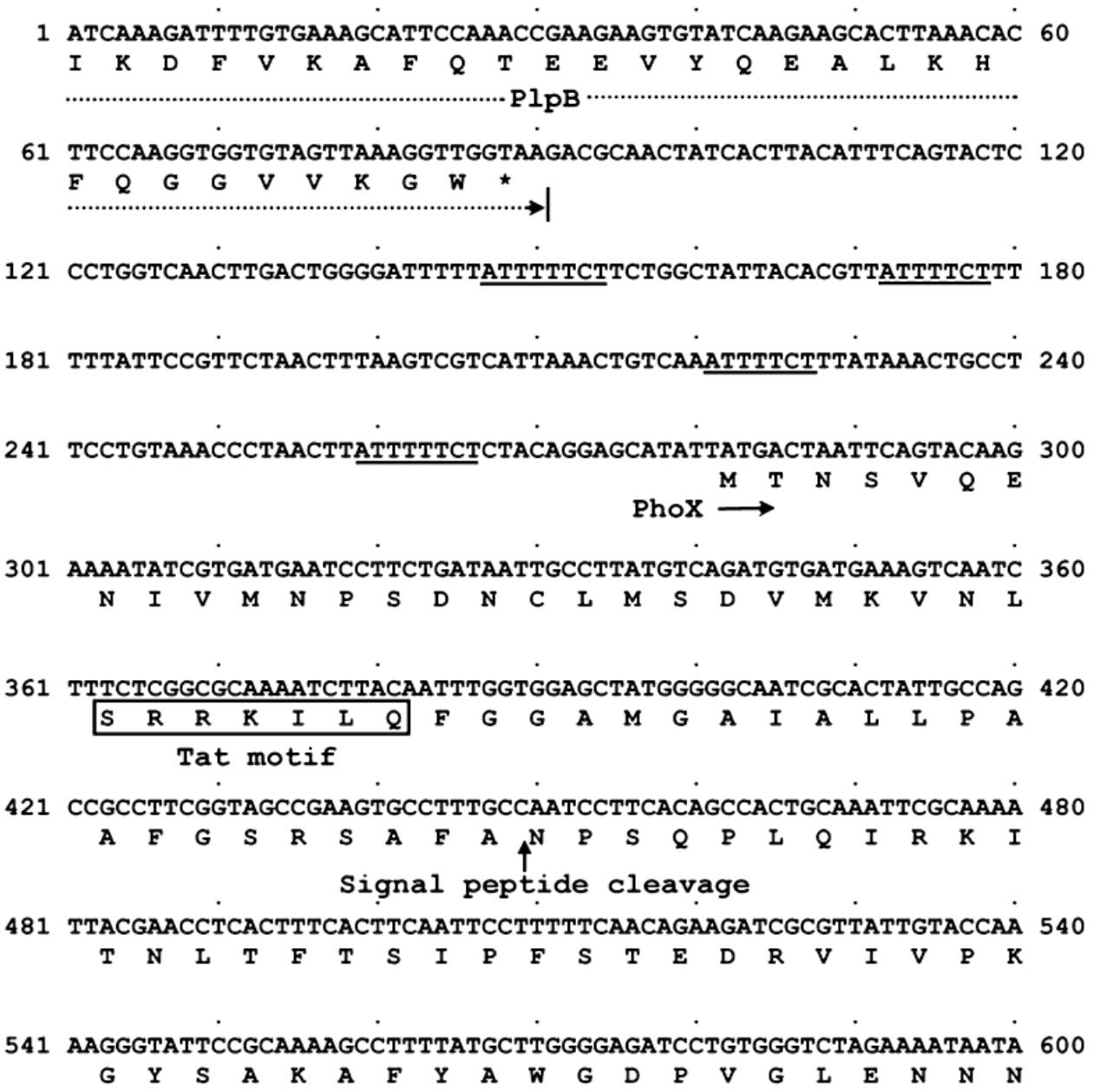

Fig. 2. Nucleotide sequences of the 3′ end of *plpB* and the region encoding the signal peptide of PhoX from *Pasteurella multocida* X-73. The protein sequences translated from each gene are shown below the nucleotide sequences. The Tat motif is boxed, and the signal peptide cleavage site is indicated with a vertical arrow. Repeated sequences present in the putative promoter region of *phoX* are underlined.

A total of four directed repeats with the consensus sequences, ATTTTCT or ATTTTTCT, were found upstream of the *phoX* initiation codon (Fig. 2). The function of these directed repeats remains unknown.

Expression and purification of recombinant PhoX

Recombinant PhoX was expressed in *E. coli* with a hexa-histidine-tag attached at its carboxyl terminus. Two forms of recombinant PhoX were expressed: full-length PhoX (r-PhoX, 72.8 kDa), and a truncated form that lacked the signal peptide (r-ΔPhoX, 66.7 kDa) (Fig. 3a). Both recombinants were highly expressed in *E. coli* and purified using nickel chromatography (Fig. 3a). Enzymatic assays using PNPP as the substrate showed that the specific activities of purified r-PhoX and r-ΔPhoX were 7780 and 5560 U mg^{-1} of protein, respectively (Fig. 3b). It should be noted that purified r-PhoX produced two bands on Commassie blue-stained sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) gels (Fig. 3a), the minor band having the expected molecular mass of 72.8 kDa, and the major band having a molecular mass similar to that of r-ΔPhoX, 66.7 kDa (Fig. 3a). It is possible that the major band of r-PhoX represents the processed form from which the signal peptide is cleaved. The two bands of r-PhoX could not be separated by chromatography and were together referred to as purified r-PhoX.

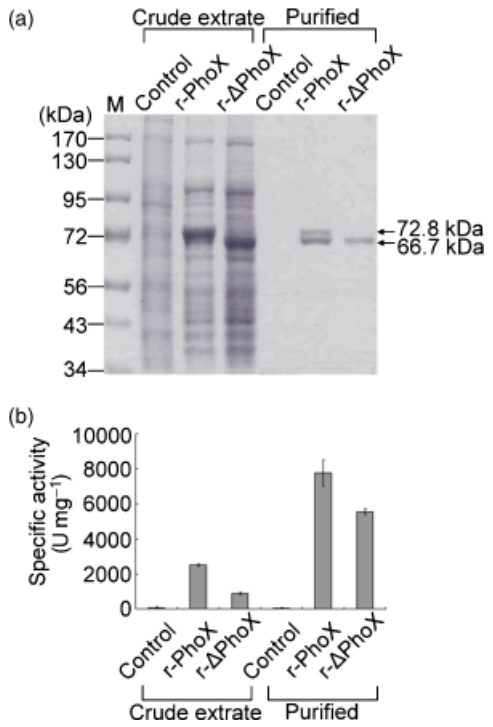

Fig. 3. Expression and purification of r-PhoX and r-ΔPhoX in *Escherichia coli*. (a) Commassie blue-stained SDS-PAGE of recombinant proteins from crude extracts or purified samples. Lane M represents the molecular mass markers. Lane marked control is the crude extracts or purified sample of *E. coli* host that harbored no recombinant plasmid. (b) Specific activity of phosphatases present in the crude extracts and purified samples. PNPP was used as the substrate for determining phosphatase activity. The results are expressed as the mean of three experiments and the SEs are shown at the top of the bar.

Molecular mass and enzymatic properties of recombinant PhoX

Gel-filtration chromatography using the Superdex HR200 column showed that the native enzymes of purified r-PhoX and r-ΔPhoX both had a molecular mass of *c.* 70 kDa, indicating that they were monomeric (data not shown). The optimal pH of both r-PhoX and r-ΔPhoX was 10.0 in 1 M Tris-HCl buffer and 11.0 in 50 mM glycine-NaOH buffer. The K_m values of r-PhoX and r-ΔPhoX were 0.095 and 0.066 mM, respectively, while the V_{max} values were 9.45 and 7.54 μmol PNPP per min per mg of protein, respectively. The activities of r-PhoX and r-ΔPhoX were completely inhibited by low levels of EDTA (0.01 mM), suggesting that they are metalloenzymes. To investigate the metal requirement of r-PhoX and r-ΔPhoX, the two enzymes were dialyzed against Tris buffer (20 mM, pH 7.5) containing 10 mM EDTA. The resulting apoenzymes were tested for phosphatase activity in the presence of various divalent metal ions. Ca^{+2} was the best metal ion to reconstitute enzyme activity, followed by Co^{+2}, Ni^{+2}, Mn^{+2}, and Mg^{+2}. Zn^{+2}, in contrast, failed to restore enzyme activity (Table 1).

Both r-PhoX and r-ΔPhoX had broad substrate specificity. The best substrates were sodium pyrophosphate and ATP, followed by glucose-1-phosphate, PNPP, AMP, and glucose-6-phosphate (Table 2). In addition to these

Table 1. The ability of various divalent metal ions to restore the enzymatic activity to the apoenzymes of r-PhoX and r-ΔPhoX

	% APase activity restored*	
Metal ion (2 mM)	r-PhoX	r-ΔPhoX
Ca^{+2}	132 ± 1.87	79 ± 0.54
Mn^{+2}	75 ± 2.16	27 ± 0.83
Co^{+2}	126 ± 0.43	62 ± 0.65
Ni^{+2}	122 ± 1.03	36 ± 0.15
Mg^{+2}	20 ± 1.46	11 ± 0.94
Zn^{+2}	2 ± 0.92	5 ± 1.05
None	1 ± 0.82	1 ± 0.54

*Results are expressed as the percentage of activity restored when compared with the enzyme that was not dialyzed against EDTA (see Materials and methods). All results are the mean of three experiments. The SEs are also provided.

Table 2. Substrate specificity of purified r-PhoX and r-ΔPhoX

	Pi liberated relative to that from PNPP*	
Substrates	r-PhoX	r-ΔPhoX
PNPP	100	100
Sodium pyrophosphate	368 ± 2.5	308 ± 4.49
β-glycerophosphate	90 ± 2.5	88 ± 4.46
ATP	366 ± 3.75	301 ± 4.46
AMP	89 ± 1.25	91 ± 3.57
Glucose-6-phosphate	90 ± 2.5	85 ± 2.68
Glucose-1-phosphate	234 ± 1.25	188 ± 1.79
bis-PNPP	93 ± 2.5	93 ± 4.92

*The Pi released from PNPP was set at 100%. All results are the mean of three experiments. The SEs are also provided.

phosphomonoesterase activities, r-PhoX and r-ΔPhoX were able to hydrolyze the phosphodiester in bis-PNPP (Table 2), indicating that r-PhoX and r-ΔPhoX have phosphodiesterase as well as phosphomonoesterase activity.

Comparison of the enzymatic properties of PhoX and PhoA$_{VC}$

Of the 180 bacterial PhoX, only PhoA$_{VC}$ has been enzymatically characterized, so it remains unknown whether other members of the PhoX family share these properties. Data presented in this study showed that PhoX from *Pasteurella multocida* X-73 shares many properties in common with PhoA$_{VC}$. Both proteins are monomeric and their apoenzymes are activated by Ca^{2+} and not by Zn^{2+}. Moreover, they are both secreted by the Tat pathway and have similar specific activity for hydrolyzing PNPP. It is possible that these properties are shared by other members of the PhoX family, helping to distinguish them from the PhoA family. Proteins of the PhoA family are dimeric enzymes that are activated by Zn^{2+} and secreted by the Sec pathway (Kim & Wyckoff, 1991).

PhoX of *Pasteurella multocida* also has properties that are distinct from Pho^{VC}. PhoX can hydrolyze ATP and AMP while PhoA$_{VC}$ cannot (Roy *et al.*, 1982). In addition, PhoX has phosphodiesterase activity and it remains unclear whether Pho^{VC} also has this activity (Roy *et al.*, 1982). These results indicate that each member of the PhoX family may have both unique and shared properties.

Identification of a potential-calcium binding site in PhoX

Databank searches using the 123D+ program (Alexandrov *et al.*, 1995) showed that all PhoX members have conserved sequences that share significant homology with the calcium-binding site of diisopropylfluorophosphatase (Fig. 4a). Diisopropylfluorophosphatase is a Ca^{2+}-dependent phosphotriesterase that was isolated from the squid, *Loligo vulgaris* (Scharff *et al.*, 2001). It was previously reported that the D229 residue of diisopropylfluorophosphatase is the ligand that binds to catalytic calcium, as a mutation in D229 completely abolished diisopropylfluorophosphatase activity (Scharff *et al.*, 2001). Downstream of D229 is a stretch of hydrophobic amino acid residues (L239-V241) that are conserved in the six β propeller blade structure of diisopropylfluorophosphatase (Fig. 4a; Scharff *et al.*, 2001). According to the alignment shown in Fig. 4a, D527 and L537-V539 in the PhoX of *Pasteurella multocida* X-73 are counterparts of D229 and L239-V241 in diisopropylfluorophosphatase. To investigate whether D527 is critical for PhoX activity, two mutant forms of r-PhoX, D527E and D527N, were created. Enzymatic assays of the mutant r-PhoX purified from *E. coli* showed that neither mutant had phosphatase activity, and suggested that D527 is critical for PhoX activity. In contrast to D527, mutations in D522, an aspartic residue near D527, had either no effect (D522E) or only a moderate effect (D522N) on the enzymatic activity of PhoX (Fig. 4b). It is likely that D527 is the calcium ligand in PhoX. To our knowledge, this is the first report of sequence homology between diisopropylfluorophosphatase and PhoX, as well as identification of the possible calcium ligand of PhoX.

A BLAST search revealed that diisopropylfluorophosphatase also shares sequence homology with two groups of proteins known as gluconolactonase and senescence marker protein-30 (SMP-30) (Scharff *et al.*, 2001). Gluconolactonase is a metalloenzyme that cleaves the lactone ring (Carper *et al.*, 1982), while SMP-30, also known as regucalcin, is a calcium-binding protein involved in aging (Fujita, 1999). The SMP-30 from mice is a gluconolactonase that participates in L-ascorbic acid biosynthesis (Kondo *et al.*, 2006). The tertiary structures of gluconolactonase and SMP-30 have not yet been resolved and the metal ligands of both proteins have not been identified. Figure 4a shows that homologous sequences shared by PhoX and diisopropylfluorophosphatase are also conserved in gluconolactonase and SMP-30 (Fig. 4a). Residues corresponding to D229 and L239-V241 in diisopropylfluorophosphatase, for example, are also present in gluconolactonase and SMP-30. These residues may participate in the metal binding and enzymatic activity of gluconolactonase and SMP-30. This finding is novel and should help elucidate the structure and function of gluconolactonase and SMP-30.

Acknowledgements

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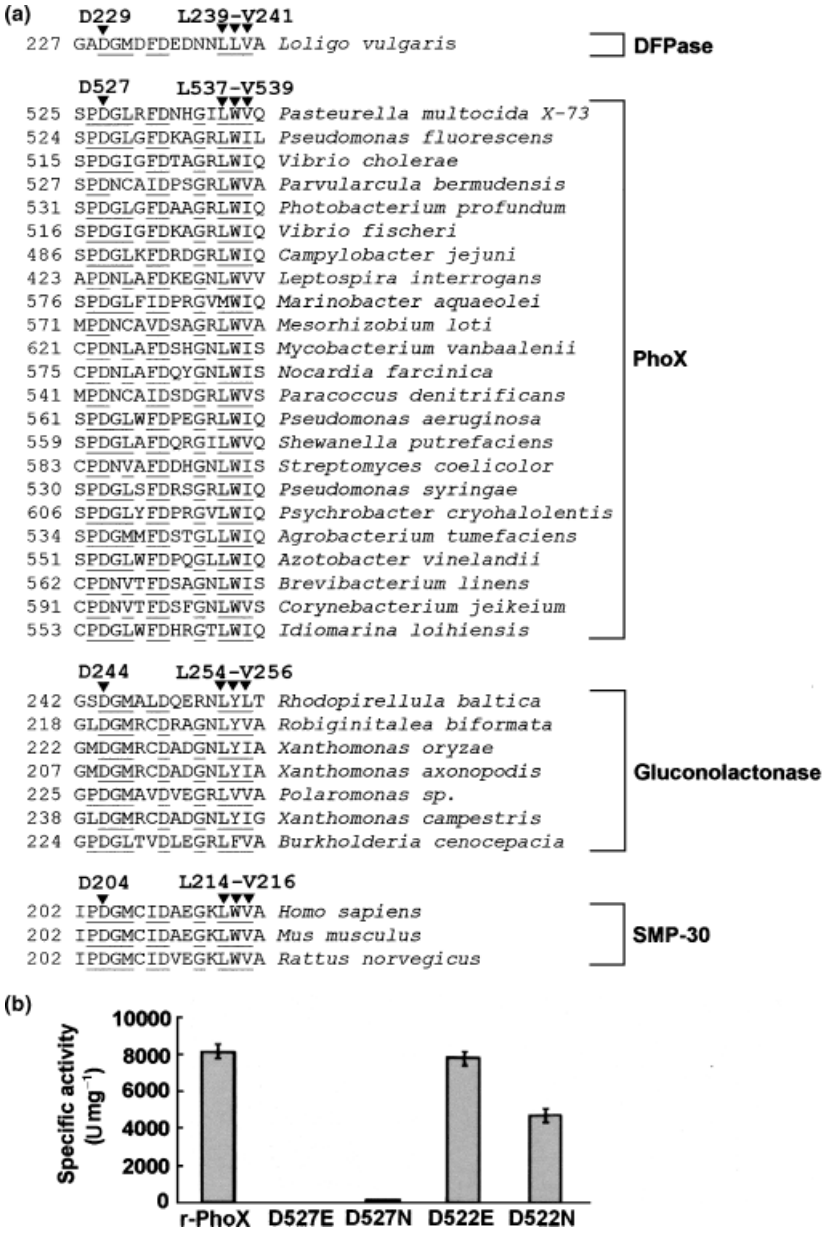

Fig. 4. (a) Amino acid sequence alignment of the calcium-binding site of diisopropylfluorophosphatase and its homologous sequences in PhoX, gluconolactonase, and SMP-30. Identical and similar residues found in diisopropylfluorophosphatase, PhoX, gluconolactonase, and SMP-30 are underlined. The possible ligand for calcium and conserved hydrophobic amino acids are indicated using arrowheads. (b) Specific activity of purified wild type and mutant r-PhoX proteins. The wild type and mutant r-PhoX proteins were all his-tagged proteins purified from *Escherichia coli*. PNPP was used as the substrate, and results are expressed as the mean of three experiments. The SEs are shown at the top of the bar.

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