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Purification, crystallization and preliminary X-ray diffraction studies of recombinant class A non-specific acid phosphatase of *Salmonella typhimurium*

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The *phoN* gene of *Salmonella enterica* sv. Typhimurium strain MD6001 was cloned in the multicopy plasmid pBluescript SK$^-$. The nucleotide sequence of the cloned gene differs from the corresponding *S. typhimurium* LT2 sequence at 23 residues, leading to 15 amino-acid differences, but was very close to the *S. typhi phoN* sequence (only three nucleotide and two amino-acid differences). The recombinant PhoN protein was purified to homogeneity. Two forms of crystals were harvested from a single crystallization condition. Diffraction intensity data were collected using a laboratory X-ray source to resolution limits of 2.5 and 2.8 Å for crystals belonging to space group $C2$ and $C222_1$, respectively. Based on non-crystallographic symmetry, four monomers of PhoN are expected to be present in the asymmetric unit of the $C2$ unit cell. Two monomers of a biologically active dimer in the asymmetric unit of the $C222_1$ unit cell are expected from the Matthews coefficient.

1. Introduction

Bacterial non-specific acid phosphohydrolases (NSAPs; EC 3.1.3.2) are secreted enzymes that function as soluble periplasmic proteins or as membrane-bound lipoproteins (Rossolini *et al.*, 1998) and exhibit optimal catalytic activity at acidic to neutral pH. They are a diverse group of monomeric or oligomeric proteins. The monomer molecular weight is in the range 25–30 kDa. Based on amino-acid sequence analysis, NSAPs have been classified into three distinct molecular families designated classes A, B and C (Thaller *et al.*, 1998). These enzymes can dephosphorylate disparate phosphate monoesters, thus enabling the organic moieties to enter the cell. The inorganic phosphate released in the periplasmic space can promote the precipitation of heavy-metal ions such as uranyl or plutonium, a property that can be exploited for the bioremediation of heavy-metal wastes (Macaskie *et al.*, 1994). The use of a *Citrobacter* species harbouring an NSAP has been proposed for the efficient bioremediation of nuclear wastes (Macaskie *et al.*, 1996). *E. coli* harbouring the *phoN* gene of *S. enterica* sv. *typhi* on a multicopy plasmid has been found to be even more efficient than *Citrobacter* for the removal/recovery of uranyl and nickel ions from dilute solution (Basnakova *et al.*, 1998; A. S. Rao & S. K. Mahajan, unpublished results). The pH optimum of the recombinant enzyme is ~5. Engineering of the cloned gene to produce a protein active in the pH range 2–3 and at high salt concentrations can make it more suitable for the remediation of real-life nuclear wastes. Detailed knowledge of the structure of the protein is crucial for this purpose. We have undertaken such a study.

The *phoN* gene of *Salmonella enterica* sv. Typhi (*S. typhi*) and *Salmonella enterica* sv. Typhimurium (*S. typhimurium*) encodes a 250 amino-acid polypeptide, 20 of which function as the signal peptide for periplasmic localization (SWISS-PROT accession Nos. P26976 and Q934J6, respectively). The 26 kDa periplasmic phosphatase is a member of the class A NSAPs (Kasahara *et al.*, 1991), having the conserved sequence motif, KX_6RP-(X_{12-54})-PSGH-(X_{31-54})-SRX_5HX_3D (Stukey & Carman, 1997). This sequence motif is also shared by several lipid phosphatases, the mammalian glucose-6-phosphatase and members of the vanadium-containing chloroperoxidase superfamily (Hemrika *et al.*, 1997).

Crystal structures of the Pho protein from *Escherichia blattae*, which has nearly 41% identity with the PhoN of *S. typhimurium* (our laboratory strain), and that of its complex with the transition-state analogue molybdate to 1.9 and 2.4 Å resolution, respectively, have been recently reported (Ishikawa *et al.*, 2000). Here, we report the cloning of the *phoN* gene and the purification, crystallization and preliminary X-ray analysis of the recombinant PhoN protein from a pathogenic *S. typhimurium* strain.

2. Cloning of the *phoN* gene

The pathogenic *S. typhimurium* strain MD6001 (our laboratory number) was obtained from the Central Research Institute (CRI), Kasauli,

India (CRI No. 5710), where it had been identified based on its biochemical and serological characters. The primers ASR1 (5′-CCGGTATGGACAGACGATAA-3′) and ASR2 (5′-CCTACGCAGTTGCACTT-CCT-3′), designed on the basis of a published sequence of this gene and its flanking regions on the *S. typhimurium* genome (Kasahara *et al.*, 1991; GenBank Accession No. NC_003197), were used to amplify the *phoN* region from the genomic DNA of our strain. The amplified fragment contained, besides the structural gene, 126 nucleotides upstream of the start codon and 61 nucleotides downstream of the stop codon. This fragment was blunt-ended with T4 DNA polymerase and ligated to *Sma*I-restricted pBluescript SK$^-$ vector. The ligation mixture was used to transform *E. coli* DH5α and the transformants were selected on LB plates supplemented with ampicillin, X-Gal and IPTG. The white clones were checked for acid phosphatase activity by transferring them to plates containing a modified version of the histochemical medium used previously by Riccio *et al.* (1997) for selecting phosphatase-positive clones on plates. Our plates contained LB agar, phenolphthalein diphosphate tetrasodium salt (1 mg ml^{-1}) and methyl green (50 g ml^{-1}). On these plates, phosphatase-positive clones turn greenish while the others remain white. Of the total of 85 clones checked, nine appeared phosphatase positive. All these clones had the correct-size insert. The specific activity of the enzyme determined for six of these clones ranged between 1900 and 2200 U (where 1 U corresponds to 1 nmol of *p*-nitrophenol being liberated from *p*-nitrophenyl phosphate per minute per milligram of protein at 310 K). The corresponding values for the phosphatase-negative controls were between 40 and 50 U. The clone giving the maximum PhoN activity (named pASK1) was chosen for further work. The sequence of the *phoN* insert in this clone was determined on an ABI automated DNA sequencer using primers ASR1 and ASR2 (GenBank accession No. AF522938). Intriguingly, this sequence was much closer (only three nucleotide and two amino-acid differences) to the published *phoN* sequence of *S. typhi* (GenBank accession Nos. AF366353, AL627282) than to that of *S. typhimurium* LT2 (23 nucleotide and 15 amino-acid differences; GenBank accession No. NC_003197). To rule out the possibility that the cloned gene was from an *S. typhi* contaminant, the chromosomal *phoN* of MD6001 was again PCR amplified and both strands of the amplification product were directly sequenced. This sequence (Fig. 1; GenBank accession No. AF521595) exactly matched that of *S. typhi*, but showed 20 base and 13 amino-acid differences from the published sequence of *S. typhimurium* LT2. This suggested a possible error in the earlier identification of the strain. To check this possibility, we amplified 16S *rRNA*, 23S *rRNA* and *aphA* genes of this isolate and determined their sequences (partial gene sequences in the case of 16S and 23S *rRNA* genes). The sequences matched with those of *S. typhimurium* LT2 (McClelland *et al.*, 2001), thus confirming that MD6001 was indeed an *S. typhimurium* strain. Why its *phoN* sequence matches that of *S. typhi* is not understood at this stage. It may have accidentally acquired this gene by horizontal transfer from *S. typhi*. Alternatively, the *phoN* gene sequence of this strain may be

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STM-KA  ATGAAAAGTCGTTATTTACTATTTTTTCTACCACTGATCGTAGCTAAATATACATCAGCA   60
STM-CL  ATGAAAAGTCGTTATTTACTATTTTTTCTACCACTGATCGTAGCTAAATATACATCAGCA
          M  K  S  R  Y  L  L  F  F  L  P  L  I  V  A  K  Y  T  S  A

STM-KA  GCAACAATGCAACCCTTTCATTCTCCTGAAGAATCAGTGAACAGTCAGTTCTACTTACCA  120
STM-CL  GCAACAATGCAACCCTTTCATTCTCCTGAAGAATCAGTGAACAGTCAGTTCTACTTACCA
          A  T  M  Q  P  F  H  S  P  E  E  S  V  N  S  Q  F  Y  L  P

STM-KA  CCACCGCCAGGTAATGATGATCCGGCTTTCCGCTATGATAAGGAGGCTTATTTTAAGGGC  180
STM-CL  CCACCGCCAGGTAATGATGATCCGGCTTTCCGCTATGATAAGGAGGCTTATTTTAAGGGC
          P  P  P  G  N  D  D  P  A  F  R  Y  D  K  E  A  Y  F  K  G

STM-KA  TATGCGATAAAGGGTTCCCCACGATGGAAACAAGCTGCTGAGGATGCAGATATAAGCGTG  240
STM-CL  TATGCGATAAAGGGTTCCCCACGATGGAAACAAGCTGCTGAGGATGCAGATATAAGCGTG
          Y  A  I  K  G  S  P  R  W  K  Q  A  A  E  D  A  D  I  S  V

STM-KA  GAAAATATAGCCAGAATATTCTCGCCAGTAGTGGGTGCTAAAATTAaTCCCAAAGATACG  300
STM-CL  GAAAATATAGCCAGAATATTCTCGCCAGTAGTGGGTGCTAAAATTAgTCCCAAAGATACG
          E  N  I  A  R  I  F  S  P  V  V  G  A  K  I  s  P  K  D  T

STM-KA  CCAGAAACCTGGAATATGTTACAGAATCTTCTGAAAaTGGGCGGCTACTACGCTACTGCT  360
STM-CL  CCAGAAACCTGGAATATGTTACAGAATCTTCTGAAAgTGGGCGGCTACTACGCTACTGCT
          P  E  T  W  N  M  L  Q  N  L  L  K  v  G  G  Y  Y  A  T  A

STM-KA  TCGGCAAAAAAATATTATATGCGTACTCGTCCCTTTGTCTTATTTAATCATTCTACCTGC  420
STM-CL  TCGGCAAAAAAATATTATATGCGTACTCGTCCCTTTGTCTTATTTAATCATTCTACCTGC
          S  A  K  K  Y  Y  M  R  T  R  P  F  V  L  F  N  H  S  T  C
                *                    *  *

STM-KA  CGTCCTGAAGATGAGAATACTTTGCGAAAAGATGGCTCTTACCCTTCCGGGCATACTGCT  480
STM-CL  CGTCCTGAAGATGAGAATACTTTGCGAAAAGATGGCTCTTACCCTTCCGGGCATACTGCT
          R  P  E  D  E  N  T  L  R  K  D  G  S  Y  P  S  G  H  T  A
                                                  *  *  *  *

STM-KA  TATAGTACACTTCTGGCATTAGTATTATCCCAGGCCAGACCGGAACGCGCGCAGGAGCTC  540
STM-CL  TATAGTACACTTCTGGCATTAGTATTATCCCAGGCCAGACCGGAACGCGCGCAGGAGCTC
          Y  S  T  L  L  A  L  V  L  S  Q  A  R  P  E  R  A  Q  E  L

STM-KA  GCCAGACGAGGATGGGAGTTCGGGCAAAGCAGAGTGATATGCGGtGCTCACTGGCAAAGC  600
STM-CL  GCCAGACGAGGATGGGAGTTCGGGCAAAGCAGAGTGATATGCGGaGCTCACTGGCAAAGC
          A  R  R  G  W  E  F  G  Q  S  R  V  I  C  G  A  H  W  Q  S
                                     *  *                 *

STM-KA  GATGTTGATGCTGGCCGTTATGTGGGAGCAGTAGAGTTTGCAAGGCTGCAAACAATCCCG  660
STM-CL  GATGTTGATGCTGGCCGTTATGTGGGAGCAGTAGAGTTTGCAAGGCTGCAAACAATCCCG
          D  V  D  A  G  R  Y  V  G  A  V  E  F  A  R  L  Q  T  I  P
          *

STM-KA  GCTTTTCAGAAGTCACTGGCAAAAGTCCGTGAGGAGCTGAACGACAAAAATAATTTATTG  720
STM-CL  GCTTTTCAGAAGTCACTGGCAAAAGTCCGTGAGGAGCTGAACGACAAAAATAATTTATTG
          A  F  Q  K  S  L  A  K  V  R  E  E  L  N  D  K  N  N  L  L

STM-KA  AGTAAAGAAGAACGCCCCGAACTTAATTACTGA  753
STM-CL  AGTAAAGAAGAACGCCCCGAACTTAATTACTGA
          S  K  E  E  R  P  E  L  N  Y  -
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Figure 1
An alignment of chromosomal *phoN* of *S. typhimurium* strain MD6001 (STM-KA) and the cloned gene (STM-CL). The chromosomal *phoN* of *S. typhimurium* strain MD6001 is observed to match exactly that of *S. typhi* (GenBank accession Nos. AF366353 and AL627282). The nucleotide bases and amino-acid residues observed to differ in the chromosomal gene from the cloned fragment are shown in bold lower case letters. The 20-residue N-terminal signal sequence is underlined. The residues of the conserved sequence motif, KX_6RP-(X_{12-54})-PSGH-(X_{31-54})-SRX_5HX_3D are identified with asterisks.

associated with its pathogenicity, a characteristic that it shares with *S. typhi*, and the sequence in the non-pathogenic strain LT2 may have diverged. It is, however, noteworthy that the differences in the *phoN* of *S. typhi* and *S. typhimurium*, as well as the mutations in the cloned gene, do not alter the important residues of the conserved class A NSAP motif.

3. Purification of PhoN protein

Purification of the expressed PhoN protein was monitored by phosphatase-activity assay using *p*-nitrophenyl phosphate (pNPP) as the substrate (Kier *et al.*, 1977). The protein was purified from 8 l of *E. coli* culture grown in a fermentor to a density of OD_{600} = 1.2 at 310 K in LB medium in the presence of 100 µg ml^{-1} ampicillin. The cells (30 g) were harvested by centrifugation at 3000*g* at 277 K and were suspended in 200 ml of lysis buffer [50 m*M* Tris–HCl pH 7.6, 200 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, one tablet of protease-inhibitor cocktail (Roche) per 100 ml]. The suspended cells were disrupted by sonication for 3 min in 30 ml portions maintained on ice. The lysate was cleared by centrifugation at 12 000*g* for 2 h and the supernatant was subjected to 20–60% ammonium sulfate saturation. The precipitate was suspended in buffer *P*1 (25 m*M* NaCl, 25 m*M* Tris–HCl pH 7.6) and dialyzed extensively against the same buffer followed by loading onto Q-Sepharose HP (Amersham-Pharmacia; 2.5 × 15 cm). Phosphatase activity was observed in the unbound fractions. The active fractions were pooled, dialyzed against buffer *P*2 (50 m*M* sodium acetate pH 5.3) and loaded onto SP-Sepharose HP (2.5 × 10 cm). The proteins were eluted with a linear gradient from 0 to 500 m*M* NaCl over six column volumes in buffer *P*2 using a Bio-Logic Lp system (Bio-Rad). The PhoN activity eluted at nearly 200 m*M* NaCl concentration. The pooled active fractions were dialyzed against buffer *P*3 (20 m*M* sodium phosphate pH 6.7), loaded onto an Econo-pac CHT-II cartridge (Bio-Rad; hydroxyapatite, 5 ml) and the bound proteins were eluted with a linear gradient from 20 to 500 m*M* sodium phosphate. A single peak of phosphatase activity eluted at 205 m*M* sodium phosphate concentration. The fractions showing phosphatase activity were pooled, mixed with ammonium sulfate (1.5 *M* final concentration) and loaded onto a phenyl-Sepharose HP column (Amersham-Pharmacia). The protein was eluted with a decreasing gradient from 1.5 to 0 *M* ammonium sulfate in buffer *P*4 (50 m*M* sodium phosphate pH 7.0). The PhoN protein eluted at 0.6 *M* ammonium sulfate. SDS–PAGE and isoelectric focusing gel electrophoresis showed that the purified protein was a homogenous preparation with a molecular weight of ∼26 kDa and a pI of ∼9. Determination of the N-terminal amino-acid sequence of the protein using a Shimadzu model PPSQ-10 protein sequencer confirmed the identity of the PhoN protein and further revealed that the 20 amino-acid signal peptide had been processed in the mature protein. The specific activity of the purified protein was very similar to that reported for wild-type protein, indicating proper translocation and folding of the recombinant protein. The protein was usually stored at 277 K during crystallization trials.

4. Crystallization

The protein was dialyzed against buffer *P*4 and concentrated to 14 mg ml^{-1} by ultrafiltration using Centricon-10 (Millipore). Initial screening for crystallization by the hanging-drop vapour-diffusion technique was performed using conditions similar to those of the sparse-matrix method (Jancarik & Kim, 1991). Typically, 2 µl protein solution and 2 µl precipitation solution were mixed on a siliconized cover slip and equilibrated against 500 µl of the same precipitation solution in a reservoir. Crystals of dimensions 450 × 210 × 90 µm were observed within 24 h in condition *S*1 (12% PEG 6000, 5% glycerol). The X-ray diffraction pattern indicated that the crystals were highly mosaic. Crystal nucleation could be retarded with the use of ionic additives such as 50 m*M* Li_2SO_4 or KNO_3, so that crystal seeds only appeared after a week. However, this did not slow crystal growth, as the microseeds grew into large crystals within a day, behaviour similar to that observed in the absence of the additives. The crystals grown in the presence of the ionic additives did not show significant changes in diffraction properties. Further optimization of the crystal-growth conditions with use of different molecular-weight polyethylene glycols, different concentrations of glycerol or the addition of detergents (0.2 m*M* Triton X-100, 1% *N*-octyl glucoside) to the precipitating solution invariably yielded a mixture of diffraction-quality monoclinic and orthorhombic crystals. The crystals used for the final diffraction intensity data acquisition were those harvested using modified *S*1 condi-

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shells.

Crystal	Monoclinic	Orthorhombic
X-ray source	Rotating-anode X-ray generator	Rotating-anode X-ray generator
Detector	R-AXIS IIC	R-AXIS IIC
Temperature (K)	298	298
Wavelength (Å)	1.5418	1.5418
Space group	*C*2	$C222_1$
Unit-cell parameters (Å, °)	*a* = 189.9, *b* = 45.1, *c* = 112.5, *β* = 111.6	*a* = 45.8, *b* = 189.6, *c* = 113.7
Dimers in asymmetric unit	2	1
V_M (Å^3 Da^{-1})	2.15	2.37
Solvent content (%)	42.8	48
No. of crystals used	1	3
Mosaicity (°)	0.5	1.1
No. of measured reflections	38104	25254
No. of unique reflections	27475	11025
Resolution range (Å)	30–2.5	30–2.8
Completeness (%)	90 (90)	88 (81)
Average $I/\sigma(I)$	7.2 (2.9)	7.7 (3.1)
Multiplicity	1.4	2.3
R_{merge}† (%)	8.8 (24.3)	9.0 (22.3)

† $R_{merge} = \sum_{hkl} |I_{hkl} - \langle I_{hkl}\rangle| / \sum_{hkl} I_{hkl}$.

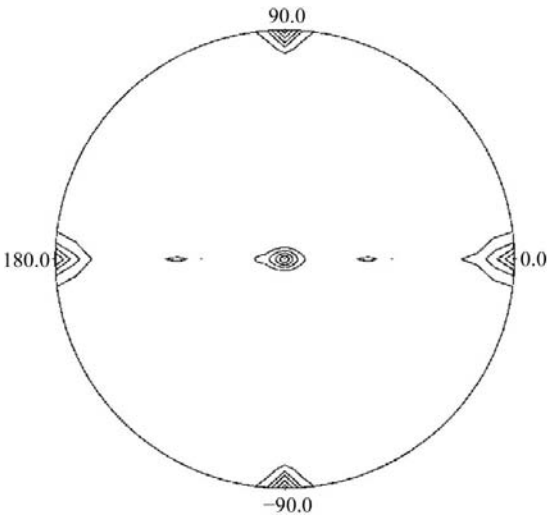

Figure 2
Self-rotation function for the monoclinic (*C*2) data, section $\kappa = 180°$. The ω angle varies in radial direction and the angle φ varies along the circumference. The radius of integration was 23 Å and resolution limits are 20–3 Å. Peaks arising from non-crystallographic symmetry axes are observed at $(\omega, \varphi, \kappa) = (90, 0, 180°)$ and $(\omega, \varphi, \kappa) = (0, 0, 180°)$. The two non-crystallographic axes are thus parallel to the crystallographic *a* and *c* axes, respectively. Contours are drawn in steps of 2σ from a starting level of 5σ above the average rotation-function values.

tions (12% PEG 6000, 2% glycerol). The crystals are expected to be composed of the functionally active form of the protein, as addition of the substrates pNPP or 5-bromo-4-chloro-3-indolyl phosphate (BCIP) turned the thoroughly washed crystals yellow or green, presumably arising from conversion to the respective products.

5. Crystallographic analysis

Single crystals from the optimized *S*1 conditions were soaked for 10 d in 28% PEG 6000 and 5% glycerol before mounting in the Lindemann glass capillary for X-ray diffraction. The diffraction intensity data for the two forms of the crystals grown in the same crystallization drop were collected at room temperature using an R-AXIS IIC imaging plate and Cu $K\alpha$ radiation from a Rigaku rotating-anode X-ray generator equipped with mirror optics. The data were indexed and integrated using *MOSFLM* (Powell, 1999). The integrated data were scaled, merged and truncated using *SCALA* and *TRUNCATE* from *CCP*4 (Collaborative Computational Project, Number 4, 1994) (Table 1). The space groups of the two crystal forms were identified as *C*2 and $C222_1$, with unit-cell parameters a = 189.9, b = 45.1, c = 112.5 Å, β = 111.6° and a = 45.8, b = 189.6, c = 113.7 Å, respectively. One monomer of molecular weight 26 kDa (as deduced from the amino-acid sequence) per asymmetric unit corresponds to Matthews coefficients (V_M; Matthews, 1968) of 8.6 and 4.74 Å^3 Da^{-1}, respectively, for the monoclinic and orthorhombic crystals.

V_M values of 2.87 and 2.15 Å^3 Da^{-1}, corresponding to three or four copies of the monomer in the asymmetric unit of the monoclinic (*C*2) cell, respectively, are in the acceptable range. However, the ambiguity in the number of monomers in the asymmetric unit could be resolved with the self-rotation function. The program *POLARRFN* from the *CCP*4 package was employed to compute the self-rotation functions using a radius of integration of 23 Å and data in the resolution range 20–3 Å. The self-rotation function clearly revealed the presence of twofold non-crystallographic symmetry (NCS) axes parallel to the a and c crystallographic axes of *C*2 (Fig. 2), corresponding to (ω, φ, κ) = (90, 0, 180°) and (ω, φ, κ) = (0, 0, 180°), respectively. The intensity of the NCS peaks with respect to the crystallographic twofold axis was observed to be 93%. The presence of two near-orthogonal twofold non-crystallographic symmetry axes and the absence of internal molecular symmetry in the monomer are in agreement with the presence of a tetramer in the crystallographic asymmetric unit of the *C*2 cell, corresponding to a V_M and solvent content of 2.15 Å^3 Da^{-1} and 42.8%, respectively.

A dimer in the crystallographic asymmetric unit of the orthorhombic ($C222_1$) cell with a reasonable V_M value of 2.37 Å^3 Da^{-1} is in agreement with the fact that the PhoN protein of *S. typhimurium* has been observed as a homodimer in the native form (Kasahara *et al.*, 1991), in contrast to the hexameric form reported for the Pho protein of *E. blattae* (Ishikawa *et al.*, 2000). The self-rotation function using data in the resolution range 20–3 Å did not show any identifiable peaks representing non-crystallographic symmetry.

In summary, we have obtained crystals of recombinant PhoN protein of *S. typhimurium*. The two forms of the crystals harvested from the same crystallization condition diffract to better than 2.8 Å resolution using a laboratory X-ray source. The asymmetric units of the monoclinic and orthorhombic cells are expected to contain four and two copies of the monomer, respectively. The structural studies of the protein have been initiated with the aim of engineering the protein for bioremediation properties suitable for treating nuclear waste under highly acidic pH (∼2.5) conditions. A combination of directed evolution based on random mutagenesis, DNA shuffling and selection, and structure/function-based site-directed mutagenesis strategies has recently been advocated for engineering proteins with improved properties (Bornscheuer & Pohl, 2001). Successful combination of both the approaches has also been demonstrated in the enhancement of nucleoside phosphorylation activity of the homologous acid phosphatase (Pho) from *E. blattae* (Ishikawa *et al.*, 2002). A similar approach to obtain a protein with efficient organophosphatase activity should be useful for bioremediation at low pH.

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