PrpE, a PPP protein phosphatase from *Bacillus subtilis* with unusual substrate specificity

Adam IWANICKI*, Anna HERMAN-ANTOSIEWICZ*, Marcin PIERECHOD*, Simone J. SÉROR† and Michał OBUCHOWSKI*¹

*Department of Molecular Biology, University of Gdańsk, ul. Kładki 24, 80-822 Gdańsk, Poland, and †Institut de Génétique et Microbiologie, UMR CNRS 8621, Université Paris-Sud, Bât. 409, 91405 Orsay cedex, France

Bacillus subtilis is a Gram-positive bacterium with a relatively large number of protein phosphatases. Previous studies have shown that some Ser/Thr phosphatases play an important role in the life cycle of this bacterium [Losick and Stragier (1992) *Nature* (London) **355**, 601–604; Yang, Kang, Brody and Price (1996) *Genes Dev.* **10**, 2265–2275]. In this paper, we report the biochemical properties of a putative, previously uncharacterized phosphatase, PrpE, belonging to the PPP family. This enzyme shares homology with other PPP phosphatases as well as with symmetrical diadenosine tetraphosphatases related to ApaH (symmetrical Ap₄A hydrolase) from *Escherichia coli*.

A His-tagged recombinant PrpE was purified from *E. coli* and shown to have Ni²⁺-dependent and okadaic acid-resistant phosphatase activity against a synthetic phosphorylated peptide and hydrolase activity against diadenosine 5',5'''-tetraphosphate. Unexpectedly, PrpE was able to remove phosphate from phosphotyrosine, but not from phosphothreonine or phosphoserine.

Key words: diadenosine tetraphosphate, diadenosine tetraphosphate hydrolase, Gram-positive bacteria, protein phosphorylation.

INTRODUCTION

Protein phosphorylation or dephosphorylation is a prominent mechanism for signal transmission found in all living organisms. For many years, the nature of protein kinases and phosphatases was thought to be different in prokaryotes and eukaryotes. Phosphorylation of prokaryotic proteins was shown to occur mainly on histidine and aspartic acid residues while, in contrast, eukaryotic proteins are usually modified on serine, threonine or tyrosine residues. However, eukaryotic-like protein kinases and phosphatases have been reported in bacteria in recent years, and conversely homologues of prokaryotic His/Asp enzymes have been discovered in eukaryotes (for reviews, see [1–7]). These studies suggest that eukaryotes and prokaryotes may share similar mechanisms for all types of signal transduction.

Protein phosphatases can be divided into two groups based on their enzymic specificity, namely Ser/Thr phosphatases and Tyr phosphatases [8,9]. The Ser/Thr phosphatases show wide specificities *in vitro* and have been grouped into four classes: PP1, PP2A, PP2B and PP2C, according to the conserved motifs, their sensitivity to different inhibitors and ion requirements [9–11]. Amino acid sequence comparisons indicated that PP1, PP2A and PP2B are members of the same PPP family [10]. The PPP family represents the largest source of protein Ser/Thr phosphatase activity in higher eukaryotes [12]. These enzymes also share sequence similarity with the symmetrical diadenosine tetraphosphatases [13]. The first prokaryotic member of the PPP family to be identified was the product of the open reading frame 221 of bacteriophage λ [14]. Currently, several members have been characterized in detail in both archaea and bacteria [15–19]. However, little data are available concerning the physiological

role of the prokaryotic PPPs. The best investigated examples of bacterial PPP phosphatases with a known physiological function come from *Escherichia coli* and *Myxococcus xanthus*. In *E. coli*, genetic experiments revealed that the PrpA and PrpB phosphatases participate in a signal transduction pathway that senses protein misfolding caused by heat shock or other stresses [18]. It was also demonstrated that the *M. xanthus* Pph1 phosphatase is involved in aggregation and formation of fruiting bodies in response to starvation conditions [20].

Dinucleoside 5',5'''-polyphosphate molecules (Np_nN; where N = A, G, C or U) were discovered over 30 years ago. This kind of nucleotide is produced in cells by several enzymes, including the aminoacyl-tRNA synthetases [21]. Currently, 14 types of dinucleoside polyphosphate are known; however, their molecular targets and their biological roles still remain unclear [22]. It has been postulated that adenosine dinucleoside 5',5'''-polyphosphate (Ap_nN; where N = G, C or U) can act as a signalling molecule generated during, for example, heat or oxidation shock response [23], during the cell cycle to control division [24] or simply as a waste product of cellular metabolism [25]. Whatever their role, it is likely that the levels of Ap_nN nucleotides are carefully regulated in relation to different cellular processes [26]. Therefore, it is not surprising that enzymes that are involved in degradation of these nucleotides are present in all living organisms from bacteria and archaeobacteria to humans [27]. Ap₄A hydrolases can be divided into two distinct families: 'nudix' asymmetrical hydrolases and symmetrical hydrolases related to the ApaH (symmetrical Ap₄A hydrolase) protein from *E. coli*. Only the second group of hydrolases shares sequence similarity with PPP phosphatases and only this group will be discussed here.

Abbreviations used: Ap₄A, diadenosine 5',5'''-tetraphosphate; Ap_nN, adenosine dinucleoside 5',5'''-polyphosphate where N = G, C or U; IPTG, isopropyl β -D-thiogalactoside; Np_nN, dinucleoside 5',5'''-polyphosphate where N = A, G, C or U; pNpp, *p*-nitrophenyl phosphate; RT-PCR, reverse transcriptase PCR.

¹ To whom correspondence should be addressed (e-mail obuchowk@biotech.univ.gda.pl).

In this paper we present the properties of an uncharacterized member of the PPP family in *Bacillus subtilis*, which we now designate PrpE (protein phosphatase E). Contrary to other PPP members, PrpE possesses unusual substrate specificity, since it releases inorganic phosphate from phosphotyrosine residues but not from phosphoserine or phosphothreonine residues. In addition, PrpE hydrolyses diadenosine 5',5'''-tetraphosphate (Ap₄A) asymmetrically and also shows ATPase activity.

MATERIALS AND METHODS

Bacterial strains and plasmids, and cloning of the *prpE* gene

Bacterial strains and plasmids are listed in Table 1.

For the cloning of the *prpE* gene, chromosomal DNA from the wild-type *B. subtilis* strain W168 was used as a template in a PCR reaction (primer 1, 5'-CATGATTCTCTCCCTGCAGATATGGGGCG-3'; primer 2, 5'-GAGGAGCATGCATGGCTTACGATATCATCAGC-3'; restriction sites for *Pst*I and *Sph*I are in bold). The PCR reaction was performed using *Pwo* polymerase (Roche). The amplified fragment was digested with restriction enzymes *Pst*I and *Sph*I and inserted into the corresponding sites of the pQE100 vector (Qiagen). The resulting plasmid, encoding an in-frame His-tag at the N-terminal end and the Tag-100 antigenic epitope at the C-terminal end of the *prpE* gene, was named pMO38. Subsequent sequencing of the construct confirmed the wild-type nucleotide sequence of the inserted *prpE* gene plus the inserted tags.

Expression and purification of the recombinant PrpE protein

The pMO38 plasmid was used to transform competent cells of the *E. coli* JM109 *lacI*^r strain. Expression of recombinant protein in JM109 strain was tested at different isopropyl β -D-thiogalactoside (IPTG) concentrations and temperatures. No inclusion bodies were observed. Maximal expression was at 37 °C with 1 mM IPTG. Transformants harbouring pMO38 were cultivated in LB medium at 37 °C to an attenuation (575 nm) of about 0.3. Overproduction of recombinant protein was initiated by the addition of IPTG to a final concentration of 1 mM, and incubation was continued for another 3 h. High levels of a 32 kDa protein were observed when the total cellular extract was analysed by SDS/PAGE and Coomassie Blue staining. The theoretical mass of the recombinant PrpE protein was predicted

as 30.9 kDa, which was in good agreement with the position of the observed band. For the purification of PrpE, induced cells were harvested by centrifugation, resuspended in 1/100 vol. of the starting vol. of buffer A (30 mM Hepes, pH 8.2, 300 mM NaCl and 10 mM imidazole) containing a protease inhibitor cocktail (Roche), and then lysozyme was added to a final concentration of 1 mg/ml; the suspension was incubated for 30 min in ice. Cells were disrupted by sonication (four times for 35 s each, with 40 W). Cell debris were removed by centrifugation (4 °C, 35000 g, 1 h) and a cell-free supernatant was loaded on to a column containing Ni-NTA Superflow Agarose (Qiagen). After washing, the protein was eluted with a linear gradient of imidazole (10–125 mM in five column vol.) as a single band. Protein from this band was concentrated and stored at –70 °C in a buffer containing 25 mM Hepes, pH 8.0, 50 mM NaCl, 0.5 mM dithiothreitol, 0.05 mM EDTA, 0.5 % Triton X-100 and 25 % glycerol. Under these conditions about 4 mg of the pure protein were obtained from 1 litre of bacterial culture.

Phosphatase activity assay

The protein phosphatase activity was tested by the ability to hydrolyse *p*-nitrophenyl phosphate (pNpp) in a buffer containing 50 mM Tris/HCl (pH 7.2), 1 mM Ni²⁺ and 10 mM pNpp at 37 °C. Phosphatase activity was determined by measurement of *p*-nitrophenol in a spectrophotometer at a wavelength of 605 nm. When phosphorylated peptides were used, the amount of released inorganic phosphate was determined by the Malachite Green method, according to the manufacturer's protocols [using the protein tyrosine phosphatase assay kit (Sigma catalogue no. PTP-101) and the serine/threonine phosphatase assay system (Promega catalogue no. V2460)].

Diadenosine polyphosphate hydrolase activity

Diadenosine oligophosphate hydrolase activity was measured as described previously [17]. Briefly, PrpE was incubated for 10 min at 37 °C in a buffer containing 1 mM diadenosine 5',5'''-polyphosphate and 3 units of alkaline phosphatase (Roche) in a total volume of 50 μ l. The reaction was stopped by addition of 50 μ l of Malachite Green solution.

Table 1 Bacterial strains and plasmid used in this study

For plasmid p-PrpE, the 400^A position is relative to the first codon of the *prpE*s gene.

Strain/plasmid	Genotype/description	Reference/origin
<i>B. subtilis</i> W168	<i>trpC</i>	Bacillus Genetic Stock Center [45]
<i>B. subtilis</i> MO1813	<i>trpC pheA1 amyE::cat</i>	Bacillus Genetic Stock Center [28]
<i>B. subtilis</i> β 17	W168 Δ <i>prpE</i>	This study
<i>B. subtilis</i> β 18	W168 Δ <i>prpE amyE::pPrpE</i>	This study
<i>B. subtilis</i> β 19	MO1813 <i>amyE::pPrpE</i>	This study
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)</i>	[46]
<i>E. coli</i> DH5 α	<i>supE44 ΔlacU169 (q80/lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	[47]
pQE100	<i>ori ColE1 bla</i>	Purchased from Qiagen
pMO38	<i>ori ColE1 bla prpE</i>	This study
pDG1728	Promotes ectopic integration into <i>amyE</i> locus	Bacillus Genetic Stock Center [28]
p-pPrpE	<i>B. subtilis</i> chromosome fragment –150 to +400 ^A inserted in front of <i>lacZ</i> in pDG1728	This study
pMTL20E	For gene inactivation by homologous recombination	From S. J. S��r (Universit�� Paris-Sud, Orsay, France)
pAHA1	pMTL20E with insertion of 1984 bp containing <i>yjbO'-yjbP-yjbQ'</i> genes	This study
pAHA2	pAHA1 with replacement of the <i>PvuII-EcoRV</i> fragment by <i>spcR</i> from pDG1728	This study

As a method of quantitative analysis for degradation products of Ap₄A and other nucleotides, anion-exchange chromatography was used. Enzymic assays were performed in a final volume of 200 μ l with appropriate ions and substrates. After 30 min incubation at 37 °C, the reaction was stopped on ice. The whole sample was loaded on to an HPLC MonoQ HR5/5 column (Amersham Bioscience). Hydrolysis products were eluted by a linear gradient of NaCl at pH 8.0. Details of chromatography were as follows: flow, 1 ml/min; sample binding and initial column washing with 2 column vol. of buffer A (20 mM Tris/HCl, pH 8.0); elution with a gradient of NaCl from 0 to 350 mM in 15 column vol., then from 350 mM to 1 M in 2 column vol. and finally washing with 1 M NaCl for 2 column vol. Results were analysed using Millennium software (Waters).

Construction of a *prpE* promoter fusion

In order to construct a transcriptional fusion of *prpE* with *lacZ*, two primers were designed: forward primer, 5'-CATAAGGAT-CCGCGCCTTTTCGTAAG-3'; reverse primer, 5'-AATCCG-AGAATTCAGAGTGTCTGAAGCC-3' (restriction sites for *Bam*HI and *Eco*RI are in bold). The amplified fragment contained in a region -440 bp to +250 bp (the first nucleotide of the ATG codon was considered to be +1) was cloned into the pDG1728 vector. The resulting plasmid, named pMO39, was linearized and integrated into the appropriate *B. subtilis* strain by a double cross-over event [28].

Construction of the Δ *prpE* strain

For the construction of the *yjbP* deletion strain, a fragment of the *B. subtilis* W168 chromosome was amplified using the following primers: 5'-AACCTGCAGCCGTATGTGTCATCA-ATCCC-3', which contains a sequence for a *Pst*I site (bold), and 5'-GGAGAATTCGGCCAAGACGAACCCATAG-3', which contains a sequence for an *Eco*RI site (bold). The product, 1984 bp in length, containing the *yjbO'*-*yjbP*-*yjbQ'* genes, was digested with *Pst*I and *Eco*RI enzymes and ligated into the vector pMTL20E digested with the same enzymes. The construct obtained was designated pAHA1. The *Eco*RV-*Eco*RV fragment (426 bp) of the *yjbP* gene was removed from pAHA1 and replaced with a *Pvu*II-*Eco*RV fragment of the pDG1728 vector containing the *spc*^R gene (1374 bp), thus giving the construct pAHA2. The *B. subtilis* W168 strain was transformed with a linearized recombinant pAHA2 plasmid, with screening for spectinomycin resistance, requiring a double cross-over. PCR and Southern blotting were performed to verify the authenticity of the final construct.

RNA isolation and reverse transcriptase PCR (RT-PCR)

Isolation of total RNA was performed using a modified hot phenol method described previously [29]. Cultures were grown in appropriate medium and cells were harvested at mid-logarithmic phase, centrifuged and the pellet suspended in 4 ml of 65 °C hot TE buffer (10 mM Tris/HCl, pH 8.0/1 mM EDTA). Then 3.5 g of glass beads (125–250 μ m) and 4 ml of hot phenol (65 °C, pH 4.8) were added. The mixture was incubated for 2 min at 65 °C, vortexed for 2 min and cooled on ice for 3 min. After cooling, the mixture was centrifuged and the aqueous phase collected. Three successive extractions were made: 1 vol. of phenol, 1 vol. of phenol/chloroform (1:1, v/v) and 1 vol. of chloroform (each for 1 min, with 1 min on ice in between). After each extraction, the mixture was centrifuged and the aqueous phase was collected. Finally, a 1/10 vol. of the starting vol. of 9 M LiCl and 3.5 vol. of cold 95% ethanol were added

to the aqueous phase. RNA was pelleted by centrifugation for 30 min, washed with cold 80% ethanol and dried in a vacuum dryer. The dry RNA pellet was dissolved before use in 20 μ l of RNase-free water.

RT-PCR experiments were performed as follows. Total RNA (100 ng) was extracted from exponential- or stationary-phase cultures growing in minimal or rich media. This was treated with RNase-free DNase I (Roche), then the DNase was thermally inactivated. The preparation was then subjected to RT-PCR (Access System; Promega), according to the protocol of the manufacturer. The products of reaction were analysed in agarose gels and quantified using a Fluoro-S MultiImager (Bio-Rad).

Analysis of origin of *prpE*

The diversity factor (D) was calculated as the product of multiplication of differences in codon usage (MCU) and differences in GC content (MGC) of specific genes. MCU and MGC were calculated using the method described previously [30]. Codon usage for genes was calculated with the Codon Frequency program from the GCG Wisconsin Package.

RESULTS AND DISCUSSION

Sequence analysis indicates that PrpE belongs to the PPP protein phosphatase family

Analysis of the amino acid sequence of YjbP from *B. subtilis*, deduced from the nucleotide sequence of the *yjbP* gene (GenBank accession number Z99110), revealed similarity with PPP protein phosphatases and diadenosine tetraphosphatases. We therefore designated the *yjbP* gene *prpE*. PrpE displays 25% identity with the diadenosine tetraphosphatase from *E. coli*, ApaH [31], 20% with the protein phosphatase from bacteriophage λ and 17% with the human phosphatase PPI [32] (Figure 1). *prpE* is apparently transcribed as a single gene at 106° on the *B. subtilis* chromosome. No paralogues have been found in this bacterium. In fact, this type of enzyme is widespread in all living organisms. The catalytic domain for the phosphatase and diadenosine tetraphosphatase has three conserved motifs [6,13,33]. The results from comparing residues in these motifs between PrpE and other PPP enzymes using three different consensus sequences, proposed previously, indicated that the motifs in PrpE were completely conserved or had a single change (Table 2). However, the changed residue is not normally critical for enzymic activity of the PPP family [33]. The presence of these invariant motifs strongly suggested that PrpE belongs to the PPP family.

Expression and purification of PrpE from *E. coli*

Overproduction of the His-tagged PrpE fusion protein was observed after 1 h at 37 °C on SDS/PAGE gels stained by Coomassie Blue (results not shown). All the protein appeared to be soluble. The level of overproduction was dependent on both the concentration of the inducer (IPTG) and the temperature. For purposes of purification, cells were grown for 3 h after induction and the protein was purified by single-step immobilized-metal-ion affinity chromatography. Western blotting with the Tag-100 antibody (Qiagen) confirmed the identity of the overproduced and purified protein (results not shown).

Phosphatase activity of PrpE

Since the sequence of PrpE indicated a relationship with PPP enzymes, a possible enzymic activity with regard to substrates that can be dephosphorylated by PPPs was examined. First, the

PP1- α	MADGELNVDSLITRLLEVRGCRPGKIVQMTEAEVRGLCIKSREIFLSQPILLELEAPLKI
orf-221	-----MRYYEKIDGSK-----YRNIWV
ApaH	-----MATYL
PrpE	-----MAYDI
PP1- α	CGDIHG QYTDLLRLFEYGGFPPEA-----NYLF- LGDYVDRG KQSLETICLLLAYKI
orf-221	VGDLHGC YTNLMNKLDTIGFDNKK-----DLLIS VGDLVDRG AEENVECLELITFP--
ApaH	IGDVHGC YDELIALLLHKVEFTPGK-----DTLWL TGDLVARG PGSLDVLRYVKSGL-
PrpE	ISDIHG CYDEMTALIQKLGTYTIKNGVPVHEEGRVLV FAGDLTDRG PKSIEVIRFVAGAYE
	motif I motif II
PP1- α	KYPENFFLLR GNHE CASINRIYGF-----YDECKRRFNIKLWKTFDTCFNCLPIAAI
orf-221	----W FRAVRGNHE QMIDGLS-----ERGNVNHWLLN-----
ApaH	---DSVRLVL GNHDL LLAVFAGISRNKPKDRLT PLLEAPDA DELLNWLRRQPL LQIDEE
PrpE	KG--AVRYVP GNHC NKLYRYLKGN-----PVKVMHGLET-----
	motif III
PP1- α	VDEKIFCCHGGLSPDLQSMEQIRRMPTD-VPDTGLLCDLLWSDPKDQVQGWGENDRGV
orf-221	-----GGGWFFNLDYKEILAKALAHK-ADELPLI IELVSKD -KKYVICHAD----
ApaH	KKLVMAHAGIT PQWDLQ TAKECARDVEAVLSSDSYPFFLDAMYGDMPNNWSPEL RGLGRL
PrpE	-----TAAELEELS KDEK SVSEQFMKLYETAPLYDILHNGELVVAHAGIRADDIG
PP1- α	SFTFGADVSVSKFLNRHDL DLICRAH QVVED-----GYEFFAKRQLV TLFSAP NYCGE
orf-221	-YPFDEYEF GKPDH QVQVWNRERISNSQN-----GIVKEIKGADTF IFGHTPA VKP
ApaH	RFTITNA FTRM RF CF PNGQLDMYSKESPEEAPAPLKPWF AI PGPVAEEYS IAFGH WASLEG
PrpE	KYTRRVKDFVLYGDVTG--ETYPDGRPIRR-----DWAAAYNGKAWV YGH TPVKEP
PP1- α	FDNAGGMSVDE ETLMCS FQILKPSEKKAKYQYGG LN SGRPVTP PPRTAN PPKKR
orf-221	LKFANQMYIDTGA VFC GNLTL IQVQ GEGA-----
ApaH	KGT PEGI YALDTGCCWGGTL TCLRWED KQYFVQPSNR HKDLGE AAAS-----
PrpE	RK-VNRTINIDTGC VFG NQLTGFR FPEI ETVSV PSSLPY DES RF PI-----

Figure 1 Comparison of the amino acid sequences of the PPP protein phosphatase family and symmetrical diadenosine tetraphosphatases with PrpE

The conserved motifs are in bold. Abbreviations: PP1- α , human protein phosphatase 1 (isoform α); orf 221, protein phosphatase from bacteriophage λ ; ApaH, Ap₄A hydrolase from *Escherichia coli*; PrpE, protein phosphatase E from *B. subtilis*. All sequences were obtained from the European Institute of Bioinformatics.

Table 2 Comparison of characteristic motifs of PPP protein phosphatases

Consensus proposed in different studies	Conserved motifs		
	I	II	III
[13]	GD(I/V/T/L)HG	GD(L/Y/F)V(D/A)RG	GNH
[48]	GD X HG	GD XV XRG	GNH
[33]	DXH	GD XXD	GNH(D/E)
PrpE [49]	SD I HG	GD L TDRG	GNHC

purified protein was assayed for its ability to cleave pNpp. PrpE hydrolysed this substrate efficiently, with an optimum at pH 7.2 at 37 °C in 50 mM Tris/HCl. As in the case of other PPPs, the enzyme displayed dependence on bivalent metal ions. In the case of PrpE, the best activity is achieved in the presence of an Ni²⁺ ion with an optimum concentration of 1 mM for pNpp hydrolysis. PrpE also displayed a 100-fold lower phosphatase activity with Mn²⁺ relative to its activity with Ni²⁺. Several other ions or combinations of ions were tested but the highest activity was observed only in the presence of Ni²⁺ (Table 3). An Ni²⁺ ion requirement is not very common, but recently the existence of several enzymes requiring nickel for activity has been reported; for example, urease, hydrogenase, CO-dehydrogenase, CO-dehydrogenase (CODH)/acetyl-CoA synthase, methyl-2-mercaptoethane-sulphonate (CoM) reductase, superoxide dismutase and glyoxalase I [34]. The utilization of such ions by

Table 3 PrpE phosphatase activity is stimulated or inhibited by different metal ions

Activity in the presence of 1 mM Ni²⁺ was taken as 100 (arbitrary units). All assays were performed at 37 °C in the presence of 10 mM pNpp. Reactions were stopped on ice and the absorbance at 405 nm was measured. All ions were tested at 1 mM.

Phosphatase activity (arbitrary units)	
Ion	
Mn ²⁺	1
Zn ²⁺	0
Cu ²⁺	0
Co ²⁺	0
Ca ²⁺	0
Ni ²⁺	100
Hg ²⁺	0
Cr ²⁺	0
Mg ²⁺	0
Ni ²⁺ , Mn ²⁺	25
Ni ²⁺ , Zn ²⁺	0
Ni ²⁺ , Cu ²⁺	0
Ni ²⁺ , Co ²⁺	0
Ni ²⁺ , Ca ²⁺	73
Ni ²⁺ , Mg ²⁺	100

protein phosphatases was also reported earlier by Zhuo et al. [33]. The average concentrations of Ni²⁺ in sea water, fresh water and soil are 30 nM, 5 nM and 16 μ g/g, respectively [35,36]. Bacteria have several systems for the specific uptake of nickel from the environment [35,37], and in bacterial cells

Table 4 Effects of potential inhibitors on the catalytic activity of PrpE

All assays were performed at pH 7.2, 1 mM Ni^{2+} , 10 mM pNpp and 37 °C. Activity without any additions was taken as 100 (arbitrary units).

Addition	Concentration	Protein phosphatase activity (arbitrary units)
EDTA	1 mM	0
NaCl	1 M	41
KCl	1 M	73
BSA	1 mg/ml	98
NaF	50 mM	46
Ammonium molybdate	50 μM	0
β -Glycerol phosphate	20 mM	36
Na_2PO_4	20 mM	2
<i>N</i> -ethylmaleimide	5 mM	67
Iodoacetic acid	10 mM	32
Okadaic acid	1 μM	97
Vanadate	100 μM	103
Hg^{2+}	10 μM	0

the Ni^{2+} concentration is higher, in the μM range [38]; however, proteins sequester most of the intracellular nickel.

The properties of PrpE as a PPP protein phosphatase were confirmed by analysing the effects of several protein phosphatase inhibitors. Sodium orthovanadate and okadaic acid did not inhibit phosphatase activity, in contrast with mercuric ions, ammonium molybdate, iodoacetic acid and EDTA, which strongly inhibited activity of this enzyme (Table 4). The presence of salt (NaCl or KCl up to 0.5 M) and BSA (up to 1 mg/ml) revealed no effect on the PrpE phosphatase activity.

Diadenosine tetraphosphatase activity of PrpE

Analysis of the amino acid sequence of PrpE shows 25 % identity with the symmetrical Ap_4A hydrolase from *E. coli*. Consequently, we analysed the enzymic activity of the tagged PrpE fusion protein with different diadenosine polyphosphates. First, using the assay buffer conditions described previously [17], we found that PrpE efficiently hydrolysed Ap_4A but not Ap_2A or Ap_6A . In addition, Ap_3A and Ap_5A were also poor substrates. To analyse the nature of Ap_4A hydrolysis catalysed by PrpE, we examined the degradation products by ion-exchange chromatography. Figure 2(A) shows that PrpE is an asymmetrical hydrolase of Ap_4A , producing both AMP and ATP. Intriguingly, between the AMP and ATP peaks, we detected a small peak of ADP, suggesting that the enzyme might have an ATPase activity (Figure 2A). Therefore, we performed another assay in which ATP was used as a substrate instead of Ap_4A . The results presented in Figure 2(B) indicate that PrpE does indeed have ATPase activity. The kinetic parameters of these activities are presented in Table 5. Importantly, both activities (Ap_4A hydrolase and ATPase) were dependent on Ni^{2+} (the optimal Ni^{2+} concentration for hydrolysis of Ap_4A was about 250 μM , with about 100 μM for ATP). In contrast, both activities were absent in the presence of Mg^{2+} or Mn^{2+} . Ap_4A and ATP are competitive substrates since an excess of ATP inhibited Ap_4A hydrolysis (results not shown). Specific ion dependence is a rule for dinucleoside polyphosphate hydrolases but different enzymes can prefer different ions. The most common is Mg^{2+} but other ions, like Mn^{2+} , Ca^{2+} and Zn^{2+} , are also used. Utilization of nickel ions was reported previously for nucleoside 5'-tetraphosphate hydrolases [39]. The Ap_4A hydrolase activity of PrpE was not very surprising, since PPP protein phosphatases and symmetrical diadenosine tetraphosphatases related to the *E. coli*

Figure 2 Ion-exchange chromatography of hydrolysis products of Ap_4A (A) and ATP (B)

PrpE protein (0.4 μg) was incubated in buffer containing of 20 mM Tris/HCl, pH 8.0/1 mM NiCl_2 plus 1 mM Ap_4A (A) or 1 mM ATP (B) for 15 min at 37 °C. After this time 200 μl of the sample was loaded on to the column. After washing, a linear gradient of NaCl (dashed line) was applied as described in the Materials and methods section. Control without enzyme is shown as a fine line. Peaks were identified by comparison of elution time with standards (mixture of 1 mM AMP, ADP, ATP and Ap_4A ; all from Sigma).

Table 5 Substrates of PrpE

EGF, epidermal growth factor; pY, phosphotyrosine; pT, phosphothreonine; pS, phosphoserine; ND, the hydrolysis of the substrate was not detectable.

Substrate	Substrate type	Biochemical parameters
TRDipYETDYYRK	Peptide; insulin receptor fragment	K_m , $76.5 \pm 1.4 \mu\text{M}$; V_{max} , $8.7 \pm 0.2 \mu\text{mol/min per mg}$; k_{cat} , $2.8 \pm 0.1 \text{ s}^{-1}$
DADEpYLIQQG	Peptide; EGF receptor fragment	K_m , $2.7 \pm 0.2 \mu\text{M}$; V_{max} , $0.8 \pm 0.1 \mu\text{mol/min per mg}$; k_{cat} , $0.3 \pm 0.1 \text{ s}^{-1}$
RRApTVA	Synthetic peptide	ND
GTlpSKIF	Peptide; myelin basic protein	ND
RTTHYGpSLPQK	Peptide; myelin basic protein	ND
Ap ₄ A	Nucleotide	K_m , $6.3 \pm 0.1 \mu\text{M}$; V_{max} , $256 \pm 9 \mu\text{mol/min per mg}$; k_{cat} , $83.4 \pm 1.7 \text{ s}^{-1}$
ATP	Nucleotide	K_m , $47.5 \pm 0.7 \mu\text{M}$; V_{max} , $185 \pm 7.1 \mu\text{mol/min per mg}$; k_{cat} , $60.2 \pm 3.2 \text{ s}^{-1}$

Figure 3 Diversity factor (D) values for the genes surrounding *prpE* (*yjbP*)

The region analysed extends from 1 229 228 to 1 248 657 bp on the *B. subtilis* chromosome. The D value for the whole region is marked as region. The codon usage value and the GC content for the whole *B. subtilis* genome were taken from Nakamura et al. [30]. The higher the D value the greater the probability that a particular gene was horizontally transferred from other species. The D values for *prpE*, the region surrounding *prpE*, *E. coli* *apaH* and human *pp1* are 0.0011, 0.0001, 0.0263 and 0.0982, respectively.

ApaH enzyme, which is the only described example of this type of activity, share the same conserved sequence features [13].

Substrate specificity of PrpE

Since the analysis of the amino acid sequence of PrpE, deduced from the nucleotide sequence of the *yjbP* gene, revealed similarity to other PPP protein phosphatases, it was of interest to analyse the enzymic activity of the PrpE fusion protein with regard to dephosphorylatable substrates. PrpE was therefore assayed for its ability to dephosphorylate individual phosphoamino acids and peptides. The results show that PrpE was not able to release inorganic phosphate from a single phosphoamino acid (results not shown) but did quantitatively release inorganic phosphate from phosphorylated peptides. The kinetic parameters for the phosphatase activity of PrpE are presented in Table 5. Unexpectedly, PrpE was able to release phosphate from phosphotyrosine but it was not able to dephosphorylate phosphothreonine or phosphoserine (Table 5). It was reported earlier that PPP phosphatases could release phosphate from tyrosine residues [10,18], but the inability to dephosphorylate phosphoserine and phosphothreonine residues is unusual. One might speculate that this effect could be provoked by the peptides themselves because peptide chains are much more flexible than protein molecules. Dephosphorylation of the peptides by PrpE was also Ni²⁺-dependent, with the optimal concentration of Ni²⁺ ions being about 25 μM . Phosphatase activity of PrpE was not observed in the presence of ions such as Mg²⁺ or Ca²⁺. In the presence of

Mn²⁺ only very low activity against the peptide from the epidermal growth factor receptor was observed.

Expression and origin of the *prpE* gene

To investigate the possible cellular role of the PrpE phosphatase, we first looked for a corresponding transcript using a pair of primers and RT-PCR. As a control for reaction specificity, we used a strain that was deleted for the *prpE* (*yjbP*) gene. The results indicated that in all conditions tested we were not able to detect the corresponding transcript in the wild-type strain, while we did detect a transcript of *yjbO* which was used as a positive control (results not shown). Similarly, in a strain carrying the putative promoter of *prpE* fused to *lacZ*, located in the non-essential *amyE* locus, no expression of β -galactosidase was detected. In order to eliminate the hypothesis that the *prpE* gene is duplicated in the genome, we performed a BLAST analysis of the *B. subtilis* chromosome but no homologues of *prpE* were detected. We also tested the possibility, using the method of Han and Zhang [40] with modifications, whether *prpE* might be horizontally acquired from another species. Data presented in Figure 3 strongly suggest that *prpE* is probably native to *B. subtilis*. The phenotype of the strain deleted for *prpE* was also examined with respect to several properties, including growth rate, sporulation efficiency, sensitivity to heat or oxidative shock, response to heavy metal ions, nucleoid organization and segregation. However, the strain Δ *prpE* did not show any significant differences in comparison with the wild-type strain W168 under all conditions tested (results not shown).

Conclusions

The product of the *yjbP* gene, designated in this paper as PrpE, shows several features indicating that PrpE is closely related to the PPP phosphatase family, as well as to Ap₄A hydrolases. The substrate specificity of PrpE in comparison with other PPP family members appears intriguing and should be confirmed with an endogenous substrate. On the basis of the present study, PrpE is the first PPP family member to be characterized that possesses specificity only for phosphotyrosine. PrpE also shows sequence similarity to the *E. coli* symmetrical diadenosine tetraphosphatase; however, the mode of cleavage by the enzyme is different (asymmetrical). Such a distinct mode of cleavage may reflect differences in the primary sequence, since PrpE and ApaH share only 30% identity. All the measured activities of PrpE were ion-dependent. However, the ion preference of PrpE is rather unusual, since few examples of enzymes utilizing Ni²⁺ have been reported so far. Transition metals such as nickel are essential for many metabolic processes and homeostatic control of their cellular concentration is crucial for life. Fluctuations in the intracellular concentration of such metal ions may lead to cell death and/or severe diseases such as human haemochromatosis [41]. Nevertheless, some types of algae, like *Chlorella emersonii* [42] or *Oscillatoria* species [43], require nickel for growth. Moreover, this ion may play a role in the biosynthesis or stabilization of the cell wall in *Methanobacterium bryantii*. Another bacterium, *Methanospirillum hungatei*, contains significant amounts of this ion in the cell membrane [36], and it was also reported that a nickel-containing pigment is involved in endospore formation in *Bacillus cereus* [44]. If we extrapolate the optimal ion concentration required for activity *in vitro* to the *in vivo* situation, this may indicate that *in vivo* PrpE displays protein phosphatase features rather than ATPase or Ap₄A hydrolase activities. The significance of these latter activities and their possible role in cell metabolism remains to be elucidated. The fact that the strain Δ *prpE* is able to grow indicates that the gene is not essential, at least under laboratory conditions. However, since under several laboratory conditions we were unable to detect RNA corresponding to *prpE*, we speculate that the expression of *prpE* may be restricted to certain specific environmental conditions.

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