The Chryseobacterium meningosepticum PafA enzyme: prototype of a new enzyme family of prokaryotic phosphate-irrepressible alkaline phosphatases?

Francesca Berlutti,1 Claudio Passariello,1 Laura Selan,1 Maria Cristina Thaller2 and Gian Maria Rossolini3

Author for correspondence: Francesca Berlutti. Tel: +39 06 4451324. Fax: +39 06 49914638. e-mail: francesca.berlutti@mail.uniroma1.it

1 Dipartimento di Scienze di Sanità Pubblica, Università di Roma ‘La Sapienza’, Piazzale A. Moro, 5, 00185 Rome, Italy
2 Dipartimento di Biologia, Università di Roma ‘Tor Vergata’, 00133 Rome, Italy
3 Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, 53100 Siena, Italy

Chryseobacterium meningosepticum is an aerobic Gram-negative rod widely distributed in natural environments. Unlike many bacteria, it produces a phosphate-irrepressible periplasmic alkaline phosphatase (AP). This work describes cloning of the gene encoding that enzyme from C. meningosepticum CCUG 4310 (NCTC 10585), and preliminary characterization of its product. The gene, named pafA, encodes a protein (PafA) of 546 amino acids with a calculated molecular mass of the mature peptide of 58682 Da. PafA exhibits high sequence identity with the PhoV AP of Synechococcus PCC 7942 (49% identity) and with the Cda Ca2+-dependent ATPase of Myroides odoratus (51% identity), while being more distantly related to the PhoD AP of Zymomonas mobilis (22% identity) and to the PhoA AP of Escherichia coli (14% identity). PafA was partially purified; it exhibits optimal activity at pH 8.5 and is active towards a broad spectrum of substrates including both phosphomonoesters and ATP, with preferential activity for the latter compound. The present findings allow definition of a new family of APs including 60 kDa, periplasmic enzymes whose expression is not influenced by freely available P$_i$ in the medium. Moreover, PafA can be considered an evolutionary intermediate between Ca$_2$+-ATPase of M. odoratus and the APs PhoV of Synechococcus PCC 7942 and PhoD of Z. mobilis.

Keywords: flavobacteria, PhoV, PhoD, Cda, Ca$_2$+-dependent ATPase

INTRODUCTION

Alkaline phosphatases (APs) constitute a large superfamily of enzymes that exhibit optimal dephosphorylating activity at alkaline pH values, with known members represented in all of the living superkingdoms. In bacteria, the paradigm for APs is represented by the Escherichia coli PhoA enzyme. PhoA is produced under conditions of inorganic phosphate (P$_i$) deprivation and is secreted into the periplasmic space, where it can release P$_i$ from organic phosphoesters that are unable to cross the cytoplasmic membrane (Wanner, 1996). The mature PhoA enzyme is a homodimeric protein containing two 48 kDa subunits, with one magnesium and two zinc ions in each active site that are essential for catalytic activity (Kim & Wyckoff, 1991). PhoA is active on a broad array of organic phosphoesters at similar rates (Heppel et al., 1962). This functional behaviour is consistent with its physiological role and with the three-dimensional structure of the molecule (Kim & Wyckoff, 1989). Production of P$_i$-repressible AP activity is a common feature among Enterobacteriaceae (Cocks & Wilson, 1972), and homologues of the E. coli PhoA enzyme have been identified and characterized in several Gram-negative and Gram-positive species, including Escherichia fergussonii (DuBose & Hartl, 1990), Serratia marcescens (DuBose & Hartl, 1990), Bacillus subtilis (Hulett et al., 1991), Bacillus licheniformis (Kim...

Abbreviations: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; PDP-MG, phenolphthalein diphosphate-methyl green medium; pNPP, p-nitrophenyl phosphate; pNP, p-nitrophenol; TBU, Terrific broth supplemented with 1% glucose; TBU XP, TBU agar containing 50 µg BCIP ml$^{-1}$.

The GenBank accession number for pafA reported in this paper is AF157621.
et al., 1998), Enterococcus faecalis (Lee et al., 1999) and Thermotoga maritima (Nelson et al., 1999).

In addition to this major lineage of ‘PhoA-like’ enzymes, a highly divergent lineage of prokaryotic APs has also been identified, including the PhoD enzyme from Zymomonas mobilis (Gomez & Ingram, 1995) and the PhoV enzyme from Synechococcus sp. (Wagner et al., 1995). These proteins are more closely related to each other (24.2% sequence identity) than to any other of the ‘PhoA-like’ enzymes, and are produced in a P-I-repressible pattern (Gomez & Ingram, 1995; Wagner et al., 1995). Both PhoD and PhoV are essentially non-specific enzymes that exhibit broad substrate specificity, with PhoD apparently being more active on ATP than on sugar phosphates. The recombinant PhoD was described as membrane-bound (Gomez & Ingram, 1995) and the recombinant PhoV as associated with the periplasmic side of the inner membrane (Wagner et al., 1995). The function of these enzymes remains to be elucidated. However, Gomez & Ingram (1995) suggested that PhoD could be involved in nucleotide turnover during primary metabolism. Interestingly, the Cda Ca2+-dependent ATPase from Myroides odoratus (formerly Flavobacterium odoratum) (Bernardet et al., 1996) also belongs to this lineage; it has not been specifically reported as an AP but as a P-type ATPase, functionally similar to sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (Desrosiers et al., 1996; Gambel et al., 1992), and involved in calcium transport for calcium homeostasis.

In this work we report evidence for the existence of a new member of the latter lineage of prokaryotic APs and describe the sequencing, partial purification and preliminary characterization of PafA enzyme from Chryseobacterium meningosepticum, which exhibits a different behaviour as compared both to PhoD of Z. mobilis and PhoV of Synechococcus, and to Ca2+-ATPase of M. odoratus.

**METHODS**

**Bacterial strains and media.** Chryseobacterium meningosepticum CCUG 4310, C. meningosepticum CCUG 214, Chryseobacterium indologenes CCUG 14556, Myroides odoratus CCUG 22182, Myroides odoratimimus CCUG 3837, Empedobacter brevis CCUG 7320, Sphingobacterium multivorum CCUG 11736, Sphingobacterium (Flavobacterium) mizuta CCUG 15907 and Sphingobacterium spirituorum CCUG 13244 were investigated for P-I-repressible AP production. E. coli MG 1655 (CGSC 6300) was used for comparison in these experiments. E. coli DH5a (Sambrook et al., 1989) was used as the host for the C. meningosepticum CCUG 4310 genomic library. Terrific broth (Sambrook et al., 1989) supplemented with 1% glucose (TBU medium) was used to grow the different flavobacterial species and E. coli MG 1655 for phosphatase assays. TBU agar medium containing 50 µg ml−1 of the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) (TBUXP medium) and phenolphthalein diphasphate-methyl green medium (PDP-MG) (Thaller et al., 1994; Riccio et al., 1997) were used for detection of AP-producing strains. When necessary, antibiotics were used at the following concentrations: ampicillin, 200 µg ml−1; chloramphenicol, 100 µg ml−1. Media components were from Difco. Chemicals were from Sigma.

**Preparation of crude cell extracts or cell fractions.** Crude cell extracts were prepared as follows. Cells were washed twice in normal saline, suspended in normal saline at OD600 2 and disrupted by sonication (three times for 30 s each time at 14 µm amplitude in a MSE Soniprep 150, while kept on ice). Cell debris was then removed by centrifugation (10000 g for 10 min at 4 °C). Periplasmic proteins were extracted from exponentially growing cells by the method of Ferro-Luzzi Ames et al. (1984).

**Protein determination.** Protein concentration in solution was determined by the method of Bradford using a commercial kit (Bio-Rad), with bovine serum albumin as the standard.

**Recombinant DNA methodology.** Basic recombinant DNA techniques were essentially as described by Sambrook et al. (1989). Construction of the genomic library of C. meningosepticum CCUG 4310 in the E. coli plasmid vector pACYC184 was described previously (Rossolini et al., 1998). The pBluescript SK vector (Stratagene) was used for subcloning procedures. Restriction and modification enzymes were purchased from Boehringer.

**Detection of recombinant AP-producing clones.** To detect AP activity in clones of the library, cells were plated onto TBUXP with chloramphenicol and incubated at 37 °C for 24–48 h; the plates were then exposed to ammonia vapour for 2–3 h at room temperature; clones expressing an AP-encoding gene were then identified by their blue colour.

AP-positive colonies were subjected to plasmid extraction (Sambrook et al., 1989) and appropriate restriction fragments were obtained and subcloned into pBluescript SK. The AP activity of recombinant subclones was detected on PDP-MG plates.

**DNA sequencing and computer analysis of sequence data.** All sequences were determined on denatured double-stranded DNA templates by the dideoxy-chain termination method (Sanger et al., 1977). The nucleotide sequence was determined for both strands. Sequences were compared using the Clustal W program (http://www2.ebi.ac.uk/clustalw/). Analysis of conserved blocks in sequences was done with the help of the program Block Maker (http://dot.imgen.bcm.tmc.edu:9331/alignmentGenebee.html).

A phylogenetic tree was generated using the Clustal W 1.75 program (http://www.genebee.msu.su/genebee.html).

**Phosphatase assays.** The phosphatase activity of crude cell extracts and of cell fractions was assayed with p-nitrophenyl phosphate (pNPP) as substrate by measuring the released p-nitrophenol (pNP) at 414.5 nm at pH ≥ 12 (ε280 = 18000 cm−1). The concentration of pNPP in the assays was 1 mM unless otherwise specified. Assays were carried out at 37 °C in a 0.3 ml volume, in 50 mM Tris/HCl buffer (pH 8.5) (assay buffer) unless otherwise specified and were initiated by addition of the substrate. The reaction was stopped by adding 0.7 ml 2 M NaOH. One unit was defined as the amount of enzyme capable of release 1 µmol pNP min−1 under the assay conditions. The activity of the partially purified PafA enzyme as a function of the pH was assayed in a similar manner, using the following 50 mM buffer systems: sodium acetate (pH 4, 5, 6 and 6.5); Tris/HCl (pH 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10). Significant differences were not observed using different buffer systems at overlapping pH values. The activity of the partially purified PafA enzyme against various substrates was tested in assay buffer by measuring the concentration of released P,
after incubation for 2 min at 37 °C, by the method of Chifflet et al. (1988) with minor modifications (the initial reaction volume was 0.1 ml and the volume of all the reagents was doubled). The substrate concentration was 1 mM. Substrate solutions were prepared as 100 mM stock solutions in double-distilled water, stored at −20 °C in small aliquots and discarded after the first thawing. Thermal stability was studied by incubating the enzyme at various temperatures in assay buffer for different periods of time. Thermal inactivation was stopped by cooling the aliquots on ice and the residual AP activity was measured at 37 °C. Reactivation was evaluated by measuring the activity of heated aliquots after 1, 24 and 48 h incubation both at 37 °C and at room temperature. The effect of inhibitors was determined by pre-incubating the enzyme in 0.3 ml assay buffer containing suitable inhibitor concentrations for 20 min at 25 °C and then starting the assay by the addition of pNPP (1 mM final concentration). Reactions were stopped after 5 min incubation at 37 °C and the released pNP was measured as described above. Control assays without the inhibitor were always run in parallel. Kinetic parameters for ATP, ADP and AMP were determined by measuring substrate hydrolysis under initial rate conditions, using the Lineweaver and Burk plot (Segel, 1976).

Protein electrophoretic techniques. SDS-PAGE was performed as described by Laemmli (1970). Zymograms were performed after SDS-PAGE using partially denaturing conditions (the sample was not heated before loading; Thaller et al., 1995) and Tris/borate-SDS as the running buffer. Following separation, the gels were washed several times with 50 mM Tris/HCl, 1% Triton X-100, pH 8.5, and AP activity was developed after overnight incubation at 37 °C in 50 mM Tris/HCl pH 8.5 with 50 µg BCIP ml−1. Bands with phosphatase activity were revealed by intense blue coloration.

Partial purification of the PafA enzyme. Purified AP was obtained from crude extracts of phosphate-producing strain E. coli DH5α (pSpessi) grown overnight in TBU with ampicillin. After sonication, the crude extracts were centrifuged at 45,000 g for 150 min and the supernatant, containing the phosphatase activity, was applied to a Sepharose DEAE-Fast Flow anion-exchange column (7 cm × 2.6 cm, Pharmacia) equilibrated with 15 mM Tris/HCl (pH 7.5) buffer at a constant flow rate of 1 ml min−1. The column was then washed with three column volumes of the equilibration buffer and the bound proteins were eluted with a linear gradient of NaCl (0–200 mM in 60 min). Fractions containing the enzymic activity were pooled, dialysed against 50 mM sodium acetate buffer (pH 5.0) clarified by centrifugation (20000 g for 30 min at 4 °C) to remove the precipitate formed following dialysis, and applied to a CM Sepharose-Fast Flow cation-exchange column (13.5 cm × 1.6 cm, Pharmacia) equilibrated with 50 mM sodium acetate buffer (pH 5.0) at a constant flow rate of 0.5 ml min−1. The column was then washed with three column volumes of the equilibration buffer and the bound proteins were eluted with a linear gradient of NaCl (0–300 mM in 60 min). The active fractions were collected, dialysed against 10 mM Tris/HCl buffer (pH 7.0), concentrated by dialysis against Spectra Gel Absorbent (Spectrum) to a final volume of 2 ml, and further purified through a HiLoad 16/60 Superdex 200 Prep Grade gel filtration column (Pharmacia), equilibrated and eluted with 100 mM sodium sulfate (pH 7.0) in 100 mM NaCl at a constant flow rate of 0.5 ml min−1. To monitor phosphatase elution, phosphatase assays were performed as described above using 1 mM pNPP as substrate.

Molecular mass determination. The apparent molecular mass of the partially purified enzyme was determined by size-exclusion HPLC, using an Ultraspherogel SEC 3000 column (7.5 mm × 30 cm) (Beckman), protected by an Ultraspherogel SEC Guard column (7.5 mm × 4 cm) (Beckman), with 100 mM sodium sulfate (pH 7.0) in 100 mM NaCl as the mobile phase, at a constant flow rate of 0.5 ml min−1. Protein elution was monitored at 215 nm and phosphatase activity was measured as described above. Molecular size markers (range 12400–200000, Sigma) were used to calibrate the column.

RESULTS

High-level production of Pₐ-irrepressible AP activity by members of rNA superfamily V

Crude extracts of cultures of various species belonging to several genera (Chryseobacterium, Myroides, Emepdobacter and Sphingobacterium) of rRNA superfamily V (Bernardin et al., 1996), grown in the presence of freely available Pₐ, were assayed for phosphatase activity at different pH values using pNPP as substrate. All the tested strains exhibited detectable phosphatase activity in the tested pH range (5–9) and in all cases the highest values were recorded at alkaline pH (Fig. 1). When the results were compared to those obtained with a crude extract of E. coli grown under the same conditions, the differences in the pattern were remarkable, especially at alkaline pH values (Fig. 1).

Pₐ-irrepressible production of AP activity by C. meningosepticum CCUG 4310

The production of AP was further investigated in different cellular preparations of C. meningosepticum CCUG 4310 grown in Pₐ-rich medium by means of a zymogram technique (Fig. 2). Identical AP activities were shown both in crude extract and in periplasmic preparations, suggesting that the Pₐ-irrepressible AP activity produced by CCUG 4310 was contributed at least in part by this periplasmic enzyme.

Cloning of the Pₐ-irrepressible AP determinant from C. meningosepticum

The C. meningosepticum gene encoding the Pₐ-irrepressible AP was isolated from a genomic library of strain CCUG 4310, constructed in the plasmid vector pACYC184 and transformed into E. coli DH5α, by means of a shotgun cloning strategy using the TBUXP medium for detection of clones producing AP activity. Using this approach, five clones that demonstrated blue coloration were identified out of approximately 10000 screened recombinants; one of them (Cme4), which showed the most intense staining with BCIP, was selected for further investigation. Zymogram analysis of a crude extract of this clone revealed an AP activity apparently identical to that observed in the extracts of C. meningosepticum CCUG 4310, and otherwise not detectable in the parent E. coli host (Fig. 2). These results suggested that the Chryseobacterium determinant of a Pₐ-irrepressible AP was carried and expressed by clone E. coli DH5α(pCme4). A periplasmic preparation from this clone contained 50–60% of the AP activity measured in the crude extract and yielded the
Phosphatase activities of crude extracts of Chryseobacterium, Myroides, Empedobacter, Sphingobacterium and E. coli strains. Cme, C. meningosepticum CCUG 4310; Cme\(^1\), C. meningosepticum CCUG 214\(^T\); Cin, C. indologenes CCUG 14556; Ec, E. coli MG 1655; Mod, M. odoratus CCUG 22182; Mmus, M. odoratimus CCUG 3837; Ebr, Emp. brevis CCUG 7320\(^T\); Smi, Sph. mizutae CCUG 15907\(^T\); Smul, Sph. multivorans CCUG 11736\(^T\); Sipi, Sph. spiritivorum CCUG 13244\(^T\). The test was performed using 5 mM pNPP as substrate in 100 mM buffers (sodium acetate pH 5 and 6; Tris/HCl pH 6, 7, 8 and 9). Significant differences were not observed using different buffer systems at overlapping pH values. The values are means of three measurements. The bars indicate the standard deviations.

**Fig. 2.** Zymogram of protein preparations revealing AP activity. Lanes: 1, C. meningosepticum CCUG 4310 periplasmic extract (10 µg); 2, C. meningosepticum CCUG 4310 crude extract (10 µg); 3, E. coli DH5\(\alpha\)(pACYC184) periplasmic extract (10 µg); 4, E. coli DH5\(\alpha\)(pCme4) periplasmic extract (10 µg).

**Sequence of the AP determinant**

A 1-9 kb NdeI–DraI fragment internal to the 2-5 kb HindIII–SpeI Chryseobacterium DNA insert of plasmid pSpesi was sequenced. A single 1641 bp open reading frame (ORF) was identified within this fragment, whose product exhibited in a BLAST search significant sequence similarity with the Ca\(^{2+}\)-ATPase of M. odoratus (51.9% identity) and with the PhoV AP of Synechococcus PCC 7942 (49.9% identity) and, although to a lower extent (22.1% identity), with the PhoD AP of Z. mobilis. Identification of this ORF as the AP determinant was also consistent with results of subcloning experiments (Fig. 3). The determinant was named pafA after phosphate-irrepressible alkaline phosphatase of *Flavobacterium* (EMBL/GenBank accession number AF157621).

The pafA coding sequence encodes a 546 aa polypeptide whose amino-terminus exhibits features typical of signal peptides targeting protein secretion into the periplasmic space. A putative terminator region was present 18 bp downstream of the TAA stop codon of pafA gene.

Analysis of the sequence with the program SIGCLEAVE (Nielsen et al., 1997) predicted the cleavage of the signal peptide after the Ala residue at position 20. This would yield a mature protein of 526 aa, with calculated molecular mass of 58682 Da and pl of 6.14.

In the sequenced region, the pafA ORF was preceded by an ORF truncated at the 5’ end by the NdeI site located at the boundary of the sequenced subclone, which encodes a polypeptide showing significant sequence similarity with the C-terminal moiety of conserved eubacterial and mammalian proteins associated with proton/sodium-glutamate symport (for example from *Bacillus stearothermophilus* and *Bacillus caldotenax*) or with glutamate/aspartate transport (for example from *Bos taurus*). Interestingly, this ORF is in the same orientation as pafA and its termination codon is separated by only 13 bp from the ATG initiation codon of pafA, suggesting that the two genes could be part of the same transcriptional unit.

The origin of the cloned determinant from a single chromosomal region of *C. meningosepticum* CCUG
C. meningosepticum alkaline phosphatase

4310 was confirmed by PCR experiments. Primers were designed to amplify the 1.9 kb Ndel–DraI fragment; a consistent amplimer was obtained using C. meningosepticum CCUG 4310 genomic DNA as template. The amplimer was restricted with EcoRV and PstI; the resulting fragments were consistent with the sequenced region. The G+C content of the cloned region was 37.8 mol%.

Structural relationships between the PafA enzyme and other bacterial APs

As already mentioned above, at the level of primary structure, the PafA AP of C. meningosepticum exhibited the closest similarity with the Cda Ca\#+–ATPase of M. odoratus (51.9% identity; 73.1% similarity) (Peiffer et al., 1996) and with the PhoV AP of Synechococcus PCC 7942 (49.9% identity; 72.2% similarity) (Wagner et al., 1995), whereas lower degrees of similarity were observed with the PhoD AP of Z. mobilis (22.1% identity; 44.4% similarity) (Gomez & Ingram, 1995) and with the PhoA AP of E. coli (14.0% identity; 30.1% similarity) (Bradshaw et al., 1981).

The analysis of the deduced amino acid sequence of pafA did not reveal outstanding homologies with the putative sites of phosphorylation of either PhoV (55% identity) (Wagner et al., 1995) or Cda (55% identity) (Peiffer et al., 1996). However, the Asp-158 residue of the Ca\#–ATPase, which is implicated in the auto-phosphorylation process of the P-type ATPases (Peiffer et al., 1996), is retained in the PafA sequence. As far as Z. mobilis PhoD is concerned, the Thr-107 residue, indicated as probably being involved in the formation of covalent phosphoryl–enzyme intermediate (Gomez & Ingram, 1993), was conserved in PafA, PhoV (Wagner et al., 1995) and Cda sequences (Peiffer et al., 1996).

Comparison of PafA and of the related PhoV, Cda and PhoD enzymes with the E. coli PhoA AP at the level of conserved blocks in the aligned sequences (Fig. 4) revealed notable similarities in some of the regions that in PhoA are known to be involved in metal binding and phosphorylation (Kim & Wyckoff, 1991). In particular, all the residues involved in Zn\# binding (Asp-51, Asp-369 and His-370) are conserved in PafA and related enzymes, while of those involved in Zn\# binding only Asp-327 and His-331 are conserved and no clear counterpart is detectable for His-412 (a conserved histidine is actually present in the C-terminal region of PafA and related enzymes, but not within a sequence block conserved with PhoA). Concerning the residues involved in Mg binding, only Asp-51 is conserved, while Thr-155 and Glu-322 are not conserved in PafA and related enzymes. Finally, the Ser-102 residue that in PhoA is involved in phosphorylation, is not conserved in PafA and related enzymes (Fig. 4).

Phylogenetic relationships between PafA and other eubacterial and archaeal APs

The phylogenetic relationships between PafA and other APs were studied on the basis of multiple alignment of amino acid sequences of several eubacterial and archaeal APs.
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Fig. 4. Comparison of segments containing metal-binding regions and the site of phosphorylation of the *E. coli* PhoA enzyme with the homologous segments of PafA and of other APs. Residues involved in metal-binding are marked with an arrow. Numbers of residues of amino acid sequences are in parentheses. Identities between the PafA enzyme and the *E. coli* PhoA enzyme are marked with an asterisk; strong similarities are marked with a colon; weak similarities are marked with full stops. Residues conserved between PafA and Cda, PhoV and PhoD are in bold. PafA, PafA of *C. meningosepticum* CCUG 4310 (Q9KJX5); Cda, *Ca*<sup>2+</sup>-ATPase of *M. odoratus* ATCC 29979 (Q47910); PhoV, PhoV of *Synechococcus* PCC 7942 (Q55320); PhoD, PhoD of *Z. mobilis* CP4 (L36230); PhoA-Ec, PhoA of *E. coli* (Bradshaw et al., 1981).

Fig. 5. Phylogenetic relationships between PafA from *C. meningosepticum* CCUG 4310 and other bacterial and archaeal APs. PhoA-Eco, PhoA AP of *E. coli* K-12 (Bradshaw et al., 1981); PhoA-Efer, PhoA AP of *E. fergusonii* ATCC 35468 (P21948); PhoA-Srm, PhoA of *Ser. marcescens* ATCC 13880 (P191477); AP-Paer, AP of *Pseudomonas aeruginosa* PAO1 (P35483); AP-Xfas, AP of *Xylella fastidiosa* 9A5C (Q9PFK0); PhoZ, PhoZ AP of *Ent. faecalis* ATCC 11700 (Q9X8W0); APIII, AP III of *B. licheniformis* JS10009 (Q9S373); APIV, AP IV of *B. licheniformis* JS10009 (Q9S373); AP-Hal, AP of *Halobacterium* sp. NRC-1 plasmid pH15000 (Q9W020); PafA, PafA of *C. meningosepticum* CCUG 4310 (Q9KJX5); Cda, *Ca*<sup>2+</sup>-ATPase of *M. odoratus* ATCC 29979 (Q47910); PhoV, PhoV AP of *Synechococcus* PCC 7942 (Q55320); PhoD, PhoD of *Z. mobilis* CP4 (L36230); PhoA-Ec, PhoA of *E. coli* (Bradshaw et al., 1981).

APs (Fig. 5). Interestingly, ORFs encoding putative proteins similar to PafA were also recognized in the genomes of *Porphyromonas gingivalis* W83 (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinished genome.html) and of *Caulobacter crescentus* CB15 (Nierman et al., 2001). These putative proteins, which
showed a 46-9% (P. gingivalis) and 44-8% (Cau. crescentus) similarity with the PafA enzyme, were also included in the phylogenetic study even though AP activities have not yet been demonstrated. The analysis of the phylogenetic tree confirmed the closest relationship of PafA with the Cda Ca²⁺-ATPase and with PhoV, and to a lesser extent with PhoD and the putative APs of P. gingivalis and Cau. crescentus. These enzymes formed a well-defined cluster separated from other representative eubacterial and archaeal APs.

Partial purification of the PafA enzyme

The PafA protein was partially purified from crude extracts of E. coli DH5α(pSpessi) by means of two ion-exchange chromatography steps and one gel-filtration step. With this procedure, a 2248-fold purification of the protein was obtained, with an overall yield of 11% and an estimated purity of approximately 85–90%, as evaluated by SDS-PAGE (data not shown). The low yield in recombinant protein was probably due to the low expression of the phosphatase in the E. coli host. The specific activity of the partially purified enzyme was 1738 units (mg protein)⁻¹.

Characterization of the PafA enzyme

The molecular mass of the polypeptide component, estimated by SDS-PAGE, was approximately 60 kDa and that of the native protein, estimated by size-exclusion chromatography, was approximately 180 kDa. Experiments using ultrafiltration were confirmatory. In fact, the partially purified enzyme was apparently completely retained by ultrafiltration membranes with a 100 kDa cut-off.

Zymograms revealed that the partially purified protein exhibited a pattern identical to that shown by the enzyme extracted from the parental strain (data not shown).

The optimum pH was studied with pNPP as substrate. Phosphatase activity was observed between pH 7-0 and pH 9-5, with optimal activity at pH 8-5. The enzyme lost 50% of its activity at pH 7-5 and 70% at pH 9-0, being virtually inactive at pH 10 and at pH 5 or lower.

Orthovanadate and EDTA were greatly inhibitory to the PafA activity (IC₅₀ 0-5 µM and 30 µM, respectively). Reactivation of enzymic activity after dialysis was not successful. Also, P₈ inhibited the PafA activity (IC₅₀ 340 µM).

The enzyme was heat-labile. Incubation of PafA for 10 min at 50 or 60 °C caused 40 and 60% inactivation, respectively. Heating at 100 °C for 2 min caused total loss of activity. No restoration of phosphatase activity was observed even upon prolonged incubation of the heat-treated enzyme at room temperature or at 37 °C.

At pH 8-5, the enzyme exhibited a broad substrate specificity, being able to dephosphorylate a great variety of phosphomonoesters with the exception of 2’:3’:cyclic AMP and phytic acid. PafA hydrolysed d-glucose 1-phosphate, d-glucose 6-phosphate, ribose 5-phosphate and 5-naphthyl phosphate at rates of 47, 15, 66 and 99% of that measured with pNPP. Moreover, it was able to dephosphorylate 3’- and 5’-nucleotides such as ADP, ATP, 3’-AMP, 3’-GMP, 3’-UMP, 5’-AMP, 5’-GMP, 5’-CMP and 5’-UMP at rates of 83, 109, 100, 60, 96, 96, 61 and 39%, respectively, of that measured with pNPP.

Kinetic parameters towards adenosine mono, di- and triphosphate are reported in Table 1. The enzyme showed a high activity against all these substrates; the highest affinity was obtained with ATP and the highest fold higher than that for AMP.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₘ (µM)</th>
<th>Vₘₐₓ (µmol min⁻¹)</th>
<th>Vₘₐₓ/Kₘ (relative)*</th>
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<tr>
<td>pNPP</td>
<td>115 ± 13</td>
<td>71 ± 12</td>
<td>100</td>
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<tr>
<td>3’-AMP</td>
<td>74 ± 4</td>
<td>206 ± 10</td>
<td>467</td>
</tr>
<tr>
<td>5’-AMP</td>
<td>90 ± 26</td>
<td>167 ± 12</td>
<td>300</td>
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<td>ADP</td>
<td>35 ± 15</td>
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<td>ATP</td>
<td>10 ± 1</td>
<td>187 ± 47</td>
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</tbody>
</table>

*The Vₘₐₓ/Kₘ values are relative to that of pNPP, which was set at 100.

DISCUSSION

Among prokaryotes, the best-known AP family encompasses PhoA of E. coli and the PhoA-like enzymes of both Gram-negative and Gram-positive bacteria and of archaea (DuBose & Hartl, 1990; Hulett et al., 1991; Kim et al., 1998; Lee et al., 1999; Nelson et al., 1999). The majority of the enzymes included in this family are not expressed in media containing P₈. In this work we demonstrate that flavobacteria and flavobacteria-like organisms produce APs when grown in the presence of freely available P₈. As a representative enzyme, we further studied that produced by C. meningosepticum, which is the most relevant species in this group from the medical standpoint (Siegmund-Igra et al., 1987). Similar to the PhoA-like enzymes, also this AP activity was apparently secreted into the periplasmic space, although at present we can not establish whether it is found as a
soluble periplasmic protein or a membrane-bound protein such as the Ca\(^{2+}\)-ATPase of *M. odoratus* (Pfeiffer *et al*., 1996) or the PhoV enzyme of *Synechococcus* (Wagner *et al*., 1995).

The *pafA* product exhibited a relatively high homology with the Cda Ca\(^{2+}\)-ATPase of *M. odoratus* and the PhoV AP of *Synechococcus* PCC 7942, a lower homology with the PhoD AP of *Z. mobilis*, and an even lower homology with the *E. coli* PhoA AP. Interestingly, the sequence of PafA demonstrates homology with *E. coli* PhoA in regions near to the metal-binding sites of PhoA, and some important residues involved in the active site of PhoA (Kim & Wyckoff, 1989; Murphy & Kantrowitz, 1994) are conserved in the PafA sequence. It could be suggested that regions important for the function of the enzymes could be derived from a common ancestor and that subsequent evolution has caused differentiation in the biochemical and functional properties of the two lineages.

PafA can be considered a broad-spectrum AP with a preferential activity on ATP. PhoD of *Z. mobilis* and the Ca\(^{2+}\)-ATPase of *M. odoratus* also demonstrated the highest activity on ATP (Gomez & Ingram, 1995; Desrosiers *et al*., 1996). However, Cda is a narrow-spectrum enzyme as it is able to hydrolyse essentially only ATP (Desrosiers *et al*., 1996), whilst PhoD is active on different substrates (Gomez & Ingram, 1995). PhoV of *Synechococcus*, on the other hand, does not show a particularly high activity on ATP, and is able to dephosphorylate several substrates at similar rates (Wagner *et al*., 1995). PafA, therefore, apparently shares some functional characteristics with both Cda and PhoV. Like both of them, PafA is partially inhibited by P_i (Gambel *et al*., 1992; Wagner *et al*., 1995); like Cda, PafA is highly sensitive to o-vanadate (Desrosiers *et al*., 1996); like PhoV, PafA is inactivated by temperature (Wagner *et al*., 1995) unlike other APs (Garen & Levinthal, 1960).

The sequenced fragment of the *pafA* region indicates that the gene is probably part of an operon as it is located downstream of another gene whose product is similar to proton/glutamate symport proteins. This fact, taken together with the high activity on ATP shown by PafA, led us to suppose that this enzyme could be involved in the energy-producing process necessary for the function of the associated transport protein(s).

The analysis of the phylogenetic tree allowed us to recognize a new enzyme family of APs evolutionarily distant from that of PhoA of *E. coli* and other PhoA-like enzymes. This new enzyme family encompasses enzymes from taxonomically unrelated species; all the enzymes share some features such as molecular mass, P_i-repressible expression and periplasmic location. Among the enzymes included in the new family, some (PafA, PhoV and PhoD) are broad-spectrum enzymes and one (Cda) is a narrow-spectrum enzyme. PafA, however, exhibits a relevant activity on ATP, at rates similar to that of Cda, indicating that it could be an evolutionary intermediate between this latter enzyme and the P_i-

irrepressible APs. Perhaps in this family could be included also the AP of *Prevotella intermedia* that was described as a tyrosine-phosphatase enzyme with molecular mass of 54 kDa, showing partial similarity with the PhoD and PhoV sequences (Ansai *et al*., 1998). However, as the complete amino acid sequence is not yet known, this enzyme was not included in the phylogenetic analysis.

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