Cloning and characterization of the NapA acid phosphatase/phosphotransferase of *Morganella morganii*: identification of a new family of bacterial acid-phosphatase-encoding genes

Maria Cristina Thaller,¹ Giovanna Lombardi,² Francesca Berlutti,¹ Serena Schippa,¹ and Gian Maria Rossolini²

Author for correspondence: G. M. Rossolini. Tel: +39 577 280903. Fax: +39 577 42011.

¹ Istituto di Microbiologia, Università 'La Sapienza', Piazzale A. Moro, 5, 00185 - Rome, Italy

² Dipartimento di Biologia Molecolare - Sezione di Microbiologia, Università di Siena, Via Laterina, 8, 53100 - Siena, Italy

The gene encoding a minor phosphate-irrepressible acid phosphatase (named NapA) of *Morganella morganii* was cloned and sequenced, and its product characterized. NapA is a secreted acid phosphatase composed of four 27 kDa polypeptide subunits. The enzyme is active on several organic phosphate monoesters but not on diesters, and is also endowed with transphosphorylating activity from organic phosphoric acid esters to nucleosides and other compounds with free hydroxyl groups. Its activity is inhibited by EDTA, inorganic phosphate, nucleosides and Ca^{2+} , but not by fluoride or tartrate, and is enhanced by Mg^{2+} , Co^{2+} and Zn^{2+} . At the sequence level, the NapA enzyme did not show similarities to any other sequenced bacterial phosphatases. However, a search for homologous genes in sequence databases allowed identification of two open reading frames located within sequenced regions of the *Escherichia coli* and *Proteus mirabilis* genomes respectively, encoding proteins of unknown function which are highly homologous to the *Morganella* enzyme. Moreover, the properties of the NapA enzyme are very similar to those reported for the periplasmic nonspecific acid phosphatase II of *Salmonella typhimurium* (for which no sequence data are available). These data point to the existence of a new family of bacterial acid phosphatases, which we propose designating class B bacterial acid phosphatases.

Keywords: *Morganella morganii*, NapA, acid phosphatase/phosphotransferase

INTRODUCTION

Enzymic cleavage of phosphoester bonds is a fundamental step in many biochemical pathways and several phosphatases, different in structure and function are typically present in the prokaryotic cell (Beacham, 1979; Dvorak *et al.*, 1967; Uerkvitz & Beck, 1981). However, even in *Escherichia coli*, which is the best studied model of such cells, the exact number of phosphatases as well as the role(s) of some of them remain to be defined. The picture is further complicated by the fact that different patterns of

phosphatase activities are found in different bacterial species, even those belonging to closely related taxa, as observed for members of the family *Enterobacteriaceae* (Cocks & Wilson, 1972; Edwards *et al.*, 1993; Groisman *et al.*, 1992; Pompei *et al.*, 1990, 1993).

Characterization of the different phosphatases present in enteric bacteria is, therefore, interesting not only in relation to microbial physiology and enzymology but also enterobacterial taxonomy and phylogenesis.

In a previous investigation on the phosphatases produced by *Morganella morganii*, which is one of the few enterobacterial species able to produce a high-level phosphate-irrepressible acid phosphatase activity (HPAP phenotype) (Pompei *et al.*, 1990, 1993), we found that *Morganella* produces two different phosphate-irrepressible acid phos-

Abbreviations: G2P, glycerol 2-phosphate; PDP, phenolphthalein diphosphate; pNP, *p*-nitrophenol; pNPP, *p*-nitrophenyl phosphate.

The GenBank/EMBL accession number for the nucleotide sequence reported in this paper is X78328.

phatases active on *p*-nitrophenyl phosphate (*p*NPP), and that the major one, named PhoC, is apparently associated with the HPAP phenotype (Thaller *et al.*, 1994). PhoC is a nonspecific acid phosphatase homologous to other bacterial acid phosphatases, including the PhoN enzymes of *Salmonella typhimurium* and *Providencia stuartii*, and the PhoC enzyme of *Zymomonas mobilis*, and we proposed designating this family of enzymes class A bacterial acid phosphatases (Thaller *et al.*, 1994).

In the present work we have studied the minor phosphate-irrepressible acid phosphatase produced by *M. morganii*, named NapA, and found that it belongs to another family of nonspecific acid phosphatases which could be widespread among the *Enterobacteriaceae* and which we propose designating class B bacterial acid phosphatases.

METHODS

Bacterial strains and genetic vectors. The *M. morganii* RS12 strain used for library construction has been previously described (Thaller *et al.*, 1994). *M. morganii* ATCC 25830^T was also used as a reference strain. *E. coli* DH5 α (Sambrook *et al.*, 1989) was used as the host for genetic vectors and recombinant plasmids. The Bluescript SK plasmid (Stratagene) was the vector used for the construction of the *M. morganii* genomic library and for subcloning procedures.

Recombinant DNA methodology. Basic recombinant DNA techniques were essentially as described by Sambrook *et al.* (1989). The procedure for construction of the *Morganella* genomic library has been previously described (Thaller *et al.*, 1994). 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. Analysis and comparison of nucleotide and amino acid sequences were performed using the UWGCG software (release 7.3) (Devereux *et al.*, 1984).

Phosphatase assays. The phosphatase activity of whole-cell or periplasmic protein preparations toward *p*NPP was assayed by measuring the released *p*-nitrophenol (*p*NP) at 414.5 nm and pH 12. The concentration of *p*NPP in the assay was 5 mM. All assays were performed in a volume of 1 ml and were initiated by addition of the substrate. Incubation was at 37 °C for 20 min. One unit of enzyme activity was defined as the amount of enzyme able to release 1 μ mol *p*NP min⁻¹ under the assay conditions.

For preparation of whole-cell proteins, bacteria were washed twice in normal saline, suspended in normal saline at an OD₆₀₀ of 10, and disrupted by sonication. Cell debris was then removed by centrifugation (10000 *g* for 10 min at 4 °C). Extraction of periplasmic proteins from *E. coli* was performed by chloroform treatment (Ferro-Luzzi Ames *et al.*, 1984).

Determination of the pH optimum of the purified NapA enzyme was performed using *p*NPP as substrate in 100 mM buffers, including sodium acetate buffer (pH 4–6), Tris/HCl buffer (pH 7–9), and glycine/NaOH buffer (pH 10). Measurement of enzyme activity in different buffer systems at overlapping pH values showed no significant buffer-related variation. Determination of the activity of the purified NapA protein using different substrates was performed as previously described (Kier *et al.*, 1977; Weppelman *et al.*, 1977) in 100 mM sodium acetate buffer, pH 6. Inhibition assays were performed in the same buffer using *p*NPP as the substrate. The enzyme was pre-

incubated at 37 °C for 30 min with each substance before starting the assay. Phosphotransferase activity was assayed in 100 mM sodium acetate buffer, pH 6, as previously described (Uerkvitz, 1988), using *p*NPP as the phosphate donor.

Protein determination. Protein concentration in solution was determined using a commercial kit (Bio-Rad Protein assay). BSA was used as the standard.

Protein electrophoretic techniques. SDS-PAGE was performed as described by Laemmli (1970). For detection of NapA enzyme activity after electrophoresis, the gel was incubated for 4 h at 37 °C in several changes of renaturation buffer to obtain renaturation of the enzyme. Renaturation buffer was 100 mM Tris/HCl, pH 7, containing 5 mM MgSO₄ and 1% (v/v) Triton X-100. After the renaturation treatment, the gel was equilibrated for 1 h in 100 mM sodium acetate buffer, pH 6, containing 5 mM MgSO₄, and then developed for phosphatase activity. For development, the gel was incubated at 37 °C for 30 min in the same buffer as used for equilibration with 5 mM *p*NPP added, washed in deionized water, and then incubated at 42 °C in a freshly prepared solution made by a 6:1 (v/v) mixture of acidified ammonium molybdate (4.2 g ammonium molybdate l⁻¹ and 28.6 ml sulphuric acid l⁻¹) and 10% (w/v) ascorbic acid, to detect the presence of inorganic phosphate (Ames, 1966). Phosphatase activities were indicated by the presence of blue-stained bands.

Purification of the NapA enzyme. When *E. coli* strains producing the *Morganella* NapA enzyme were grown in liquid medium at 37 °C, a consistent amount of the protein was released into the medium. The NapA protein was purified from the supernatant of a 24-h-old stationary-phase culture of clone PM9 (see below) grown at 37 °C in Brain Heart Infusion broth supplemented with carbenicillin (0.2 mg ml⁻¹). The culture supernatant was dialysed against 10 mM Tris/HCl, pH 7.4, and loaded on a DEAE-Sephacrose CL-6B (Pharmacia) column (1.5 × 11.5 cm) equilibrated with the same buffer. The enzyme did not bind to the resin and eluted as a single peak. Fractions containing the eluted enzyme were collected, pooled, and dialysed against 10 mM HEPES, pH 7.4. The enzyme was then loaded on a CM-Sephadex C-50 (Pharmacia) column (1.5 × 11.5 cm) equilibrated with 10 mM HEPES, pH 7.4. Again, the enzyme did not bind to the resin and eluted as a single peak. Fractions containing the eluted enzyme were pooled, dialysed against 100 mM sodium acetate buffer, pH 6, and concentrated by ultrafiltration in a Centricon 10 concentrator (Amicon). At this point the enzyme was > 95% pure, as assayed by SDS-PAGE, and only two additional bands were evident. Removal of these contaminants was obtained by gel-filtration. For this purpose, SDS was added to a final concentration of 2% (w/v) and the enzyme was immediately loaded (maximum loading volume 0.3 ml) onto a Sephacryl S-200 (Pharmacia) column (1 × 40 cm) equilibrated with 100 mM sodium acetate buffer, pH 6. The enzyme eluted as a single peak and the fractions containing it were pooled, dialysed against 100 mM sodium acetate buffer, pH 6, and concentrated by ultrafiltration as described above.

For determination of the molecular mass of the NapA protein by means of gel-filtration, the purified protein was applied to a Sephacryl S-200 column (1 × 40 cm) equilibrated and eluted with 100 mM sodium acetate buffer, pH 6. The Sephacryl S-200 column was calibrated with protein standards (Pharmacia) dissolved in elution buffer. The peak fraction of each protein was determined by A₂₈₀. A linear plot of the partition coefficients (Reiland, 1971) versus the logs of the molecular masses of the protein standards was used to estimate the molecular mass of the phosphatase.

NH₂-terminal amino acid sequencing. The purified protein preparation was subjected to SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon, Millipore). The transferred protein was stained with Coomassie brilliant blue R-250 and the band was excised and subjected to sequence analysis using an Applied Biosystems model 470A gas-phase sequencer (Matsudaira, 1987).

RESULTS

Cloning of the *M. morganii* genetic determinant encoding the minor phosphate-irrepressible acid phosphatase (NapA)

The *M. morganii* gene coding for the minor phosphate-irrepressible acid phosphatase (named NapA) was isolated from a genomic library of *M. morganii* RS12, constructed in the plasmid vector Bluescript SK and transformed in *E. coli* DH5 α by the same shotgun cloning strategy previously used for isolation of the *Morganella phoC* gene (Thaller *et al.*, 1994).

Using this strategy, two clones (PM2 and PM9) were identified, which showed acid phosphatase activity much higher than *E. coli* DH5 α (pBluescript) (data not shown) and produced an acid phosphatase composed of a 27 kDa polypeptide which was not detectable in *E. coli* (Fig. 1a) and apparently corresponded to the minor phosphate-irrepressible acid phosphatase detectable in *Morganella* by

a zymogram technique (Fig. 1b). The acid phosphatase produced by the two clones could be extracted by a chloroform treatment suitable for extraction of the periplasmic proteins from *E. coli* (Ferro-Luzzi Ames *et al.*, 1984) (Fig. 1c) and the ratio of phosphatase activity between a periplasmic and a whole cell protein preparation was consistent with a location of this protein in the periplasmic space (data not shown).

Restriction mapping and cross-hybridization experiments performed using the insert carried by clone PM9 as a probe showed that the two clones carried overlapping DNA fragments (Fig. 2), which a Southern blot analysis confirmed to be derived from the *M. morganii* RS12 genome (data not shown).

The above results suggested that the *Morganella* gene encoding the minor phosphate-irrepressible acid phosphatase had been cloned. Definitive confirmation that the enzyme produced by PM2 and PM9 was actually encoded by the cloned DNA was obtained by comparison of the NH₂ terminal sequencing data of the enzyme with the nucleotide sequence of the cloned DNA (see below).

Characterization of the NapA enzyme

Purification of the *Morganella* NapA enzyme from the culture supernatant of *E. coli* PM9 was achieved by means of two ion-exchange chromatography steps followed by a gel-filtration step (see Methods for details of the purification procedure). The two ion-exchange steps were able to remove most protein contaminants from the initial preparation (the NapA enzyme did not bind to either of the two matrices under the conditions adopted), while the final gel-filtration step allowed purification to homogeneity of the NapA protein, as judged by SDS-PAGE (Fig. 3a). The specific activity of the purified protein, assayed against *p*NPP at pH 6, was 46.2 U mg⁻¹.

The NH₂-terminal sequence of the NapA protein was determined as NH₂-KVYMPEKVS DGVTV AQLAEQ.

The molecular mass of the protein, estimated by gel-filtration chromatography, was approximately 105 kDa. This finding was confirmed by ultrafiltration experiments, which showed that the protein was completely retained by filters which excluded globular proteins of sizes larger than 100 kDa.

The NapA enzyme was completely denatured to the monomeric form following heating in the presence of 2% SDS (Fig. 3b). However, avoiding the heat treatment before loading the SDS-PAGE gel resulted in only partial denaturation of the protein, part of which migrated as an approximately 100 kDa band (Fig. 3b). It was possible to vary the ratio between the 100 kDa and the 27 kDa forms by varying the duration of the exposure to SDS and/or the degree of heat treatment of the protein sample before loading the gel (data not shown). If heating was avoided and, at the same time, either the SDS concentration was lowered to 0.01%, or 1% Triton X-100 was added, the protein migrated almost exclusively as a 100 kDa band (Fig. 3b). Results of partially denaturing SDS-PAGE, which gave a molecular mass of approximately 100 kDa

Fig. 1. SDS-PAGE analysis of proteins of *E. coli* PM9 and *E. coli* DH5 α (pBluescript). (a) Coomassie-blue-stained whole-cell proteins of *E. coli* DH5 α (pBluescript) (lane 1) and PM9 (lane 2); proteins were prepared from late stationary phase cells grown in terrific broth (Sambrook *et al.*, 1989). (b) Same samples as in (a) but in this case a lower amount of protein was loaded and, after electrophoresis, the gel was subjected to renaturation treatment and developed for phosphatase activity at pH 6 as described in Methods; the lane labelled MM12 was loaded with a whole-cell protein preparation of *M. morganii* MM12, to show for comparison the two phosphate-irrepressible acid phosphatases that can be resolved in *M. morganii* by the same zymogram technique (Thaller *et al.*, 1994). (c) Coomassie-blue-stained periplasmic proteins of *E. coli* DH5 α (pBluescript) (lane 1) and PM9 (lane 2). Protein size markers are given in kDa on the left. Results obtained with clone PM2 were the same as those obtained with PM9 and are not shown.

Fig. 2. Restriction endonuclease map of the DNA inserts of the recombinant plasmids carried by clones PM2 and PM9 (respectively named pPM2 and pPM9) and subcloning strategy. SB, *Sau3AI/BamHI* junction; RI, *EcoRI*; Sm, *SmaI*; Sc, *SacII*; B, *BamHI*; A, *ApaI*. Thick lines represent *Morganella* DNA while thin zigzag lines represent vector sequences. The location of the putative *NapA* ORF identified on the basis of sequencing data is shown below the map. Production of the *NapA* protein was assayed both by measuring *pNPP*-hydrolysing activity at pH 6, and by SDS-PAGE analysis of periplasmic proteins of different subclones.

Fig. 3. SDS-PAGE analysis of the purified *NapA* protein. (a) Purified *NapA* protein (approximately 5 µg); the gel was stained with a silver staining technique (Heukeshoven & Dernick, 1985) allowing a detection sensitivity of at least 5 ng of protein per band. (b) Coomassie-blue-stained preparation of the purified *NapA* protein subjected to SDS-PAGE after different treatments before loading: the protein was mixed with Laemmli's sample buffer in the presence of 2% SDS and boiled for 5 min (lane 1), mixed with Laemmli's sample buffer in the presence of 2% SDS and 1% Triton X-100 and incubated at 25 °C for 5 min (lane 2), mixed with Laemmli's sample buffer in the presence of 0.01% SDS and incubated at 25 °C for 5 min (lane 3), or mixed with Laemmli's sample buffer in the presence of 2% SDS and incubated at 25 °C for 5 min (lane 4). Protein size markers are given in kDa on the left.

for the partially denatured *NapA* protein, were consistent with gel-filtration and ultrafiltration data concerning the size of the native protein.

The purified protein preparation was used to analyse some of its functional properties. Using *pNPP* as substrate, the *NapA* enzyme showed a pH optimum around 6, retaining

Table 1. *pNPP*-hydrolysing activity of the *NapA* phosphatase at different pH values

All assays were performed using 0.3 µg of the purified enzyme in a volume of 1 ml.

pH	<i>pNPP</i> production (nmol min ⁻¹ ml ⁻¹)*	Relative activity
4	7.86 ± 0.13	0.60
4.5	10.22 ± 0.15	0.78
5	10.74 ± 0.15	0.82
5.5	11.53 ± 0.17	0.88
6	13.10 ± 0.18	1.00
6.5	12.45 ± 0.19	0.95
7	11.40 ± 0.17	0.87
8	5.11 ± 0.10	0.39
9	1.70 ± 0.05	0.13
10	0.39 ± 0.04	0.03

* Mean value of three experiments ± SE.

more than 50% of maximal activity in the pH range 4–7 (Table 1). The enzyme showed a broad substrate specificity which did not include diesters (Table 2). The activity of the enzyme was inhibited by EDTA, inorganic phosphate and Ca²⁺, stimulated by Mg²⁺, Co²⁺ and Zn²⁺, and apparently unaffected by fluoride and tartrate (Table 3). *NapA* also showed transphosphorylating activity from organic phosphoric acid esters to nucleosides and other compounds with free hydroxyl groups (Table 4). Results of these experiments also showed that the activity of *NapA* was inhibited by nucleosides and stimulated by high concentrations of ethanol and glycerol.

The functional properties of the *NapA* enzyme, as well as the sizes of the native protein and of its polypeptide component, are very similar to those of the *S. typhimurium* *NapII* enzyme (Uerkvitz, 1988; Uerkvitz & Beck, 1981).

Table 2. Relative activities of the NapA phosphatase toward various substrates

All assays were performed using 0.3 µg of the purified enzyme in a volume of 1 ml.

Substrate	P _i production (nmol min ⁻¹ ml ⁻¹)*	Relative activity
5'-AMP	10.37 ± 0.21	1.00
5'-UMP	2.90 ± 0.08	0.28
3'-AMP	12.34 ± 0.23	1.19
3'-UMP	6.95 ± 0.14	0.67
pNPP	12.86 ± 0.25	1.24
PDP	11.82 ± 0.20	1.14
G2P	5.08 ± 0.11	0.49
Glucose 6-P	2.07 ± 0.06	0.20
Ribose 5-P	0.62 ± 0.05	0.06
2':3'-Cyclic AMP	< 0.1	< 0.01
2':3'-Cyclic UMP	< 0.1	< 0.01

* Mean value of three experiments ± SE.

Table 3. Effect of various substances on the pNPP-hydrolysing activity of the NapA enzyme

All assays were performed using 0.3 µg of the purified enzyme in a volume of 1 ml.

Substance (mM)	pNPP production (nmol min ⁻¹ ml ⁻¹)*	Relative activity
None	12.50 ± 0.19	1.00
EDTA (1)	1.25 ± 0.05	0.10
EDTA (20)	< 0.1	< 0.01
P _i (20)	12.75 ± 0.18	1.02
P _i (50)	10.25 ± 0.16	0.82
P _i (100)	7.75 ± 0.14	0.62
Tartrate (1)	12.88 ± 0.18	1.03
Tartrate (10)	12.38 ± 0.19	0.99
F ⁻ (1)	11.88 ± 0.17	0.95
F ⁻ (10)	11.50 ± 0.18	0.92
Ca ²⁺ (1)	1.25 ± 0.07	0.10
Mg ²⁺ (1)	21.63 ± 0.31	1.73
Co ²⁺ (1)	30.25 ± 0.41	2.42
Zn ²⁺ (1)	17.25 ± 0.26	1.38

* Mean value of three experiments ± SE.

Table 4. Phosphotransferase activity of the NapA phosphatase in the presence of pNPP (5 mM) as donor and various organic phosphate acceptors containing free hydroxyl groups

All assays were performed using 0.15 µg of the purified enzyme in a volume of 1 ml.

Acceptor (mM)	Production (nmol min ⁻¹ ml ⁻¹)* of:		Phosphate transfer (%)
	pNP	P _i	
H ₂ O	5.95 ± 0.12	5.99 ± 0.13	0
Ethanol (200)	9.70 ± 0.15	7.08 ± 0.15	27
Ethanol (1000)	18.35 ± 0.29	3.90 ± 0.10	79
Ethanol (2000)	17.55 ± 0.27	1.90 ± 0.08	89
Glycerol (200)	7.95 ± 0.14	7.10 ± 0.16	11
Glycerol (1000)	10.30 ± 0.16	2.95 ± 0.09	71
Adenosine (0.1)	2.95 ± 0.08	2.15 ± 0.07	27
2'-Deoxyadenosine (0.1)	3.65 ± 0.11	2.80 ± 0.09	23
Cytidine (0.1)	4.10 ± 0.10	3.85 ± 0.11	6
2'-Deoxycytidine (0.1)	3.30 ± 0.10	2.50 ± 0.08	24
Uridine (0.1)	2.20 ± 0.07	1.70 ± 0.06	22
2'-Deoxyuridine (0.1)	2.20 ± 0.08	1.55 ± 0.06	29

* Mean value of three experiments ± SE.

protein (Fig. 4). Since the insert of pPM9 terminated at a *Sau3AI* site located a short distance upstream of the start codon (Fig. 4), part of the insert of pPM2 was also sequenced to determine the structure of the 5'-flanking region of the *napA* gene.

The *napA* ORF has the potential to code for a polypeptide of 236 amino acids with a predicted molecular mass of 26686 Da. The deduced amino acid sequence of the NH₂-terminal region of the polypeptide showed features resembling those of prokaryotic signal sequences for protein export to the periplasmic space (Oliver, 1985) and, on the basis of NH₂-terminal sequencing data, it actually appeared to function as a signal sequence which is cleaved by signal peptidase after the alanine residue at position 23. A putative ribosome-binding site resembling those of *E. coli* is located upstream of the start codon.

All *E. coli* strains harbouring recombinant plasmids which included the *napA* ORF and flanking sequences showed strong pNPP-hydrolysing activity and were able to produce the NapA protein. On the other hand, strains harbouring recombinant plasmids which contained only a portion of the *napA* ORF showed pNPP-hydrolysing activity comparable to that of *E. coli* DH5α(pBluescript) and were not able to produce the NapA protein (Fig. 2). These data were in agreement with the hypothesis that the above ORF actually encoded the NapA protein.

The presence of a similar genomic region including the *napA* ORF was also analysed in the *M. morganii* strain ATCC 25830^T by means of PCR using primers MMNAP-

Structure of the *napA* gene and deduced amino acid sequence of the NapA protein

The nucleotide sequence of the *Morganella* DNA insert of plasmid pPM9 was determined, and a computer analysis of possible coding regions allowed identification of a single ORF whose size was compatible with the results of SDS-PAGE analysis, and which was able to code for a polypeptide containing an amino acid sequence corresponding to the NH₂-terminal sequence of the NapA

Comparison of the deduced amino acid sequence of the NapA protein with those of other sequenced bacterial periplasmic phosphatases including the *E. coli* PhoA (Chang *et al.*, 1986), UshA (Burns & Beacham, 1986a),

Fig. 5. Comparison of the deduced amino acid sequences of the NapA phosphatase of *M. morganii* (Mmo NapA) with those of the hypothetical proteins of *E. coli* (Eco ORF; EMBL accession number Z26592) and *P. mirabilis* (Pmi ORF; Charles *et al.*, 1985; EMBL accession number M11587) showing sequence homology to NapA. Numbers on the left correspond to the residue numbers of the proteins. Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a colon. In the comparison between the NapA enzyme and the *P. mirabilis* hypothetical protein, homology is followed through different reading frames (indicated as 1°, 2° and 3°, respectively) and frameshifts introduced to follow homology are indicated; X indicates a stop codon.

When the deduced amino acid sequence of the *M. morganii* NapA enzyme was compared with all sequences present in the EMBL database (release 37) by means of the TFASTA program (Pearson, 1990), a high degree of homology was found with two hypothetical proteins of unknown function encoded by sequenced regions of the *E. coli* and *Proteus mirabilis* genomes respectively. The hypothetical *E. coli* NapA homologue is encoded by an ORF located at 92 min, in the *tyrB-wrA* intergenic region (EMBL accession number Z26592), and shows an overall degree of homology of 60.6% (rising to 90.7% when conservative amino acid substitutions are allowed for) with the *Morganella* NapA enzyme (Fig. 5). Interestingly, the opposite strand of this ORF entirely encompasses another ORF encoding a DNA-binding protein specific for the *E. coli* origin of replication (cited in EMBL entry Z26592). With regard to the *P. mirabilis* NapA homologue, it is apparently encoded by an ORF located 0.4 kb upstream of the chromosomal *cat* gene which is involved in chloramphenicol resistance (Charles *et al.*, 1985). In this case, however, only part of the sequence is available (corresponding to amino acids 1–157 of the *Morganella* NapA protein) and the ORF encoding the NapA homologue is

prematurely interrupted by a termination codon (Fig. 5). However, since the homology can be followed in alternative reading frames (Fig. 5), and since a NapA-like phosphatase is actually present in *P. mirabilis* (M. C. Thaller and others, unpublished), it seems more likely that the interruptions in the *P. mirabilis* ORF are due to sequencing errors than to actual mutations that led to a silencing event. Considering the alignment that can be obtained after frameshifts have been introduced to follow homology, the *P. mirabilis* NapA homologue shows, in the sequenced region, an overall degree of homology of 79.9% (rising to 95.6% when conservative amino acid substitutions are allowed for).

DISCUSSION

Phosphatase activities of enteric bacteria have been studied from several viewpoints, but current knowledge on these enzymes is still incomplete.

With regard to periplasmic acid phosphatases, several different enzymes have been described and characterized at the sequence level, and different molecular classes of these enzymes can be recognized on the basis of sequence data and functional properties. These include: (a) the class A acid phosphatases, which are EDTA-resistant non-specific acid phosphatases composed of low molecular mass polypeptides (~25–27 kDa), thus far described in *S. typhimurium* (Groisman *et al.*, 1992; Weppelman *et al.*, 1977), *M. morganii* (Thaller *et al.*, 1994) and *P. stuartii* (M. L. Riccio and others, unpublished, EMBL accession number X64820); (b) the Agp–AppA family of enzymes described in *E. coli*, which includes two enzymes with somewhat different properties [an acid glucose phosphatase (Agp) and an acid phosphatase with a very low pH optimum (AppA)] which are homologous at the sequence level and are probably derived from the same ancestor gene (Dassa *et al.*, 1990); (c) the periplasmic UDP-sugar hydrolases, thus far characterized and sequenced in *E. coli* and *S. typhimurium* (Burns & Beacham, 1986a, b) (in the latter species the gene is silent), but probably present in several enteric bacteria (Neu, 1968); (d) the cyclic phosphodiesterases, thus far sequenced in *E. coli* (Liu *et al.*, 1986), but apparently present in several enteric bacteria (Neu, 1968).

Characterization and sequencing of the *M. morganii* NapA acid phosphatase, previously identified as a minor phosphate-irrepressible acid phosphatase (Thaller *et al.*, 1994), enabled its recognition as a new molecular species of bacterial periplasmic acid phosphatase. Since an enzyme with similar properties has been described in *S. typhimurium* (Uerkvitz, 1988; Uerkvitz & Beck, 1981), it is reasonable to hypothesize that this enzyme represents a NapA homologue produced by *S. typhimurium*. Moreover, genes homologous to *napA* are present in the *E. coli* and *P. mirabilis* genomes and, although their products have not been characterized, we have recently described in *E. coli* a nonspecific acid phosphatase composed of a 27 kDa polypeptide component (Rossolini *et al.*, 1994) which could correspond to the product of the *napA* homologue found in this species, and we have also found a NapA-like

protein in *P. mirabilis* (M. C. Thaller and others, unpublished). The *Morganella* NapA enzyme, therefore, appears to be a member of a family of conserved enzymes which we propose designating class B bacterial acid phosphatases, and which could be widespread among enteric bacteria. We are currently investigating the presence of NapA homologues in other enteric bacteria, and preliminary results indicate a widespread distribution of such enzymes among members of this family (M. C. Thaller and others, unpublished).

The class B bacterial acid phosphatases thus far characterized (i.e. the *Morganella* NapA and the *Salmonella* NapII enzymes) are nonspecific acid phosphatases composed of low molecular mass polypeptides similar to class A enzymes, but unlike the latter they apparently require a metal cofactor for activity and are more susceptible to phosphate inhibition. Interestingly, these enzymes are also endowed with phosphotransferase activity, a property that could be relevant in cell physiology, especially for an enzyme located in the periplasmic space. The physiological role of these enzymes remains to be established. In *S. typhimurium*, the NapII enzyme has been considered as the major periplasmic 5'-nucleotidase (Uerkvitz & Beck, 1981). It would be interesting to confirm a similar role in other bacterial species and to investigate whether other functions are dependent on these class B enzymes.

ACKNOWLEDGEMENTS

This work was supported in part by grant nos 92.01195.PF70 and 93.01030.PF70 from the Italian National Research Council (CNR), targeted project 'Biotecnologie e Biostrumentazione', and no. 92.02779.CT04 from the CNR. We should like to thank Giuseppe Satta for helpful suggestions and discussions and Annalisa Santucci for NH₂-terminal sequencing of the NapA enzyme. Thanks are also due to Francesco Lissi and Elena Sestini for their skilful editorial assistance.

REFERENCES

- Ames, B. N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* **8**, 115–118.
- Beacham, I. R. (1979). Periplasmic enzymes in Gram-negative bacteria. *Int J Biochem* **10**, 877–883.
- Burns, D. M. & Beacham, I. R. (1986a). Nucleotide sequence and transcriptional analysis of the *E. coli ushA* gene, encoding periplasmic UDP-sugar hydrolase (5'-nucleotidase): regulation of the *ushA* gene, and the signal sequence of its encoded protein product. *Nucleic Acids Res* **14**, 4325–4342.
- Burns, D. M. & Beacham, I. M. (1986b). Identification and sequence analysis of a silent gene (*ushA*⁰) in *Salmonella typhimurium*. *J Mol Biol* **192**, 163–175.
- Chang, C. N., Kuang, W.-J. & Chen, E. Y. (1986). Nucleotide sequence of the alkaline phosphatase gene of *Escherichia coli*. *Gene* **44**, 121–125.
- Charles, I. G., Keyte, J. W. & Shaw, W. V. (1985). Nucleotide sequence analysis of the *cat* gene of *Proteus mirabilis*: comparison with the type I (Tn9) *cat* gene. *J Bacteriol* **164**, 123–129.
- Cocks, G. T. & Wilson, A. C. (1972). Enzyme evolution in the *Enterobacteriaceae*. *J Bacteriol* **110**, 793–802.

- Dassa, J., Marck, C. & Boquet, P. L. (1990). The complete nucleotide sequence of the *Escherichia coli* gene *appA* reveals significant homology between pH 2.5 acid phosphatase and glucose-1-phosphatase. *J Bacteriol* **172**, 5497–5500.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Dvorak, H. F., Brockman, R. W. & Heppel, L. A. (1967). Purification and properties of two acid phosphatase fractions isolated from osmotic shock fluid of *Escherichia coli*. *Biochemistry* **6**, 1743–1751.
- Edwards, C. J., Innes, D. J., Burns, D. M. & Beacham, I. R. (1993). UDP-sugar hydrolase isozymes in *Salmonella enterica* and *Escherichia coli*: silent alleles of *ushA* in related strains of Group I *Salmonella* isolates, and of *ushB* in wild-type and K12 strains of *E. coli*, indicate recent and early silencing events, respectively. *FEMS Microbiol Lett* **114**, 293–298.
- Falkow, S., Ryman, I. R. & Washington, O. (1962). Deoxyribonucleic acid base composition of *Proteus* and Providence organisms. *J Bacteriol* **83**, 1318–1321.
- Ferro-Luzzi Ames, G., Prody, C. & Kustu, S. (1984). Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J Bacteriol* **160**, 1181–1183.
- Groisman, E. A., Saier, M. H., Jr & Ochman, H. (1992). Horizontal transfer of a phosphatase gene as evidence for mosaic structure of the *Salmonella* genome. *EMBO J* **11**, 1309–1316.
- Heukeshoven, J. & Dernick, R. (1985). Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* **6**, 103–112.
- Kier, L. D., Weppelman, R. & Ames, B. N. (1977). Resolution and purification of three periplasmic phosphatases of *Salmonella typhimurium*. *J Bacteriol* **130**, 399–410.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Liu, J., Burns, D. M. & Beacham, I. R. (1986). Isolation and sequence analysis of the gene (*cpdB*) encoding periplasmic 2',3'-cyclic phosphodiesterase. *J Bacteriol* **165**, 1002–1010.
- Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**, 10035–10038.
- Neu, H. C. (1968). The 5'-nucleotidases and cyclic phosphodiesterases (3'-nucleotidases) of the *Enterobacteriaceae*. *J Bacteriol* **95**, 1732–1737.
- Oliver, D. (1985). Protein secretion in *Escherichia coli*. *Annu Rev Microbiol* **39**, 615–618.
- Pearson, W. R. (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol* **183**, 63–98.
- Pompei, R., Cornaglia, G., Ingiani, A. & Satta, G. (1990). Use of a novel phosphatase test for simplified identification of species of the tribe *Proteae*. *J Clin Microbiol* **28**, 1214–1218.
- Pompei, R., Ingiani, A., Foddìs, G., Di Pietro, G. & Satta, G. (1993). Patterns of phosphatase activity among enterobacterial species. *Int J Syst Bacteriol* **43**, 174–178.
- Pond, J. L., Eddy, C. K., Mackenzie, K. F., Conway, T., Borecky, D. J. & Ingram, L. O. (1989). Cloning, sequencing, and characterization of the principal acid phosphatase, the *phoC*⁺ product, from *Zymomonas mobilis*. *J Bacteriol* **171**, 767–774.
- Pradel, E., Marck, C. & Boquet, P. L. (1990). Nucleotide sequence and transcriptional analysis of the *Escherichia coli* *agp* gene encoding periplasmic acid glucose-1-phosphatase. *J Bacteriol* **172**, 802–807.
- Reiland, J. (1971). Gel filtration. *Methods Enzymol* **22**, 287–321.
- Rossolini, G. M., Thaller, M. C., Pezzi, R. & Satta, G. (1994). Identification of an *Escherichia coli* periplasmic acid phosphatase containing a 27 kDa-polypeptide component. *FEMS Microbiol Lett* **118**, 167–174.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Thaller, M. C., Berlutti, F., Schippa, S., Lombardi, G. & Rossolini, G. M. (1994). Characterization and sequence of PhoC, the principal phosphate-irrepressible acid phosphatase of *Morganella morganii*. *Microbiology* **140**, 1341–1350.
- Uerkvitz, W. (1988). Periplasmic nonspecific acid phosphatase II from *Salmonella typhimurium* LT2. *J Biol Chem* **263**, 15823–15830.
- Uerkvitz, W. & Beck, C. F. (1981). Periplasmic phosphatases in *Salmonella typhimurium* LT2. A biochemical, physiological, and partial genetic analysis of three nucleoside monophosphate dephosphorylating enzymes. *J Biol Chem* **256**, 382–389.
- Weppelman, R., Kier, L. D. & Ames, B. N. (1977). Properties of two phosphatases and a cyclic phosphodiesterase of *Salmonella typhimurium*. *J Bacteriol* **130**, 411–419.

Received 22 April 1994; revised 9 August 1994; accepted 21 September 1994.