Characterization and sequence of PhoC, the principal phosphate-irrepressible acid phosphatase of Morganella morganii

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Phosphatase activities were investigated in Morganella morganii, which is one of the few enterobacterial species producing high-level phosphate-irrepressible acid phosphatase activity (HPAP phenotype), and the gene encoding the major phosphate-irrepressible acid phosphatase was cloned, sequenced, and its product characterized. Using p-nitrophenyl phosphate as substrate, Morganella produced a major phosphate-irrepressible acid phosphatase (named PhoC) which is associated with the HPAP phenotype, a minor phosphate-irrepressible acid phosphatase, and a phosphate-repressible alkaline phosphatase. The presence of the PhoC activity prevented induction of alkaline phosphatase when a PhoC-hydrolysable organic phosphate ester, such as glycerol 2-phosphate, was the sole phosphate source. PhoC is a secreted nonspecific acid phosphatase apparently composed of four 25 kDa polypeptide subunits. The enzyme is resistant to EDTA, P2, fluoride and tartrate. The M. morganii PhoC showed 84.6% amino acid sequence identity to the PhoN nonspecific acid phosphatase of Providencia stuartii, 45.3% to the PhoN nonspecific acid phosphatase of Salmonella typhimurium, and 37.8% to the principal acid phosphatase (PhoC) of Zymomonas mobilis. Comparison of sequence data and of regulation of these enzymes suggested a different phylogeny of members of this gene family within the Enterobacteriaceae.

Keywords: Morganella morganii, PhoC, phosphatase activities

INTRODUCTION

In members of the family Enterobacteriaceae, production of phosphatase activity is a constant feature (Cocks & Wilson, 1972; Satta et al., 1988), but different enzymic patterns have been observed in different species (Cocks & Wilson, 1972; Pompei et al., 1990, 1993). Such differences have been confirmed at the molecular level in the case of Escherichia coli and Salmonella typhimurium, which are the best characterized enterobacterial species from this point of view. In fact, although some activities are present in both species, such as 2',3'-cyclic phosphodiesterases and acid hexose phosphatases (Dvorak et al., 1967; Kiee et al., 1977a), S. typhimurium is apparently lacking a counterpart for the alkaline phosphatase and periplasmic UDP-sugar hydrolase found in E. coli (Cocks & Wilson, 1972; Neu, 1968; Schlesinger & Olsen, 1968), while E. coli is apparently lacking a counterpart for the PhoN nonspecific acid phosphatase found in S. typhimurium (Kasahara et al., 1991). Differences between these species could also extend to other acid phosphatases, but the limited information concerning properties of a nonspecific acid phosphatase fraction described in E. coli (Dvorak et al., 1967), and the lack of information on the possible presence in S. typhimurium of an enzyme active at very low pH values similar to the E. coli AppA enzyme (Dassa et al., 1982), do not allow a precise comparison in this case.

Since they show diverse patterns of expression, characterization of the different phosphatases that have evolved in the Enterobacteriaceae could provide a relevant contribution, not only to the knowledge of the physiology and evolution of these enzymes and of their regulation, which is still limited, but also to evolutionary studies in the enterobacterial genome (see for instance Groisman et al., 1992).

Abbreviations: G2P, glycerol 2-phosphate; HPAP, high-level phosphate-irrepressible production of acid phosphatase activity; pNPP, p-nitrophenyl phosphate.

The GenBank accession number for the sequence data reported in this paper is X64444.
Among the various patterns of phosphatase activity observed in *Enterobacteriaceae*, a peculiar one consists in a high-level phosphate-irrepressible production of acid phosphatase activity (HPAP phenotype), which has thus far been observed in *Morganella morganii* and *Providencia stuartii* (Pompei et al., 1990, 1993). For this reason, we have started to investigate the phosphatases of the above species.

In this report we have studied in some detail the phosphatases of *M. morganii* (as a representative HPAP* species) and found some differences in their pattern as compared to that of other *Enterobacteriaceae*.

*M. morganii* is an enterobacterial species occurring in low frequency in the intestines of mammals and some reptiles (Phillips, 1955; Winslow et al., 1919) and, recently, the calf intestine has been reported as a major source of this species (Hawkey et al., 1986). In humans, *Morganella* can be responsible for urinary tract infections as a primary pathogen, and can also occur as an opportunistic secondary invader in infections of various parts of the body (von Graevenitz & Spector, 1969; Williams et al., 1983).

**METHODS**

**Culture media and conditions.** To study phosphatase activities produced by *M. morganii* under different conditions of P available, SP medium (devised on the basis of preliminary experiments) was used. Composition (in g 1⁻¹ of the SP medium was as follows: NaCl, 0.5; KCl, 0.4; MgSO₄·7H₂O, 0.2; CaCl₂, 2H₂O, 0.265; NH₄Cl, 0.535; glucose, 4; L-arginine, 0.126; L-cysteine, 0.012; L-glutamine, 0.292; L-histidine, 0.042; L-isoleucine, 0.052; L-leucine, 0.052; L-lysine, 0.0725; L-methionine, 0.05; L-phenylalanine, 0.0325; L-threonine, 0.048; L-tryptophan, 0.01; L-tyrosine, 0.036; L-valine, 0.046; biotin, 0.001; folic acid, 0.001; nicotinic acid, 0.001; pantothentic acid, 0.001; pyridoxal, 0.001; riboflavin, 0.0001; thiamin, 0.001. The medium was buffered with 200 mM Tris/HCl, pH 7.2. Phosphate sources (P₁ or G₂P) were added to the desired final concentration.

For studying production of phosphatase activities under different conditions of P available, bacteria exponentially growing in SP medium containing 1 mM P₁ were washed in sterile saline (at 37 °C) and inoculated in prewarmed SP medium containing 1 mM P₁, 1 mM G₂P, or 0.005 mM P₁. Bacteria grown in the above assay media were then collected at the mid exponential phase of growth. In the SP medium containing 0.005 mM P₁, bacterial growth was limited to only a few generations and in this case cells were always collected after a 5 h incubation time.

All strains were grown aerobically at 37 °C.

**Bacterial strains and genetic vectors.** The *M. morganii* strains used in this study were from the strain collection at the Institute of Microbiology, University 'La Sapienza', Rome, and had been identified according to standard procedures (Farmer & Kelly, 1991). All strains were clinical isolates from humans with urinary tract infections.

*E. coli* DH5α (Sambrook et al., 1989) was used as the host for genetic vectors and recombinant plasmids.

The Bluescript SK plasmid (Stratagene) was used for the construction of the *M. morganii* RS12 genomic library and for subcloning procedures.

**Phosphatase assays.** The phosphatase activity of whole cell or periplasmic protein preparations toward p-nitrophenyl phosphate (pNPP) was assayed by measuring the released p-nitrophenol at 414.5 nm at pH 12. The concentration of pNPP 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 nmol of p-nitrophenol per min under the assay conditions. The assays were performed in 100 mM buffers, including sodium acetate buffer (pH 5 and 6), Tris/HCl buffer (pH 7–9), and glycine/NaOH buffer (pH 10). Measurement of enzymic activity in different buffer systems at overlapping pH values showed no significant buffer-related variation.

For preparation of whole-cell proteins, bacteria were washed twice in normal saline, resuspended in normal saline at an A₅₅₀ 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 activity of the purified PhoC protein using different substrates was performed as previously described (Kier et al., 1977a; Weppelman et al., 1977) in 100 mM sodium acetate buffer, pH 6. Inhibition assays were performed in the same buffer using pNPP as the substrate. The enzyme was preincubated at 37 °C for 30 min with each substance before starting the assay.

**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 previously described (Laemmli, 1970). After electrophoresis the gels were either stained with Coomassie brilliant blue R250 or incubated for 4 h at 37 °C in several changes of renaturation buffer to obtain renaturation of enzymes. Renaturation buffer was 100 mM Tris/HCl, pH 7, containing 5 mM MgSO₄ and 1% (v/v) Triton X-100. After the renaturation treatment, gels were equilibrated for 1 h in either 100 mM sodium acetate buffer, pH 6, or 100 mM glycine/NaOH buffer, pH 10, both 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 used for equilibration with 5 mM pNPP 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 1⁻¹ and 28.6 ml sulphuric acid 1⁻¹) and 10% (w/v) ascorbic acid, to detect the presence of P₁ (Ames, 1966). Phosphatase activities were indicated by the presence of blue-stained bands.

**Purification of the PhoC enzyme.** When *E. coli* strains producing the *Morganella* PhoC enzyme were grown in liquid medium at 37 °C, a consistent amount of the protein was released in the culture medium. The PhoC protein present in the medium of 24-h-old stationary phase cultures in Brain Heart Infusion broth supplemented with carbenicillin (0.2 mg ml⁻¹) represented approximately 50% of the total proteins present in the medium, as evaluated by a densitometric scan of a Coomassie-blue-stained polyacrylamide gel (data not shown); its estimated amount was approximately 0.1 mg ml⁻¹. A similar culture supernatant was dialysed against 10 mM sodium acetate buffer, pH 6 (buffer A), until complete removal of P₁, and then used as the starting material for purification of the PhoC protein. For phosphocellulose chromatography, the dialysed supernatant was washed onto a phosphocellulose (Whatman P11) column (Poly-Prep chromatography column; Bio-Rad)
previously equilibrated with buffer A. The PhoC enzyme was then eluted with 20 mM sodium phosphate buffer, pH 6.

For determination of the molecular mass of the PhoC protein by means of gel filtration, the culture supernatant or the purified protein was applied to a Sephacryl S-200 column (1 x 20 cm) equilibrated and eluted with buffer A. The Sephacryl S-200 column was calibrated with protein standards dissolved in elution buffer. The peak fraction of each protein was determined by measuring the absorbance 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.

Amino-terminal amino acid sequencing. The purified protein preparation was subjected to SDS-PAGE and transferred electrothermally 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).

Recombinant DNA methodology. Basic recombinant DNA techniques were essentially as described by Sambrook et al. (1989). High molecular mass chromosomal DNA was extracted from M. morganii as described by Frankel et al. (1989), with the addition of a phenol deproteinization step. For construction of the Morganella genomic library, chromosomal DNA was partially digested with Sau3AI restriction endonuclease to obtain restriction fragments more abundantly represented in the range from 1 to 10 kb; following removal of the smaller DNA fragments by PEG precipitation (Lis, 1980), the restriction fragments were ligated to BamHI-linearized and dephosphorylated plasmid Bluescript SK. Unidirectional deletions of DNA fragments using exonuclease I11 and nuclease S1 were performed techniques were essentially as described by Sambrook et al., 1989. 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. Comparison and alignment of sequences was performed using the CLUSTAL program (Higgins & Sharp, 1988). The minimum value for conservative amino acid substitutions according to the log-odds matrix of Dayhoff was fixed at 8.

RESULTS AND DISCUSSION

Analysis of phosphatase activities in M. morganii under conditions of different P, availability

As previously mentioned, M. morganii is one of the enterobacterial species showing an HPAP' phenotype (Pompei et al., 1990, 1993). However, Morganella strains are occasionally found which apparently lack this phenotype (Thaller et al., 1992a). To characterize the phosphatase activities produced by M. morganii under conditions of different P, availability, two HPAP' strains (RS12 and RS24) and one HPAP' strain (RS31) were selected.

Using pNPP as substrate, phosphatase activity produced by the above strains was analysed, at different P, values, in whole cell protein preparations obtained from cultures growing in media of different composition. The HPAP' strains RS12 and RS24 showed high-level production of phosphatase activity under all growth conditions. Activity peaked at acidic pH value when cells were grown in SP medium either containing abundant P, (1 mM) or G2P (1 mM) as the sole phosphate source, the activity pattern being similar in both conditions (Fig. 1a). In P,-starved cultures with no addition of any organic phosphate source (SP medium containing 0.005 mM P,) high-level phosphatase activity at acidic P values was still present, but a peak of phosphatase activity appeared at alkaline pH values (Fig. 1a). The HPAP' strain RS31 showed a much lower phosphatase activity, as compared to that of the two HPAP' strains, when cells were grown in the SP medium containing abundant P,. In this strain, growth on G2P as the sole phosphate source caused a moderate increase of phosphatase activity, peaking at alkaline P, values, while P,-starvation with no available organic phosphate source caused a remarkable increase of phosphatase activity, peaking at alkaline P, values (Fig. 1b).

To analyse in more detail the phosphatase activities produced by M. morganii under the above culture conditions, zymograms were performed on whole cell protein preparations obtained from cultures grown under different conditions, using pNPP as the substrate. This analysis showed that, in HPAP' Morganella strains, a major and a minor acid phosphatase activity, constituted by 25 and 27 kDa polypeptide units, respectively, were produced under any culture conditions, while an alkaline phosphatase activity, constituted by a 48 kDa polypeptide unit, was produced only when cells were grown under conditions of absolute P,-deprivation. The major acid phosphatase, named PhoC, retained part of its activity also at alkaline P, values, and its production was apparently somewhat reduced when cells were P,-starved and alkaline phosphatase was also produced (Fig. 2). In the HPAP' strain, the dominant feature was represented by a complete lack of production of the PhoC enzyme under any culture conditions, while the minor acid phosphatase activity was still produced. Expression of alkaline phosphatase appeared to be P,-regulated, as in the HPAP' strains. In this case, however, alkaline phosphatase was also produced when G2P was the sole phosphate source, although in a lower amount compared to that observed in P,-starved cultures (data not shown).

The data from zymograms were consistent with those obtained from the measurement of total enzymic activity and indicated that at least two P,-irrepressible acid phosphatases, constituted by low molecular mass polypeptide chains, are produced by M. morganii and may contribute to the HPAP' phenotype typical of this species. Of the two enzymes, PhoC is likely to be necessary for, as well as the principal (if not the only) enzyme responsible for, this phenotype, since it was by far the major activity and was lacking in the RS31 HPAP' strain. Confirmation of these assumptions will be achieved by the construction and analysis of isogenic mutants for the genes encoding each enzyme.

Under conditions of low-P, availability, as previously reported (Cocks & Wilson, 1972), Morganella is also able to produce an alkaline phosphatase which, according to its regulation and the molecular mass of its polypeptide component, is probably similar to that of E. coli (Bradshaw et al., 1981; Garen & Levinthal, 1960; Torriani, 1960). The fact that in Morganella, unlike in E. coli (Torriani,
**Fig. 1.** Total phosphatase activity against pNPP, at different pH values, of (a) *M. morganii* RS12 and (b) *M. morganii* RS31. The assay was performed using whole cell protein preparations from cultures growing in media of different composition. •, SP medium + 1 mM Pi; □, SP medium + 1 mM G2P; ○, SP medium + 0.005 mM Pi. The values are reported in U (mg total protein)^-1 and represent mean values of three different experiments (standard error bars are also shown; for points closely spaced with other points, only the upper or the lower half of the bar is shown). The activity profiles observed were reproducible in the three experiments. Results obtained with strain RS24 were the same as those obtained with strain RS12, and are not shown.

**Fig. 2.** Zymograms developed for phosphatase activity at pH 6 (a) or 10 (b) after renaturing SDS-PAGE. Lanes: 1-3, whole-cell proteins (approximately 0.1 mg) from strain RS12 growing in SP medium + 1 mM Pi, SP medium + 1 mM G2P and SP medium + 0.005 mM Pi, respectively; 4, periplasmic proteins from the PM11 *E. coli* clone. Protein size markers are reported in kDa on the left. These zymogram patterns were reproducible in triplicate experiments and representative results are shown. Results obtained with strain RS24 were the same as those obtained with RS12, and are not shown.

**Fig. 3.** SDS-PAGE analysis of proteins of the PM11 clone. (a) Coomassie-blue-stained whole cell protein preparations of *E. coli* DH5α (pBluescript) (lane 1) and PM11 (lane 2). (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 against pNPP at pH 6. (c) Coomassie-blue-stained periplasmic proteins of PM11. Protein size markers are reported in kDa on the left.

1960), production of alkaline phosphatase remains fully repressed when an organic phosphate ester such as G2P becomes the sole phosphate source, is likely to be due to the presence of the two phosphate-irrepressible acid phosphatases, both of which are able to hydrolyse G2P (see below, and M. C. Thaller and others, unpublished) and, in doing so, would provide enough Pi to prevent induction of alkaline phosphatase. A relevant role of PhoC in this phenomenon is suggested by the fact that, in the HPAP<sup>−</sup> RS31 strain which does not produce PhoC, alkaline phosphatase is actually produced when G2P is the only phosphate source.

The zymogram technique appeared to be very useful for a rapid and rather comprehensive analysis of the phosphatase activities produced under different growth conditions. The study of bacterial phosphatases, in fact, is complicated by the fact that many such enzymes are produced in the cell and their production can be regulated by multiple mechanisms. Therefore, the classical approach of selection of mutants altered in production of a given phosphatase may be hampered by the presence of other overlapping activities, while the classical procedure of purification and characterization of different enzymic
activities can be rendered even more cumbersome by the need of repetition in several different culture conditions. The renaturing SDS-PAGE technique was preferred to native electrophoresis techniques since it allowed a good resolution of activities, providing at the same time information on the molecular mass of the polypeptide which constitutes the protein.

The fact that we were not able to detect other phosphatase activities, such as cyclic phosphodiesterase, 5'-nucleotidase, or hexose-phosphatase, in zymograms should not be surprising since the above experiments were performed using pNPP as substrate (which may not be hydrolysed by some of the above enzymes) and using growth conditions which may not induce some of the above enzymes. Using the same zymogram technique in combination with appropriate culture conditions and substrates, we were actually able to detect putative hexose-phosphatase and phosphodiesterase activities in *M. morganii* (Thaller and others, unpublished). Since *Morganella* phosphatase activity was not assayed at very low pH values, the presence of enzymic activities similar to the *E. coli* AppA enzyme (Dassa et al., 1982) could not have been detected. It should finally be noted that the use of a renaturing SDS-PAGE technique could have also resulted in missing enzymic activities which are either heteropolymeric in nature or not able to renature following SDS-PAGE.

### Cloning of the *M. morganii* genetic determinant coding for the major phosphate-irrepressible acid phosphatase (PhoC)

The *M. morganii* gene coding for the major phosphate-irrepressible acid phosphatase was isolated by a shotgun-cloning strategy which allows the isolation of different phosphatase-encoding genes in one step (M. L. Riccio and others, unpublished) using an indicator medium that does not detect *E. coli* phosphatase activity (Satta et al., 1979, 1988). Briefly, a genomic library of RS12, constructed in the plasmid vector Bluescript SK and transformed in *E. coli* DH5α, was screened for clones able to grow as green-stained colonies on tryptose-phosphate agar plates containing the phosphatase substrate phenolphthalein diphosphate (2 mg ml⁻¹), methyl green (0.05 mg ml⁻¹) and carbenicillin (0.1 mg ml⁻¹) for plasmid selection.

A green-stained clone (PM11) was thus identified, which showed a much higher acid phosphatase activity than *E. coli* DH5α (pBluescript) (data not shown) and produced a 25 kDa polypeptide, not present in *E. coli* (Fig. 3a), endowed with phosphatase activity at acidic pH after allowing in-gel renaturation of proteins (Fig. 3b). Comparison of the acid phosphatase produced by the PM11 clone to the *Morganella* phosphatases resolved by the zymogram technique showed that it apparently corresponded to the PhoC acid phosphatase (Fig. 2). The acid phosphatase produced by the PM11 clone could be extracted by a chloroform treatment suitable for extraction of the periplasmic proteins from *E. coli* (Ferro-Luzzi Ames et al., 1984) (Fig. 3c), and the ratio of phosphatase activity between a periplasmic and a whole cell protein preparation from this clone was consistent with a location of this protein into the periplasmic space.

The cloned DNA fragment harboured by the PM11 clone was mapped (Fig. 4), and its origin was confirmed by digestion of chromosomal DNA of *M. morganii* RS12 with several restriction endonucleases and analysis of restriction fragments by Southern blotting, using the cloned fragment as the hybridization probe (data not shown).

The above results suggested that the *Morganella* gene encoding the major phosphate-irrepressible acid phosphatase had been cloned. Definitive confirmation that the enzyme produced by the PM11 clone was actually encoded...
The amino-terminal sequence of the PhoC protein was determined as NH$_2$-AIPAGNDATTKPDLYYLKNE.

The molecular mass of the protein, estimated by gel-filtration chromatography, was approximately 95 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 30 kDa, while being only partially retained by filters which excluded globular proteins of sizes larger than 100 kDa. The above data, along with results of renaturing SDS-PAGE, suggest that the native protein is a homotetramer of the 25 kDa polypeptide.

The purified protein preparation was used to analyse some of its functional properties. Using pNPP as substrate, the PhoC enzyme had a pH optimum around 6, retaining part of its activity within a broad pH range (data not shown; see also Fig. 2). The enzyme showed a broad substrate specificity which did not include diesters (Table 1). The activity of the PhoC enzyme against pNPP was fully retained in the presence of EDTA (up to 20 mM was assayed), fluoride (up to 10 mM was assayed) or tartrate (up to 10 mM was assayed). Enzymic activity was also fully retained in the presence of up to 10 mM Pi, while a slight inhibition (approximately 10%) was observed in the presence of 100 mM Pi.

The functional properties of the PhoC protein are similar overall to those of the PhoN nonspecific acid phosphatase of S. typhimurium, the similarity extending also to the size of the polypeptide component (Uerkvitz & Beck, 1981; Weppelman et al., 1977). Unlike the Salmonella enzyme, however, the Morganella PhoC protein is apparently more active on 5'-nucleotides than on 3'-nucleotides and is not inhibited by fluoride ions. Moreover, a difference in the quaternary structure of the two enzymes is suggested by the fact that the molecular mass of the Morganella PhoC enzyme is higher (approximately twofold) than that of the Salmonella PhoN protein (Uerkvitz & Beck, 1981; Weppelman et al., 1977). It should be noted that the two enzymes are also similar at the level of the primary structure (see below), so the functional and quaternary structure differences are likely to be related to discrete differences between the two amino acid sequences. These two enzymes, therefore, could represent a valuable starting model for studies on the structure-function relationship in this class of enzymes.

### Structure of the phoC gene

The phoC gene was preliminarily located within the 1.2 kb Sau3AI–EcoRI DNA fragment of the cloned insert by generating different subclones and assaying their ability to produce the PhoC protein (Fig. 4).

The nucleotide sequence of this Morganella DNA fragment was determined (Fig. 6), and computer analysis for possible coding regions identified a single open reading frame (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 amino-terminal sequence of the PhoC protein (see above). This ORF has the potential to code...
All *E. coli* strains harbouring recombinant plasmids which included the *phoC* ORF and flanking sequences showed strong pNPP hydrolysing activity and were able to produce the PhoC protein, independently of the insert orientation and of the presence of *E. coli* promoter sequences located near the insert junctions. On the other hand, strains harbouring recombinant plasmids which contained only a portion of the *phoC* ORF, obtained by deletion of the insert of plasmid pPM12R using exonuclease III and nuclelease S1, showed a pNPP hydrolysing activity comparable to that of *E. coli* DH5α (pBluescript) and were no longer able to produce the 25 kDa polypeptide (Fig. 4). These data were in agreement with the hypothesis that the above ORF actually encoded the PhoC protein, and also suggested that the *Morganella* DNA sequences located upstream of the *phoC* gene could promote transcriptions of the same gene in *E. coli*.

The G+C contents of the sequenced region (47%) and of the *phoC* ORF (52%), as well as at the third position of codons (54%), are consistent with the value reported (50%) for the *Morganella* genome (Falkow et al., 1962).

### Comparison of the primary structure of the *Morganella PhoC* enzyme to that of other bacterial acid phosphatases

The deduced amino acid sequence of the *M. morganii* PhoC enzyme was compared to all protein sequences present in the SwissProt database (release 24.0) by means of the FASTA program (Pearson, 1990). A significant degree of sequence homology was found between this enzyme and three other bacterial acid phosphatases, i.e. the PhoC principal acid phosphatase of *Zymomonas mobilis* (Pond et al., 1989), the PhoN nonspecific acid phosphatase of *S. typhimurium* (Groisman et al., 1992; Kasahara et al., 1991) and the PhoN acid phosphatase of *P. stuartii* (M. L. Riccio, G. Lombardi, A. Chiesurin et al., unpubl.) from the *M. morganii* genome (Falkow et al., 1962). The deduced amino acid sequence of the *M. morganii* PhoC enzyme (526 residues) is compared with the sequences of three other bacterial acid phosphatases of *Z. mobilis* (Pond et al., 1989) and two *E. coli* enzymes, respectively, while the degree of similarity increased to 94.4% when the *M. morganii* PhoC sequence was compared to the *P. stuartii* PhoC enzyme, and to 84.6%, 45.3% and 37.8% when the *M. morganii* PhoC enzyme was aligned with the *P. stuartii*, *S. typhimurium*, and *Z. mobilis* enzymes, respectively, while the degree of similarity increased to 94.4%, 80.6% and 77.1%, respectively, after allowing for conservative amino acid substitutions. The homology among the same regions at the nucleotide sequence level was 71.8%, 52.1%, and 46.9% when the *Morganella* *phoC* sequence was compared to the *P. stuartii*, *S. typhimurium*, and *Z. mobilis* sequences, respectively.

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**Fig. 6.** Nucleotide sequence of the *phoC* ORF and flanking regions. Number 1 represents the first base of the start codon of the *phoC* ORF. Putative sequences involved in transcriptional control, identified on the basis of similarity with *E. coli* consensus sequences, are underlined (−10 regions) or overlined (−35 regions). Two convergent arrows indicate the downstream inverted repeat possibly functioning as a transcriptional terminator. The putative ribosomal-binding site is boxed. The deduced amino acid sequence of the *PhoC* protein is reported under the nucleotide sequence. The signal sequence, which is cleaved by signal peptidase after the alanine residue at position 20, is shown in bold. A bacterial phosphatases, showed the existence of several conserved regions shared by the four proteins, including two cysteine residues and a perfectly conserved 8-amino-acid sequence, GSYPSGHT (Fig. 7). At the sequence level, the enzymes from *M. morganii* and *P. stuartii* are more similar to each other than to the other two proteins. Considering the 201-amino-acid region that could be aligned virtually without gaps in all sequences (corresponding to amino acids 44–244 of the *Morganella* enzyme; see Fig. 7), the overall amino acid identity was 84.6%, 45.3%, and 37.8% when the *M. morganii* PhoC enzyme was aligned with the *P. stuartii*, *S. typhimurium*, and *Z. mobilis* enzymes, respectively, while the degree of similarity increased to 94.4%, 80.6% and 77.1%, respectively, after allowing for conservative amino acid substitutions. The homology among the same regions at the nucleotide sequence level was 71.8%, 52.1%, and 46.9% when the *Morganella* *phoC* sequence was compared to the *P. stuartii*, *S. typhimurium*, and *Z. mobilis* sequences, respectively.

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**Fig. 6.** Nucleotide sequence of the *phoC* ORF and flanking regions. Number 1 represents the first base of the start codon of the *phoC* ORF. Putative sequences involved in transcriptional control, identified on the basis of similarity with *E. coli* consensus sequences, are underlined (−10 regions) or overlined (−35 regions). Two convergent arrows indicate the downstream inverted repeat possibly functioning as a transcriptional terminator. The putative ribosomal-binding site is boxed. The deduced amino acid sequence of the *PhoC* protein is reported under the nucleotide sequence. The signal sequence of the PhoC protein is underlined.
The high level of homology between the *M. morganii* and the *P. stuartii* class A acid phosphatases at the nucleotide sequence level is consistent with previous results, which showed that a probe made from a fragment of the *P. stuartii* phoN gene was able to hybridize with *M. morganii* genomic DNA under low stringency conditions (Thaller et al., 1992b). On the basis of these data it could be speculated that also in *P. stuartii*, which is the other HPAP+ enterobacterial species thus far described, this phenotype is associated with a phosphate-irrepressible production of the respective class A phosphatase.

**Vertical phylogeny versus horizontal transfer of class A acid phosphatases in enteric bacteria**

From studying base composition and codon usage data, and the presence of a nucleotide sequence showing similarity to the oriT region of incFII plasmids and located upstream of the gene, it has recently been suggested that the *phoN* gene of *S. typhimurium* was acquired by lateral transmission from another species with a low G+C genomic content in a plasmid-mediated event (Groisman et al., 1992). This is apparently not the case for the *M. morganii* *phoC* gene. In fact, the occurrence of highly homologous class A acid phosphatase genes in more than one member (*M. morganii* and *P. stuartii*) of the enteroccluster 3 lineage (Ahmad et al., 1990), along with the fact that the G+C contents of the above genes (also at the third position of the codons) is consistent with the overall G+C contents of the respective species (Falkow et al., 1962), suggest that these genes are vertically derived from a common ancestor present in the corresponding lineage before divergence of the above species. Moreover, considering the sequence data, it appears unlikely that the *S. typhimurium* *phoN* gene has been derived by lateral transfer from *M. morganii* or *P. stuartii*. In fact, of the two latter species, only *P. stuartii*, which has a low G+C content both at the genomic level (41% versus 50% of *Morganella*; Falkow et al., 1962) and in its own *phoN* gene (43% versus 52% of the *Morganella* *phoC* gene), could be a suitable donor candidate, given the low G+C content of the *Salmonella* *phoN* gene (Groisman et al., 1992). In this case, however, a significantly higher degree of similarity with the *Salmonella* gene would have been expected for the *P. stuartii* gene than for the *M. morganii* gene, and an overall higher degree of similarity would have also been expected between the *Salmonella* gene and the genes of the two enteroccluster 3 members.

Unlike the PhoN enzyme of *S. typhimurium*, the production of which is regulated by several environmental conditions and is moderately induced by Pi starvation (Kier et al., 1977b), the *M. morganii* class A acid phosphatase is produced in a Pi-irresistible fashion and Pi starvation actually seemed to somewhat repress its production while inducing alkaline phosphatase. The production of class A acid phosphatases, therefore, appears to be under the control of different regulatory systems in these two enterobacterial species, and this is consistent with a different evolutionary history of these genes.

We are currently investigating the presence of homologous genes in other bacterial species to ascertain the intriguing evolutionary history of this gene family.

**Concluding remarks**

Results of this study indicate that the set of phosphatases of *M. morganii* is different from both that of *E. coli* and that of *S. typhimurium*, while sharing with them some common features. *Morganella*, in fact, is able to produce both an alkaline phosphatase (similarly to *E. coli*) and a class A nonspecific acid phosphatase (similarly to *Salmonella* and unlike *E. coli*). Regulation of the former enzyme is apparently similar to that of *E. coli*, while the class A acid phosphatase is apparently regulated in a different way as compared to the *S. typhimurium* homologue. These findings therefore support the hypothesis that different sets of phosphatases have evolved in *Enterobacteriaceae*.

The role of class A acid phosphatases in microbial physiology, as well as the significance of their different regulation in different species, remains to be determined. In members of the genus *Salmonella* this gene appears to be widespread, but strains are found which are no longer able to produce the PhoN protein owing to recent mutational events (Groisman et al., 1992). PhoN production is apparently dispensable under laboratory conditions (Uerkvitz & Beck, 1981), and is also not important for microbial pathogenicity notwithstanding the recruitment of the *phoN* gene under the *phoP*-*phoQ* virulence regulon (Fields et al., 1989; Miller et al., 1989). A similar situation is consistent with the evolutionary origin of the *phoN* gene which has been acquired by horizontal transfer.
(Groisman et al., 1992). Since in Morganella the evolutionary history of the pbcC gene and its regulation are apparently different from those of the homologous Salmonella phoN gene, it would be of interest to understand the role of the PhoC enzyme and its high-level production in this species.

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