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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, P_i, 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; Kier *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.

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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_i availability, SP medium (devised on the basis of preliminary experiments) was used. Composition (in g l^{-1}) of the SP medium was as follows: NaCl, 0·5; KCl, 0·4; $MgSO_4.7H_2O$, 0·2; $CaCl_2.2H_2O$, 0·265; NH_4Cl, 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; pantothenic 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 (P_i or G2P) were added to the desired final concentration.

For studying production of phosphatase activities under different conditions of P_i availability, bacteria exponentially growing in SP medium containing 1 mM P_i were washed in sterile saline (at 37 °C) and inoculated in prewarmed SP medium containing 1 mM P_i, 1 mM G2P, or 0·005 mM P_i. 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 P_i 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 (*p*NPP) was assayed by measuring the released *p*-nitrophenol at 414·5 nm at 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 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 A_{590} of $\simeq 10$, 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 *p*NPP as the substrate. The enzyme was pre-incubated 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 $MgSO_4$ 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 $MgSO_4$, 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 *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 P_i (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 P_i, 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 × 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 A_{280}. 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 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).

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 *Sau*3AI 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 *Bam*HI-linearized and dephosphorylated plasmid Bluescript SK. Unidirectional deletions of DNA fragments using exonuclease III and nuclease S1 were performed as previously described (Henikoff, 1984). 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_i 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_i availability, two $HPAP^+$ strains (RS12 and RS24) and one $HPAP^-$ strain (RS31) were selected.

Using *p*NPP as substrate, phosphatase activity produced by the above strains was analysed, at different pH 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_i (1 mM) or G2P (1 mM) as the sole phosphate source, the activity pattern being similar in both conditions (Fig. 1a). In P_i-starved cultures with no addition of any organic phosphate source (SP medium containing 0·005 mM P_i) high-level phosphatase activity at acidic pH 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_i. In this strain, growth on G2P as the sole phosphate source caused a moderate increase of phosphatase activity, peaking at alkaline pH values, while P_i-starvation with no available organic phosphate source caused a remarkable increase of phosphatase activity, peaking at alkaline pH 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 *p*NPP 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_i-deprivation. The major acid phosphatase, named PhoC, retained part of its activity also at alkaline pH values, and its production was apparently somewhat reduced when cells were P_i-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_i-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_i-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_i-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_i 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,

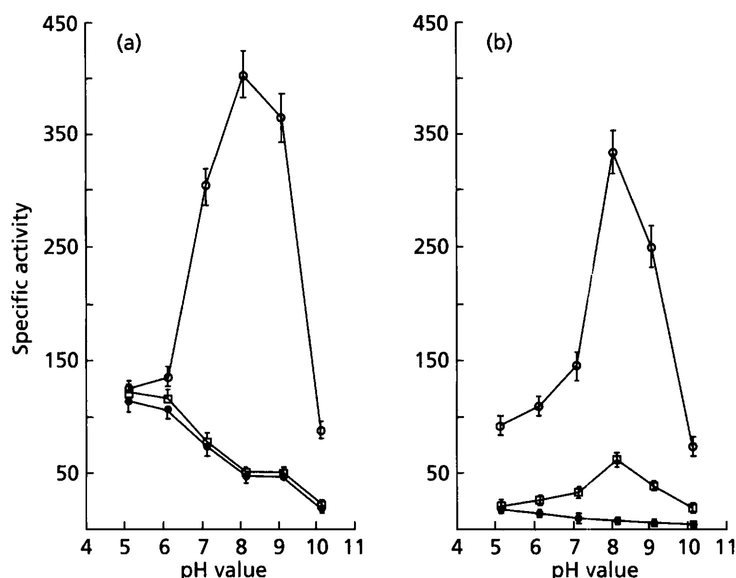

Fig. 1. Total phosphatase activity against *p*NPP, at different pH values, of (a) *M. morganii* RS12 and (b) *M. morganii* RS31. The assay was performed using whole cell protein preparations from cultures growing in media of different composition. ●, SP medium + 1 mM P_i; □, SP medium + 1 mM G2P; ○, SP medium + 0·005 mM P_i. 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.

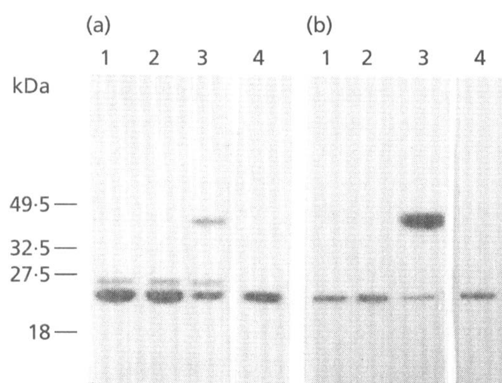

Fig. 2. Zymograms developed for phosphatase activity at pH 6 (a) or 10 (b) after renaturing SDS-PAGE. Lanes: 1–3, whole-cell proteins (approximately 0·1 mg) from strain RS12 growing in SP medium + 1 mM P_i, SP medium + 1 mM G2P and SP medium + 0·005 mM P_i, respectively; 4, periplasmic proteins from 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.

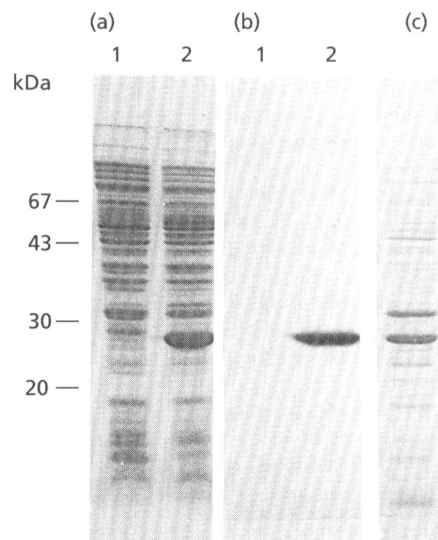

Fig. 3. SDS-PAGE analysis of proteins of the PM11 clone. (a) Coomassie-blue-stained whole cell protein preparations of *E. coli* DH5α (pBluescript) (lane 1) and PM11 (lane 2). (b) Same samples as in (a) but in this case a lower amount of protein was loaded and, after electrophoresis, the gel was subjected to renaturation treatment and developed for phosphatase activity against *p*NPP 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 P_i to prevent induction of alkaline phosphatase. A relevant role of PhoC in this phenomenon is suggested by the fact that, in the HPAP$^-$ 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

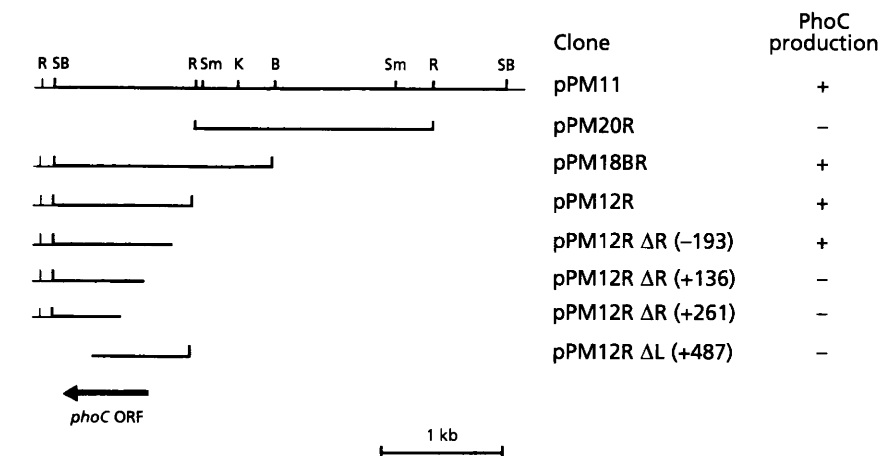

Fig. 4. Restriction endonuclease map of the DNA insert of the recombinant plasmid carried by PM11, subcloning strategy, and deletion analysis. Abbreviations: B, *Bam*HI; R, *Eco*RI; K, *Kpn*I; SB, *Sau*3AI/*Bam*HI junction; Sm, *Sma*I. Thick lines represent *Morganella* DNA, while thin lines represent vector sequences. The location of the putative *PhoC* ORF, identified on the basis of sequencing data and deletion analysis, is shown below the map. Production of the PhoC protein was assayed both by measuring *p*NPP-hydrolysing activity at pH 6, and by SDS-PAGE analysis of periplasmic proteins of different subclones. PhoC production by *E. coli* (pPM12R) occurred independently of insert orientation. Deletion derivatives of pPM12R are indicated as ΔR or ΔL according to the extremity (right or left, respectively) of the insert from which the deletion process was started. Numbers in parentheses indicate the position of the first undeleted nucleotide according to the numbering reported in Fig. 6.

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 *p*NPP 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

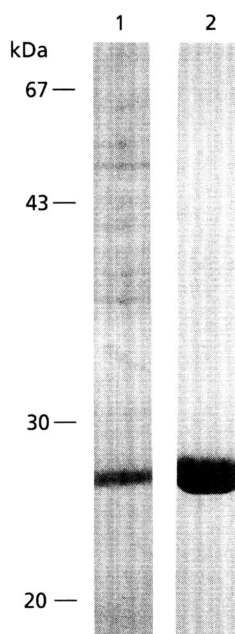

Fig. 5. **SDS-PAGE analysis of the purified PhoC protein. Lanes: 1, culture supernatant (0·08 ml) of the PM11 *E. coli* clone which was used as the starting material for purification; 2, purified PhoC protein (approximately 0·08 mg).**

Table 1. Relative activities of the PhoC enzyme toward various substrates

Substrate	Relative activity
5′-UMP	1·00
5′-AMP	1·17
3′-UMP	0·07
3′-AMP	0·05
*p*NPP	2·45
Phenolphthalein diphosphate	1·51
G2P	0·19
Glucose 6-phosphate	1·44
bis-*p*NPP	< 0·01
2′:3′-cyclic UMP	< 0·01
2′:3′-cyclic AMP	< 0·01

by the cloned *Morganella* fragment was obtained by comparison of amino-terminal sequencing data of the enzyme to the nucleotide sequence of the cloned insert (see below).

Characterization of the PhoC enzyme

Purification of the *Morganella* PhoC enzyme produced by the *E. coli* PM11 clone was obtained in a single step using phosphocellulose column chromatography (Fig. 5; see Methods for details on the purification procedure). The specific activity of the purified protein, assayed against *p*NPP at pH 6, was 7487 U mg^{-1}.

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 *p*NPP 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 *p*NPP 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 P_i, while a slight inhibition (approximately 10%) was observed in the presence of 100 mM P_i.

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 *Sau*3AI–*Eco*RI 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

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-315 GAATTCCGGAAAATTTCATTCATTTTAATTGTTAAGAATATGCTGGCAAAAACAAAACCC

-255 AATGCTTTATATTTTCTTATAATATCTGTGTGTTATCTTTTTCAATACTATCGGTCAGGT

-195 CTTATTTATCCGTTCGTTAACAAAAGCCATGCTGTTTCTGTCAAATTATCTGAAAATCAT

-135 CATCAAAAATACTTACCTGTCTTCCGTCTGTTTCGTCACACTTTTTTGAAAGAGTTAACA

 -75 TCAATTTGCATCTCTCCGCCCTACACTGGCAAACAGGTTTCTGAGTAATACTGTTGTATC

 -15 TGATAAGGAGATGTCATGAAGAAGAATATTATCGCCGGTTGTCTGTTCTCACTGTTTTCC
                      M  K  K  N  I  I  A  G  C  L  F  S  L  F  S

 +46 CTTTCCGCACTGGCCGCGATCCCGGCAGGCAACGATGCCACCACCAAGCCGGATTTATAT
      L  S  A  L  A  A  I  P  A  G  N  D  A  T  T  K  P  D  L  Y

+106 TATCTGAAAAATGAACAGGCTATCGACAGCCTGAAACTGTTACCGCCACCGCCGGAAGTC
      Y  L  K  N  E  Q  A  I  D  S  L  K  L  L  P  P  P  P  E  V

+166 GGCAGTATTCAGTTTTTAAATGATCAGGCAATGTATGAGAAAGGCCGTATGCTGCGCAAT
      G  S  I  Q  F  L  N  D  Q  A  M  Y  E  K  G  R  M  L  R  N

+226 ACCGAGCGCGGAAAACAGGCACAGGCAGATGCTGACCTGGCCGCAGGGGGGGTGGCAACC
      T  E  R  G  K  Q  A  Q  A  D  A  D  L  A  A  G  G  V  A  T

+286 GCATTTTCAGGGGCATTCGGCTATCCGATAACCGAAAAAGACTCTCCGGAGCTGTATAAA
      A  F  S  G  A  F  G  Y  P  I  T  E  K  D  S  P  E  L  Y  K

+346 CTGCTGACCAATATGATTGAGGATGCCGGTGACCTTGCCACCCGCTCCGCCAAAGAACAT
      L  L  T  N  M  I  E  D  A  G  D  L  A  T  R  S  A  K  E  H

+406 TACATGCGCATCCGTCCGTTTGCGTTTTACGGCACAGAAACCTGTAATACCAAAGATCAG
      Y  M  R  I  R  P  F  A  F  Y  G  T  E  T  C  N  T  K  D  Q

+466 AAAAAACTCTCCACCAACGGATCTTACCCGTCAGGTCATACGTCTATCGGCTGGGCAACG
      K  K  L  S  T  N  G  S  Y  P  S  G  H  T  S  I  G  W  A  T

+526 GCATTAGTGCTGGCGGAAGTGAACCCGGCAAATCAGGATGCGATTCTGGAACGGGGTTAT
      A  L  V  L  A  E  V  N  P  A  N  Q  D  A  I  L  E  R  G  Y

+586 CAGCTCGGACAGAGCCGGGTGATTTGCGGCTATCACTGGCAGAGTGATGTGGATGCCGCG
      Q  L  G  Q  S  R  V  I  C  G  Y  H  W  Q  S  D  V  D  A  A

+646 CGGATTGTCGGTTCAGCCGCTGTCGCGACATTACATTCCGATCCGGCATTTCAGGCGCAG
      R  I  V  G  S  A  A  V  A  T  L  H  S  D  P  A  F  Q  A  Q

+706 TTAGCGAAAGCCAAACAGGAATTTGCACAAAAATCACAGAAATAAGCAGTGATATCTGGT
      L  A  K  A  K  Q  E  F  A  Q  K  S  Q  K  *

+766 CAGGGCAGTGCAATATCTGCCCTGAAATCCCTGTTTATTCCCACATCCAGCGGTCTTCCC

+826 GATC
```

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.**

for a polypeptide of 249 amino acids with a predicted molecular mass of 27002 Da. The deduced amino acid sequence of the amino-terminal region of the polypeptide showed features resembling those of procaryotic signal sequences for protein export to the periplasmic space (Oliver, 1985) and, on the basis of amino-terminal sequencing data, it actually appeared to function as a signal sequence which is cleaved by signal peptidase after the alanine residue at position 20. A putative ribosomal-binding site resembling those of *E. coli* is located just upstream of the ATG codon. The *phoC* ORF is preceded by putative promoter sequences, and a 9 bp inverted repeat, which could function as a rho-independent transcription terminator, is located downstream of the ORF (Fig. 6).

All *E. coli* strains harbouring recombinant plasmids which included the *phoC* ORF and flanking sequences showed strong *p*NPP 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 inset of plasmid pPM12R using exonuclease III and nuclease S1, showed a *p*NPP 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 transcription 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 *M. morganii* 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 & G. Satta, unpublished results; EMBL accession number X64820). The result of multiple sequence alignment analysis within this family of enzymes, which we propose to indicate as class A bacterial acid 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.

```
PhoC Zmo    1 MIKVPRFICMIALTSGVLASGLSQSVSAHTEKSEPSSTYHFHS-----DPLLYLAPPP
PhoN Sty    1 MKSRYLVF----F----LPLIVAKYTSAE---TVQP----FHSPEESVNSQFYLPPPP
PhoN Pst    1 MKKLLAVFCAGAF----VSTSVFAAIPPGNDVTTKPDLYYLKNSQA-IDSLALLPPPP
PhoC Mmo    1 MKKNIIAGCLFSL----FSLSALAAIPAGNDATTKPDLYYLKNEQA-IDSLKLLPPPP

             54 TSGSPLQAHDDQTFNSTRQLKGSTRWALATQDADLHLASVLKDYACAAGMNLDIAQLP
             44 GNDDPAYRYDKEAYFKGYAIKGSPRWKQAAEDADVSVENIARIFSPVVGAKINPKDTP
             54 EVGSILFLNDQAMYEKGRLLRNTERGEQAAKDADLAAGGVANAFSEAFGYPITEKDAP
             54 EVGSIQFLNDQAMYEKGRMLRNTERGKQAQADADLAAGGVATAFSGAFGYPITEKDSP

            112 HLANLIKRAL-RTEYDDIGRAKNNWNRKRPFVDTDQPICTEKDREGLGKQGSYPSGHT
            102 ETWNMLKNLLTMGGYYATASAKKYYMRTRPFVLFNHSTCRPEDENTLRKNGSYPSGHT
            112 EIHKLLTNMIEDAGDLATRSAKEKYMRIRPFAFYGVATCNTKDQDKLSKNGSYPSGHT
            112 ELYKLLTNMIEDAGDLATRSAKEHYMRIRPFAFYGTETCNTKDQKKLSTNGSYPSGHT

            169 TIGWSVALILAELIPDHAANILQRGQIFGTSRIVCGAHWFSDVQAGYIMASGEIAALH
            160 AYGTLLALVLSEARPERAQELARRGWEFGQSRVICGAHWQSDVDAGRYVGAVEFARLQ
            170 AIGWASALVLSEINPENQDKILKRGYELGQSRVICGYHWQSDVDAARIVASGAVATLH
            170 SIGWATALVLAEVNPANQDAILERGYQLGQSRVICGYHWQSDVDAARIVGSAAVATLH

            227 GDADFRRDMELARKELEKARTSAHTPDDLLCKIEQSAR--
            218 TIPAFQKSLAKVREELND-------KNNLLSKEDHPKLNY
            228 SNPEFQKQLQKAKDEFA--------------KLKK-----
            228 SDPAFQAQLAKAKQEFAQ-------------KSQK-----
```

Fig. 7. **Comparison of the deduced amino acid sequence of the PhoC phosphatase of *M. morganii* RS12 (PhoC *Mmo*) with those of the PhoC phosphatase of *Z. mobilis* CP4 (PhoC *Zmo*) (Pond *et al.*, 1989), the PhoN phosphatase of *S. typhimurium* ATCC 14028 (PhoN *Sty*) (Groisman *et al.*, 1992), and the PhoN phosphatase of *P. stuartii* PV81 (PhoN *Pst*) (M. L. Riccio, G. Lombardi, A. Chiesurin & G. Satta, unpublished results; EMBL accession number X64820). Numbers located at the left correspond to the residue numbers of the respective proteins. Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a colon.**

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 enterocluster 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 enterocluster 3 members.

Unlike the PhoN enzyme of *S. typhimurium*, the production of which is regulated by several environmental conditions and is moderately induced by P_i starvation (Kier *et al.*, 1977b), the *M. morganii* class A acid phosphatase is produced in a P_i-irrepressible fashion and P_i 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 unlike *Salmonella*) 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 *phoC* 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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REFERENCES

Ahmad, S., Weisburg, W. G. & Jensen, R. A. (1990). Evolution of aromatic amino acid biosynthesis and application to the fine-tuned phylogenetic positioning of enteric bacteria. *J Bacteriol* **172**, 1051–1061.

Ames, B. N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* **8**, 115–118.

Bradshaw, R. A., Cancedda, F., Ericsson, L. H., Neuman, P. A., Piccoli, S. P., Schlesinger, M. J., Schrifer, K. & Walsh, K. A. (1981). Amino acid sequence of *Escherichia coli* alkaline phosphatase. *Proc Natl Acad Sci USA* **78**, 3473–3477.

Cocks, G. T. & Wilson, A. C. (1972). Enzyme evolution in the *Enterobacteriaceae*. *J Bacteriol* **110**, 793–802.

Dassa, E., Cahu, M., Desjoyaux-Cherel, B. & Boquet, P. L. (1982). The acid phosphatase with optimum pH of 2·5 of *Escherichia coli*: physiological and biochemical study. *J Biol Chem* **257**, 6669–6676.

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.

Falkow, S., Ryman, I. R. & Washington, O. (1962). Deoxyribonucleic acid base composition of *Proteus* and Providence organisms. *J Bacteriol* **83**, 1318–1321.

Farmer, J. J. III & Kelly, M. T. (1991). *Enterobacteriaceae*. In *Manual of Clinical Microbiology*, 5th edn, pp. 360–383. Edited by A. Balows, W. J. Hausler Jr, K. L. Herrmann, H. D. Isenberg & H. J. Shadomy. Washington DC: American Society for Microbiology.

Ferro-Luzzi Ames, G., Prody, C. & Kustu, S. (1984). Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J Bacteriol* **160**, 1181–1183.

Fields, P. I., Groisman, E. A. & Heffron, F. (1989). A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243**, 1059–1062.

Frankel, G., Newton, S. M. C., Schoolnik, G. K. & Stocker, B. A. D. (1989). Unique sequences in the region VI of the flagellin gene of *Salmonella typhi*. *Mol Microbiol* **3**, 1379–1383.

Garen, A. & Levinthal, C. (1960). A fine-structure genetic and biochemical study of the enzyme alkaline phosphatase of *E. coli*. *Biochim Biophys Acta* **38**, 470–483.

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.

Hawkey, P. M., Penner, J. L., Linton, A. H., Hawkey, C. A., Crisp, L. J. & Hinton, M. (1986). Speciation, serotyping, antimicrobial susceptibility and plasmid content of *Proteeae* from the environment of calf-rearing units in south west England. *J Hyg* **97**, 405–417.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359.

Higgins, D. G. & Sharp, P. M. (1988). CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237–244.

Kasahara, M., Nakata, A. & Shinagawa, H. (1991). Molecular analysis of the *Salmonella typhimurium phoN* gene, which encodes nonspecific acid phosphatase. *J Bacteriol* **173**, 6770–6775.

Kier, L. D., Weppelman, R. & Ames, B. N. (1977a). Resolution and purification of three periplasmic phosphatases of *Salmonella typhimurium*. *J Bacteriol* **130**, 399–410.

Kier, L. D., Weppelman, R. & Ames, B. N. (1977b). Regulation of two phosphatases and a cyclic phosphodiesterase of *Salmonella typhimurium*. *J Bacteriol* **130**, 420–428.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lis, J. T. (1980). Fractionation of DNA fragments by polyethylene glycol induced precipitation. *Methods Enzymol* **65**, 347–353.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**, 10035–10038.

Miller, S. I., Kukral, A. M. & Mekalanos, J. J. (1989). A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci USA* **86**, 5054–5058.

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.

Phillips, J. E. (1955). *In vitro* studies of *Proteus* organisms of animal origin. *J Hyg* **53**, 26–31.

Pompei, R., Cornaglia, G., Ingianni, 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., Ingianni, A., Foddis, 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.

Reiland, J. (1971). Gel filtration. *Methods Enzymol* **22**, 287–321.

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.

Satta, G., Grazi, G., Varaldo, P. E. & Fontana, R. (1979). Detection of bacterial phosphatase activity by means of an original and simple test. *J Clin Pathol* **32**, 391–395.

Satta, G., Pompei, R., Grazi, G. & Cornaglia, G. (1988). Phosphatase activity is a constant feature of all isolates of all major species of the family *Enterobacteriaceae*. *J Clin Microbiol* **26**, 2637–2641.

Schlesinger, M. J. & Olsen, R. (1968). Expression and localization of *Escherichia coli* alkaline phosphatase synthesized in *Salmonella typhimurium* cytoplasm. *J Bacteriol* **96**, 1601–1605.

Thaller, M. C., Berlutti, F., Pantanella, F., Pompei, R. & Satta, G. (1992a). Modified MacConkey medium which allows simple and reliable identification of *Providencia stuartii*. *J Clin Microbiol* **30**, 2054–2057.

Thaller, M. C., Berlutti, F., Riccio, M. L. & Rossolini, G. M. (1992b). A species specific DNA probe for *Providencia stuartii* identification. *Mol Cell Probes* **6**, 417–422.

Torriani, A. (1960). Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. *Biochim Biophys Acta* **38**, 460–469.

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.

Von Graevenitz, A. & Spector, H. (1969). Observations on indole positive *Proteus*. *Yale J Biol Med* **41**, 434–445.

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.

Williams, E. W., Hawkey, P. M., Penner, J. L., Senior, B. W. & Barton, L. (1983). Serious nosocomial infections caused by *Morganella morganii* and *Proteus mirabilis* in a cardiac surgery unit. *J Clin Microbiol* **18**, 5–9.

Winslow, C.-E. A., Kliger, I. J. & Rothberg, W. (1919). Studies on the classification of the colon-typhoid group of bacteria with special reference to their reactions. *J Bacteriol* **4**, 429–503.

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