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Review

Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology

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Abstract. Bacterial nonspecific acid phosphohydrolases (NSAPs) are secreted enzymes, produced as soluble periplasmic proteins or as membrane-bound lipoproteins, that are usually able to dephosphorylate a broad array of structurally unrelated substrates and exhibit optimal catalytic activity at acidic to neutral pH values. Bacterial NSAPs are monomeric or oligomeric proteins containing polypeptide components with an M_r of 25–30 kDa. On the basis of amino acid sequence relatedness, three different molecular families of NSAPs can be distinguished, indicated as molecular class A, B and C, respectively. Members of each class share some common biophysical and functional features, but may also exhibit functional differences. NSAPs have been detected in several microbial taxa, and enzymes of different classes can be produced by the same bacterial species. Structural and phyletic relationships exist among the various bacterial NSAPs and some other bacterial and eucaryotic phosphohydrolases. Current knowledge on bacterial NSAPs is reviewed, together with analytical tools that may be useful for their characterization. An overview is also presented concerning the use of bacterial NSAPs in biotechnology.

Key words. Acid phosphohydrolases; bacteria; genetics; physiology; molecular evolution; microbial biotechnology.

Introduction

Bacteria have several enzymes able to dephosphorylate organic compounds, which play various essential or accessory roles in cell physiology. Most dephosphorylating reactions known to occur in the procaryotic cell involve the hydrolysis of phosphoester or phosphoanhydride bonds and are catalysed by a group of enzymes indicated overall as phosphohydrolases or phosphatases [1]. Some of these enzymes are secreted outside the plasma membrane, where they are either released in a soluble form or retained as membrane-bound proteins. These enzymes, which henceforth will be referred to as secreted phosphohydrolases, are believed to function essentially in scavenging organic phosphoesters (such as nucleotides, sugar phosphates, phytic acid etc.) that cannot cross the cytoplasmic membrane. Inorganic phosphate (Pi) and organic by-products are released,

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that can be transported across the membrane, thus providing the cell with essential nutrients [2–4]. Some secreted phosphohydrolases have evolved specialized functions relevant to microbial virulence (e.g. the respiratory burst-inhibiting acid phosphatases of *Legionella micdadei* [5] and *Francisella tularensis* [6], and the protein-tyrosine phosphatases of *Yersinia* spp. [7, 8] and *Salmonella enterica* ser. *typhimurium* [9]). Other phosphohydrolases are found in the cytosolic compartment, where they may be involved in dephosphorylating reactions occurring in signal transduction [10] as well as in several metabolic pathways.

The interest in bacterial phosphohydrolases is not only related to their multiple roles in the biology of the procaryotic cell and to their occasional involvement in microbial pathogenicity but also to the possibility of exploiting these enzymes as (i) investigative tools in enzymology (see, for instance, refs 11, 12) and in regulation of gene expression (see, for instance, refs 13, 14); (ii) paradigms for molecular evolution (see, for instance, refs 15, 16); (iii) markers for bacterial taxonomy and identification (see, for instance, refs 17–19); (iv) reporters in immunology and molecular biology (see, for instance, refs 20–21); (v) tools for bioremediation in environmental microbiology [22, 23].

Current knowledge on bacterial phosphohydrolases is far from being complete. Most of the available information is derived from studies performed in the *Escherichia coli* or *S. enterica* ser. *typhimurium* models of procaryotic cell, and even in these models, the exact number of phosphatase activities remains to be established and the roles of known enzymes are only partially understood [4]. Information on phosphohydrolases of other bacterial species is considerably more limited. Notions derived from comparative studies suggest that the phosphatase pattern can be variable even within closely related bacterial species [15, 24–27], so that results obtained studying one species may not necessarily be valid for others. The study of microbial phosphatase, therefore, remains an active investigational field, with relevance to various aspects of microbial physiology and biotechnology.

Classification of phosphatases, including the bacterial ones, was initially based on the biochemical and biophysical properties of the enzyme such as pH optimum (acid, neutral or alkaline), substrate profile (nonspecific vs. specific for certain substrates) and molecular size (high vs. low molecular weight). As molecular sequence data for different enzymes became available, it was recognized that, like other proteins, phosphatases could be grouped into different molecular families according to similarity at the level of primary structure. This structural criterion has led to the definition of various molecular families and superfamilies of phosphatases, and signature sequence patterns specific for each family have been identified [28] which are useful for a tentative identification of the function of newly discovered genes from large-scale sequencing projects.

The objective of this article is to review current information on bacterial nonspecific acid phosphohydrolases (NSAPs). This term refers to a group of secreted enzymes which are usually able to hydrolyse a broad array of structurally unrelated organic phosphoesters and exhibit optimal catalytic activity at acidic to neutral pH values. Some of these enzymes were purified and characterized several years ago [29–31], but only recently have further investigations provided additional insights concerning the structure, function and distribution of bacterial NSAPs [27, 32–42]. An overview is first presented, in which the research done on bacterial NSAPs is briefly outlined, and current knowledge on these enzymes is summarized. Analytical tools useful for studying bacterial NSAPs are then reviewed. A detailed description of known bacterial NSAPs follows, in which structural and functional features of the various enzymes, and their distribution, are reviewed and compared. The final section discusses the use of some bacterial NSAPs as tools for applications in biotechnology.

Bacterial NSAPs: an overview

The term 'NSAP' was originally adopted to indicate bacterial enzymes which, unlike alkaline phosphatase, show optimal catalytic activity at acidic to neutral pH values and, unlike specific phosphohydrolases (e.g. 3′-nucleotidases, 5′-nucleotidases, hexose-phosphatases and phytases), do not exhibit a marked substrate specificity, retaining activity towards several different and structurally unrelated phosphoesters.

The existence of NSAP activity in the *E. coli* periplasm was reported in the late sixties [43], but this enzyme was not purified to homogeneity and further characterized. The first bacterial NSAPs purified and characterized in detail were the periplasmic PhoN (or nonspecific acid phosphatase I) and AphA (or nonspecific acid phosphatase II) enzymes produced by *S. enterica* ser. *typhimurium* [29–32]. Both enzymes were made by polypeptides of relatively low molecular mass (around 25 kDa), but showed different biophysical and functional properties.

Subsequent studies, performed on different bacterial species, demonstrated that production of acid phosphohydrolases containing low molecular mass polypeptides (i.e. in the 25–30-kDa range) and showing properties similar to the *Salmonella* NSAPs was not restricted to members of the latter genus, being actually widespread among several different microbial taxa [27, 33, 36–40]. At the same time, cloning of some NSAP-encoding genes allowed identification, on the basis of amino acid sequence relatedness, of the existence of two different molecular families of NSAPs that we proposed to designate as molecular class A and molecular class B bacterial NSAPs, respectively [37, 38]. According to this criterion, the *Salmonella* PhoN enzyme [34, 35] turned out to be a member of molecular class A, while the AphA enzyme belonged to molecular class B [37, 38].

Most recently, further investigation on NSAPs produced by nonenterobacterial species led to the discovery of a third molecular family of bacterial NSAP that we have proposed to designate as molecular class C [42]. Enzymes of this class appear to be distantly related to class B NSAPs from the structural and evolutionary standpoint, but unlike the latter, which are secreted across the cytoplasmic membrane, yielding soluble periplasmic proteins, they carry an amino-terminal signal sequence typical of bacterial lipoproteins and are found as membrane-bound lipoproteins.

Current knowledge on bacterial NSAPs can be summarized as follows: (i) bacterial NSAPs are widespread enzymes that can be found in several different microbial taxa; (ii) all the bacterial NSAPs thus far identified and characterized are secreted enzymes, of which some are produced as soluble periplasmic proteins, while others are membrane-bound lipoproteins; (iii) at least three different molecular families of bacterial NSAPs can be identified on the basis of relatedness at the sequence level, and members of these families are designated as molecular class A, B and C NSAPs, respectively; signature sequence motifs specific for each molecular class have been defined that can be useful for a tentative identification of new hypothetical proteins; in addition to sequence similarity, members of each molecular class share some common functional and biophysical features which can be exploited as phenotypic markers for presumptive classification of newly discovered enzymes; (iv) notwithstanding the existence of common features, members of each molecular class of NSAPs may exhibit functional differences suggesting that, within a molecular class, enzymes with different functions have evolved; in fact, although most NSAPs are active against a broad spectrum of substrates, some of them show a narrower substrate profile; (v) production of NSAPs of different molecular families can occur in the same bacterial species supporting the view that, at least in these cases, different physiological roles are played by enzymes of different classes; (vi) conserved structural motifs are shared among the various bacterial NSAPs and some other bacterial and eucaryotic phosphohydrolases, rendering the former enzymes interesting also for studies of molecular evolution and comparative enzymology.

Analytical tools for studying bacterial NSAPs

Enzyme assays with crude preparations

Screening for production of NSAP activity is complicated by the fact that the bacterial cell normally contains multiple phosphohydrolases with overlapping substrate profiles, whose production can be differentially regulated. Therefore, the simple measurement of phosphatase activity of whole cells or of crude cell extracts using chromogenic substrates is not expected to be highly informative. However, gross differences in the pattern of phosphatase production can also be detected using this simple approach [18, 26, 44], which is suitable for analysing a considerable number of strains. In fact, measurement of total phosphatase activity produced by different members of the family *Enterobacteriaceae* showed that *Providencia stuartii* and *Morganella morganii* are able to produce a high-level P_i-irrepressible acid phosphatase (HPAP) activity (indicated as HPAP phenotype), unlike most other enterobacterial species, which produce only low to moderate levels of acid phosphatase activity under similar conditions [18, 26]. This feature has also been exploited for rapid identification of the above species by means of suitable indicator media [18, 45].

Enzyme purification and characterization

The classical approach for characterization of individual bacterial phosphohydrolases is represented by enzyme purification followed by biophysical and biochemical characterization of the purified protein. This approach has been successfully pursued for the analysis of some NSAPs [29, 31] but can be rather complex and is not suitable for screening several strains. The purification procedure has to be adjusted for each new enzyme and may be complicated by the presence, in the starting material, of several enzymes active on the substrate used to monitor the purification steps.

Zymogram assays

A further alternative beyond enzymatic assays with crude preparations and enzyme purification is represented by the analysis of phosphatase activities using zymogram techniques. In this case crude extracts are first subjected to an electrophoretic separation, and phosphatase activities are subsequently detected in situ by means of chromogenic reactions. Such reactions are based either on substrates which yield coloured products upon dephosphorylation [27, 33, 36, 46] or on the detection of the released P_i by means of the acidified ammonium molybdate method, which yields a blue precipitate [37, 47].

Electrophoretic separation in zymograms is classically done under nondenaturing conditions, using either isoelectric focusing or gel electrophoresis. Although useful in separating the various activities, these procedures do not provide precise information on the molecular size of the enzyme. An interesting alternative is to conduct zymograms after sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [48] followed by a treatment which allows enzyme renaturation in the gel matrix (renaturing SDS-PAGE) [27, 36]. With this approach, proteins are separated on the basis of the size of their polypeptide component, and the migration distance at which the band of activity is detected depends on the molecular mass of the polypeptide component of the enzyme. Limitations of renaturing SDS-PAGE are represented by its intrinsic inability to detect heteropolymeric enzymes, and by the possibility that the boiling step in the presence of SDS may cause irreversible denaturation of the protein. According to our experience, NSAPs of all molecular classes (A, B and C) as well as other secreted bacterial phosphohydrolases, including alkaline phosphatases, 2′:3′-cyclic phosphodiesterases and acid-hexose phosphatases, can be zymographically detected after renaturing SDS-PAGE [27, 36–38, 41, 42, 49 and unpublished results].

Zymogram analysis is suitable in analysing several bacterial strains while providing significantly more information than simple enzymatic assays with crude preparations. Since different substrates can be used for the development of phosphatase activity, zymograms can be useful in determining the substrate profile of the various enzymes [27, 36, 49]. Moreover, enzyme inhibitors (e.g. EDTA, tartrate, fluoride ions, SDS etc.) can be added to the equilibration buffer at the desired concentration to assay their effect on the enzyme activity [27]. Densitometric analysis of the bands of activity can be employed for quantitative measurements that can be useful for comparing enzyme activity against different substrates or for studying regulation of production of the enzyme [36].

Expression-cloning of bacterial phosphatase-encoding genes

An alternative approach to studying bacterial phosphatases, which has proven to be invaluable for NSAPs, is that of expression-cloning of phosphatase-encoding genes followed by characterization of the cloned genes and their products [33, 37, 38, 42, 49, 50]. This approach is based on screening bacterial genomic libraries constructed in a multicopy plasmid vector for clones overproducing phosphatase activity detectable on plates of an indicator medium on which the host used for cloning has a phosphatase-negative phenotype. Of the various systems employed for expression-cloning of bacterial phosphatase-encoding genes, that based on the tryptose-phosphate phenolphtalein methyl green (TPMG) indicator medium has proven particularly useful, allowing the isolation of several different such genes [49] (table 1).

Class A bacterial acid phosphatases

Molecular class A acid phosphatases are a group of bacterial secreted phosphohydrolases which contain a polypeptide component with an M_r of 25 to 27 kDa and show conserved sequence motifs. Six different class A phosphatase-encoding genes have been cloned and sequenced (table 1, fig. 1), and their products have been characterized to various extents.

The *S. enterica* ser. *typhimurium* PhoN enzyme, also indicated as nonspecific acid phosphatase I, was the first class A enzyme purified and characterized in detail. PhoN-Se is a homodimeric protein containing two 27-kDa subunits. It is active against a very broad array of substrates including 3′- and 5′-nucleoside monophosphates, nucleoside diphosphates, nucleoside triphosphates, hexose and pentose phosphates, α- and β-glycerophosphate, p-nitrophenyl phosphate (pNPP), phenolphthalein diphosphate (PDP), α-naphthyl phosphate and pyrophosphate, but not diesters. Reaction velocities are similar overall for the various hydrolysable substrates. K_m values for the various substrates are in the 1–2 mM range. The pH optimum is around 5.5, using 5′-adenosine monophosphate (AMP) as substrate. PhoN-Se activity is inhibited by fluoride and mercuric ions, while being unaffected by EDTA and various other divalent cations including Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+} and Zn^{2+}. P_i partially inhibits enzyme activity at high concentrations (0.1 M), the inhibitory effect being more evident with 5′-nucleotides than with pNPP as substrates [29–31]. The *phoN-Se* gene was apparently acquired by *S. enterica* following a recent horizontal transfer of genetic material [35]. The gene is found in different *Salmonella* serovars, indicating that the transfer event occurred prior to diversification of the present-day salmonellae. In some strains the gene has been silenced by point mutations [35]. Expression of *phoN-Se* is under the control of the *phoP-phoQ* two-component regulatory system [51, 52], which promotes transcription of *phoN* and other PhoP-activated genes under low environmental Mg^{2+} concentrations [53]. Unlike other products of genes which are part of the *phoP-phoQ* regulon, PhoN-Se is not involved in *Salmonella* virulence [54].

The *Zymomonas mobilis* PhoC-Zm enzyme represents the major P_i-irrepressible acid phosphohydrolase produced by this species, and was the first sequenced class A enzyme [33]. The PhoC-Zm protein has not been purified and further characterized.

Table 1. NSAPs detected in bacteria.

Bacterial species (strain)[a]	NSAPs	Genes (EMBL accession #)[e]	References
Cedecea davisae (CIP 8034^T)	class A (subclass A1)[b]	-	27
Cedecea neteri (ATCC 33855^T)	class A (subclass A1)[b]	-	27
Chryseobacterium meningosepticum (CCUG 4310)	class C	*olpA* (Y12759)*	42
Citrobacter amalonaticus (ATCC 25405^T)	class B[b]	-	27
Citrobacter freundii (ATCC 8090^T)	class B[b]	-	27
Citrobacter koseri (CIP 7214)	class B[b]	-	27
Enterobacter aerogenes (CIP 6086^T)	class A (subclass A1)[b]	-	27
Enterobacter agglomerans (ATCC 29904)	-[c]		27
Enterobacter amnigenus (ATCC 33072^T)	-[c]		27
Enterobacter cloacae (CIP 6085^T)	-[c]		27
Enterobacter sakazaki (ATCC 29544^T)	-[c]		27
Enterobacter taylorae (ATCC 35317^T)	-[c]		27
Escherichia coli (MG1655)	class B	*aphA-Ec* (X86971)	27, 36, 41
Escherichia fergusonii (ATCC 35469^T)	class B[b]	-	27
Escherichia hermannii (ATCC 33650^T)	-[c]		27
Hafnia alvei (ATCC 29926)	class A (subclass A1)[b]	-	27
	class B[b]	-	27
Haemophilus influenzae (Rd)	class B	*napA-Hi* (Y07615)[f]	60
	class C	*hel* (M68502)	42, 62
Klebsiella oxytoca (CIP 666)	class A (subclass A1)[b]	-	27
Klebsiella planticola (CIP 8131)	class A (subclass A1)[b]	-	27
Klebsiella pneumoniae (CIP 52144)	class A (subclass A1)	*phoC-Kp**[g]	27
	class B[d]	*napA-Kp**[h]	27
Klebsiella terrigena (CIP 8007^T)	class A (subclass A1)[b]	-	27
Kluyvera ascorbata (ATCC 33434)	-[c]		27
Leclercia adecarboxylata (CIP100921)	-[c]		27
Leminorella grimontii (ATCC 33999^T)	-[c]		27
Moellerella wisconsensis (ATCC 35017^T)	-[c]		27
Morganella morganii (ATCC 25830^T)	class A (subclass A1)	*phoC-Mm* (X64444)*[i]	27, 37
	class B	*napA-Mm* (X78328)*[i]	27, 38
Proteus mirabilis (ATCC 29906^T)	class B[b]	-	27
Proteus penneri (ATCC 33519^T)	-[c]		27
Proteus vulgaris (ATCC 8427)	-[c]		27
Providencia alcalifaciens (CIP 5862)	class B[b]	-	27
Providencia rettgeri (ATCC 29944^T)	class B[b]	-	27
Providencia rustigianii (ATCC33673^T)	class B[b]	-	27
Providencia stuartii (ATCC 29914^T)	class A (subclass A1)	*phoN-Ps* (X64820)*[j]	27, 49
	class B[b]	-	27
Salmonella enterica ser. *typhi* (Ty2)	class A (subclass A2)[b]	-	27
	class B	*aphA-Se* (X96552)[k]	27
Salmonella enterica ser. *typhimurium* (LT2)	class A (subclass A2)	*phoN-Se* (X59036)	27, 29–31, 34, 35
	class B	-	31, 32
Serratia fonticola (CIP 7864^T)	-[c]		27
Serratia liquefaciens (CIP 674)	-[c]		27
Serratia marcescens (CIP 6755)	-[c]		27
Serratia odorifera (CIP 7901^T)	-[c]		27
Serratia plymutica (CIP 7712)	class A (subclass A2)[b]	-	27
Shigella flexneri (YSH 6000)	class A (subclass A1)	*phoN-Sf* (D82966)[l,m]	40
(clinical isolate, serotype 2a)	class A (subclass A3)	*apy – Sf* (U04539)[l,n]	39
(CIP 8248)	class B[b]	-	27
Yersinia enterocolitica (CIP 8027^T)	-[c]		27
Yersinia kristensenii (CIP 8030^T)	-[c]		27
Yersinia pseudotuberculosis (Yss133)	-[c]		27
Yokenella regensburgei (ATCC 35313)	class A (subclass A1)[b]	-	27
Zymomonas mobilis (CP4)	class A	*phoC-Zm* (M24141)	33

[a]CIP, Collection of the Institut Pasteur; ATCC, American Type Culture Collection; CCUG, Culture Collection of the University of Goteborg. [b]The enzyme has been detected in zymogram assays, and the class (and subclass) attribution was based on distinctive zymogram properties (see text for further details). The gene has not been cloned, nor has the protein been purified. [c]No NSAP activity was detected in zymogram assays performed as described in ref. 27. [d]The class B NSAP was not detectable in zymogram assays performed as described in ref. 27, either in this or in other *K. pneumoniae* strains including ATCC 13883^T. [e]Genes marked with an asterisk were isolated using the TPMG expression cloning procedure [49]. A minus sign indicates that the gene corresponding to the enzyme detected in zymograms or purified has not been cloned. [f]The accession number refers to the *napA-Hi* gene cloned and resequenced from strain CCUG 7317/A. [g]The gene was cloned from *K. pneumoniae* ATCC 13883^T (Passariello et al., unpublished results). [h]The gene was cloned from *K. pneumoniae* ATCC 13883^T. When expressed in *E. coli*, it yields a functional product (Passariello et al., unpublished results). [i]The gene was cloned from *M. morganii* RS12 [37]. [j]The gene was cloned from *P. stuartii* PV81 [49]. [k]The gene was cloned from *S. enterica* ser. *typhi* Sty4. [l]The gene is carried on the large virulence-associated plasmid. [m]Homologous plasmid-borne genes have also been detected in some clinical isolates of *Shigella* spp. and enteroinvasive *E. coli*. [n]An apyrase activity similar to that encoded by the *apy* gene has also been detected in other clinical isolates of *Shigella* spp. and enteroinvasive *E. coli*.

A

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   |PhoC-Mm  AIPAGNDATTKPDLYYLKNEQAIDSLKLLPPPPEVGSIQFLNDQAMYEKGRMLRNTERGKQAQADADLAAGGVATA
A1 |PhoN-Ps  AIPPGNDVTTKPDLYYLKNSQAIDSLALLPPPPEVGSILFLNDQAMYEKGRLLRNTERGEQAAKDADLAAGGVANA
   |PhoN-Sf  SIPPGNDVTTKPDLYYLTNDNAIDSLALLPPPPQIGSIAFLNDQAMYEKGRLLRNTERGKLAAEDANLSSGGVANV
A2  PhoN-Se  ----------ETVQPFHSPEESVNSQFYLPPPPGNDDPAYRYDKEAYFKGYAIKGSPRWKQAAEDADVSVENIARI
A3   Apy-Sf  ---------LKAEG-FLTQQTSPDSLSILPPPPAENSVVFQADKAHYEFGRSLRDANRVRLASEDAYYENFGLA--
    PhoC-Zm  -----HTEKSEPSSTYHFHS---DPLLYLAPPPTSGSPLQAHDDQTFNSTRQLKGSTRWALATQDADLHLASVLKD
                           .           * ***        *   .    .. . *  *  **      .

   |PhoC-Mm  FSGAFGYPITEKDSPELYKLLTNMIEDAGDLATRSAKEHYMRIRPFAFYGTETCNTKDQKKLSTNGSYPSGHTSIG
A1 |PhoN-Ps  FSEAFGYPITEKDAPEIHKLLTNMIEDAGDLATRSAKEKYMRIRPFAFYGVATCNTKDQDKLSKNGSYPSGHTAIG
   |PhoN-Sf  FSAAFGSPITAKDSPELHKLLTNMIEDAGDLATRSAKEYYMRIRPFAFYGVSTCNTKEQDTLSRNGSYPSGHTSIG
A2  PhoN-Se  FSPVVGAKINPKDTPETWNMLKNLLTMGGYYATASAKKYYMRTRPFVLFNHSTCRPEDENTLRKNGSYPSGHTAYG
A3   Apy-Sf  FSDAYGMDISRENTPILYQLLTQVLQDSHDYAVRNAKEYYKRVRPFVIYKDATCTPDKDEKMAITGSYPSGHASFG
    PhoC-Zm  YACAAGMNLDIAQLPHLANLIKRALRTEYDDIGR-AKNNWNRKRPFVDTDQPICTEKDREGLGKQGSYPSGHTTIG
             ..   *  .    *    ..  .          **   * ***      *       .  *******..*

   |PhoC-Mm  WATALVLAEVNPANQDAILERGYQLGQSRVICGYHWQSDVDAARIVGSAAVATLHSDPAFQAQLAKAKQEFAQKSQ
A1 |PhoN-Ps  WASALVLSEINPENQDKILKRGYELGQSRVICGYHWQSDVDAARIVASGAVATLHSNPEFQKQLQKAKDEFAKLKK
   |PhoN-Sf  WATALVLSEINPARQDTILKRGYELGDSRVICGYHWQSDVDAARIVGSAIVATLHSNPVFQAQLQKAKDEFANNQK
A2  PhoN-Se  TLLALVLSEARPERAQELARRGWEFGQSRVICGAHWQSDVDAGRYVGAVEFARLQTIPAFQKSLAKVREELNDKNN
A3   Apy-Sf  WAVALILAEINPQRKAEILRRGYEFGESRVICGAHWQSDVEAGRLMGASVVAVLHNTPEFTKSLSEAKKEFEELNT
    PhoC-Zm  WSVALILAELIPDHAANILQRGQIFGTSRIVCGAHWFSDVQAGYIMASGEIAALHGDADFRRDMELARKELEKART
                **.*.* *     .  **  * **..** ** *** *   . .  * *.    *  .   . *

   |PhoC-Mm  K----------------
A1 |PhoN-Ps  -----------------
   |PhoN-Sf  K----------------
A2  PhoN-Se  LLSKEDHPKLNY-----
A3   Apy-Sf  PTN-ELTP---------
    PhoC-Zm  SAHTPDDLLCKIEQSAR
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B

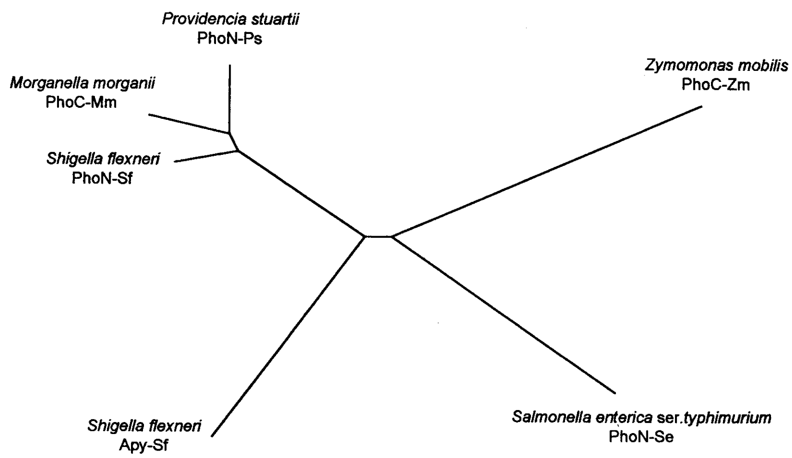

Figure 1. (*A*) Comparison of the amino acid sequences of the six known molecular class A NSAPs. The sequences of the mature proteins are reported. PhoC-Mm, PhoC protein of *M. morganii* [37]; PhoN-Ps, PhoN protein of *P. stuartii* [49, EMBL accession # X64820]; PhoN-Sf, PhoN protein of *S. flexneri* [40]; PhoN-Se, PhoN protein of *S. enterica* ser. *typhimurium* [35]; Apy-Sf, apyrase of *S. flexneri* [39]; PhoC-Zm, PhoC protein from *Z. mobilis* [33]. The proposed distinction in subclasses A1, A2 and A3 is also indicated. Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a dot. The region corresponding to the signature sequence motif for this family of enzymes is indicated by a horizontal bar. (*B*) Unrooted tree showing phylogenetic relationships among the various class A proteins.

The two class A genes of *M. morganii* (*phoC-Mm*) and *P. stuartii* (*phoN-Ps*) were shotgun-cloned via the TPMG expression-cloning procedure while studying the molecular bases of the HPAP phenotype exhibited by isolates of these species [37, 49]. In *Morganella* isolates the class A enzyme represents the major P_i-irrepressible acid phosphatase activity, and the HPAP phenotype is associated with PhoC-Mm production. In this species, the presence of PhoC is apparently able to prevent induction of alkaline phosphatase when PhoC-hydrolysable organic phosphosters represent the sole phosphate source in the medium, probably as a consequence of PhoC-mediated P_i release from the substrate [37]. Although not specifically investigated, a similar scenario is likely to occur also in *P. stuartii*. The *Morganella* PhoC-Mm enzyme has been purified and characterized. PhoC-Mm is a homotetrameric protein containing four 25-kDa subunits. It exhibits a broad substrate specificity including 5′- and 3′-nucleoside monophosphates, glucose 6-phosphate, β-glycerophosphate and aryl-phosphates (*p*NPP and PDP), but not diesters. The highest reaction velocities are observed with 5′-nucleotides, glucose 6-phosphate and aryl-phosphates. The pH optimum is around 6, using *p*NPP as substrate. PhoC-Mm activity is not inhibited by EDTA, tartrate or fluoride, and is only slightly inhibited by high (0.1 M) P_i concentrations [37]. The *P. stuartii* class A enzyme has not been purified and characterized in detail. However, being very similar to that of *M. morganii* at the sequence level (84% of identical amino acid residues, fig. 1) it is expected to retain similar properties; partial characterization of this enzyme by means of zymograms yielded results consistent with this hypothesis [27, 49]. The occurrence of these highly homologous class A genes in more than one member of the enterocluster 3 lineage [55], along with their values of G + C contents which are consistent with those of the respective species, suggest that these genes are vertically derived from a common ancestor present in the corresponding lineage before divergence of the above species. Analysis of sequence data also suggests that the *S. typhimurium phoN-Se* gene was not acquired from any the above species. In fact, only *P. stuartii*, which has a low G + C content both at the genomic level (41%) [56] and in its own class A gene (43%), could have been a suitable donor candidate, given the low (43%) G + C content of the *Salmonella phoN* gene [35]. In this case, however, a significantly higher degree of similarity with the *Salmonella* gene would have been expected for the *P. stuartii* than for the *M. morganii* allele, and an overall higher degree of similarity would also have been expected between the *Salmonella* gene and those carried by the two members of enterocluster 3.

Considering that the degree of sequence divergence between the class A enzyme of *Salmonella* and those of *M. morganii* and *P. stuartii* is substantially higher than that between the two latter proteins (fig. 1), and that the *Salmonella* enzyme also differs from them as far as quaternary structure (homodimeric vs. homotetrameric) and susceptibility to fluoride (susceptible vs. resistant) are concerned, we have proposed to further distinguish class A enzymes into at least two subclasses indicated as A1 (prototype enzyme: PhoC-Mm) and A2 (prototype enzyme: PhoN-Se) [27].

The two class A enzymes found in *S. flexneri*, PhoN-Sf and Apy-Sf, are both encoded by genes carried on the large virulence-associated plasmid harboured by clinical isolates of this species [39, 40].

The *Shigella* PhoN-Sf protein is an NSAP which exhibits a broad substrate profile including nucleotides (like the *Morganella* class A enzyme, PhoN-Sf appears to be more active on 5′-nucleotides than on 3′-nucleotides), *p*NPP, glucose 6-phosphate and *β*-glycerophosphate. The pH optimum is at 6.6. The enzyme activity is not inhibited by chelators of divalent ions (EDTA, *o*-phenanthroline), fluoride, tartrate, cysteine, L-phenylalanine, L-tryptophan, benzamidine and soybean trypsin inhibitor, while being inhibited by *N*-bromosuccinimide, dithiothreitol and diisopropylfluorophosphate, suggesting that serine and tryptophan residues, as well as disulphide bonds, are relevant to PhoN-Sf activity [40]. At the sequence level, it exhibits a higher degree of similarity to the *M. morganii* and *P. stuartii* class A enzymes than to the other members of this class (fig. 1). This enzyme, therefore, can be classified as a subclass A1 NSAP. The PhoN-Sf protein is produced by only some *Shigella* and enteroinvasive *E. coli* (EIEC) strains, and is apparently not involved in the virulence phenotype of these bacteria [40].

The *Shigella* Apy-Sf protein shows some distinctive features as compared with the other class A enzymes. The native Apy-Sf enzyme is a 25-kDa monomer. It exhibits a marked preferential activity on nucleoside triphosphates (NTPs), which are hydrolysed sequentially to the corresponding diphosphates and monophosphates through release of P_i. It is also active on pyrophosphate and, although to a lower extent, on *p*NPP, but not on AMP. This enzyme can therefore be considered essentially as an ATP diphosphohydrolase or apyrase (EC 3.6.1.5). The optimal pH value for activity is between 7 and 7.5 [39]. Similarly to the other class A enzymes, the Apy-Sf activity is not inhibited by EDTA, while it is inhibited by fluoride (like enzymes of subclass A2), *o*-vanadate, sodium azide and various divalent cations including Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} [39]. Considering the peculiar functional and structural features of the Apy protein, along with the degree of divergence observed at the sequence level with the other class A enzymes (fig. 1), we propose to distinguish a further molecular subclass for class A enzymes, subclass A3 (prototype enzyme: Apy-Sf). The *apy-Sf* gene or closely related alleles are carried by virulent *Shigella* spp. and enteroinvasive *E. coli* strains and are expressed in a thermoregulated manner [39], like many other virulence-associated genes of *Shigella* [57]. This observation, together with the localization of the enzyme in the periplasmic space, the specific activity of the enzyme on NTPs and the dramatic decrease of the NTPs pool in eucaryotic cells invaded by *Shigella* [58], suggests that Apy-Sf could be involved in the virulence phenotype of these pathogens [39, 58].

Comparison of the amino acid sequences of the six known class A enzymes shows the existence of various conserved domains (fig. 1), and a signature sequence motif for this family of enzymes has been defined as G-S-Y-P-S-G-H-T (PROSITE PDOC00891; [28]). This motif was defined before the sequence of the Apy-Sf enzyme was available. Considering also the latter enzyme, the class A acid phosphatase signature motif could be modified as G-S-Y-P-S-G-H-[TA]. The existence of a conserved sequence motif, K-X(6)-R-P-X (12,54)-P-S-G-H-X(31,54)-S-R-X(5)-H-X(2)-D, among class A enzymes, a neutral phosphatase of *Treponema denticola*, some lipid phosphatases (including bacterial phosphatidylglycerol phosphate phosphatases and mammalian phosphatidic acid phosphatases), mammalian glucose-6-phosphatases and a yeast diacylglycerol pyrophosphatase, has recently been identified, suggesting that all these enzymes could be mechanistically related and that the conserved residues are likely essential for enzyme function and possibly part of the catalytic site [59].

Class A acid phosphatases belonging to subclasses A1 and A2 can be zymographically detected by renaturing SDS-PAGE using various substrates, including the chromogenic substrates 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), or PDP in combination with methyl-green [27]. When renaturing SDS-PAGE is used for zymogram detection of these enzymes, BCIP (which

A

```
AphA-Ec  LASSPSPLNPGTNVARLAEQAPIHWVSVAQIENSLAGRPPMAVGFDIDDTVLFSSPGFWRGKKTFSPESE
AphA-Se  LVSSPSTLNPGTNVAKLAEQAPVHWVSVAQIENSLTGRPPMAVGFDIDDTVLFSSPGFWRGKKTYSPDSD
NapA-Mm  KVYMPEKVSDGVTVAQLAEQHAIHWISVEQIEESLKGQ-PMAVGFDIDDTVLFSSPGFYRGKLEYSPNDY
NapA-Hi  -GKTEPYTQSGTNAREMLQEQAIHWISVDQIKQSLEGKAPINVSFDIDDTVLFSSPCFYHGQQKFSPGKH
                   * .   . ..  .**.** **  ** *. *. * ************ * .*.  .**

AphA-Ec  DYLKNPVFWEKMNNGWDEFSIPKEVARQLIDMHVRRGDAIFFVTGRSPTKTETVSKTLADNFHIPATNMN
AphA-Se  DYLKNPAFWEKMNNGWDEFSIPKEVARQLIDMHVRRGDSIYFVTGRSQTKTETVSKTLADNFHIPAANMN
NapA-Mm  SYLKNPEFWEKMNNEWDKFSMPKKSGMELVQMHLKRGDTVYFITGRSKTKTETVTKYVQEGLRIPADKMN
NapA-Hi  DYLKNQDFWNEVNAGCDKYSIPKQIAIDLINMHQARGDQVYFFTGRTAGKVDGVTPILEKTFNI--KNMH
          ****  **  .*   * .*.**     *. **  *** ..*.***.  * . *.  .     *    *.

AphA-Ec  PVIFAG--DKPGQNTKSQWLQDKNIRIFYGDSDNDITAARDVGARGIRILRASNSTYKPLPQAGAFGEEV
AphA-Se  PVIFAG--DKPGQNTKVQWLQEKNMRIFYGDSDNDITAARDCGIRGIRILRAANSTYKPLPQAGAFGEEV
NapA-Mm  PVIFAG--DEEGQNNKVSWMRDHKLKIYYGDADADIAAARELNIRGIRVLRASNSSYQPLPKAGQFGEEV
NapA-Hi  PVEFMGSRERTTKYNKTPAIISHKVSIHYGDSDDDVLAAKEAGVRGIRLMRAANSTYQPMPTLGGYGEEV
         ** * *  .   . .*   .  . . * ***.* *. **.. . ****..**.**.*.*.*  * .****

AphA-Ec  IVNSEY
AphA-Se  IVNSEY
NapA-Mm  VINSEY
NapA-Hi  LINSSY
         ..** *
```

B

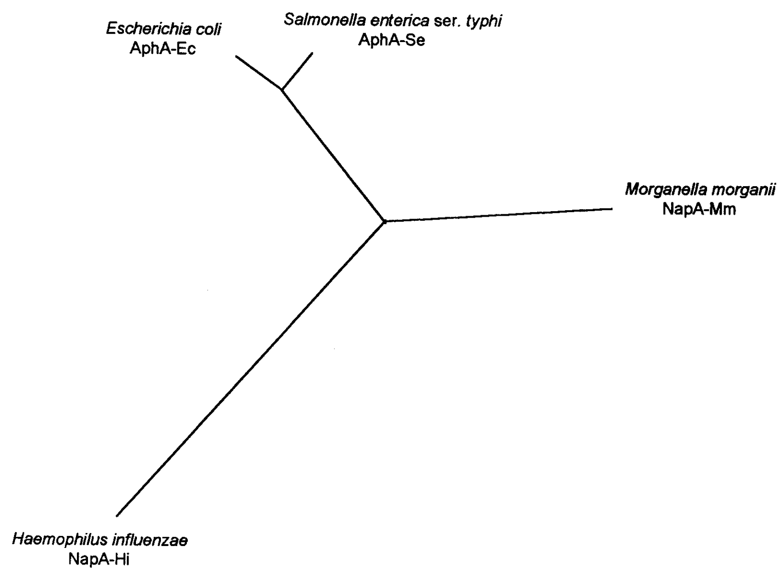

Figure 2. (*A*) Comparison of the amino acid sequences of the four known molecular class B NSAPs. The sequences of the mature proteins are reported. AphA-Ec, AphA protein of *E. coli* [41]; AphA-Se, AphA protein of *S. enterica* ser. *typhi* [EMBL accession # X96552]; NapA-Mm, NapA protein of *M. morganii* [38]; NapA-Hi, NapA protein of *H. influenzae* (EMBL accession # Y07615). Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a dot. The region corresponding to the proposed signature sequence motif for this family of enzymes is indicated by a horizontal bar. (*B*) Unrooted tree showing phylogenetic relationships among the various class B proteins.

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OlpA-Cm    MKKILLTG----GLILSFISCSAQ---KADHDTKDLVNATA------------------WMQNAGEYKALTIQ
e(P4)-Hi   MKTTLKMT-ALAALSAFVLAGCGSHQMKSEEHANMQLQQQAV-------------LGLNWMQDSGEYKALAYQ
LlpC-Seq   MKTKQVASVISLALSLFLVTGCAQLDHKANVNSKEVKQTKVTYSDEQLRSNENTMSVLTWYQRAAEAKALYLQ

HP1285     ---------------------------MSVLNAKECVSPITRS--------------VKYHQQSAEIRALQLQ
                                       .   .   .                        * . * .**  *

OlpA-Cm    AYQLAQIRLAQILTQEVSEKPRAIVLDIDETVLDNSPYQAYQIENKKNFNQEDWSKWTRLAQAEPIAGALNFL
e(P4)-Hi   AYNAAKVAFDHA--KVAKGKKKAVVADLDETMLDNSPYAGWQVQNNKPFDGKDWTRWVDARQSRAVPGAVEFN
LlpC-Seq   GYQLATDRLKNQLGQA-TDKPYSIVLDIDETVLDNSPYQAKNILEGTSFTPESWDVWVQKKEAKPVAGAKEFL

HP1285     SYKMAKMALDNNL-KLVKDKKPAVILDLDETVLNTFDYAGYLIKNCIKYTPETWDKFEKEGSLTLIPGALDFL
            *  *         .    *  ... *.***.* .  *    .     .    *           . **  *

OlpA-Cm    NFTKNNGVEIFYVSNRSEA-ERVPTLENLQKKNFPYADNDHLI-LKTDKSSKESRRQKL-SEKYNIVLFFGDN
e(P4)-Hi   NYVNSHNGKVFYVTNRKDSTEKSGTIDDMKRLGFN-GVEESAFYLKKDKSAKAARFAEIEKQGYEIVLYVGDN
LlpC-Seq   QFADQNGVQIYYISDRAVS-QVDATMENLQKEGIPVQGRDHLLFLEEGVKSKEARRQKV-KETTNLIMLFGDN

HP1285     EYANSKGVKIFYISNRTQ-KNKAFTLKTLKSFKLP-QVSEESVLLKEKGKPKAVRRELVAK-DYAIVLQVGDT
            .    .  ..*.. *        *.  ..         .    *      *  *   .      ...  **.

OlpA-Cm    LSDFSD-MYYYNNEGKTSSEKVLEHPELFGSKFIILPNAMYGDWESSMYKKN--TDKKLSNEQVKMKSLRSFT
e(P4)-Hi   LDDFGNTVY--GKLNADRRAFVDQNQGKFGKTFIMLPNANYGGWEGGLAEG-YFKKDTQGQIKARLDAVQAWD
LlpC-Seq   LVDFAD-FSKKSEEDRTA--LLSELQEEFGRQFIIFPNPMYGSWESAVYKGD--KLDASHQLKERRKALESFE

HP1285     LHDFDAIFAKDAKNSQEQRAKVLQNAQKFGTEWIILPNSLYGTWED-----------------EPIKAWQNKK
           * **                 . .    **   *. **  ** **                      .

OlpA-Cm    TQNINQ
e(P4)-Hi   GK----
LlpC-Seq   K-----

HP1285     ------
```

Figure 3. Comparison of the amino acid sequences of known or putative molecular class C NSAPs. The sequences of the native proteins are reported. OlpA-Cm, OlpA protein of *C. meningosepticum* [42; EMBL accession # Y12759]; *e*(P4)-Hi, *e*(P4) outer membrane lipoprotein of *H. influenzae* [62]; LlpC-Seq, LlpC membrane lipoprotein of *S. equisimilis* [63]. The sequence of the hypothetical protein of *H. pylori* (HP1285) [64] showing significant similarity with class C NSAPs is also shown. Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a dot. The lipoproteins signal peptides are underscored.

apparently is not hydrolysed by molecular class B NSAPs, see below) is useful for their differentiation from class B enzymes, which contain a polypeptide component of similar size. Additional features useful in differentiating class A from class B activities in zymograms are represented by resistance to inhibition by EDTA, and inactivation by low SDS concentrations. Resistance to inhibition by fluoride can be tested to differentiate enzymes of subclass A1 from those of subclass A2 [27].

A screening of representative strains of various *Enterobacteriaceae* for the presence of subclass A1 and A2 NSAPs, conducted using zymograms after renaturing SDS-PAGE, showed that production of similar enzymes is not restricted to *S. enterica* ser. *typhimurium*, *M. morganii*, *P. stuartii* or *Shigella* strains carrying the virulence plasmid. In fact, production of a putative subclass A1 NSAP was also detected in representative strains of *Cedecea* spp., *Enterobacter aerogenes*, *Hafnia alvei*, *Klebsiella* spp. and *Yokenella regensburgei*, while production of a putative subclass A2 NSAP was also detected in a *Serratia plymuthica* strain (table 1). In the same study no such enzymes were detected in representative strains of *Citrobacter* spp., *Enterobacter* spp. other than *E. aerogenes*, *Escherichia* spp., *Kluyvera ascorbata*, *Leclercia adecarboxylata*, *Leminorella grimontii*, *Moellerella wisconsensis*, *Proteus* spp., *Providencia* spp. other than *P. stuartii*, *Serratia* spp. other than *S. plymuthica* and *Yersinia* spp. (table 1). However, since it cannot be excluded that the growth conditions might have been nonpermissive for enzyme production in some species, a confirmation for the above class A-negative patterns should be sought, both under different growth conditions, and at the genetic level.

According to current knowledge on class A NSAP distribution in enteric bacteria, enzymes of subclass A1 appear to be scattered among various enterobacterial lineages, while the occurrence of enzymes of subclass A2 seems to be rather exceptional. This scenario suggests that subclass A1 genes have circulated among members of some lineages during enterobacterial evolution. The finding of a subclass A1 gene (*phoN-Sf*) on the large plasmid carried by some *Shigella* and EIEC strains [40] actually raises the possibility that other subclass A1 genes could be also located on extrachromosomal elements, and that similar alleles could have been exchanged among the ancestors of some enterobacterial species via plasmid-mediated transfer. On the other hand, the rare subclass A2 genes found in enteric bacteria would have been acquired by recent horizontal transfer from nonenterobacterial members, their phylogeny being distinct from that of subclass A1 alleles. The more specialized *apy-Sf* gene (proposed as a member of a new subclass) carried by the large virulence-associated plasmid of *Shigella* and EIEC strains [39] may have been acquired during plasmid evolution or evolved as a virulence-associated gene from a plasmid-borne subclass A1 ancestor following a duplication event. The contemporary presence of the *phoN-Sf* gene on the same plasmid could represent the trace of a similar event.

Apart from the *S. flexneri* apyrase, which has evolved a rather specific substrate profile and for which a role in microbial pathogenicity has been hypothesized, the physiological roles of the other class A NSAPs remain to be established.

Class B bacterial acid phosphatases

Molecular class B acid phosphatases include a group of secreted bacterial phosphohydrolases which contain a polypeptide component with an M_r of approximately 25 kDa and share conserved sequence motifs. Although the polypeptide size is similar to that of class A NSAPs, class B enzymes are completely unrelated to the former at the sequence level.

In their native form, class B NSAPs are 100-kDa homotetrameric proteins comprising four polypeptide subunits. Unlike polymeric class A NSAPs, class B enzymes tend to be quite resistant to depolymerization by SDS and, in SDS-PAGE, migrate at least in part as 100-kDa bands if the sample, prepared in Laemmli's buffer [48], is not subjected to the boiling treatment [27, 32, 38, 41]. Unlike class A NSAPs, class B enzymes are apparently unable to dephosphorylate the chromogenic substrate BCIP, retain their activity in the presence of low SDS concentrations and are inhibited by EDTA [27, 31, 38, 41]. These features can be exploited for putative identification of class B NSAPs in zymograms following renaturing SDS-PAGE [27].

Four different class B phosphatase-encoding genes have been cloned and sequenced (table 1, fig. 2), and their products have been characterized to various extents.

The *S. enterica* AphA-Se enzyme was the first class B NSAP purified and characterized in detail [31, 32]. It was originally purified from *S. enterica* ser. *typhimurium* LT2 and was also named nonspecific acid phosphatase II [31] to distinguish it from the class A NSAP (PhoN-Se, also named nonspecific acid phosphatase I) which had already been identified in this strain [29, 30]. The AphA-Se enzyme is active on various organic phosphomonoesters, including 5′- and 3′-uridine monophosphate (UMP), *p*NPP and α-naphthyl phosphate, but not on diesters. The highest-reaction velocities are observed with 3′-nucleotides and *p*NPP. The pH optimum for the phosphatase activity is in the acidic range and appears to be substrate-dependent, being lower (5 to 5.5) with 3′-UMP or *p*NPP and higher (around 6.5) with 5′-UMP. The K_m value of the enzyme for 5′-UMP was calculated to be 0.3 mM. The phosphatase activity of the AphA-Se enzyme is inhibited by EDTA, by high P_i concentrations (50% of activity reduction in the presence of 0.1 M of P_i), and by nucleosides. The inhibitory effect of nucleosides is higher with 2′-deoxyribonucleosides than with the corresponding ribonucleosides, is evident even at low concentrations (28–79% of activity reduction, depending on the nucleoside type, in the presence of a 0.1 mM concentration), and appears to be concentration-dependent [31, 32]. The AphA-Se enzyme is also able to function as a phosphotransferase if suitable organic compounds carrying a free hydroxyl group are present as phosphate acceptors together with a hydrolysable phosphoester which can function as a phosphate donor. This low-energy phosphotransferase activity was demonstrated using *p*NPP as a phosphate donor and either alkylalcohols (methanol, ethanol, ethylene glycol or glycerol) at high concentrations (0.2 to 2 M) or nucleosides at low concentrations (0.1 mM) as phosphate acceptors. With alcohols, the transphosphorylation rate increases by increasing the acceptor concentration, and the transphosphorylation activity is associated with an increase of the *p*NPP-splitting activity, without affecting the rate of release of P_i. With nucleosides as acceptors, the transphosphorylation reaction is seen at thousandfold lower concentrations and is not associated with an increase but actually a decrease of the *p*NPP-splitting activity (due to the inhibitory effect of nucleosides on the enzyme activity), so that nucleosides appear to be more effective than alcohols as phosphate acceptors. The products of transphosphorylation of nucleosides are mostly represented by 3′-nucleotides, with only minor amounts of 5′- and 2′-nucleotides [32]. The AphA-Se enzyme tends to adhere to plastic and glass surfaces, but the immobilized protein is inactive. This phenomenon accounts for the apparently low stability of the enzyme in diluted solutions, and can be prevented by the presence of nonionic detergents (such as Triton X-100 or *n*-octyl glucoside) or of polyethylene glycol 6000 over a broad concentration range. Nonionic detergents are also able to redissolve and reactivate the immobilized enzyme when present at concentrations near or above their critical micelle value [32]. Crystals of the purified AphA-Se enzyme have also been obtained [32], but the three-dimensional structure of the protein has not been solved. The gene encoding the AphA-Se enzyme has recently been cloned from a *S. enterica* ser. *typhi* strain (table 1). Polymerase chain reaction (PCR) amplification of the corresponding genetic locus from *S. enterica* ser. *typhimurium* LT2 and restriction analysis of the amplimer demonstrated that the *aphA* genes carried by the two serovars are highly conserved at the sequence level (M. C. Thaller et al., unpublished results).

The *M. morganii* NapA-Mm enzyme was the first class B NSAP to be cloned and sequenced [38]. NapA-Mm was initially identified as a minor P_i-irrepressible NSAP produced by this species, in addition to the major P_i-irrepressible PhoC-Mm enzyme [37, 38]. Characterization of the NapA-Mm protein purified from an *E. coli* strain carrying the cloned *napA-Mm* gene showed that its biophysical and functional properties were similar to those of the *S. enterica* AphA-Se enzyme, and consequently the definition of molecular class B NSAPs was proposed, with the NapA-Mm sequence being the prototypic one [38]. The *Morganella* NapA-Mm enzyme is active on various organic phosphomonoesters, including 5′- and 3′-nucleoside monophosphates, aryl-phosphates (*p*NPP and PDP), β-glycerophosphate and sugar phosphates (glucose 6-phosphate and ribose 5-phosphate), but not on diesters. The highest reaction velocities are observed with purine nucleotides, *p*NPP and PDP. With *p*NPP as substrate, the pH optimum of the phosphatase activity is about 6 [38]. Substrate-dependency of the pH optimum has not been investigated with this enzyme. The phosphatase activity of the NapA-Mm enzyme is inhibited by EDTA, by high P_i concentrations (P_i does not decrease enzyme activity up to a 20 mM concentration, while a partial inhibitory effect becomes apparent at higher P_i concentrations), by Ca^{2+} and by nucleosides and 2′-deoxynucleosides (31–63% of activity reduction, depending on the nucleoside type, in the presence of a 0.1 mM concentration). The phosphatase activity of the NapA-Mm enzyme is unaffected by tartrate and fluoride and stimulated by low concentrations (1 mM) of Mg^{2+}, Co^{2+} and Zn^{2+}. Similarly to the *Salmonella* AphA-Se enzyme, NapA-Mm is also able to function as a phosphotransferase using *p*NPP as a phosphate donor and either alkylalcohols or nucleosides as phosphate acceptors. Although not studied in comparable detail, transphosphorylation properties of the NapA-Mm enzyme were similar, overall, to those of the *Salmonella* class B enzyme [38].

In *E. coli*, the presence of a periplasmic acid phosphatase with features typical of a class B NSAP was initially detected by zymogram assays [27, 36]. As soon as the sequence of the *Morganella* class B enzyme became available, an unknown open reading frame, located in the *tyrB-uvrA* intergenic region (at approximately 92 min of the genetic map) of the *E. coli* chromosome was putatively identified as the gene encoding this enzyme on the basis of the sequence similarity of its product with the *Morganella* NapA-Mm protein [37]. The identity of this gene, named *aphA-Ec*, was subsequently confirmed by cloning and expression experiments [41]. The *E. coli* class B NSAP was purified from an *E. coli* strain engineered for overexpression of the *aphA-Ec* gene. Its biophysical and functional properties are similar, overall, to those of the *S. enterica* AphA-Se enzyme and of the *M. morganii* NapA-Mm enzyme. The *E. coli* AphA-Ec enzyme is active against a broad array of organic phosphomonoesters, including 5′- and 3′-nucleoside monophosphates, aryl-phosphates (*p*NPP, PDP, phenyl phosphate and *O*-phospho-L-tyrosine), nonaromatic phospho-amino acids (*O*-phospho-L-serine and *O*-phospho-L-threonine), β-glycerophosphate, ribose 5-phosphate and phytic acid, showing the highest reaction velocities with aryl-phosphates and nucleotides. No activity was detectable against adenosine triphosphate (ATP), glucose 1-phosphate, glucose 6-phosphate or diesters. Similarly to the *Salmonella* AphA-Se enzyme, the pH optimum for the phosphatase activity of the *E. coli* class B NSAP is around 6–6.5 for 5′-nucleoside monophosphates and lower (5.5–6) for *p*NPP. The phosphatase activity of the NapA-Mm enzyme is inhibited by EDTA, by P_i (in this case a slight decrease of the enzyme activity is evident at 5 mM P_i concentration and increases progressively with the P_i concentration), by Ca^{2+} and by nucleosides (67–80% of reduction of activity, depending on the nucleoside type, in the presence of a 0.1 mM concentration). The phosphatase activity of the AphA-Ec enzyme is unaffected by fluoride and stimulated by low concentrations of Mg^{2+}. Similarly to the other class B enzymes, AphA-Ec is also able to function as a phosphotransferase using *p*NPP as a phosphate donor and either alkylalcohols at high concentrations or nucleosides at low concentrations as phosphate acceptors [41]. In *E. coli*, production of the AphA-Ec enzyme is detectable when cells are grown on carbon sources other than glucose, being undetectable when glucose is available as a carbon source [36].

In *H. influenzae*, the presence of a chromosomal gene encoding a hypothetical protein similar to other class B NSAPs was identified at complement of nucleotides 511018–510313 [60]. In the original sequence data this open reading frame was interrupted by frameshifts, which were solved after cloning and resequencing of the corresponding region (fig. 2). The *H. influenzae* class B gene was named *napA-Hi*. When subcloned into an *E. coli* expression vector, the *napA-Hi* gene was actually able to direct production of a recombinant protein endowed with acid phosphatase activity and showing zymogram properties typical of class B NSAPs (G. M. Rossolini et al., unpublished results).

Molecular class B NSAPs appear to be quite conserved at the sequence level, the percent of identical amino acid residues ranging from 91%, when comparing the *E. coli* and *Salmonella* proteins, to 46% when comparing the enzymes from *Enterobacteriaceae* to that of *Haemo-*

philus (fig. 2). Comparison of amino acid sequences shows the existence of various highly conserved domains. The sequence motif F-D-I-D-D-T-V-L-F-S-S-P could be proposed as a signature sequence pattern for bacterial class B NSAPs (fig. 2). At the sequence level, class B NSAPs also appear to be distantly related to molecular class C bacterial NSAPs and to some plant acid phosphatases (see below).

Class B acid phosphatases can be zymographically detected after renaturing SDS-PAGE using various substrates, including the chromogenic substrate PDP in combination with methyl green [27, 36, 38, 41]. Distinctive features useful in identifying class B enzymes in zymograms performed following renaturing SDS-PAGE have been previously described (see above).

A screening of representative strains of various *Enterobacteriaceae* for the presence of class B acid phosphatases, performed by renaturing SDS-PAGE, showed that production of similar enzymes is not restricted to *S. enterica*, *M. morganii* or *E. coli*. In fact, production of a putative class B NSAP was also detected in representative strains of *Citrobacter* spp., *Escherichia fergusonii*, *Hafnia alvei*, *Proteus mirabilis*, *Providencia* spp. and *Shigella* spp. (table 1). In the same study, no class B enzymes were detected in representative strains of *Cedecea* spp., *Enterobacter* spp., *Escherichia hermanii*, *Klebsiella* spp., *Kluyvera ascorbata*, *Leclercia adecarboxylata*, *Leminorella grimontii*, *Moellerella wisconsensis*, *Proteus* spp. other than *P. mirabilis*, *Serratia* spp., *Yersinia* spp. and *Yokenella regensburgei* (table 1). However, since production of class B NSAPs can be regulated [36], the class B-negative zymogram pattern observed in some species could also have resulted from growth conditions nonpermissive for enzyme production at levels detectable by the zymogram assay. This point is being currently investigated by searching the genomic DNAs of strains that showed a class B-negative zymogram pattern for the presence of class B genes by means of polymerase chain reaction (PCR) amplification using degenerate primers for two highly conserved regions of known class B NSAPs. Preliminary results suggest that class B alleles are also carried by at least some of the species showing a class B-negative zymogram pattern (M. C. Thaller et al., unpublished results). Moreover, a class B NSAP-encoding gene has been cloned from *K. pneumoniae* via the TPMG expression-cloning procedure (table 1), indicating that, in this species, a class B gene is actually present and can also be functional.

The widespread distribution of class B alleles among enteric bacteria suggests that a class B gene was likely present in the enterobacterial ancestor or was acquired early in the lineage. During subsequent evolution of *Enterobacteriaceae*, this gene may have undergone mutations or rearrangements accounting for the present-day distribution and expression pattern. The phylogeny of class B NSAPs in enteric bacteria, therefore, appears to be substantially different from that of class A NSAPs.

Concerning the physiological role of class B NSAPs, it was initially proposed that in *S. enterica* ser. *typhimurium* the AphA-Se enzyme could represent the major periplasmic 5′-nucleotide-splitting enzyme, and possibly also substitute for alkaline phosphatase which is lacking in this species. This proposal was based both on the results of physiological studies performed with some mutants (altough not genetically characterized) and on kinetic data (a relatively low K_m value for 5′-UMP), and was also supported by the knowledge that, as compared to *E. coli*, *S. enterica* ser. *typhimurium* is lacking a counterpart for both the UshA periplasmic 5′-nucleotidase and the PhoA alkaline phosphatase [31]. However, it was recently shown that most *S. enterica* serovars other than *typhimurium* do produce a functional UshA homologue [24], and that production of a class B acid phosphatase is not restricted to *Salmonella* but also occurs in *E. coli* and in several other enterobacterial species [27], including those able to produce 5′-nucleotidase and alkaline phosphatase activities [15, 25]. This updated knowledge on enterobacterial periplasmic phosphatases, therefore, would suggest reconsideration of the above hypothesis, leaving the physiological function of class B enzymes an open issue.

Class C acid phosphatases and the superfamily of DDDD phosphohydrolases

Molecular class C acid phosphatases have recently been identified as a group of secreted bacterial lipoproteins endowed with NSAP activity that contain a polypeptide component with an M_r of approximately 30 kDa and share conserved sequence motifs. At the sequence level class C enzymes appear to be related, although distantly, to class B NSAPs and also to some plant acid phosphohydrolases.

The first identified class C NSAP was the OlpA enzyme of *Chryseobacterium* (formerly *Flavobacterium*) *meningosepticum* which, among the ex-flavobacterial species, is the most relevant from the clinical standpoint [61]. This enzyme was discovered as a zymographically detectable NSAP activity containing an approximately 30-kDa polypeptide, while screening nonenterobacterial species for the presence of NSAPs [42]. The gene encoding OlpA was isolated from a genomic library of *C. meningosepticum* CCUG 4310 via the TPMG expression-cloning procedure, and sequence analysis yielded a protein whose primary structure did not resemble either class A or class B NSAPs, and contained a signal peptide typical of bacterial lipoproteins (fig. 3). Consequently, the existence of a new molecular class of bacterial NSAPs (molecular class C) was proposed, with OlpA-Cm being the prototype enzyme of this class [42].

OlpA-Cm was found to share significant sequence similarity with two other bacterial lipoproteins for which a phosphatase activity had not been previously demonstrated: the *e*(P4) outer membrane lipoprotein of *H. influenzae* [62], and a cytoplasmic membrane lipoprotein of *Streptococcus equisimilis* [63] (fig. 3). Cloning and expression of the *H. influenzae* gene (*hel*) encoding the *e*(P4) lipoprotein in *E. coli* has recently confirmed that this lipoprotein also exhibits acid phosphatase activity against various phosphomonoesters [42]; hence *e*(P4) can be classified also as a member of class C NSAPs. Inclusion of the *S. equisimilis* LlpC membrane lipoprotein into the family of molecular class C NSAPs is awaiting the demonstration of a NSAP activity of the above protein.

Comparison of the amino acid sequences of known or putative class C NSAPs allowed identifying various conserved domains (fig. 3). An overall sequence similarity was also observed between these proteins and a hypothetical secreted protein encoded by an open reading frame (HP1285) located at complement of nucleotides 1362349–1361660 of the *Helicobacter pylori* chromosome [64], which could represent another member of this molecular family (fig. 3).

The recent discovery of class C NSAPs has not allowed enough time for a detailed analysis of their enzymatic properties. Concerning the physiological role, the *e*(P4) lipoprotein of *H. influenzae* was recently demonstrated to be essential for haemin uptake by this species [65]. The relationship between this function, which is carried out by a domain located near the amino-terminus of the protein that contains sequences putatively involved in haemin binding and/or transport [65], and the NSAP activity of the protein remains to be clarified.

```
   AphA-Ec   65-VGFDIDDTVLFSSPGFWRGKKTFSPESEDYLKNPVFWEKMNNGWDEFSIPKEVARQLIDMHV
   AphA-Se   64-VGFDIDDTVLFSSPGFWRGKKTYSPDSDDYLKNPAFWEKMNNGWDEFSIPKEVARQLIDMHV
   NapA-Mm   64-VGFDIDDTVLFSSPGFYRGKLEYSPNDYSYLKNPEFWEKMNNEWDKFSMPKKSGMELVQMHL
   NapA-Hi   63-VSFDIDDTVLFSSPCFYHGQQKFSPGKHDYLKNQDFWNEVNAGCDKYSIPKQIAIDLINMHQ

   OlpA-Cm   71-IVLDIDETVLDNSP--YQAYQIENKKNF----NQEDWSKWTRLAQAEPIA--GALNFLNFTK
  e(p4)-Hi   80-IVADLDETMLDNSP--YAGWQVQNNKPF----DGKDWTRWVDARQSRAVP--GAVEFNNYVN
  LlpC-Seq   95-IVLDIDETVLDNSP--YQAKNILEGTSF----TPESWDVWVQKKEAKPVA--GAKEFLQFAD
    HP1285   53-VILDLDETVLNTFD--YAGYLIKNCIKY----TPETWDKFEKEGSLTLIP--GALDFLEYAN

 APS1-Lyce  105-WIFDVDETLLSNLP--YYSDHRYGLEVF----DDVEFDKWVENGTAPALG--SSLKLYQEVL
 AP-Glymax  100-WVFDIDETTLSNLP--YYADHGFGVELY----NETSFNKWVDLGEAPALP--ESLKLYKKLL
                   *.*.* *                                       .


   AphA-Ec      RRGDAIFFVTGRSPTKTETVSKTLADNFHIPATNMNPVIFA------G--DKPGQNTKSQWL
   AphA-Se      RRGDSIYFVTGRSQTKTETVSKTLADNFHIPAANMNPVIFA------G--DKPGQNTKVQWL
   NapA-Mm      KRGDTVYFITGRSKTKTETVTKYVQEGLRIPADKMNPVIFA------G--DEEGQNNKVSWM
   NapA-Hi      ARGDQVYFFTGRTAGKVDGVTPILEKTFNI--KNMHPVEFM------GSRERTTKYNKTPAI

   OlpA-Cm      NNGVEIFYVSNRSEA-ERVPTLENLQKKNFPYADNDHLI-L-------KTDKSSKESRRQKL
  e(P4)-Hi      SHNGKVFYVTNRKDSTEKSGTIDDMKRLGFN-GVEESAFYL-------KKDKSAKAARFAEI
  LlpC-Seq      QNGVQIYYISDRAVS-QVDATMENLQKEGIPVQGRDHLLFL-------EEGVKSKEARRQKV
    HP1285      SKGVKIFYISNRTQK-NKAFTLKTLKSFKLP-QVSEESVLL-------KEKGKPKAVRRELV

 APS1-Lyce      KLGFKVFLLTGRSER-HRSVTVE--NLMNAGFHDWHKLILR-GSDDHGKTATTYKSERRNAM
 AP-Glymax      SLGIKIVFITGRPLD-QKAVTATNLNLKLAGYHTWEKLITKNTSEYHGKTAVTYKSTERKKL
                  .  .   . *        .                                  .      .


   AphA-Ec      QDKNIRI--FYGDSDNDI-40
   AphA-Se      QEKNMRI--FYGDSDNDI-40
   NapA-Mm      RDHKLKI--YYGDADADI-40
   NapA-Hi      ISHKVSI--HYGDSDDDV-40

   OlpA-Cm      -SEKYNIVLFFGDNLSDF-72
  e(P4)-Hi      EKQGYEIVLYVGDNLDDF-68
  LlpC-Seq      -KETTNLIMLFGDNLVDF-65
    HP1285      -AKDYAIVLQVGDTLHDF-52

 APS1-Lyce      VEEGFRIVGNSGDQWSDL-20
 AP-Glymax      EEKGYKIIGNIGDQWSDL-20
                      .    **   *
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Figure 4. Comparison of the amino acid sequences of known or putative molecular class C NSAPs with those of class B NSAPs and of two plant acid phosphatases. APS1-Lyce, tomato acid phosphatase [Swiss-Prot accession # P27061]; AP-Glymax, soybean acid phosphatase [EMBL accession # AJ223074]; for the names of other sequences see legends to figs 2 and 3. Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a dot. Only the relevant protein domains are shown in this alignment; numbers at the beginning of each sequence indicate the number of residues from the N-terminus of the native protein; numbers at the end of each sequence indicate the number of residues from the C-terminus of the protein.

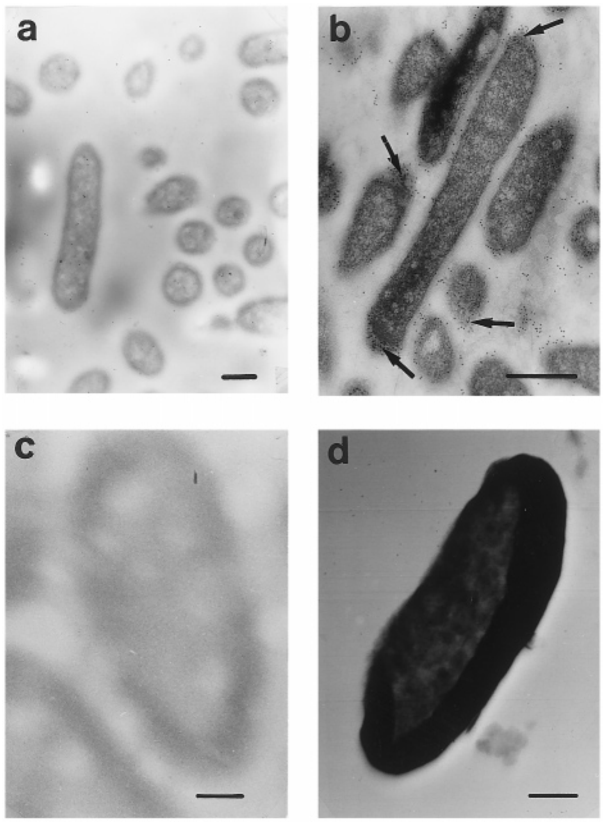

Figure 5. Phosphatase production and uranyl phosphate accumulation by the N14 strain. (*a*, b) Immunogold labelling to show phosphatase production and localization [86]. (a) Cells of a phosphatase deficient mutant show no immunogold label. (b) The parent strain shows surface and periplasmically localized enzyme (arrowed). The use of cell sections shows negligible intracellular enzyme. Bars are 500 nm. (*c*, d) Uranium accumulation by whole cells in the presence of UO_2^{2+} and the phosphatase substrate glycerol 2-phosphate. (*c*) Uranium uptake by the phosphatase deficient mutant or by the parent strain in the absence of UO_2^{2+} (control). (*d*) Uranium uptake by the parent strain following exposure to UO_2^{2+} for several hours. The accumulated precipitate was identified as HUO_2PO_4 using energy dispersive X-ray analysis, proton induced X-ray emission analysis, infrared spectroscopy, solid-state magic angle spinning ^{31}P NMR and X-ray powder diffraction analysis [23, 77, 82].

Comparison of class C enzymes with other sequenced proteins allowed identifying conserved sequence motifs between the former enzymes and other bacterial or eucaryotic proteins, including class B bacterial NSAPs and some plant acid phosphatases (fig. 4). These findings suggest that class B and class C bacterial NSAPs, together with their plant homologues, are members of the same superfamily of phosphohydrolases that we propose to indicate as "DDDD" after the presence of four invariant aspartate residues within the most conserved domains (fig. 4). All these enzymes could be mechanistically and phylogenetically related, and the highly conserved residues are likely essential for enzyme function and could be part of the catalytic site.

Bacterial NSAPs as tools in biotechnology

Similarly to alkaline phosphatase, which has been successfully used as a reporter in enzyme immunoassays [20] or as a probe for protein topology [21], bacterial NSAPs could also be exploitable for biotechnological applications. To date, this objective has been pursued with some class A NSAPs that have been successfully used for biotechnological applications as outlined below.

Use of NSAPs as tools for environmental bioremediation

In 1982 an environmental Gram-negative, rod-shaped, oxidase-negative, fermenting strain (N14), identified as *Citrobacter* sp., which was able to tolerate cadmium and accumulate it when grown in the presence of Cd, was isolated from metal-polluted soil [66]. Such a potential was present in cells pregrown in the absence of the metal, and was retained by cells subsequently exposed to metals, either in a resting or immobilized state [67, 68]. The requirement for the presence of suitable organic phosphoesters for metal accumulation to occur, along with the time-dependent metal accumulation, and the consistency of metal uptake with high-level production of a periplasmic acid phosphatase activity by the N14 strain, suggested an enzyme-mediated metal-uptake mechanism possibly involving the cleavage of the phosphoester bond to yield inorganic phosphate which precipitates stoichiometrically with available heavy metal cations, so that the metal phosphates are tightly bound as $MHPO_4$ (M = metal) at the cell surface [67, 69, 70]. This hypothesis was verified by X-ray microanalysis and magic angle spinning ^{31}P nuclear magnetic resonance (NMR) analysis: the heavy metal precipitation occurs initially at discrete loci at the cell surface of resting cells, being followed by a heavy cellular deposition of the same material [71]. Moreover, a phosphatase-deficient mutant of the N14 strain (lp4a) was ineffective at accumulation of heavy metals [72, 73].

Further studies demonstrated an effective phosphatase-mediated accumulation, by the N14 strain, of various heavy metals and actinides, including Am, Pu and U, in the form of their insoluble hydrogen phosphates or phosphates (fig. 5) [22, 72, 74–77]. Removal of Th(IV) is poor per se but can be facilitated if La(III) is also incorporated into the solution, and the same method is effective in enhancing removal of Pu(IV) [78]. The product of uranyl biocrystallization, cell-bound hydrogen uranyl phosphate (HUP) [23, 77], is a polycrystalline, lamellar material with intercalative cation-exchange ability (i.e. cations of other metals could displace protons from within the interlamellar space) [79–82]. In contrast to other heavy metals, Ni is not removed by the metal phosphate deposition reaction, but the intercalative ion exchange property of HUP could be successfully exploited in the removal of Ni from dilute aqueous solutions, since Ni^{2+} ions are reversibly incorporated into cell-bound HUP to form nickel uranyl phosphate $[Ni(UO_2PO_4)_2]$. This was designated as microbially enhanced chemisorption of heavy metals [80–82].

Removal of heavy metals from aqueous wastes via microbially generated precipitant ligands ('biomineralization'), whose formation is dependent on the production of a secreted acid phosphatase, has therefore become a valuable alternative to classical and biosorptive methods of waste water treatment [72, 74, 83–85].

Purification of the acid phosphatase produced by strain N14 and determination of the amino-terminal sequence and of some internal amino acid sequences showed significant similarities with known class A NSAPs [86], suggesting that similar enzymes could be involved in the biomineralization process. In fact, an *E. coli* strain engineered to overproduce the *Salmonella* class A NSAP demonstrated a high efficiency for accumulating uranyl ions and acting as a bioorganic ion exchanger via the accumulation of HUP and subsequent removal of Ni (G. Baskanova et al., unpublished). This finding represents the first example of an acid phosphatase-mediated metal biomineralization process by a microorganism other than N14, and opens the possibility of future engineering of improved strains for specific industrial applications.

Use of NSAP-encoding genes as insertional inactivation targets in cloning vectors

Class A NSAP-encoding genes have been exploited as targets for insertional inactivation in cloning vectors that allow direct identification of recombinants. Using these vectors, recombinants are easily identified on the basis of their phosphatase-negative phenotype, while clones containing an empty vector exhibit a phosphatase-positive phenotype [87, 88].

The major advantages of similar vectors, as compared to the most popular *lacZ* α-complementation-based cloning vehicles (i.e. the pUC series and derivatives [89, 90]), are represented by the possibility of using them in any *E. coli* host, independently on its *lac* genotype, and by the significantly lower cost of the indicator medium as compared with that used for the β-galactosidase plate assay.

The problem of engineering a versatile multiple cloning site (MCS) into the phosphatase gene, without disturbing the activity of its product, has been solved by replacing the region encoding the phosphatase signal peptide with a modified amino-terminal moiety of the *E. coli lacZ* gene, derived from a *lacZ* α-complementation-based cloning vector [88]. With this approach, cloning vectors have been constructed that allow identification of recombinants based on phosphatase inactivation while retaining all the MCS facilities that made so popular the *lacZ* α-complementation-based vectors [88].

Concluding remarks and future work

Although the existence of bacterial NSAPs has been known for a relatively long time, only recently has knowledge on these enzymes undergone a considerable advancement concerning their primary structure, genetics, enzymology, distribution and phylogeny. Notwithstanding this advanced knowledge, the role of NSAPs in microbial physiology remains an open issue, at least for most of them. Indeed, the widespread distribution and high-level conservation of some of these enzymes (e.g. the molecular class B NSAPs in enteric bacteria) suggest an involvement in functions relevant to procaryotic cell physiology, but these functions have yet to be identified with certainty. On the other hand, contemporaneous production of NSAPs of different molecular classes by some bacteria (e.g. production of both a class A and a class B enzyme by *M. morganii*, *H. alvei*, *P. stuartii* and *S. enterica*, or production of both a class B and a class C enzyme in *H. influenzae*) suggests that, at least in such instances, NSAPs of different classes play different roles. To understand this essential point, additional investigations will be required, concerning various fundamental aspects. An evaluation of the kinetic parameters of these enzymes could be useful to determine their catalytic efficiencies toward different hydrolysable substrates. In fact, kinetic parameters have not been investigated for most NSAPs, while the approximate functional data available for several enzymes are often not fully comparable due to differences in the experimental conditions adopted. A comprehensive comparative analysis of the functional properties of NSAPs of each molecular class would also be interesting for understanding the molecular evolution and structure-function relationships of these enzymes. Regulation of the various NSAP-encoding genes, and the effects of gene inactivation or overexpression on cell physiology, represent additional aspects that would deserve investigation to understand the physiological role of these enzymes.

Another interesting topic for additional investigation is represented by production of NSAPs throughout the microbial kingdom. In fact, all NSAPs thus far described are from representative strains of enteric bacteria or of a few other Gram-negative species (with the exception of the hypothetical class C enzyme of *S. equisimilis*). Identification and characterization of NSAPs from other bacterial taxa would help at understanding their role, while providing valuable information in the field of bacterial and molecular evolution. Concerning the potential utility of bacterial NSAPs in the sector of applied microbiology and biotechnology, the successful exploitation of some class A NSAPs for similar purposes should encourage further investigation in this field, also with enzymes of other molecular families.

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