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Acid Phosphatase/Phosphotransferases from Enteric Bacteria

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We have investigated the enzymatic phosphorylation of nucleosides and found that *Morganella morganii* phoC acid phosphatase exhibits regioselective pyrophosphate (PP_i)-nucleoside phosphotransferase activity. In this study, we isolated genes encoding an acid phosphatase with regioselective phosphotransferase activity (AP/PTase) from *Providencia stuartii*, *Enterobacter aerogenes*, *Escherichia blattae* and *Klebsiella planticola*, and compared the primary structures and enzymatic characteristics of these enzymes with those of AP/PTase (PhoC acid phosphatase) from *M. morganii*. The enzymes were highly homologous in primary structure with *M. morganii* AP/PTase, and are classified as class A1 acid phosphatases. The synthesis of inosine-5′-monophosphate (5′-IMP) by *E. coli* overproducing each acid phosphatase was investigated. The *P. stuartii* enzyme, which is most closely related to the *M. morganii* enzyme, exhibited high 5′-IMP productivity, similar to the *M. morganii* enzyme. The 5′-IMP productivities of the *E. aerogenes*, *E. blattae* and *K. planticola* enzymes were inferior to those of the former two enzymes. This result underlines the importance of lower K_m values for efficient nucleotide production. As these enzymes exhibited a very high degree of homology at the amino acid sequence level, it is likely that local sequence differences in the binding pocket are responsible for the differences in the nucleoside-PP_i phosphotransferase reaction.

[**Key words:** acid phosphatase, pyrophosphate, nucleoside, phosphotransferase, 5′-nucleotide, enteric bacteria]

Inosine-5′-monophosphate (5′-IMP) and guanosine-5′-monophosphate (5′-GMP) are commercially important nucleotides that are used as flavour potentiators in various foods. We have investigated a nucleoside phosphorylation reaction using the food additive pyrophosphate (PP_i) in order to establish a novel 5′-nucleotide production process, which consists of fermentation of nucleosides such as inosine (1) and guanosine (2), and the enzymatic phosphorylation of these nucleosides. We screened for a C5′-selective nucleoside phosphorylation reaction using PP_i as shown in the following equation (3): nucleoside+PP_i → nucleoside-5′-monophosphate+P_i. We purified a nucleoside-phosphorylating enzyme from a crude extract of *Morganella morganii* NCIMB10466, and found that the enzyme was an acid phosphatase with regioselective PP_i-nucleoside phosphotransferase activity (4). Then, we isolated the acid phosphatase/phosphotransferase (AP/PTase) gene from *M. morganii*, and improved its phosphotransferase activity by random mutation (5). The *M. morganii* AP/PTase gene was identical to the *M. morganii* PhoC acid phosphatase gene, which is classified as a class A nonspecific acid phosphatase (6). To date, enzymes of this class have been isolated from several bacterial species, including *Salmonella typhimurium* (7), *Zymomonas mobilis* (8), *M. morganii* (6), *Shigella flexneri* (9, 10), and *Prevotella intermedia* (11). The phosphatase activities of bacteria have been studied from several viewpoints, but their phosphotransferase activity has received very little attention. During the course of screening for phosphotransferase activity, regioselective PP_i-nucleoside phosphotransferase activity was found to be widely distributed among enteric bacteria (3).

In this study, we have cloned several AP/PTases from enteric bacteria and compared their 5′-nucleotide productivity as a starting point for investigating further improvement of phosphotransferase activity as well as the structure-function relationships of these enzymes. This paper describes cloning of the AP/PTase genes from *Providencia stuartii*, *Enterobacter aerogenes*, *Escherichia blattae* and *Klebsiella planticola*, and the comparison of the primary structure and enzymatic characteristics of the enzymes from these bacteria with those of AP/PTase from *M. morganii*.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions *P. stuartii* ATCC 29851, *E. aerogenes* IFO 14930, *E. blattae* JCM1650 and *K. planticola* IFO 14939, which were previously selected as 5′-nucleotide-producing strains using PP_i as the phosphate source (3), were used as DNA donors. *Escherichia coli* JM109 (12) was used as the host strain for DNA manipulation and expression. The plasmids used in this study are listed in Table 1. Plasmids pUC118, pUC18 and pUC19 (Takara Shuzo, Kyoto) were used as vectors for *E. coli*. Luria-Bertani (LB) medium (13) was used for the culture of these microorganisms. The bacteria were grown aerobically at 37°C. For

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the selection of *E. coli* transformants, ampicillin (50 μg/ml) was added to the medium.

Cloning and nucleotide sequencing of the AP/PTase gene All basic recombinant DNA procedures such as isolation and purification of DNA, restriction enzyme digestion, ligation of DNA and transformation of *E. coli* were performed as described by Sambrook *et al.* (13).

Amplification of a DNA fragment containing the *P. stuartii* phoC gene was carried out by 30 cycles of PCR using *P. stuartii* chromosomal DNA as the template and two specific primers, PR1 (5′-CTGGATCCTGTGGCTATCATCACCT) and PR2 (5′-CTGGATCCGACGCGATTTTACCATA) (the *Bam*HI site is underlined). Each cycle was carried out at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 90 s. The synthesized fragment was digested with *Bam*HI, and then cloned into pUC19.

Chromosomal DNA libraries of *E. aerogenes*, *E. blattae*, and *K. planticola* were constructed by inserting partially *Sau*3A1-digested fragments of 3 to 6 kilobases (kb) into the *Bam*HI site of pUC118. *E. coli* JM109 transformants were grown on LB plates containing 50 μg/ml ampicillin and 1 mM β-D-thiogalactopyranoside (IPTG) for 16 h. Phosphotransferase-positive clones were screened as described previously (5) and used for further study.

DNA was sequenced by the dideoxynucleotide chain termination method using a Dye Terminator Cycle sequencing kit (Perkin-Elmer, Norfolk, Connecticut) and a DNA sequencer (model 373A, Perkin-Elmer). Synthesized universal primers and oligonucleotides specific for each cloned fragment were used as primers.

Enzyme assay Phosphotransferase activity was assayed in a standard reaction mixture containing 100 μmol of sodium acetate buffer (pH 5.0), 40 μmol of inosine, 100 μmol of tetrasodium pyrophosphate, and the enzyme solution in a total volume of 1 ml. The reaction mixture was incubated for 10 min at 30°C and then the reaction was terminated by adding 0.2 ml of 2 N HCl. Quantitative determination of inosine and 5′-IMP was carried out by HPLC as described previously (4). One unit of phosphotransferase activity was defined as the amount of enzyme that produces 1 μmol of 5′-IMP per min under the assay conditions. As the solubility of inosine is limited, kinetic constants for inosine were determined with a substrate concentration range from 0.5 to 80 mM.

Enzyme purification Each AP/PTase was purified from harvested cells of *E. coli* JM109 transformants harboring the relevant plasmid by ammonium sulfate fractionation and ion-exchange, hydrophobic and gel-filtration column chromatographies as described previously (4). The purity of the recovered samples was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed by the method of Laemmli (14), with 14% acrylamide. The protein concentration was assayed by the method of Bradford (15) using a dye reagent concentrate (Bio-Rad Laboratories) with bovine serum albumin as the standard. The molecular weight of the enzyme was estimated by gel filtration, and amino-terminal amino acid sequences were determined using an automatic protein sequencer (Prosequencer 6625; Milligen/Biosearch) as described previously (5).

Synthesis of 5′-IMP by *E. coli* overproducing each phosphatase/phosphotransferase Each *E. coli* JM109 transformant was cultured and harvested as described previously (5). The reaction mixture for 5′-IMP synthesis contained 40 g/*l* (148 mM) inosine, 150 g/*l* (676 mM) disodium hydrogen pyrophosphate, 1 mM sodium acetate buffer (pH 4.0) and 1 g/*l* (dry weight) of each type of cells in 10 ml. The reaction was carried out at 30°C with moderate shaking and terminated by adding 1 ml of 2 N HCl. The synthesized 5′-IMP was calculated as IMP·2Na·7.5H_2O (MW 527).

Nucleotide sequence accession numbers The nucleotide sequences of the AP/PTase genes from *E. aerogenes*, *E. blattae* and *K. planticola* reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases with the accession numbers AB044338, AB020481 and AB044345, respectively.

RESULTS

Isolation of the genes encoding AP/PTases from enteric bacteria and their expression in *E. coli* Genes encoding an acid phosphatase with PP_i-nucleoside phosphotransferase (AP/PTase) activity were isolated. As *M. morganii* PhoC acid phosphatase exhibited AP/PTase activity, *P. stuartii* PhoC acid phosphatase gene (unpublished results; EMBL accession no. X64820), with significant homology to the *M. morganii* PhoC gene, was isolated by PCR. As expected, the *P. stuartii* PhoC acid phosphatase also exhibited AP/PTase activity. AP/PTase genes were also isolated by a shotgun-cloning strategy from chromosomal DNA libraries of *E. aerogenes*, *E. blattae*, and *K. planticola*. Phosphatase-positive clones were selected and then phosphotransferase-positive clones were selected from among them. Subcloning revealed the fragments on which each AP/PTase activity was retained, and the fragments were subcloned into pUC19 or pUC18 as listed in Table 1.

When grown in LB broth, phosphotransferase activity was not detected in *E. coli* JM 109 harboring pUC19 or pUC18. On the other hand, the specific activities of phosphotransferase in *E. coli* JM 109 harboring pPRP100, pEAP120, pEBP120, and pKPP120 were 1.75, 0.357, 0.286 and 0.603 unit/mg, respectively. These activities were about 110- to 280-fold higher than those of the wild type strains of *P. stuartii* (6.23×10^{-3} units/mg), *E. aerogenes* (3.12×10^{-3} units/mg), *E. blattae* (2.30×10^{-3} units/mg) and *K. planticola* (4.57×10^{-3} units/mg).

Comparison of the amino acid sequences of the cloned AP/PTases with those of other related enzymes The nucleotide sequence of each fragment (given in the database) showed an ORF considered to encode the 744 to 747 bp AP/PTase. The *E. blattae* AP/PTase gene appeared to be identical to the gene we recently isolated as a nonspecific

TABLE 1. Plasmids in which AP/PTase genes were cloned in this study

Plasmid	Relevant characteristic	Reference or source
pMPI501	Containing 1.1 kb *Hin*dIII-*Eco*RI fragment derived from *M. morganii* cloned in pUC18	5
pPSP100	Containing 1.1 kb *Bam*HI fragment encoding *P. stuartii phoC* in pUC18	Unpublished data X64820
pEAP100	Containing 7.6 kb *Sau*3AI fragment derived from *E. aerogenes* cloned in pUC118	This study
pEAP120	Containing 1.6 kb *Sal*I-*Kpn*I fragment derived from *E. aerogenes* cloned in pUC19	This study
pEBP100	Containing 7.6 kb *Sau*3AI fragment derived from *E. blattae* cloned in pUC118	This study
pEBP120	Containing 1.2 kb *Pst*I-*Hin*cII fragment derived from *E. blattae* cloned in pUC19	This study
pKPP100	Containing 3.6 kb *Sau*3AI fragment derived from *K. planticola* cloned in pUC118	This study
pKPP120	Containing 2.2 kb *Kpn*I-*Eco*RI fragment derived from *K. planticola* cloned in pUC19	This study

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Mmo  :MKKNIIAGCLFSLFSLSALA AIPAGNDATTKPDLYYLKNE QAIDSLKLLPPPPEVGSIQF LNDQAMYEKGRMLRNTERGK  80
Pst  :MKKLLAVFCAGAFVSTSVFA AIPPGNDVTTKPDLYYLKNS QAIDSLALLPPPPEVGSILF LNDQAMYEKGRLLRNTERGE  80
Eae  :MKKRVLALCLASLFSVNAFA LVPAGNDATTKPDLYYLKNA QAIDSLALLPPPPEVGSIAF LNDQAMYEKGRLLRNTERGK  80
Ebl  :MKKRVLAVCFAALFSSQALA LVATGNDTTTKPDLYYLKNS EAINSLALLPPPPAVGSIAF LNDQAMYEQGRLLRNTERGK  80
Kpl  :MKKRVLALCLASLFSVSAFA LVPAGNDATTKPDLYYLKNA QAIDSLALLPPPPEVGSIAF LNDQAMYEKGRLLRATARGK  80
      ***    *    *   *     *** ******* ****  ** ** ****** **** * ******** ** ** * **

                                                                    QXXXXXR P
Mmo  :QAQADADLAAGGVATAFSGA FGYPITEKDSPELYKLLTNM IEDAGDLATRSAKEHYMRIR PFAFYGTETCNTKDQKKLST 160
Pst  :QAAKDADLAAGGVANAFSEA FGYPITEKDAPEIHKLLTNM IEDAGDLATRSAKEKYMRIR PFAFYGVATCNTKDQDKLSK 160
Eae  :LAAEDANLSAGGVANAFSSA FGSPITEKDAPQLHKLLTNM IEDAGDLATRSAKEKYMRIR PFAFYGVSTCNTTEQDKLSK 160
Ebl  :LAAEDANLSSGGVANAFSGA FGSPITEKDAPALHKLLTNM IEDAGDLATRSAKDHYMRIR PFAFYGVSTCNTTEQDKLSK 160
Kpl  :LAAEDANLSAGGVANAFSAA FGSPISEKDAPALHKLLTNM IEDAGDLATRGAKEKYMRIR PFAFYGVSTCNTTEQDKLSK 160
       * ** *  **** *** * ** ** *** *   ****** ********** **  ***** ******  ****  * ***

          PSGH                                    S RXXXXXHXXXD
Mmo  :NGSYPSGHTSIGWATALVLA EVNPANQDAILERGYQLGQS RVICGYHWQSDVDAARIVGS AAVATLHSDPAFQAQLAKAK 240
Pst  :NGSYPSGHTAIGWASALVLS EINPENQDKILKRGYELGQS RVICGYHWQSDVDAARIVAS GAVATLHSNPEFQKQLQKAK 240
Eae  :NGSYPSGHTSIGWATALVLA EINPQRQNEILKRGYELGES RVICGYHWQSDVDAARIVGS AVVATLHTNPAFQQQLQKAK 240
Ebl  :NGSYPSGHTSIGWATALVLA EINPQRQNEILKRGYELGQS RVICGYHWQSDVDAARVVGS AVVATLHTNPAFQQQLQKAK 240
Kpl  :NGSYPSGHTSIGWATALVLA EINPQRQNEILKRGYELGES RVICGYHWQSDVDAARIVGS AVVATLHTNPAFQQQLQKAK 240
      ********* **** ****  * **  *  ** *** ** * ***************** * *   *****  * ** ** ***

Mmo  :QEFAQKSQK 249
Pst  :DEFA-KLKK 248
Eae  :DEFAKTQK- 248
Ebl  :AEFAQHQKK 249
Kpl  :DEFAKQQK- 248
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FIG. 1. Amino acid alignment of the deduced amino acid sequences of several AP/PTases. Numbering of the amino acid residues starts at the amino-terminus of the proproteins. The signal sequences are underlined. Asterisks show identical amino acid residues. Three phosphatase motif domains are indicated above the deduced amino acid sequence. The glycine-92 and isoleucine-171 residues that were previously reported to be important for the improvement of *M. morganii* AP/PTase (5) are boxed. The sequences shown are: Mmo, *Morganella morganii*; Pst, *Providencia stuartii*; Eae, *Enterobacter aerogenes*; Ebl, *Escherichia blattae*; Kpl, *Klebsiella planticola*.

acid phosphatase gene (16). The deduced sequence of each AP/PTase was compared to those of proteins in the SWISS-PROT and NBRF-PIR databases. These AP/PTases appeared to be highly homologous to bacterial nonspecific acid phosphatases, such as *M. morganii* PhoC. When *M. morganii* AP/PTase (PhoC acid phosphatase) was compared with the *P. stuartii*, *E. aerogenes*, *E. blattae* and *K. planticola* enzymes, the overall amino acid identities were found to be 79.9, 80.4, 77.1 and 79.2%, respectively. Multiple alignment of the deduced sequences of these five AP/PTases is shown in Fig. 1. These enzymes were highly homologous with each other, and shared the three conserved sequence motifs, KXXXXXXPR, PSGH and SRXXXXXH XXXD, identified by Stukey and Carman (18).

The amino-terminal amino acid sequence of each purified enzyme was detected in each deduced amino acid sequence, as indicated in Fig. 1. The amino-terminal sequencing data revealed that these enzymes become mature after the cleavage of a signal sequence by signal peptidase. The molecular weights of each AP/PTase, deduced from the amino acid sequence excluding the signal peptide removed by post-translational modification, are in good agreement with the value of 25,000 estimated by SDS-PAGE.

A phylogenetic tree of the five AP/PTases used in this study and other phosphatases that share the same conserved sequence motif is shown in Fig. 2. It appears that these five enzymes are closely related, but they could be further divided into two groups. The *P. stuartii* enzyme was the most closely related to the *M. morganii* enzyme, and the *E. aerogenes*, *E. blattae*, and *K. planticola* enzymes were also closely related to each other.

Characterization of acid phosphatase/phosphotransferases AP/PTases from *P. stuartii* (AP/PT-Ps), *E. aerogenes* (AP/PT-Ea), *E. blattae* (AP/PT-Eb) and *K. planticola* (AP/PT-Kp) were purified and their enzymatic characteristics were compared with those of AP/PTase from *M. morganii* (AP/PT-Mm). All enzymes exhibited PP_i-nucleoside phosphotransferase activity, and their phosphorylation reaction was strictly regioselective at the C5′ position of the nucleoside. The relative molecular weight of the subunit of each enzyme was estimated to be about 25,000 by SDS-PAGE analysis. The relative molecular weight of each enzyme was calculated to be about 160,000 by gel filtration (data not shown). From these results, the enzymes appear to consist of six identical subunits, similar to the *M. morganii* enzyme.

The optimum reaction conditions for each AP/PTase were investigated by measurement at various pH values in several buffers. All enzymes exhibited optimal phosphotransferase activity at pH 5.2. The enzyme activities were also measured at various temperatures from 4 to 70°C. AP/PT-Mm and AP/PT-Ps had maximal activity at approximately

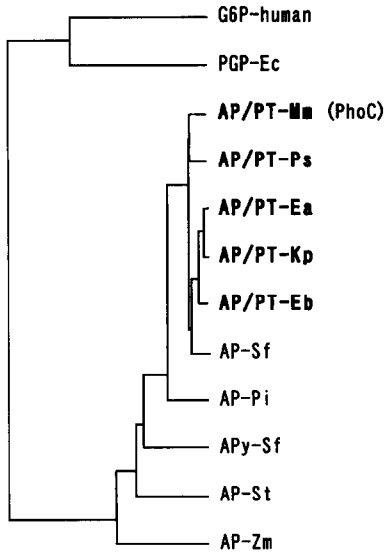

FIG. 2. Phylogenetic relationships among proteins belonging to the class A acid phosphatase family. The phylogenetic tree was constructed on the basis of the multiple alignment of amino acid sequences by the unweighted pair-group method with arithmetic mean (UPGMA) procedure with the GENETIX-MAC ver. 10.0 program. Abbreviations are as follows: G6P-human, human glucose-6-phosphatase; PGP-Ec, *E. coli* phosphatidyl glycerol phosphate phosphatase; AP-Sf, *S. flexneri* nonspecific acid phosphatase; AP-Pi, *P. intermedia* acid phosphatase; Apy-Sf, *S. flexneri* apyrase; AP-St, *S. typhimurium* nonspecific acid phosphatase; AP-Zm, *Z. mobilis* acid phosphatase.

30°C, whereas AP/PT-Ea, AP/PT-Eb and AP/PT-Kp had maximal activity at approximately 35°C. None of the enzymes were inhibited by the addition of various chelating reagents. Enzyme activities were fully retained in the presence of 1 mM EDTA, *o*-phenanthroline, 2,2'-dipyridyl, 8-hydroxyquinoline and sodium fluoride.

To evaluate the phosphotransferase activity of the various AP/PTases, the K_m value for inosine in the transphosphorylation reaction was determined for each enzyme. As shown in Table 2, the K_m values for inosine of the enzymes are very high. The K_m value of AP/PT-Mm is approximately three-fourth that of AP/PT-Ps, and is almost half of those of AP/PT-Ea, AP/PT-Eb and AP/PT-Kp. Conversely, the V_{max} values of AP/PT-Mm and AP/PT-Ps are almost two fold those of AP/PT-Ea, AP/PT-Eb and AP/PT-Kp.

Synthesis of 5'-IMP by *E. coli* overproducing each AP/PTase The time course of 5'-IMP synthesis from inosine and PP_i by *E. coli* overproducing each AP/PTase is shown in Fig. 3. Hydrolysis was directed primarily towards PP_i, and inosine was phosphorylated to form 5'-IMP. The 5'-IMP productivity of *E. coli* JM109 (pMPI501) was the highest. *E. coli* JM109 (pPSP100) produced almost the same amount of 5'-IMP as *E. coli* JM109 (pMPI501). The 5'-IMP productivities of *E. coli* JM109 (pEAP120), *E. coli* JM109 (pEBP120) and *E. coli* JM109 (pKPP120) were inferior to those of the two former strains, and about half the amount of 5'-IMP was produced. As the reaction time was prolonged, the dephosphorylation was directed towards 5'-IMP and all of the synthesized 5'-IMP was hydrolyzed to inosine. The phosphotransferase reactions of the three latter strains were slower, and the dephosphorylation of the 5'-IMP synthesized by these strains was also slower.

TABLE 2. Kinetic constants for transphosphorylation reactions

Origin of the AP/PTase	K_m (mM)	V_{max} (u/mg)
M. morganii	117	6.09
P. stuartii	156	6.21
E. aerogenes	218	3.75
E. blattae	200	2.78
K. planticola	231	2.65

The enzyme activities were assayed as described in the Materials and Methods section. Initial velocities were determined, and the steady-state kinetic constants were calculated using a Lineweaver-Burk plot.

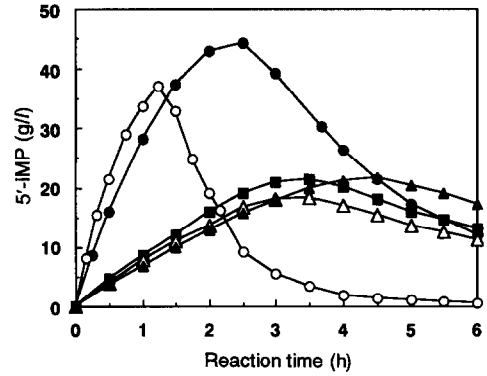

FIG. 3. 5'-IMP synthesis by *E. coli* overproducing each AP/PTase. The time course of 5'-IMP synthesis by resting cells of *E. coli* JM109 (pMPI501) (solid circle), *E. coli* JM109 (pPSP100) (open circle), *E. coli* JM109 (pEAP120) (solid triangle), *E. coli* JM109 (pEBP120) (open triangle) and *E. coli* JM109 (pKPPI700) (solid square) was measured. The reaction was carried out at pH 4.0 and 30°C in a reaction mixture consisting of 0.1 M sodium acetate buffer (pH 4.0) containing 40 g/*l* (148 mM) inosine, 150 g/*l* (676 mM) disodium hydrogen pyrophosphate and 1 g/*l* (dry weight) of each type of cells.

DISCUSSION

In this study, we isolated, cloned, and analyzed acid phosphatases with regioselective PP_i-nucleoside phosphotransferase activity from enteric bacteria. Bacterial nonspecific acid phosphatases (NSAPs) are divided into three classes, designated as A, B and C, on the basis of amino acid sequence similarity (17, 18). Class A NSAPs are further classified into three subclasses designated as A1 (prototype enzyme: PhoC of *M. morganii* (PhoC-Mm)), A2 (prototype enzyme: PhoN of *S. typhimurium* (PhoN-St) and A3 (prototype enzyme: Apy of *S. flexneri* (Apy-Sf)). We have found that PhoC-Mm exhibits PP_i-nucleoside phosphotransferase activity and have investigated its application for 5'-nucleotide production (4, 5). Class A1 enzymes are resistant to fluoride, whereas enzymes of the other classes are inhibited by fluoride. On the basis of these diagnostic criteria, AP/PT-Eb, AP/PT-Ea and AP/PT-Kp can be classified as class A1 enzymes, similar to AP/PT-Mm and AP/PT-Ps. Class A1 NSAPs are thought to exhibit regioselective PP_i-nucleoside phosphotransferase activity. It will be interesting to determine if other NSAPs of this group also exhibit phosphotransferase activity. Several members of the NSAP family have been used for biotechnological applications, *e.g.*, as tools for environmental bioremediation (19) and as inser-

tional inactivation targets in cloning vectors (20), and regioselective phosphotransferase activity appears to be another useful feature of this phosphatase family.

Groisman *et al.* examined the *phoN* gene of *Salmonella typhimurium* (PhoN-St) in order to evaluate the genetic basis for the structure and organization of enteric bacterial genomes. The 1.4 kb region containing *phoN* had an overall guanine-plus-cytosine (G+C) content of 43%, much lower than that of the *Salmonella* chromosome, which averages 52%. They suggested that *Salmonella* acquired the *phoN* gene from low G+C content, phosphatase-producing species, such as *M. morganii* and *P. stuartii*, by lateral transfer prior to the diversification of *Salmonella* (21). However, the G+C contents of the AP/PTase gene open reading frames from *E. aerogenes*, *E. blattae* and *K. planticola* are 52, 55 and 55%, respectively, higher than those of *M. morganii* and *P. stuartii*. Therefore, lateral transmission does not appear to be the case with the AP/PTase genes of these three strains.

In terms of phylogenetic relationships, these AP/PTases are divided into two groups, one of which contains the enzymes from *E. aerogenes*, *E. blattae* and *K. planticola*, and the other contains the enzymes from *M. morganii* and *P. stuartii*. As expected from such phylogenetic relationships, enzymes in the same group showed similar properties, and when expressed in *E. coli* the AP/PTases in each group produced almost the same amount of 5′-IMP under optimal conditions. The reaction mechanism of phosphotransferase activity catalyzed by a phosphatase is thought to operate via a phosphate-enzyme intermediate. Therefore, a lower K_m value for the phosphate acceptor is thought to be advantageous for the transphosphorylation reaction. The results for 5′-IMP production strongly suggest the importance of a lower K_m value for nucleotide production. Although these enzymes exhibited a very high degree of homology at the amino acid sequence level, the K_m values for inosine differ by about two fold from a high of 231 mM of AP/PT-Kp to a low of 117 mM of AP/PT-Mm. This suggests that local sequence differences in the binding pocket are responsible for the difference in the PP_i-nucleoside phosphotransferase reaction. As previously reported, the phosphotransferase reaction yield of *M. morganii* AP/PTase was much improved by introducing the two mutations of glycine-92 to aspartate and isoleucine-171 to threonine (5). These two residues are conserved in the other AP/PTases. Therefore, it would be possible to use the same approach to improve these AP/PTases and increase their phosphotransferase reaction yields.

It will be interesting to determine which residues contribute to the affinity for the phosphate acceptor, and to further investigate the relationship between primary structure and K_m value, in order to optimize nucleotide production by these enzymes. Recently, we have also determined the structure of AP/PT-Eb (16), and further studies on structure-activity relationships and improvement of phosphotransferase activity are in progress on the basis of these results.

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