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 (PPi)-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 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 phosphotransferase reaction.

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

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 PPi-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

For further details, please refer to the provided text.
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'-CTGGATATGGGCTATACATACC) and PR2 (5'-CTGGAGTCCGACGCGATTTTACCATA) (the BamHI 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 BamHI, and then cloned into pUC19.

Chromosomal DNA libraries of E. aerogenes, E. blattae, and K. planticola were constructed by inserting partially SaciAl-digested fragments of 3 to 6 kilobases (kb) into the BamHI 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 colonies 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). 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 pmol of 5'-IMP per min under the assay conditions. As the solubility of inosine is limited, kinetic constants for inosine were determined using an automatic protein sequencer (Prosequencer 6618; 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-2Na7.5Hz0 (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 PPi-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. Phosphotransferase-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 JM109 harboring pPRA120, 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 × 10³ units/mg), E. aerogenes (3.12 × 10³ units/mg), E. blattae (2.30 × 10³ units/mg) and K. planticola (4.57 × 10³ 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) was compared with those of other related enzymes. The nucleotide sequences of the cloned genes were identical to the gene we recently isolated as a nonspecific AP/PTase. The cloned AP/PTase gene appeared to encode the 744 to 747 bp sequence accession numbers.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristic</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMP1501</td>
<td>Containing 1.1 kb HindIII-EcoRI fragment derived from M. morganii cloned in pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pSP100</td>
<td>Containing 1.1 kb BamHI fragment encoding P. stuartii phoC in pUC18</td>
<td>Unpublished data X64820</td>
</tr>
<tr>
<td>pEAP120</td>
<td>Containing 7.6 kb Sau3AI fragment derived from E. aerogenes cloned in pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pEA100</td>
<td>Containing 1.6 kb Sau3AI fragment derived from E. aerogenes cloned in pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pEBP110</td>
<td>Containing 3.6 kb Sau3AI fragment derived from E. blattae cloned in pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pEBP120</td>
<td>Containing 1.2 kb PstI-HindII fragment derived from E. blattae cloned in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pKPP100</td>
<td>Containing 3.6 kb Sau3AI fragment derived from K. planticola cloned in pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pKPP120</td>
<td>Containing 2.2 kb PstI-EcoRI fragment derived from K. planticola cloned in pUC19</td>
<td>This study</td>
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</table>

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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 phosphatasas, 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 genes; Ebl, AP/PT'ase was compared to those of proteins in the

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.

A phylogenetic tree of the five AP/PTases used in this study and other phosphatases that share the same conserved sequence motifs, KXXXXXXPR, PSGH and SRXXXXXH were constructed from the deduced sequences. The tree is shown in Fig. 2. 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 for each enzyme was calculated to be about 160,000 by gel filtration analysis. The relative molecular weight of each enzyme was determined to be about 160,000 by gel filtration analysis.

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-M), 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~ nucleoside phosphatase activity, and their phosphorylation reaction was strictly regioslective 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
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 $K_m$ values for inosine in the transphosphorylation reaction were determined for each enzyme. The $V_{max}$ values for AP/PT-Mm are approximately three-fold higher than 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

<table>
<thead>
<tr>
<th>Origin of the AP/PTase</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (u/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. morgani</td>
<td>117</td>
<td>6.00</td>
</tr>
<tr>
<td>P. stuartii</td>
<td>156</td>
<td>6.21</td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>218</td>
<td>3.75</td>
</tr>
<tr>
<td>E. blattae</td>
<td>200</td>
<td>2.78</td>
</tr>
<tr>
<td>K. planticola</td>
<td>231</td>
<td>2.65</td>
</tr>
</tbody>
</table>

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.

**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. morgani (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-

![FIG 3. 5'-IMP synthesis by *E. coli* overproducing each AP/PTase.](image-url)
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-Sf) 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, phosphate-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 transfer 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.

REFERENCES


