Product-Induced Gene Expression, a Product-Responsive Reporter Assay Used To Screen Metagenomic Libraries for Enzyme-Encoding Genes

Taku Uchiyama1 and Kentaro Miyazaki1,2*

Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan,1 and Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Tsukuba, Ibaraki 305-8566, Japan2

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A reporter assay-based screening method for enzymes, which we named product-induced gene expression (PIGEX), was developed and used to screen a metagenomic library for amidases. A benzoate-responsive transcriptional activator, BenR, was placed upstream of the gene encoding green fluorescent protein and used as a sensor. Escherichia coli sensor cells carrying the benR-gfp gene cassette fluoresced in response to benzoate concentrations as low as 10 μM but were completely unresponsive to the substrate benzamide. An E. coli metagenomic library consisting of 96,000 clones was grown in 96-well format in LB medium containing benzamide. The library cells were then cocultivated with sensor cells. Eleven amidase genes were recovered from 143 fluorescent wells; eight of these genes were homologous to known bacterial amidase genes while three were novel genes. In addition to their activity toward benzamide, the enzymes were active toward various substrates, including β- and γ-aminocarboxylic acids, and displayed enantioselectivity. Thus, we demonstrated that PIGEX is an effective approach for screening novel enzymes based on product detection.

Environmental microorganisms carry great potential as sources of industrial enzymes (5, 21); however, the majority of these organisms are difficult to culture in the laboratory (1, 29). Metagenomics has emerged as a credible alternative to conventional, cultivation-dependent microbial screening. It allows exhaustive screening of microbial genomes in their natural environments by directly cloning environmental DNA in a surrogate host; however, functional screening of metagenomic libraries often suffers from low hit rates, largely as a result of insufficient expression of anonymous foreign genes in the host, which is commonly Escherichia coli (26).

One approach to overcoming this problem is to use a reporter assay (23), a strategy that is frequently employed in molecular biology (e.g., β-galactosidase assay and chloramphenicol acetyltransferase [CAT] assay) and biotechnology (e.g., to detect environmental pollutants [18] and food additives [17]). Previously, we developed a substrate-induced gene expression (SIGEX) method (25) to capture genetic elements that respond to exogenous compounds. By using green fluorescent protein (GFP) as a reporter and performing the screen using a fluorescence-activated cell sorter, we achieved high screening efficiency. The method was successfully used to retrieve genetic elements that sense exogenous compounds (e.g., benzamide and naphthalene).

In the present study, we extended our reporter assay system to specifically retrieve enzymes. To this end, we utilized the previously identified transcriptional activator BenR, which was placed upstream of gfp. We assumed that E. coli cells harboring the benR-gfp cassette would fluoresce in the presence of a benzoate precursor compound (substrate) if they expressed an enzyme capable of actively transforming the precursor into benzoate (Fig. 1). This reporter assay system, which we named product-induced gene expression (PIGEX), should allow the identification of desired enzymatic activities by linking product formation to reporter gene expression. Using this system, we targeted amidases that convert benzamide to benzoate.

Amidases (EC 3.5.1.4) catalyze the conversion of amides to their corresponding carboxylic acids (with ammonia being produced as a by-product) according to the following reaction: RCONH₂ + H₂O → RCOOH + NH₃. Given that the function of amidases is to hydrolyze amide bonds, the biological consequences of this reaction are many and varied. In addition to their diverse biological roles, amidases have found a number of industrial applications, notably in the synthesis of chiral compounds (7, 20, 28). To date, numerous bacterial amidases have been purified and characterized, but no efforts have been made to recover these enzymes via culture-independent approaches. To assess the possibility of identifying novel amidases in metagenomes, we used PIGEX screening to search for amidases that use benzamide as a substrate. By coupling benzamidase activity to benzoate-responsive fluorescence, we successfully identified 11 amidase genes, including three genes with low sequence similarity to known amidases.

MATERIALS AND METHODS

Reagents. Restriction and DNA modification enzymes and pHSG398 were purchased from Takara Bio (Shiga, Japan). Aromatic compounds, acetamide, acrylamide, and propionamide were purchased from Tokyo Chemical Industry (Tokyo, Japan), while cis,cis-muconic acid was purchased from Sigma-Aldrich

* Corresponding author. Mailing address: Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan. Phone: (81) 29 861 6060. Fax: (81) 29 861 6413. E-mail: miyazaki-kentaro@aist.go.jp.
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Bacterial strains, media, and growth conditions. The E. coli strains used in this study were JM109 (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δlac-proAB [F' traS6 proAB' lacIq lacZΔM15]) and EPI300 (F' mcrA qmvr-hsdR(MS-mcrBC) Δ(lac-proAB) lacIq Δ(lacZΔM15) F' traS6 proAB'). Cells were cultured at 37°C in LB broth or on LB agar plates. Antibiotics (100 μg/ml ampicillin [Amp] and 34 μg/ml chloramphenicol [Cm]) were added to the culture medium as appropriate.

Construction of a benzoate-responsive sensor plasmid. A DNA fragment containing the benzamide-responsive transcriptional regulator BenR upstream of gfp was excised from phn028 using EcoRI and SphI (25). The same restriction fragment was ligated into pHSG398, yielding pCmGFPbenR.

PCR genotyping. Cells were removed from the positive wells and cultured in 1 ml of LB-Cm medium at 37°C for 18 h. They were then centrifuged, washed twice with water, and suspended in 200 μl of water. Oligonucleotide primers were designed to amplify part of the ampicillin resistance gene (25) and were used in a PCR reaction mixture consisting of 10 mM sodium phosphate buffer (pH 7.0), 0.2 mM dNTPs, 2.5 U of Taq DNA polymerase (QIAGEN), and 0.5 μM of each primer. The reaction was performed in a DNA Engine gradient (MJ Research, Waltham, MA). After the initial denaturation at 98°C for 2 min, 35 cycles of 98°C for 30 s, 50°C for 30 s, and 72°C for 1 min were performed. PCR products were analyzed by agarose gel electrophoresis.

Substrate specificities of the retrieved amidases. E. coli JM109 cells carrying the metagenomic amidase genes were cultivated at 37°C for 18 h in 250 ml of LB-Amp medium. Cells were then harvested and washed twice with 10 mM sodium phosphate buffer (pH 7.0), resuspended in 5 ml of 10 mM sodium phosphate buffer (pH 7.0), and disrupted using a cell disrupter (One Shot Model; Bio-Rad, Richmond, CA) with bovine gamma globulin as the standard. The supernatants were discarded, and the cell pellets were washed in distilled water and resuspended in 200 μl of distilled water. Cell suspension (100 μl) from each well was transferred to the wells of a black 96-well microplate. GFP fluorescence was measured as described above.

Isolation of individual positive fosmid clones from hit wells. Because each well contained ideally 100 fosmid clones, a second screen was performed to isolate individual positive clones from the hit wells. Cells were removed from each well and were screened as described above. When several colonies were retrieved from the same well, their individual identities were determined by restriction fragment length polymorphism (RFLP) analysis using EcoRI and/or PstI.

Metagenomic clone

CONH₂

1. Amidase

Sensor

GFP

2. BenR

3. GFP

FIG. 1. Schematic representation of the PIGEX (product-induced gene expression) method using amidase as the target enzyme. The amidase-positive clone catalyzes the conversion of benzamide to benzoate (step 1). Benzoate activates the transcriptional regulator BenR, which in turn activates the benA promoter (P_{benA}) (step 2) and induces the expression of a reporter gene (gfp; step 3). Enzymatic benzoate production is measured as GFP fluorescence.
to the culture medium while those lacking this genetic unit fluoresced upon the addition of benzoate. In contrast, benzaamide did not yield significant fluorescence, making it a suitable sensor for PIGEX screening. The second factor that needs to be considered is the absence of endogenous inducers. The E. coli strains used in this study did not produce detectable levels of benzoate when grown in LB medium in the presence or absence of benzamide, allowing us to isolate clones capable of catalyzing the conversion of benzamide to benzoate. Third, products generated by amidase reactions must be inert in vitro to ensure constant induction of the sensor. From this point of view, substrates such as amino acid amides are unsuitable because the products of their metabolism, amino acids, are readily metabolized in E. coli cells. The use of benzamide as the sole substrate may potentially have limited our ability to retrieve functionally diverse amidases although it should be noted that amidases are known to be catalytically promiscuous.

**RESULTS AND DISCUSSION**

**PIGEX.** The PIGEX method is based on the reporter assay illustrated in Fig. 1. This system consists of a sensor gene paired with a reporter gene. To screen for amidase genes, we used as the sensor a benzoate-responsive BenR transcriptional regulator that we ourselves had previously retrieved from a metagenome (25). As the reporter, we used GFP. Cells carrying this genetic unit fluoresced upon the addition of benzoate to the culture medium while those lacking BenR did not. An important factor in the choice of sensor is the ability to distinguish between substrate and product. To confirm the suitability of BenR for use as a sensor, we tested its substrate specificity. A range of benzoate analogs were tested for their ability to activate BenR (Table 1) by separately adding them to LB medium at a concentration of 1 mM. Significant fluorescence was observed for benzoate (3,682 ± 160 arbitrary fluorescence units [AU]), 2-hydroxybenzoate (2,215 ± 177 AU), 3-hydroxybenzoate (631 ± 103 AU), and benzaldehyde (840 ± 64 AU). In contrast, benzamide did not yield significant fluorescence (39 ± 1 AU), making it a suitable sensor for PIGEX screening.

**TABLE 1. Induction of green fluorescence in E. coli JM109/pCMGFPbenR by various compounds**

<table>
<thead>
<tr>
<th>Inducing compound</th>
<th>Fluorescence (AU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>Benzoate and derivatives</td>
<td></td>
</tr>
<tr>
<td>Benzoate</td>
<td>3,682 ± 160</td>
</tr>
<tr>
<td>2-Hydroxybenzoate</td>
<td>2,215 ± 177</td>
</tr>
<tr>
<td>3-Hydroxybenzoate</td>
<td>631 ± 103</td>
</tr>
<tr>
<td>4-Hydroxybenzoate</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>3,4-Hydroxybenzoic acid</td>
<td>47 ± 1</td>
</tr>
<tr>
<td>2,5-Hydroxybenzoic acid</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>Benzene and derivatives</td>
<td></td>
</tr>
<tr>
<td>Benzene</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>Toluene</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Benzy alcohol</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>840 ± 64</td>
</tr>
<tr>
<td>Aniline</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>Benzyamine</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>Benzonitrile</td>
<td>40 ± 0</td>
</tr>
<tr>
<td>Benzamide</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>Acetophenone</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>Benzoic acid methyl ester</td>
<td>41 ± 2</td>
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<tr>
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</tr>
<tr>
<td>Phenol</td>
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</tr>
<tr>
<td>Catechol</td>
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</tr>
<tr>
<td>o-Cresol</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>Polyaromatics</td>
<td></td>
</tr>
<tr>
<td>Naphthalene</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>1,2-Dihydroxynaphthalene</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>47 ± 1</td>
</tr>
<tr>
<td>2,3-Dihydroxybiphenyl</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>cis,cis-Muconic acid</td>
<td>43 ± 1</td>
</tr>
</tbody>
</table>

*Fluorescence intensity values were normalized to cell densities. All values are the means of five measurements. Arbitrary fluorescence units (AU) represent the fluorescence due to GFP production.

**FIG. 2.** Growth- and dose-dependent induction of GFP by benzoate. Error bars represent 1 standard deviation from the mean (n = 4). (A) Growth phase dependence of GFP fluorescence. E. coli JM109/pCMGFPbenR sensor cells were grown to the log or stationary phase, and then benzoate was added to the culture medium (final concentration of 0.5 mM). Cell density (left panel) and fluorescence intensity (right panel) were monitored after induction. Open circle, log-phase cells (no benzoate induction); filled circle, log-phase cells induced with 0.5 mM benzoate; closed triangle, stationary-phase cells induced with 0.5 mM benzoate. (B) GFP fluorescence in sensor cells (E. coli JM109/pCMGFPbenR) induced with different concentrations of benzoate. The inset panel shows a close-up of the graph in the 0 to 100 μM benzoate region.
of 0.5 mM benzoate to the culture medium, the cells began to fluoresce within 1 h. Fluorescence peaked at 6 h and persisted for at least 24 h. In contrast, when cells were grown to the stationary phase before induction, they did not fluoresce, even after the addition of 0.5 mM benzoate, suggesting that *gfp* expression is growth phase dependent in our system. Thus, our subsequent experiments employed mid-log-phase cells as a sensor. We also examined the benzoate dose dependence of *gfp* fluorescence (Fig. 2B). The fluorescence intensity of *gfp* sensor. We also examined the benzoate dose dependence of expression is growth phase dependent in our system. Thus, our

<table>
<thead>
<tr>
<th>Amidase name or type</th>
<th>Fluorescent well(s) (identification no.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>8D-type</td>
<td>1A5, 1D7, 1G1, 1G8, 1G12, 1H5, 1H7, 1H9, 1H12, 2B1, 2D3, 2E4, 2F1, 2F4, 2F11, 2H6, 3A10, 3B1, 3B8, 3C12, 3D1, 3D4, 3D7, 3E2, 3E3, 3E4, 3F3, 3F7, 3F10, 3H1, 4A1, 4A4, 4A8, 4B4, 4B6, 4B8, 4F8, 4G11, 4H6, 4H9, 4H11, 5A9, 5B1, 5B4, 5C5, 5C3, 5D3, 5D7, 5E6, 5F6, 5G8, 5G10, 6A2, 6A11, 6B1, 6B10, 6B12, 6C4, 6E3, 6F2, 6H4, 7A10, 7B1, 7B2, 7B4, 7B10, 7C8, 7D1, 7F3, 7F4, 7F9, 7G1, 7G2, 7G10, 7H1, 7H9, 8B10, 8C2, 8D11, 8E7, 8F5, 8F12, 8G3, 8H5, 9A4, 9A5, 9A8, 9A11, 9C7, 9D10, 9E11, 9F1, 9F3, 9F4, 9G5, 9G6, 9G7, 9H9, 10A7, 10C6, 10C12, 10D2, 10D5, 10D9, 10E3, 10F6, 10F7, 10F11, 10G3, 10G9, 10H6</td>
</tr>
<tr>
<td>3D8-type</td>
<td>1D11, 3D8, 4A10, 5A1, 5B9, 6D9, 6E7, 7F1, 7G5, 8B11, 8G12, 9D12, 9E6, 10A11</td>
</tr>
<tr>
<td>6H10-type</td>
<td>1H4, 2H2, 5F2, 5G9, 6H10, 7F7</td>
</tr>
<tr>
<td>10A5-type</td>
<td>8C3, 8D3, 10A5</td>
</tr>
<tr>
<td>3B10-type</td>
<td>1F5, 3B10, 6E2</td>
</tr>
<tr>
<td>1B9</td>
<td>1B9</td>
</tr>
<tr>
<td>3B3</td>
<td>3B3</td>
</tr>
<tr>
<td>4E7</td>
<td>4E7</td>
</tr>
<tr>
<td>5C8</td>
<td>5C8</td>
</tr>
<tr>
<td>9D11</td>
<td>9D11</td>
</tr>
<tr>
<td>10H1</td>
<td>10H1</td>
</tr>
</tbody>
</table>

* A metagenomic library (96,000 clones divided in 10 96-well plates) was used for screening. Wells in boldface were used for single clone isolation.

Table 2. Summary of hit wells identified in the first screen

- **PIGEX screening of a metagenomic library.** Metagenomic library cells (96,000 clones divided among 96 wells) were grown overnight and combined with an equal volume of sensor cells grown separately to mid-log phase with benzamide. After the mixed culture was grown overnight, we identified 143 fluorescent wells (Table 2) (maximum fluorescence intensity, 2,625 AU [well 10D9]; minimum, 152 AU [well 3E2]). Of these, four wells (2B1, 3E2, 9C7, and 10D9) were selected, and their contents were subjected to a second round of screening to isolate individual clones. Fosmid DNA (insert length, 30 to 40 kb) was purified from the amidase-positive clones obtained from each well. We then created fosmid shotgun libraries (insert length, 2 to 5 kb) and functionally screened them by PIGEX. Short DNA fragments responsible for the observed benzamidase activity were subjected to DNA sequencing.

Unexpectedly, all four clones shared a nearly identical DNA sequence, containing a putative amidase gene (pAD; open reading frame 3 [ORF3]) (see Fig. S1 and Table S1 in the supplemental material). This bias could be due to the use of activated sludge (which has the specific biological function of degrading compounds in wastewater) as a source of DNA. A similar bias was noted in a screen for extradiol dioxygenase genes using the same library. Of 43 extradiol dioxygenase genes identified, 20 shared nearly identical sequences (22). Alternatively, the bias may be a consequence of using *E. coli* as the host. It has been suggested that host genetic systems (i.e., transcription and translation systems) can affect the expression of foreign genes and may limit the diversity of retrieved genes (26).

To explore the types of amidase genes in the 143 fluorescent wells we initially identified, we performed PCR-based genotyping. PCR primers were designed to target genes homologous to 8D amidase. A set of primers specific for the internal region of the amidase gene (nucleotide positions 262 to 279) and its 64-bp downstream region was designed. As a result, 8D-type PCR products were obtained from 111 wells, indicating a predominance of this type of gene in our metagenomic library. The remaining 32 wells yielded no product, suggesting the presence of different types of amidase genes. We applied PIGEX screening to these 32 wells to recover individual fosmid clones; RFLP analysis confirmed their uniqueness. We produced pUC-based shotgun libraries for these fosmids, screened the libraries by the PIGEX procedure, and then determined the nucleotide sequences of the inserts of the positive plasmids by DNA sequencing. Genes showing high similarity were identified in a number of clones (3D8-type, 14 clones; 6H10-type, 6 clones; 10A5-type, 3 clones; and 3B10-type, 3 clones); unique genes were found in 6 clones (1B9, 3B3, 4E7, 5C8, 9D11, and 10H1) (Table 2). In total, we recovered 11 different benzamidase genes. We confirmed the activity of these amidases (1B9, 3B3, 3B10, 3D8, 4E7, 5C8, 6H10, 8D, 9D11, 10A5, and 10H1) by the appearance of benzoate and disappearance of benzamide in HPLC analysis.

In this study, our screening process consisted of three stages: (i) well screening, (ii) individual isolation from hit wells, and (iii) gene identification through functional screening of fosmid libraries. This apparently complex procedure was necessitated by the use of a fosmid-based library with approximately 100 clones per well (22). One could screen a plasmid-based library with one clone per well in a single step.

Comparison of PIGEX and other reporter assay methods. Reporter assay systems similar to PIGEX have been described...
by Williamson et al. (30), Mohn et al. (14), and van Sint Fiet et al. (27). Williamson et al. (30) used a recombinant
*E. coli*
carrying *luxR* and *gfp* genes on a single reporter plasmid, which fluoresced in the presence of bacterial quorum-sensing com-
pounds. They screened a metagenomic library for quorum-
sensing compounds using an intracellular system in which both
sensor and reporter vectors were included in a single cell. They
identified 11 quorum-sensing genes, only one of which was
identified as positive in their extracellular system. Based on
these results, they concluded that their intracellular system was
superior to their extracellular one in terms of sensitivity.

We also performed—in parallel—amidase screening using
an intracellular system and observed cross talk between posi-
tive and negative clones (data not shown). When we coculti-
vated *E. coli* cells carrying amidase-positive fosmids and the
benzoate sensor on agar plates, we found that negative col-
onies in close proximity with positive colonies themselves
fluoresced. This phenomenon most likely resulted from
the diffusion of benzoate produced by amidase-expressing
clones. We believe that such cross talk may be a common
problem in intracellular screens performed using compounds
that freely permeate cell membranes. Thus, determining which
system works better is not straightforward.

**Sequence analysis of metagenomic amidases.** Plasmids were
purified from the 11 positive clones, and their nucleotide se-
quencies were determined. The genetic organization of the
plasmid inserts is shown in Fig. S1 in the supplemental mate-
rial. The amidase genes carried by eight of the clones (1B9,
3B3, 3D8, 4E7, 6H10, 8D, 9D11, and 10A5) were readily iden-
tified on the basis of amino acid sequence identity (see Table
S1). In contrast, 3B10, 5C8, and 10H1 had no obvious identity
to known amidase-coding genes, indicating that PIGEX was
effective for screening our metagenome for novel enzymes.

The last three clones contained a single ORF in their inserts.
We assumed that these ORFs were responsible for their
amidase activity. The ORF of 3B10 shared 54% amino acid
sequence identity with a putative isochorismatase hydrolase
from *Streptomyces* sp. strain AA4. Although this enzyme
bore no apparent relationship to benzamidase, a conserved
domain database search (13) revealed homology between the
ORF and the cysteine hydrolase superfamily, which includes
nicotinamidase. Thus, it is likely that this ORF is responsible
for the observed benzamidase activity. The ORF included in
the 5C8 clone shared 45% primary amino acid sequence iden-
tity with a hypothetical protein. The ORF included in 10H1
shared 57% primary amino acid sequence identity with a trans-
membrane ABC transporter signature motif-containing protein, a
component of the periplasmic binding protein-dependent ABC
transporter involved in the uptake of branched-chain amino ac-
ids. Despite its apparent independence from amidases, one
well-characterized ε-stereospecific alanine amidase from *Bre-
vibacillus borstelensis* exhibits 44% amino acid sequence iden-
tity to an ABC transporter (2). Therefore, it is possible that the
ORF encodes a polypeptide with amidase activity.

A primary sequence alignment of eight canonical metagenomic
benzamidases and functionally characterized bacterial ami-
dases is shown in Fig. S2 in the supplemental material, and
their phylogenetic relationships are shown in Fig. 3. Our meta-
genomic amidases are sparsely distributed in the amidase fam-
ily. Chebrou et al. (3) compared the amino acid sequences of
22 amidases from both prokaryotes and eukaryotes and iden-
tified a highly conserved GGSS(G/S/A)G sequence. This motif

![FIG. 3. Phylogenetic (neighbor-joining) tree of the metagenomic and representative amidases. The topology of the tree was determined by
1,000 bootstrap replicates, and the distances were corrected by the Kimura method (9). Amidases from isolated bacteria are represented with their
organismal origin, and accession numbers are in parentheses. The numbers above the nodes represent the bootstrap values. The scale bar indicates
the expected number of substitutions per residue. The acetamidase of *Aspergillus oryzae* (EA2 subfamily) was used as the outgroup (3). Abbreviations for the distinct enzyme subfamilies are as follows: IAAH, indole-acetamide hydrolases; AH, 6-aminohexanoate-cyclic-dimer hydrolases; AMD, bacterial amidases.
](http://aem.asm.org/cgi/doi/10.1128/AEM.02859-12)
is conserved in canonical metagenomic benzamidases though the second Gly is replaced with Val in compounds 1B9 (residue 154), 3D8 (residue 154), and 9D11 (residue 154). The active site (residues Asp191 and Ser195 of Rhodococcus rhodochrous J1 [10]) containing the motif G/A-XDX-G/A-G/A-S-I/V/LRXP-A/S (residues 173 to 184 of 9D11) is also conserved in our metagenomic amidases.

**Substrate specificities of metagenomic amidases.** The substrate specificities of our metagenomic amidases were determined using *E. coli* cell extracts (Table 3). All of the clones, including those that lacked sequence homology to known amidases (3B10, 5C8, and 10H1), showed benzamide activity, but clone-to-clone variations were observed for other substrates. Recently, the crystal structure of an amidase from *Rhodococcus* sp. strain N-771 (identical to *Rhodococcus* sp. strain N-774 amidase) was determined (15). This amidase was found to prefer benzamide over all other substrates tested (acetamide, acrylamide, propionamide, and benzamide). Hydrophobic residues in the active site (Phe146, Ile227, Trp328, Leu447, and Ile450) were proposed to be the basis for the specificity of the substrate amidase. Amidases from *Delftia acidovorans* 1B9, 3D8, and 9D11 share 53% with known D-amino acid amidases, whereas the amidase from *V. paradoxus* prefers the D-enantiomers of alanine amide, leucine amide, and phenylalanine amide, whereas the amidase from *V. paradoxus* prefers the d-enantiomers of alanine amide, leucine amide, and phenylalanine amide. For the other metagenomic amidases, hydrophilic residues were found in the active site. Notably, the residue corresponding to Leu447 in *Rhodococcus* sp. N-771 amidase was changed to Asp (1B9, 3D8, 4E7, and 9D11) or Met (8D), and those metagenomic amidases with Asp in place of Leu tended to have higher activity against substrates other than benzamide. From a biotechnological point of view, the stereospecific hydrolysis of racemic amino acid amides is particularly useful as a cost-effective approach for the production of optically pure l- and d-amino acids (2, 6, 8, 11, 12). Of our metagenomic amidases, 1B9, 3D8, and 9D11 share ~68% amino acid sequence identity with each other and ~53% with known d-amino acid amidases from *Delftia acidovorans* (8) and *Variovorax paradoxus* (12). The amidase from *D. acidovorans* is highly selective for the d-enantiomers of proline amide, leucine amide, and phenylalanine amide, whereas the amidase from *V. paradoxus* prefers the d-enantiomers of alanine amide, leucine amide, and phenylalanine amide and the l-enantiomer of proline amide. In our study, the 3D8 and 9D11 amidases preferred the D-enantiomer of leucine amide, while the 1B9 amidase, the closest homolog of the 9D11 enzyme (84% primary sequence identity) lacked this selectivity (Table 3). All three of these enzymes lacked selectivity for phenylalanine and proline amides, and the 3D8 amidase would not metabolize proline amide. Interestingly, the 8D amidase had the opposite selectivity, favoring the l-enantiomers of leucine and phenylalanine amides. The 6H10 and 10A5 amidases share high amino acid sequence identity (76%), and their substrate specificities were similar. The 5C8 and 10H1 amidases also showed similar activity profiles despite a lack of sequence similarity. Therefore, the relationship between sequence and selectivity is not straightforward.

We used benzamide as the sole substrate for screening. However, owing to the intrinsic catalytic promiscuity of am-
dases, our metagenomic amidases displayed various substrate specificities and stereospecificities. They may form a unique starting point for further protein engineering.

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