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Transformation of Isopropylamine to L-Alaninol by \textit{Pseudomonas} sp. Strain KIE171 Involves N-Glutamylated Intermediates

Susana I. de Azevedo Wäsch, Jan R. van der Ploeg, Tere Maire, Alice Lebreton, Andreas Kiener, and Thomas Leisinger

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\textit{Pseudomonas} sp. strain KIE171 was able to grow with isopropylamine or L-alaninol \([5-(+)-2\text{-amino-1-propanol}]\) as the sole carbon source, but not with \(\Delta\)-alaninol. To investigate the hypothesis that L-alaninol is an intermediate in the degradation of isopropylamine, two mini-Tn5 mutants unable to utilize both isopropylamine and L-alaninol were isolated. Whereas mutant KIE171-BI transformed isopropylamine to L-alaninol, mutant KIE171-BII failed to do so. The two genes containing a transposon insertion were cloned, and the DNA regions flanking the insertions were sequenced. Two clusters, one comprising eight \(ipu\) (isopropylamine utilization) genes \((ipuABCDEFGH)\) and the other encompassing two genes \((ipuI and orf259)\), were identified. Comparisons of sequences of the deduced proteins suggested that isopropylamine is transported into the cytoplasm by a putative permease, IpuG. The next step, the formation of \(\gamma\)-glutamyl-isopropylamide from isopropylamine, ATP, and L-glutamate, was shown to be catalyzed by IpuC, a \(\gamma\)-glutamylamidase synthetase. \(\gamma\)-Glutamyl-isopropylamide is then subjected to stereospecific monooxygenation by the hypothetical four-component system IpuABDE, thereby yielding \(\gamma\)-glutamyl-L-alaninol \([(\gamma\text{-glutamyl})\text{-L-hydroxy-isopropylamide}]\). Enzymatic hydrolysis of a hydrolase, IpuE, was shown to finally liberate L-alaninol and to regenerate L-glutamate. No gene(s) encoding an enzyme for the next step in the degradation of isopropylamine was found in the \(ipu\) clusters. Presumably, L-alaninol is oxidized by an alcohol dehydrogenase to yield L-2-aminopropanol. This compound, and a degradative pathway involving L-alaninol would be of interest for the biotransformative production of this versatile synthon from the cheap prochiral compound isopropylamine. This prompted us to isolate an organism, \textit{Pseudomonas} sp. strain KIE171, which grows with isopropylamine and L-alaninol but not with \(\Delta\)-alaninol. In the present work we provide genetic and biochemical evidence that isopropylamine degradation in this strain proceeds via \(\gamma\)-glutamyl-isopropylamide and \(\gamma\)-glutamyl-L-alaninol to L-alaninol.

\textbf{MATERIALS AND METHODS}

\textbf{Materials}. Reagents for molecular biology were obtained from Fermentas (Vilnius, Lithuania) and New England Biolabs. \(\gamma\text{-}(\text{L-glutamyl})\text{-L-alaninol}\) and \(\gamma\text{-}(\text{L-glutamyl})\text{-L-hydroxy-isopropylamide}\) were obtained from Bachem (Bubendorf, Switzerland). All other chemicals were reagent grade or better and were obtained from Aldrich, Fluka, or Sigma.

\textbf{Growth media and bacterial strains}. \textit{Pseudomonas} sp. strain KIE171 and its derivatives were cultivated aerobically at 30°C in the mineral salts medium described by Kulla et al. (15) with a 10 or 20 mM carbon source. Cells were grown in 500-ml Erlenmeyer flasks containing 100 ml of liquid medium and shaken at 140 rpm. \textit{Escherichia coli} was grown aerobically with shaking (140 rpm) in Luria-Bertani medium (22). Growth was monitored as turbidity at 650 nm.

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\textit{Pseudomonas} sp. strain KIE171 has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 12360. The isopropylamine-utilizing \(\Delta\)-alaninol-nonutilizing mutants KIE171-B, KIE171-BL, and KIE171-BII carry the Deutsche Sammlung von Mikroorganismen und Zellkulturen numbers DSM 11521, DSM 11629, and DSM 13380, respectively. \textit{E. coli} strains DH5\(\alpha\) (GIBCO/BRL Life Technologies) and XLI-Blue (Stratagene) were used for cloning, and \textit{E. coli} BL21(DE3) (Novagen) was used as the host for the overexpres-
of IpuC as an N-terminal histidine-tagged fusion protein, the codon for histidine was cloned into the vector PET28a (+), resulting in plasmid pME4751. To confirm that no changes had been introduced during PCR amplification, the ipuF gene of plasmid pME4751 was sequenced. Plasmid pME4756, which expresses wild-type IpuF, was obtained by cloning the Nhel and SacI restriction sites into the expression vector PET24b (+).

Enrichment and isolation of an isopropylamine-utilizer. Ten milliliters of sewage sludge from the Lonza AG wastewater treatment plant was mixed with 90 ml of mineral salts medium containing 20 mM isopropylamine, and the mixture was incubated at 30 °C under nonsterile conditions without shaking. After growth had occurred, 1 ml aliquots of the enrichment were transferred into 99 ml of the same medium and incubated under the above-mentioned conditions. After four subcultures in sterile medium with shaking, the final enrichment was serially diluted and spread onto plates containing mineral salts medium with 20 mM isopropylamine. The majority of the colonies were of one type. Purification of a typical colony yielded a pure culture of the isopropylamine-utilizer Pseudomonas sp. strain KIE171.

Mutagenesis. Chemical mutagenesis of strain KIE171 was performed with N-methyl-N-nitro-N-nitrosoguanidine (MNGG) according to the protocol of Foster (10). A suspension of mutagenized cells containing approximately 50% survivors was plated on minimal medium containing 20 mM L-glutamate. A total of 2,000 colonies were replica plated on minimal plates containing 20 mM isopropylamine or 20 mM L-alanine as the sole carbon source. One mutant, strain KIE171-B, unable to grow with these compounds, was chosen for further study.

Transposon mutagenesis was carried out by using the mini-Tn5 system (7). The transposon was introduced into strain KIE171 by plate conjugation on Luria-Bertani medium at 30 °C for 8 h with a donor/recipient ratio of 1:1. E. coli S17-1 apr (pUT-miniTn5Km) was used as the donor strain. Exconjugants were selected on minimal salts agar containing 10 mM L-lactate and 10 mM L-alanine plus 50 μg of kanamycin per ml. A total of 3,000 kanamycin-resistant strain KIE171 exconjugants were then replicated onto minimal salts medium containing 50 μg of kanamycin per ml and a 20 mM concentration of either isopropylamine, L-alanine, or L-lactate as the sole carbon source. Mutants impaired in growth with one or several of these carbon sources were chosen for further study.

DNA manipulations. Isolation of plasmids and of genomic DNA, restriction enzyme digestion, agarose gel electrophoresis, Southern analysis, and transformation of E. coli were carried out using standard methods (2).

RNA isolation and primer extension analysis. Isolation of RNA and primer extension were performed as described before (3). Oligonucleotide primers (5′-CAGATCATATTCTTTGGCGTTGCCTCAT-3′) were used to prime the reverse transcription reaction. The plasmid used for generation of a sequencing ladder was pME4771, which was constructed by insertion of a 0.7-kb ApaI/PstI fragment containing the ipu promoter region in pBluescript II KS.

Construction of an ipuH:zeFle fusion strain. Plasmid pME4268 contains a 2.7-kb Smal/SalI fragment harboring the ipuH gene (S. L. de Azevedo Wach, unpublished data). For the construction of an ipuH:zeFle fusion in strain KIE171, the 2.4-kb Smal fragment from pX1918GT (24) was inserted in the blunted BamHI site of pME4268 to give pME4762. The Smal/HindIII fragment from pME4762 was then cloned in the vector pEX18Tc (24), resulting in pME4763. Plasmid pME4763 was introduced in strain KIE171 by conjugation, and integrants were selected on Luria-Bertani plates containing tetracycline (15 μg/ml). To check for second crossover events, integrants were subsequently plated on Luria-Bertani medium containing 5% sucrose. Success-resistant colonies were obtained, and one such colony was designated KIE171-BV. PCR was used to verify whether correct replacement had occurred.

Construction of ipuC and ipuF expression plasmids. For the production of IpuC, as an N-terminal histidine-tagged fusion protein, the ipuC gene was amplified by PCR from genomic DNA of strain KIE171 with the oligonucleotide primers IPCU-NT (5′-AACAGGTGATACTATATGCGGAAG-3′) and IPCU-CT (5′-TTTGAAGCTTGGACGTCCG-3′), with the changes to introduce Ndel and HindIII restriction sites, respectively, underlined. The 1.4-kb PCR product was digested with Ndel and HindIII, and the resulting fragment encompassing ipuC was ligated into Ndel/HindIII-digested pET-28a (+) (Novagen), resulting in plasmid pME4275, in which ipuC is under the control of the T7 polymerase promoter. The ipuC sequence of plasmid pME4275 was sequenced to confirm that no changes had been introduced during PCR amplification. For the production of the wild-type IpuF, the Ndel/HindIII ipuF insert of pME4275 was placed under the control of the T7 promoter of vector pET24a (+) (Novagen), generating plasmid pME4277.

For the production of IpuF as an N-terminal histidine-tagged fusion protein, the ipuF gene was amplified by PCR from plasmid pME4746 with the primers 22-24-kb HindIII fragment harboring ipuF, cloned into the expression vector pET3d (Stratagene). The oligonucleotide primers used were IMPU-NT (5′-GGGCCAAGTACAGGCTGAGAAGTTCG-3′) and IMPU-CT (5′-TCTAGAAGCTTGAATACGCT-3′), with the changes to introduce Nhel and SacI restriction sites, respectively, underlined. The 909-bp PCR product was digested with Nhel and SacI, and the resulting 891-bp fragment containing the ipuF sequence was cloned into the vector PET28a (+), resulting in plasmid pME4751. To confirm that no changes had been introduced during PCR amplification, the ipuF gene of plasmid pME4751 was sequenced. Plasmid pME4756, which expresses wild-type IpuF, was obtained by cloning the Nhel and SacI restriction sites into the expression vector PET24b (+).

Enzyme assays. γ-Glutamylamidase synthetase (Ipuc) activity was assayed by measuring the substrate-dependent formation of inorganic phosphate from ATP as described for the assay of γ-glutamylamidase synthetase and isopropylamine phosphatase (14). The reaction mixture (0.4 ml) contained 10 mM ATP, 10 mM substrate, 10 mM L-glutamate, 50 mM MgCl2, 50 mM imidazole-HCl (pH 7.0), 3.5 mM NaCl, and 25 μg of enzyme preparation. The reaction was started by the addition of enzyme, and it was run at 25 °C. To stop the reaction, 0.9 ml of ferrous sulfate reagent (0.8% FeSO4·7H2O in 15 mM H2SO4) and 0.075 ml of ammonium molybdate reagent [6.6% (NH4)6Mo7O24·4H2O in 7.5 M H2SO4] were added to 0.1 ml of incubation mixture. The sample was mixed vigorously and color was allowed to develop to carry out the assay. The reaction mixture pME4751 was performed at 18 °C for 1 h to a final A650 of 1.5. The same procedure was used for production of wild-type IpuC in E. coli strain BL21(DE3) harboring the expression plasmid pME4277. To obtain His6-IpuF, the induction of strain BL21(DE3) carrying the expression plasmid pME4751 was performed at 18 °C for 5 h to a final A600 of 1. The same procedure was used for the production of wild-type IpuF from the expression plasmid pME4756.

Induced E. coli BL21(DE3) cells (0.5 g) expressing His6-IpuC or His6-IpuF were suspended in 4 ml of the appropriate lysis buffer containing DNase I (10 μg/ml). Cell extract was obtained by two passages through a French pressure cell at 5.5 MPA and subsequent centrifugation at 40,000 × g for 30 min. His6-IpuC was precipitated by metal chelation chromatography on a column of 25-μl HisBlue beads (Novagen) according to the manufacturer's instructions, and the pure protein was stored at −20 °C in 200 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 20% (vol/vol) glycerol (pH 8.0).

Crude extract containing His6-IpuF was supplemented with glycerol to a final concentration of 15% (vol/vol). His6-IpuF was purified on a 1-ml Ni-NTA-agarose gel column (Qiagen) as described by the manufacturer and stored at −20°C in 250 mM imidazole, 300 mM NaCl, 50 mM Na2HPO4, 15% (vol/vol) glycerol (pH 8.0).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Mini-PROTEIN II system (Bio-Rad) with 12% polyacrylamide gels under denaturing conditions (22). The broad range molecular weight markers used were from Bio-Rad or from New England Biolabs. Protein concentrations were measured using the method of Bradford (4) with Bio-Rad dye concentrate. Bovine serum albumin was used as a standard. HPLC. HPLC was carried out with an alliance HPLC system (Waters), using MILLIENNUM software. Phenylisothiocyanate (PITC)-derivatized amine derivatives, including isopropylamine, L-alanine, γ-glutamylamides, and ATP, and ADP were separated on a Nucleosil C18 reversed-phase column (250 by 4.6 mm; particle size, 7 μm) by applying a step gradient from 5 mM potassium phosphate buffer, pH 6.5, at the start to 80% (vol/vol) methanol–20% (vol/vol) 5 mM potassium phosphate buffer, pH 6.5, at 27 min. The flow rate was 1 ml/min. Compounds eluting from the column were detected by measuring the A254 nm and identified by cochromatography. For derivatization with PITC, 15 μl of sample was mixed with 15 μl of ethyl alcohol and subsequently with 140 μl of derivatization mixture [70% (vol/vol) ethanol, 20% (vol/vol) triethylammonium, 10% (vol/vol) PITC]. After 10 min at room temperature, the sample was lyophilized, reconstituted in 0.1 M NaOH and stored at −20 °C until use.

ISOPROPYLAMINE TO L-ALANINOL 2369

VOL. 68, 2002
RESULTS

Properties of Pseudomonas sp. strain KIE171. Pseudomonas sp. strain KIE171 was isolated from sludge of the Lonza AG wastewater treatment plant by enrichment on minimal medium containing 20 mM isopropylamine as the sole carbon source. Isopropylamine, l-alaninol, l-alanine, d-alanine, l-lactate, proline acid, aminoethanol, propane-1,2-diol, and d-glutamate supported growth, whereas l-alaninol, d,l-alaninol, and acetone did not. Based on its 16S ribosomal DNA (rDNA) sequence and its profile of fatty acids, strain KIE171 was classified within the γ-subdivision of the proteobacteria into rDNA similarity group 1 of Pseudomonas (18). The 16S rDNA sequence of strain KIE171 was 99% identical to that of Pseudomonas citronellolis, whereas its physiological characteristics matched most closely those of Pseudomonas aeruginosa (95.8% 16S rDNA identity). A clear attribution of strain KIE171 to a particular species of RNA group 1 thus was not obtained.

Accumulation of l-alaninol by an isopropylamine-nonutilizing mutant. To establish that l-alaninol is an intermediate in the degradation of isopropylamine, a mutant defective in the utilization of these compounds was generated by mutagenesis with MNNG and isolated as described in Materials and Methods. This mutant, strain KIE171-B, exhibited no growth after 5 days of incubation in minimal medium containing either 20 mM isopropylamine or 20 mM l-alaninol. Whole cells of strain KIE171-B suspended in minimal medium with 20 mM isopropylamine formed 8 mM l-alaninol within 40 h. Based on HPLC analysis, the supernatant of the medium after 40 h of incubation did not contain d-alaninol. Incubation of the cell suspension for more than 40 h resulted in the disappearance of l-alaninol (not shown). This effect is believed to be due to the activity of a putative aldehyde dehydrogenase encoded by ipuH (see below).

Identification of genes involved in isopropylamine utilization. For the identification of the genes encoding the enzymes of the isopropylamine degradation pathway, we isolated mini-Tn5 transposon insertion mutants of Pseudomonas sp. strain KIE171 that are unable to grow with isopropylamine. Two Ipu− (isopropylamine utilization-negative) mutants were obtained that displayed no growth with isopropylamine or l-alaninol as the sole carbon source but displayed normal growth with all other carbon sources tested. Of these mutants, strain KIE171-BI transformed isopropylamine to l-alaninol, whereas strain KIE171-BII failed to do so. The genes whose insertional inactivation caused loss of the ability to grow with isopropylamine were isolated by selection of the kanamycin resistance gene present on the minitransposon (7). The DNA fragments carrying a transposon insertion were sequenced and found to represent two apparently unlinked loci. These were termed cluster A (mutant KIE171-BI) and cluster B (mutant KIE171-BII) and appear to constitute two transcriptional units comprising two and eight genes, respectively. A schematic representation of the 10 open reading frames (ORFs) identified in the DNA sequences of clusters A and B is shown in Fig. 1.

With the exception of that encoded by orf259, the polypeptides encoded in clusters A and B displayed significant sequence identity to proteins of known function (Table 1). Based on the function of their homologs, these proteins can be arranged in a hypothetical pathway for the degradation of isopropylamine that is presented in Fig. 2. In this pathway isopropylamine is thought to be transported into the cytoplasm by IpuG, which shows weak sequence similarity to amino acid permeases of the amino acid-polyamine-organocation (APC) superfamily (13). The degradation of isopropylamine is then initiated by IpuC. This protein exhibits about 30% sequence identity to glutamine synthetases. Preliminary characterization revealed that IpuC catalyzes the ATP-dependent conversion of isopropylamine and l-glutamate to γ-glutamyl-isopropylamide and that ammonium is not a substrate (see below). IpuC thus is a γ-glutamyl-isopropylamide synthetase.

The next step in the hypothetical pathway is catalyzed by a four-component monooxygenase system that is proposed to stereospecifically hydroxylate γ-glutamyl-isopropylamide to γ-glutamyl-l-alaninol. It is thought to be composed of IpuD, which in its sequence is 30% identical to P-450CAM of Pseudomonas putida (23), and of IpuA, IpuB, and IpuE. IpuA exhibits 28% sequence identity to various thioredoxin reductases. Sequence alignments revealed that the two active-site cysteines typical for thioredoxin reductases (29) are absent in IpuA. Since the two flavin adenine dinucleotide binding domains and the NAD(P) binding site are present, it seems to be a NAD(P)-dependent ferredoxin reductase, but not a thioredoxin reductase. IpuB and IpuE show strong sequence identity (48 and 40%, respectively) to ferredoxins. A [4Fe-4S] and a [3Fe-4S]
cluster are present in IpuB, whereas IpuE contains a single [3Fe-4S] cluster. This suggests that IpuB and IpuE function as electron transport components and, together with IpuA and IpuD, constitute a four-component cytochrome P-450-based monooxygenase system responsible for the stereospecific hydroxylation of γ-glutamyl-isopropylamide.

In a further step of the proposed pathway, γ-glutamyl-L-alaninol is hydrolyzed to L-alaninol and L-glutamate. This reaction was shown to be catalyzed by purified IpuC (see below). IpuF is related to the N-terminal part of guanosine 5′-phosphate synthetase. This enzyme catalyzes the final step in guanine ribonucleotide biosynthesis, and its N-terminal domain is responsible for the hydrolysis of glutamine, thus providing ammonium for the ATP-dependent formation of guanosine 5′-phosphate from xanthosine 5′-phosphate by the C-terminal synthetase domain (31).

None of the ORFs encoded in clusters A and B is similar to an enzyme reacting with an amino-alcohol such as L-alaninol. The reactions proposed to be involved in the degradation of this compound (Fig. 2) thus are entirely speculative. L-Alaninol appears to involve two aldehyde dehydrogenases. This view is supported by our observation that, in order to obtain a strain stably accumulating L-alaninol from isopropylamine, it is necessary to inactivate both ipuH and ipuI (de Azevedo Wäsch et al., unpublished).

### Evidence for the formation of γ-glutamyl-isopropylamide by IpuC.

IpuC. In the pathway suggested by analysis of the ipu genes (Fig. 2), IpuC is postulated to catalyze the ATP-dependent formation of γ-glutamyl-isopropylamide from isopropylamine and L-glutamate. To verify this reaction, IpuC was expressed in E. coli as an N-terminally histidine-tagged fusion protein (His₆-IpuC) and purified in one step by metal chelate-affinity chromatography (Fig. 3). A crude E. coli extract containing His₆-IpuC had a specific activity of 0.67 U/mg of protein, while a crude extract from E. coli expressing wild-type IpuC exhibited an activity of 0.75 U/mg of protein (Fig. 4). When purified His₆-IpuC was incubated for 5 h in 1 ml of standard incubation mixture, 6.5 μmol of isopropylamine, 7.1 μmol of L-glutamate, and 4.7 μmol of ATP were consumed, and the products detected were 5.6 μmol of γ-glutamyl-isopropylamide, 4.9 μmol of ADP, and 5.6 μmol of inorganic phosphate. Except for inorganic phosphate, which was determined by a colorimetric assay, substrates and products were measured by HPLC analysis and identified by cochromatography with pure compounds (see Materials and Methods). 4-Aminobutyrate, glutarate, D-glutamate, L-aspartate, and L-2-aminoadipate were not accepted as substrates by the enzyme. However, IpuC showed a broad substrate range with respect to primary amines. Amino-alkanes; amino-alcohols, including L-alaninol and D-alaninol; and amino-esters were substrates (Table 2). Ammonium and primary amines with a positive or a negative charge on the side chain were not.

IpuF is a γ-glutamyl-L-alaninol hydrolase. IpuF catalyzes the third step in the proposed pathway, the hydrolysis of γ-glutamyl-L-alaninol to L-alaninol and L-glutamate (Fig. 2).

### Table 1. Genes in DNA regions associated with isopropylamine utilization

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<th>Gene or ORF</th>
<th>Length (amino acids)</th>
<th>Gene Start (bp)</th>
<th>Gene End (bp)</th>
<th>Inferred function</th>
<th>Sequence comparison of representative hit</th>
<th>% Identity</th>
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<td></td>
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<tr>
<td>ipuI</td>
<td>327</td>
<td>1362</td>
<td>2342</td>
<td>NAD(P)H-dependent reductase</td>
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<td>2342</td>
<td>2680</td>
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<td>FdxA (Streptomyces griseus) (Q01839)</td>
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<td>ipuC</td>
<td>460</td>
<td>2743</td>
<td>4122</td>
<td>γ-Glutamyl-isopropylamide synthetase</td>
<td>GlnA (Thermotoga maritima) (P36205)</td>
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<td>ipuD</td>
<td>387</td>
<td>4194</td>
<td>5354</td>
<td>γ-Glutamyl-isopropylamide monooxygenase</td>
<td>CamA (Pseudomonas putida) (P00183)</td>
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<tr>
<td>ipuE</td>
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<td>5371</td>
<td>5565</td>
<td>Ferredoxin</td>
<td>FdKA (Pyrococcus furiosus) (P36205)</td>
<td>40.3 (64)</td>
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<td>ipuF</td>
<td>296</td>
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<td>6476</td>
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<td>ipuH</td>
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<td>9574</td>
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<td>ALDH2 (Homo sapiens) (P05091)</td>
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<td>43.9 (481)</td>
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* Accession numbers from PIR, SwissProt, and Trembl databases are shown in parentheses.

* The numbers of amino acids considered for comparison are given in parentheses.
FIG. 2. Postulated pathway for the degradation of isopropylamine by *Pseudomonas* sp. strain KIE171. Arrows with broken lines indicate hypothetical reactions, for which no experimental evidence is available. Abbreviations: GIPA, \(\gamma\)-glutamyl-isopropylamide; GALO, \(\gamma\)-glutamyl-L-alaninol.
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**FIG. 3.** Purification of histidine-tagged \(\gamma\)-glutamylamidase synthetase (His\(_6\)-IpuC) from \(E.\ coli\) BL21(DE3)(pME4275). Protein samples (15 \(\mu\)g) were analyzed by SDS-PAGE on 12\% acrylamide gels and stained with Coomassie brilliant blue. Lane M, molecular mass markers; lane 1, crude extract of uninduced \(E.\ coli\) BL21(DE3); lane 2, crude extract of uninduced \(E.\ coli\) BL21(DE3)(pME4275); lane 3, crude extract of induced \(E.\ coli\) BL21(DE3)(pME4275); purified His\(_6\)-IpuC after metal chelate-affinity chromatography.

\(\gamma\)-glutamyl-isopropylamide as crude extract containing His\(_6\)-IpuF, that is, 1.44 and 1.35 U/mg of protein, respectively. Purified His\(_6\)-IpuF was stable for several weeks upon storage at \(-20^\circ\text{C}\) in buffer containing 20\% (vol/vol) glycerol. However, during incubation at 30\(^{\circ}\text{C}\), the activity of the enzyme dropped with a half-life of 19 min. The enzyme showed a Michaelis-

**FIG. 4.** Liberation of inorganic phosphate from ATP under standard conditions for the assay of \(\gamma\)-glutamylamide synthetase activity with isopropylamine as substrate. Each vial contained in a total volume of 400 \(\mu\)l of complete assay mixture 25 \(\mu\)g of protein from one of the following crude extracts: \(E.\ coli\) BL21(DE3)(pME4277) (\(\times\)), \(E.\ coli\) BL21(DE3)(pME4275) (\(\bullet\)), or \(E.\ coli\) BL21(DE3) (\(\Delta\)).

**TABLE 2.** Substrate range of purified histidine-tagged \(\gamma\)-glutamyl-amidase synthetase (His\(_6\)-IpuC)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylamine</td>
<td>90</td>
</tr>
<tr>
<td>Ethylamine</td>
<td>145</td>
</tr>
<tr>
<td>Propylamine</td>
<td>72</td>
</tr>
<tr>
<td>Butylamine</td>
<td>62</td>
</tr>
<tr>
<td>Isoamylamine</td>
<td>100</td>
</tr>
<tr>
<td>Isobutylamine</td>
<td>100</td>
</tr>
<tr>
<td>(t)-Butylamine</td>
<td>74</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>146</td>
</tr>
<tr>
<td>1-Amino-2-propanol</td>
<td>186</td>
</tr>
<tr>
<td>3-Amino-2-propanol</td>
<td>140</td>
</tr>
<tr>
<td>(\alpha)-2-Amino-1-propanol</td>
<td>142</td>
</tr>
<tr>
<td>(\delta)-2-Amino-1-propanol</td>
<td>140</td>
</tr>
<tr>
<td>(\delta)-2-Amino-1-butanol</td>
<td>133</td>
</tr>
<tr>
<td>(R)-2-Amino-1-butanol</td>
<td>132</td>
</tr>
<tr>
<td>Glycine methyl ester</td>
<td>50</td>
</tr>
<tr>
<td>4-Aminobutyric acid methyl ester</td>
<td>42</td>
</tr>
<tr>
<td>2-Amino-1,3-propanediol</td>
<td>79</td>
</tr>
</tbody>
</table>

\(^a\) A relative specific activity of 100\% corresponds to 1.16 U/mg of protein.

Menten-type saturation curve in response to increasing concentrations of \(\gamma\)-glutamyl-isopropylamide, with an estimated \(K_m\) of 65 mM and a \(V_{\text{max}}\) of 13.4 U/mg of protein. For \(\gamma\)-glutamyl-isopropylamide the \(K_m\) was 65 mM, with a \(V_{\text{max}}\) of 1.5 U/mg of protein. IpuF thus catalyzed the hydrolysis of \(\gamma\)-glutamyl-L-alaninol postulated in the pathway (Fig. 2), although with a high \(K_m\) and at a rate 1 order of magnitude below that observed for \(\gamma\)-glutamyl-isopropylamide.

**Regulation of expression of the ipu4BCDEFGH genes.** In order to investigate the regulation of expression of the ipu4ABC DEFGH genes, we constructed strain KIE171-BV, which harbors a chromosomally encoded transcriptional ipuH:xyE fusion, and measured the levels of catechol-2,3-dioxygenase activity. KIE171-BV exhibited an ~22-fold-higher catechol-2,3-dioxygenase specific activity when grown with glutamate plus isopropylamine than when grown with glutamate alone (not shown).

Primer extension analysis confirmed that expression of ipu is regulated at the level of transcription. The transcriptional start of the ipu gene cluster was located 28 bp upstream of the translational start of the ipu4 gene (Fig. 5).

**DISCUSSION**

Analysis of the genes responsible for the utilization of isopropylamine as a carbon source by *Pseudomonas* sp. strain KIE171 leads us to propose a pathway that converts the substrate in three enzyme-catalyzed steps to L-alaninol. This compound is then further degraded by as yet unknown reactions, of which at least one appears to be catalyzed by two aldehyde dehydrogenases, whose genes have been identified (Fig. 2). The pathway from isopropylamine to L-alaninol involves two \(N\)-glutamylated intermediates, and its occurrence was supported by the following lines of physiological and biochemical evidence. Resting cells of a mutant of strain KIE171, unable to grow with L-alaninol, quantitatively accumulated L-alaninol from isopropylamine. The purified enzymes \(\gamma\)-glutamyl-isopropylamide synthetase (IpuC) and \(\gamma\)-glutamyl-L-alaninol hydro-
FIG. 5. Identification of the transcriptional start of the ipuABCDEFGH operon by primer extension analysis. RNA was isolated from glutamate (Glu)-grown cells or from cells grown with glutamate and isopropylamine (Glu + IPA) as carbon sources and reverse transcribed. The sequencing ladder obtained with plasmid pME4771 as a template is shown. The major primer extension product is indicated by an arrow, and the position of the transcriptional start is marked by an asterisk.

The pathway from isopropylamine to L-alaninol consumes 1 mol of ATP per mol of substrate degraded. A more energy-efficient version of the pathway would involve transfer of the glutamyl residue of γ-glutamyl-L-alaninol onto isopropylamine, rather than its hydrolytic removal. Based on amino acid sequence analysis it is unlikely that IpuF is able to catalyze such a glutamyl-transfer reaction. However, it cannot be excluded that a host enzyme encoded outside of the ipu gene clusters acts as glutamyl-transferase and thereby participates in isopropylamine degradation. The IpuF enzyme is about half the size of guanosine 5′-phosphate synthetase of E. coli, and its sequence aligns with the N-terminal part, the amidotransferase domain (31), of the latter. The catalytically important residues in amidotransferases, Cys-86 and His-181 (sequence numbering), of the latter. The catalytically important residues in amidotransferases, Cys-86 and His-181 (sequence numbering), are present in IpuF as Cys-104 and His-200, thus suggesting that the mechanism of hydrolysis of γ-glutamyl-L-alaninol is similar to that for the hydrolysis of glutamine of amidotransferase enzymes (20).

The ipuABCDEFGH gene cluster was found to be flanked by two putative IS elements (Fig. 1), features reminiscent of a catabolic transposon for isopropylamine degradation. ISL, the left-hand element, exhibited sequence similarity to IS 2 (8), but inverted repeats and the start codon of a putative transposase gene could not be identified. The sequence of ISR was similar to that of IS 3 (8). It consisted of a 1,334-bp DNA sequence with similarity to genes encoding transposition proteins. This sequence was interrupted by several frameshifts, and it contained flanking 30-bp imperfect inverted repeats. These features and the absence of target site duplications resulting from transposon insertion make it appear unlikely that the apparent ipu transposon is functional. However, the possibility that it becomes transposable by site-specific recombination with an element containing a functional transposase gene cannot be excluded (28). Support for the view that the ipu genes have been introduced into Pseudomonas sp. strain KIE171 by lateral transfer is provided by their GC content of 51%. This value differs significantly from the overall GC content of members of the genus Pseudomonas, which varies between 58 and 68% (19).

Sequencing of the genes involved in aniline degradation by P. putida (11) and Acinetobacter sp. strain YAA (26) has revealed ORFs that encode, in addition to a putative aniline oxygenase, a protein with homology to glutamine synthetases (TdnQ and AtdA1, respectively) and one with homology to the amidotransferase domain of GMP synthetases (TdnT and AtdA2, respectively). These proteins were proposed to transfer the amino group of the substrate to an unknown acceptor or to release ammonia (11). However, by analogy to the isopropylamine degradation pathway described in the present study, it seems more likely that they are involved in the N-glutamyl-ligation of aniline and in the subsequent hydrolytic removal of glutamate, respectively. The principle of protecting an amino

![Diagram](image-url)
group by a glutamyl residue prior to hydroxylation of a neighboring carbon atom thus appears to be followed in at least one other degradative pathway for a primary amine.

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REFERENCES


