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Isopropylamine degradation in strain *Pseudomonas* sp. KIE171.

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Table of Contents

Abbreviations of chemicals

Am	Ampicillin
Cm	Chloramphenicol
GALO	γ-(L-glutamyl)- L-1-hydroxy-isopropylamide
GIPA	γ-(L-glutamyl)-isopropylamide
Gm	Gentamycin
IPA	isopropylamine
IPTG	isopropyl thio-β-D-1-galactoside
Kan	Kanamycin
L-Alaninol	S(+)-2-amino-1-propanol
MNNG	*N*-methyl-*N'*-nitro-N-nitrososguanidine
Tc	Tetracycline
Theanine	γ-(L-glutamyl)-ethylamide

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1. Summary

Pseudomonas sp. KIE171, an organism enriched and isolated from the Lonza wastewater treatment plant, was able to grow with isopropylamine or with L-alaninol as the sole carbon source. To investigate the hypothesis that L-alaninol is an intermediate in the degradation pathway of isopropylamine, the degradation of isopropylamine was mutationally blocked and the mutants obtained were examined for L-alaninol formation from isopropylamine. Two mutants (KIE171-B and KIE171-BI), unable to utilize both L-alaninol and isopropylamine were isolated. They transformed isopropylamine into L-alaninol with an enantiomeric excess of more than 99%. However, both strains were unsuitable for the production of L-alaninol, since L-alaninol accumulated but transiently in the medium and was further metabolized.

Transposon mutagenesis was used to create mutants that could not grow on isopropylamine. Genes containing the transposon insertion were cloned and the DNA regions flanking the insertions were sequenced. Two clusters, one comprising eight *ipu* (isopropylamine utilization) genes (*ipuABCDEFGH*), the other encompassing two *ipu* genes (*ipuIJ*), were identified. Sequence comparisons of the deduced Ipu proteins to protein sequences stored in the database suggested that isopropylamine degradation is initiated by a putative permease, IpuG, which transports the compound into the cytoplasm. The next step, the formation of γ-glutamylisopropylamide from isopropylamine, ATP and L-glutamate, is catalyzed by IpuC, a γ-glutamylamide synthetase. This compound is then subjected to stereospecific monooxygenation by a hypothetical three-component system IpuABD, thereby yielding γ-glutamyl-L-alaninol. Enzymatic hydrolysis by a hydrolase, IpuF, finally liberates L-alaninol and regenerates L-glutamate. No gene(s) encoding an enzyme for the next step in the breakdown of isopropylamine was found in the *ipu*-clusters. Presumably L-alaninol is oxidized by an alcohol dehydrogenase to yield L-2-aminopropionaldehyde, or it is deaminated by an ammonia lyase to yield propionaldehyde. The aldehyde formed is probably further oxidized by the hypothetical aldehyde

dehydrogenases IpuI and IpuH to either L-alanine or propionic acid, compounds which can be processed by reactions of the intermediary metabolism.
Genes *ipuC* and *ipuF* were overexpressed in *Escherichia coli*. Their products were purified and partially characterized. IpuC was found to be a γ-glutamylamide synthetase with a broad substrate range, possibly of interest for the production of γ-glutamylethylamide (theanine) and other γ-glutamylamide compounds. Purified IpuF catalyzed the hydrolysis of various γ-glutamylamides.

To obtain strains that stably accumulate L-alaninol from isopropylamine, two approaches were followed. In a first approach, the effect of inactivating both genes, *ipuI* and *ipuH*, on the accumulation of L-alaninol was examined by construction of strain KIE171-BIII. L-Alaninol produced by biotransformation with this strain remained stable in growing cell cultures and in cell suspensions. The maximum concentration obtained was 1.2 g L-alaninol per liter. In the second approach, the genes required for L-alaninol formation from isopropylamine were expressed in *Escherichia coli*, a bacterium unable to grow with L-alaninol. The recombinant plasmid pME4755, which carries the genes *ipuABCDEFG*, conferred on *Escherichia coli* the ability to transform isopropylamine to L-alaninol without further degradation of the product. Results of the present study provide a starting point for the development of an industrial process for the production of L-alaninol by biotransformation of the cheap prochiral compound isopropylamine.

Zusammenfassung

Pseudomonas sp. KIE171 wurde mit Hilfe von Anreicherungskulturen aus industriellem Klärschlamm isoliert und war fähig, sowohl auf Isopropylamin als auch auf L-Alaninol als einziger Kohlenstoffquelle zu wachsen. Um die Hypothese zu untersuchen, L-Alaninol sei ein Metabolit im Isopropylamin-Abbau, wurden die enzymatischen Schritte im Isopropylamin-Metabolismus durch Mutationen unterbrochen. Die erhaltenen Mutanten wurden auf die Synthese von L-Alaninol aus Isopropylamin hin untersucht. Die Mutanten KIE171-B und KIE171-BI waren nicht mehr in der Lage, Isopropylamin sowie L-Alaninol als Kohlenstoffquelle zu verwenden, konnten aber Isopropylamin mit einem Enantiomer-Überschuss von mehr als 99% zu L-Alaninol umsetzen. Beide Stämme waren zur Produktion von L-Alaninol ungeeignet, da sie zwischenzeitlich akkumuliertes L-Alaninol wieder abbauten.

Um die Gene des Isopropylamin-Abbauwegs zu isolieren, wurde Transposon Mutagenese eingesetzt. Gene mit Transposon-Insertionen, die zum Verlust der Fähigkeit zu Wachstum auf Isopropylamin geführt hatten, wurden isoliert, und die DNA Regionen im Bereich der Insertionen wurden sequenziert. Wir fanden zwei Gruppen von *ipu* (isopropylamine utilization) Genen. Eine Gruppe umfasst acht Gene (*ipuABCDEFGH*) und die andere zwei Gene (*ipuIJ*). Die Analyse der Translationsprodukte der *ipu* Gene liess folgenden Abbauweg für Isopropylamin vermuten: Isopropylamin wird mittels der hypothetischen Permease, IpuG durch die cytoplasmatische Membran in die Zelle transportiert. In der Zelle katalysiert die γ-Glutamylisopropylamid-Synthetase IpuC die Synthese von γ-Glutamylisopropylamid aus Isopropylamin, ATP und L-Glutamat. Diese Verbindung wird nachfolgend stereospezifisch durch den hypothetischen 3-Komponenten-Monooxygenase-Komplex IpuABD hydroxyliert, wobei das Produkt γ-Glutamyl-L-Alaninol entsteht. Dieses wird durch die Hydrolase IpuF hydrolytisch gespalten, wodurch L-Alaninol und L-Glutamat freigesetzt werden. Unter den *ipu* Genen konnten keine Gene für den Abbau von L-Alaninol gefunden werden. Es wird angenommen, dass L-Alaninol entweder von einer Alkohol-Dehydrogenase zu L-2-Aminopropionaldehyd oxidiert oder von einer

Ammonium-Lyase zu Propionaldehyd umgesetzt wird. Das dabei gebildete Aldehyd könnte dann durch die hypothetischen Aldehyd-Dehydrogenasen IpuI und IpuH zu den entsprechenden Produkten L-Alanin oder Propionsäure oxidiert werden - Produkte, die im Intermediärmetabolismus verwertet werden können. Die Gene *ipuC* und *ipuF* wurden in *Escherichia coli* überexprimiert, die Proteine wurden gereinigt, und es wurde eine erste Charakterisierung unternommen. IpuC ist eine γ-Glutamylamid-Synthetase mit einem breiten Substratspektrum, mit der eine Vielzahl von γ-Glutamylamiden hergestellt werden kann, zum Beispiel auch γ-Glutamylethylamid (Theanin). IpuF katalysiert die Hydrolyse verschiedener γ-Glutamylamid-Verbindungen in L-Glutamat und das entsprechende Amin.

Die Herstellung eines Stammes, der L-Alaninol produziert, es aber nicht abbaut, wenn es sich im Biotransformations-Medium angereichert hat, wurde auf zwei Wegen realisiert.

Einerseits wurde die Mutante KIE171-BIII hergestellt, in der die beiden Gene *ipuI* und *ipuH* inaktiviert sind. L-Alaninol, das durch Biotransformationen mit diesem Stamm produziert wurde, erwies sich in wachsenden Zellkulturen und in Zellsuspensionen als stabil. Die maximale Ausbeute betrug 1.2 g L-Alaninol pro Liter. Andererseits wurden die Gene, welche für die Synthese von L-Alaninol aus Isopropylamin notwendig sind, in *Escherichia coli* exprimiert, einem Organismus, der nicht auf L-Alaninol wachsen kann. Dazu wurde das rekombinante Plasmid pME4755 konstruiert, das die Gene *ipuABCDEFG* trägt. Man konnte damit bestätigen, dass es möglich ist, in *Escherichia coli* L-Alaninol aus Isopropylamin herzustellen. Diese Ergebnisse eröffnen einen Weg zur Gewinnung von L-Alaninol mittels eines biokatalytischen Prozess.

2. Introduction

Microorganisms and enzymes are used as biocatalysts in the manufacture of a wide variety of substrates of industrial and pharmaceutical interest (Schmid et al., 2001). Particularly interesting are regioselective and stereospecific biocatalytic reactions like for example the stereospecific hydroxylation of inactive centers in hydrocarbons. The present study reports on a stereospecific biocatalytic process for the synthesis of S(+)-2-amino-1-propanol (L-alaninol). L-Alaninol will be shown to be an intermediate in the degradation pathway of isopropylamine (IPA). IPA is used as solvent in organic chemistry and it occurs as an intermediate in the biodegradation of herbicides like *s*-triazine (Topp et al., 2000; Karns, 1999; Sadowsky et al., 1998; de Souza et al., 1998) or propachlor (Martin et al., 1999).

2.1 L-Alaninol.

Biocatalytic synthesis of L-alaninol.

Dr. A. Kiener at Lonza AG (Visp, CH) proposed to study the possibility of using a biocatalytic process to produce the chiral compound L-alaninol. He suggested to synthesize L-alaninol from the cheap prochiral compound IPA by stereospecific hydroxylation (Figure 1). Accordingly, it was the aim of the present project to develop a cost-effective procedure for obtaining L-alaninol in high enantiomeric excess from IPA.

As a first step the microbial degradation of IPA was investigated. Organisms were sought, which were able to grow with IPA and with L-alaninol but not with its stereoisomer R(-)-2-amino-1-propanol (D-alaninol). An organism, *Pseudomonas* sp. KIE171, with these features was enriched from sludge of the Lonza wastewater treatment plant on a medium containing IPA as the sole carbon and nitrogen source (A. Kiener, unpublished). Strain KIE171 utilized IPA and L-alaninol but not D-alaninol as a carbon and nitrogen source. Furthermore,

its growth with L-alaninol was inhibited by 10 mM D-alaninol (de Azevedo Wäsch, 1996). These preliminary results suggested that in strain KIE171 IPA was metabolized via L-alaninol. With a view to establish a procedure for the biocatalytic synthesis of L-alaninol, the degradation pathway of IPA was investigated in this thesis.

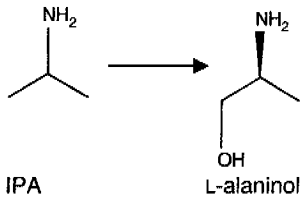


Fig. 1. Hypothetical stereospecific microbial hydroxylation of IPA into L-alaninol.

Chemical synthesis of L-alaninol.

Commercially available L-alaninol is at present synthesized chemically in a costly procedure involving chemical blocking and deblocking steps. L-alaninol can be synthesized by borane reduction of L-alanine after protection of the amino group (Stanfield et al., 1981; Kelly et al., 1986) (Figure 2). This process involves three reaction steps and starts from a chiral compound.

First, the amino group of L-alanine has to be protected with *t*-butoxycarbonyl azide, $(CH_3)_3COC(O)N_3$, in order to form the *t*-butoxycarbonyl (Boc) derivative. In the second step the carbonyl group of the protected L-alanine is reduced with borane-tetrahydrofurane (BH_3•THF) in THF at 0°C in an N_2-atmosphere. Other reduction procedures, like the reduction with $LiAlH_4$, are not suitable for protected amino acids because the Boc group will consume primarily the hydride. The third step, the deblocking step, is performed by standard procedures such as catalytic hydrogenolysis or with a mixture of hydrochloride and acetic acid.

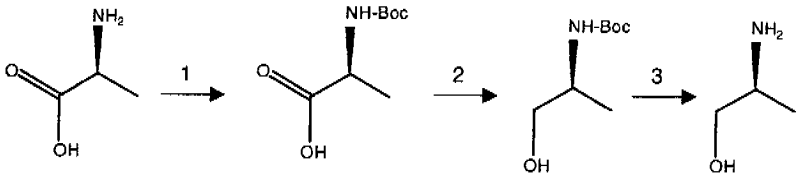


Fig. 2. Chemical synthesis of L-alaninol. 1; Protection of the amino group of L-alanine with Boc, 2; reduction with BH_3•THF, 3; deblocking step yielding L-alaninol.

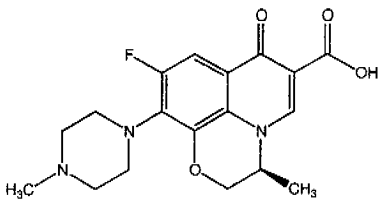
Uses of L-alaninol.

L-Alaninol is used as a building block for the synthesis of the antibacterial agent (-)-ofloxacin (levofloxacin) (Figure 3; Kang et al., 1997). It has been shown that (-)-ofloxacin is 8-128 times more active *in vitro* than (+)-ofloxacin and twice as active as the racemate (Mitscher et al., 1987). For economic reasons ofloxacin is administrated as a racemate.

The use of L-alaninol as a building block for the synthesis of beta-blocking agents is also conceivable, since beta-blockers have an amino-alcohol structure with at least one chiral center in the side chain (Egginger et al., 1993).

The L-2-propanolamide-moiety can be found in nature as a substituent of the alkaloid derivative lysergic acid, thereby forming the compound D-lysergic-acid-L-2-propanolamide (Beyer & Walter, 1988). This compound is better known by its trivial names Ergometrin and Ergobasin (Figure 3). It is produced by the ascomycete *Claviceps purpurea*, which grows on cereals and grasses. In the middle ages it was the cause of the widespread epidemically occurring disease 'Holy fire', where patients suffered from gangrene, cramps, hallucinations, up to loss of their limbs (Esser & Düvell, 1984). Because of its impact on the smooth muscles of the uterus, D-lysergic-acid-L-2-propanolamide is used in obstetrics to control postpartum hemorrhage.

A



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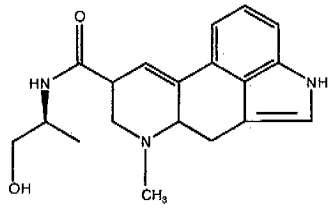


Fig. 3. (A) Levofloxacin and (B) D-Lysergic-acid-L-2-propanolamide.

2.2 Degradation of isopropylamine.

Conceivable pathways for the degradation of IPA.

At the beginning of this work, different degradation pathways for IPA were conceivable. They are based on reports in the literature on the degradation of IPA, acetone, L-alaninol and methylamine. In the following, five hypothetical pathways for the degradation of IPA are presented. In three of these pathways IPA degradation is initiated by oxidation or deamination of the compound. In two pathways IPA first combines with L-glutamate to produce N-isopropyl-derivates, which are further metabolized.

Hypothetical pathways initiated by oxidation or deamination of IPA.

The first proposed pathway for IPA degradation is based on a combination of the knowledge obtained from investigations of organisms growing on IPA

(Cerniglia & Perry, 1975) and acetone (Taylor et al., 1980). Cerniglia and Perry investigated *Mycobacterium convolutum* strain NPA-1, which grew with IPA as sole source of carbon and nitrogen. The presence of isocitrate lyase activity in *Mycobacterium convolutum* strain NPA-1 after growth on IPA suggested that IPA was metabolized through a 2-carbon intermediate. Furthermore an inducible amine dehydrogenase was present in cell extracts after growth on IPA. Amine dehydrogenase activity with IPA could be measured in the supernatant fraction of crude extracts after high-speed centrifugation in the presence of the electron acceptor phenazine methyl sulfate. Therefore, IPA degradation was proposed to occur via deamination followed by cleavage into a C_2 and a C_1 moiety.

Taylor et al. (1980) identified four Gram-positive organisms, A1, A2, SA1 and SP1, which showed characteristics of *Corynebacterium* species. These strains were able to grow on isopropanol, acetone, acetol and methylglyoxal as sole carbon source. It was demonstrated that cell extracts of strain A1 contained NAD^+-dependent isopropanol dehydrogenase, acetol dehydrogenase and methylglyoxal dehydrogenase activities. Acetone monooxygenase activity could not be measured, but the presence of [^{14}C]pyruvate and [^{14}C]acetol after the growth of strain A1 on [2-^{14}C]acetone provided evidence for the conversion of acetone in acetol. Degradation of isopropanol was therefore proposed to occur via the pathway: isopropanol → acetone → acetol → methylglyoxal → pyruvate. By combining the findings of Cerniglia and Perry (1975) with those of Taylor et al., (1980), a possible degradation pathway for IPA can be proposed. The first step would be performed by an isopropylamine dehydrogenase. Hydroxylation of acetone by an acetone monooxygenase would then yield acetol. In a further step acetol would be oxidized by an acetol dehydrogenase to methylglyoxal. The last step would be catalyzed by a methylglyoxal dehydrogenase yielding pyruvate (Figure 4, pathway 1).

A second possible IPA degradation pathway is postulated to be initiated by hydroxylation of IPA to L-alaninol (Figure 4, reaction 2,3a). This reaction could be performed by a monooxygenase or in a two-step mechanism, comprising dehydrogenation to an α,β–unsaturated compound and subsequent hydration,

as identified in the asymmetric hydroxylation of iso-butyric acid by *Candida rugosa* (Ohashi & Hasegawa, 1992) (see 2.3).

The next step could be performed by an alaninol ammonia lyase, similar to ethanolamine ammonia lyase (Figure 4, reaction 2b). Both L-alaninol and D-alaninol are substrates for ethanolamine ammonia lyase and yield propionaldehyde and ammonia as products (Graves et al., 1980). This enzyme reaction utilizes a mechanism of propagation of a radical center between its cofactor adenosylcobalamin, its substrate and the product (LoBrutto et al., 2001). Oxidation of propionaldehyde by a hypothetical propionaldehyde dehydrogenase is postulated to yield propionic acid, which would subsequently be hydroxylated by a hypothetical propionic acid monooxygenase (Figure 4, reaction 2c and 2d). Lactate would then be oxidized by lactate dehydrogenase to pyruvate.

As a third possibility, alaninol formed from IPA could be oxidized by a hypothetical alaninol dehydrogenase to yield 2-aminopropionaldehyde, which could be further oxidized by a hypothetical aldehyde dehydrogenase to alanine. Pyruvate could then be formed from alanine by alanine dehydrogenase (Figure 4, pathway 3).

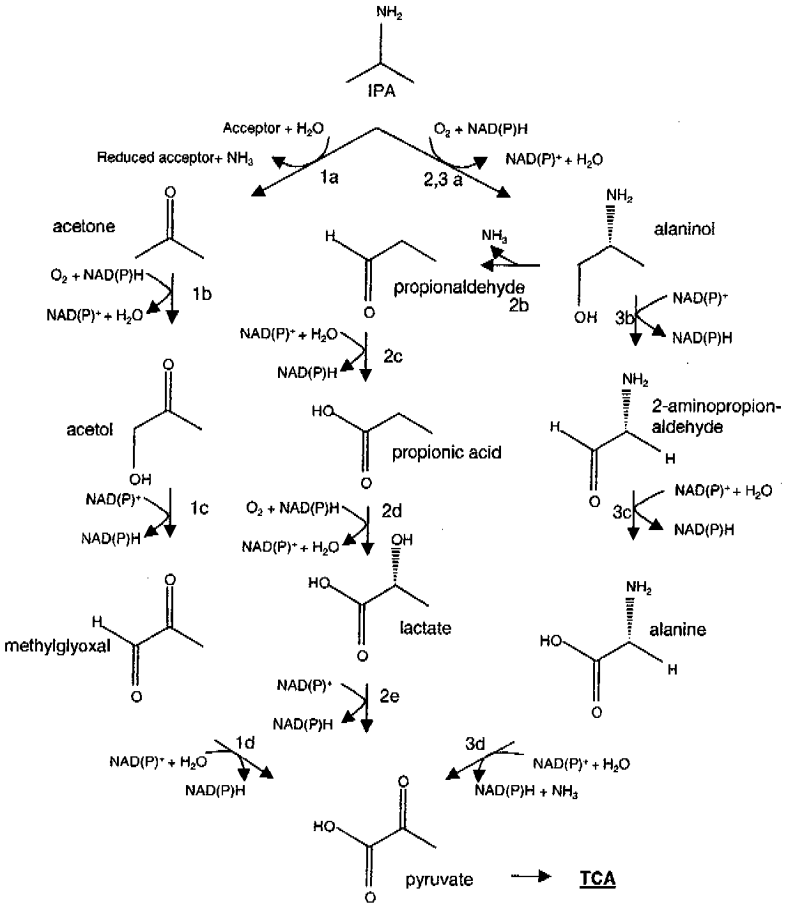


Fig. 4. Hypothetical pathways initiated by oxidation or deamination of IPA. (1a), *IPA dehydrogenase*; (1b), *acetone monooxygenase*; (1c), acetol dehydrogenase; (1d), methylglyoxal dehydrogenase (EC 1.2.1.23/49); (2a), *IPA monooxygenase*; (2b), *alaninol ammonia-lyase* or ethanol ammonia-lyase (EC 4.3.1.7); (2c), *propionaldehyde dehydrogenase*; (2d), *propionic acid monooxygenase*; (2e), lactate dehydrogenase (EC 1.1.1.27); (3a), *IPA monooxygenase*; (3b), *alaninol dehydrogenase*; (3c), *2-aminopropyl-aldehyde dehydrogenase*; (3d), alanine dehydrogenase (EC 1.4.1.1). Enzyme activities written in italics are hypothetical. Enzymes that have been characterized in crude extracts or have been purified are in regular font.

Hypothetical pathways involving reactions of IPA with glutamate.

Methylamine, a compound related to IPA, is utilized by various methylotrophic bacteria as the sole source of carbon and nitrogen. Four different pathways for methylamine oxidation have been described in these organisms. Since mechanisms similar to those involved in methylamine oxidation might be operative in the degradation of IPA, the background of methylamine degradation by methylotrophs is summarized in the following.

Two methylamine utilization pathways are provided by enzymes that catalyze the direct oxidation of methylamine to formaldehyde (Figure 5). Members of the genus *Arthrobacter* contain an amine oxidase with covalently-bound topaquinone (TPQ), which converts methylamine and molecular oxygen to formaldehyde, ammonia and hydrogen peroxide (Figure 5, pathway 4; Anthony, 1996; Levering et al., 1981). Alternatively, many methylotrophic bacteria use a tryptophan tryptophyl-quinone (TTQ)-containing methylamine dehydrogenase, which catalyzes the formation of ammonia and formaldehyde from methylamine (Figure 5, pathway 3, Anthony, 1996; Large, 1990, 1983; Eady & Large, 1971, 1968).

The other two pathways employ enzymes that catalyze the indirect oxidation of methylamine to formaldehyde involving L-glutamate in the reaction. One pathway uses *N*-methyl-L-glutamate synthase, an inducible enzyme, found in several species of *Pseudomonas* grown on methylamine (Hersh, 1985; Pollock & Hersh, 1973, 1971; Shaw et al., 1966). The enzyme functions in a cycle in which free methylamine first reacts with L-glutamate to yield *N*-methyl-L-glutamate and ammonia. The *N*-methyl-L-glutamate is then oxidized by *N*-methyl-L-glutamate dehydrogenase to release formaldehyde for growth of the microorganism (Figure 5, pathway 2, Hersh et al., 1971).

Pathway 1 (Figure 5) involves γ-glutamylmethylamide synthetase, an enzyme which like glutamine synthetases requires ATP and Mg^{++} (Levitch, 1977a; Levitch, 1977b; Kung & Wagner, 1969). For a long time no clear evidence was found for the metabolism of this compound and it was suggested that γ-glutamylmethylamide serves as temporary pool for methylamine under conditions of low oxygen (Jones & Bellion, 1991). Only recently the pathway of

methylamine metabolism via γ-glutamylmethylamide was elucidated in *Methylophaga* sp. AA-30 (Kimura et al., 1995, 1992, 1990). A γ-glutamylamide synthetase as well as a γ-glutamylmethylamide-dissimilating enzyme-system from *Methylophaga* sp. AA-30 were purified and characterized. It was demonstrated that γ-glutamylmethylamide was oxidized to α-ketoglutarate, ammonia and formaldehyde by this enzyme system, which required $NAD(P)^+$.

By analogy to the indirect pathways for the oxidation of methylamine, oxidation of IPA could involve the formation of γ-glutamylisopropylamide (GIPA) or/and *N*-isopropylglutamate (N-GIPA) (Figure 6). In these pathways glutamate would function as a protecting group for the amino group of IPA. It is conceivable that the isopropyl-moiety of these intermediates is subsequently hydroxylated by a monooxygenase with formation of the corresponding alcohol. Degradation would then continue by the action of an alcohol dehydrogenase, followed by an aldehyde dehydrogenase yielding the acid. At that point hydrolysis of the γ-glutamyl-moiety could occur, yielding glutamate and alanine (Figure 6, pathway 4e) or a dehydrogenase could release glutamate and pyruvate (Figure 6, pathway 5e).

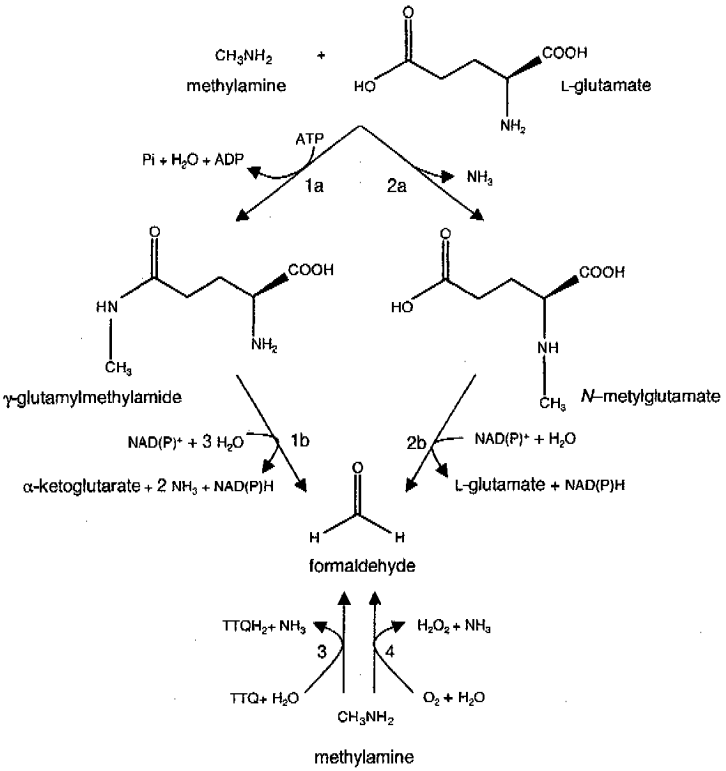


Fig. 5. Enzymatic pathways of methylamine oxidation. The enzymes are: (1a), γ-glutamylmethylamide synthetase (EC 6.3.4.12); (1b), γ-glutamylmethylamide dissimilating enzyme system; (2a), *N*-methylglutamate synthase (EC 2.1.1.21); (2b), *N*-methylglutamate dehydrogenase (EC 1.5.99.5); (3), methylamine dehydrogenase (EC 1.4.99.3); (4) methylamine oxidase (EC 1.4.3.6).

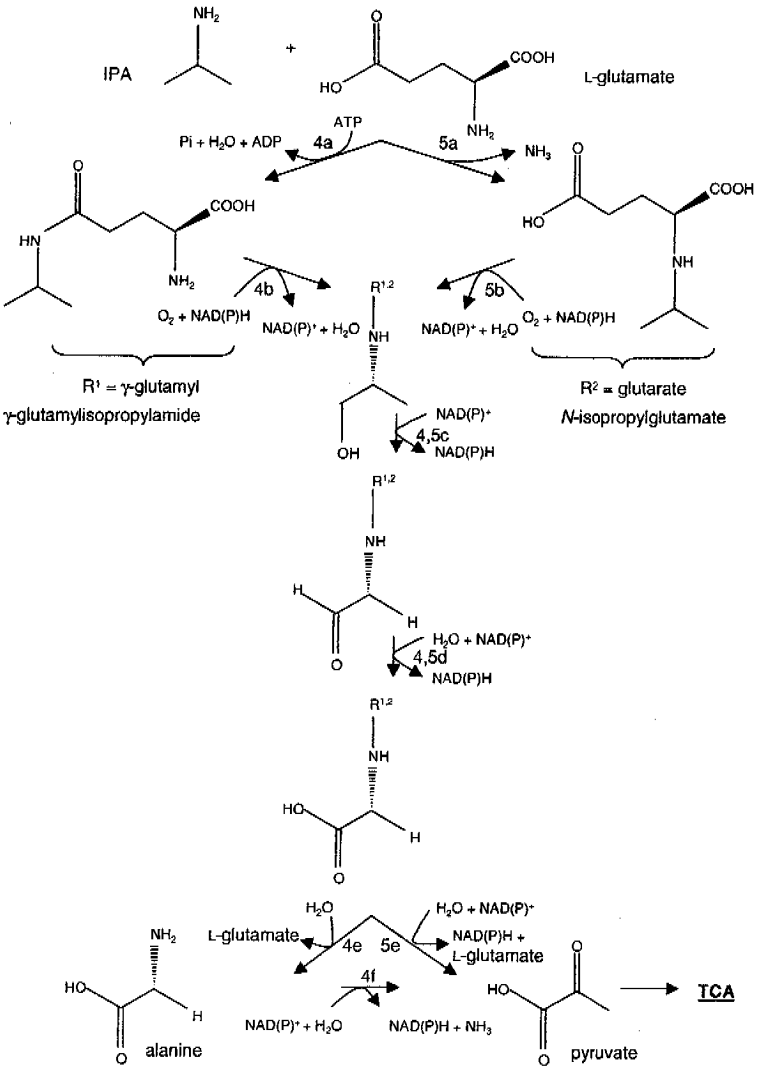


Fig. 6. Hypothetical pathways involving reactions of IPA with L-glutamate. The postulated enzymes are written in italic. The enzymes are: (4a), *γ-glutamylisopropylamide synthetase*; (4b), *γ-glutamylisopropylamide monooxygenase*; (4,5c), *alcohol dehydrogenase*; (4,5d), *aldehyde dehydrogenase*; (4e), *hydrolase*; (4f), alanine dehydrogenase; (5a), *N-isopropylglutamate synthase*; (5b), *N-isopropylglutamate monooxygenase*; (5e), *dehydrogenase*.

2.3 Two hydroxylation processes catalyzed by bacteria.

In the context of the present work, the stereospecific hydroxylation of IPA to L-alaninol is the decisive step of IPA degradation. This step could conceivably involve a dehydrogenation followed by subsequent hydration or it could be catalyzed by a monooxygenase. In this chapter, applications correlated to hydroxylation by these two enzymatic processes are presented, including a short overview of cytochrome P-450$_{CAM}$ and cytochrome P-450$_{BM3}$.

Hydration of unsaturated carbon bonds.

Due to the structural similarity of IPA and iso-butyric acid the hydroxylation of IPA into L-alaninol might proceed similarly to the asymmetric hydroxylation of the prochiral compound iso-butyric acid. Such a hydroxylation is catalyzed by *Candida rugosa* which provides an efficient process for the preparation of both isomers of β-hydroxy-iso-butyric acid (Figure 7). This process proceeds in two steps: an initial dehydrogenation to the α,β-unsaturated carboxylic acid is followed by subsequent hydration. The enzymes utilized for these reactions are involved in the β-oxidation pathway of lipid catabolism, or they are implicated in the catabolism of valine. Therefore, the β-hydroxy acids produced have generally the L(+)-form. For example, optically active L(+)-β-hydroxy-iso-butyric acid has been used as a starting material for the synthesis of the vitamin α-tocopherol, the flavour muscone and the antibiotic calcimycin (Ohashi & Hasegawa, 1992).

Hydroxylation by cytochrome P-450.

In bacteria enzyme-catalyzed mono-hydroxylation reactions are often carried out by cytochrome P-450 monooxygenase systems. Most P-450 monooxygenase systems have three components: the FAD-containing

flavoprotein (NAD(P)H-dependent reductase), an iron-sulfur protein and the terminal oxidase cytochrome P-450 (Harayama et al., 1992). Two classes of cytochromes P-450 are defined: the bacterial/mitochondrial (type I) and the microsomal (type II), whereof the type I cytochromes P-450 are soluble and the type II are, with the exception of cytochrome $P\text{-}450_{BM3}$, membranous. All known cytochromes P-450 show a conserved cysteine residue that provides for anchoring the haem, which is responsible for the activation of molecular oxygen, into the active site (Munro & Lindsay, 1996; Degtyarenko, 1995). The catalytic cycle of cytochrome P-450 performing the insertion of activated oxygen into an unactivated aliphatic carbon-hydrogen bond has recently been elucidated (Wong, 1998; Montellano, 2000). Cytochromes P-450 are often involved in the oxygenation of xenobiotics and their presence in some pathogenic bacteria might be correlated to drug resistance (e.g. *Mycobacterium tuberculosis*; Nelson, 1999). Cytochrome $P\text{-}450_{CAM}$ of *Pseudomonas putida* and cytochrome $P\text{-}450_{BM3}$ of *Bacillus megaterium* are two examples that show how research on bacterial cytochromes P-450 can be applied to improve regio- and/or stereoselectivity as well as engineer the substrate range of these enzymes.

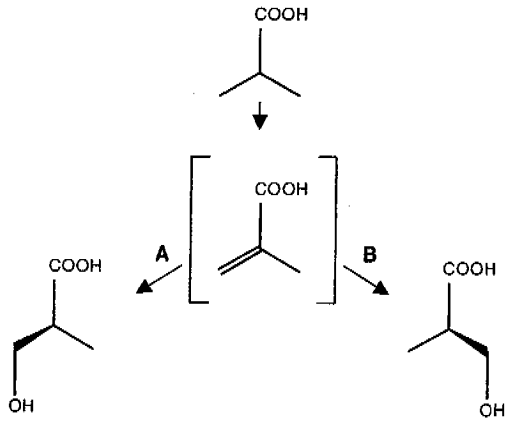


Fig. 7. Asymmetric microbial hydroxylation of iso-butyric acid. A; *Candida rugosa* IFO 0750 and B; *Candida rugosa* IFO 1542.

Cytochrome P-450$_{CAM}$.

Pseudomonas putida cytochrome P-450$_{CAM}$ catalyzes the 5-exo hydroxylation of the monoterpene D-camphor (Figure 8A), and it is the best characterized cytochrome P-450. The cytochrome P-450$_{CAM}$ system has three components: NADH-putidaredoxin reductase, putidaredoxin and cytochrome P-450$_{CAM}$. In this system, NADH reduces FAD to $FADH_2$ of putidaredoxin reductase, and $FADH_2$ subsequently reduces the [2Fe-2S] center of the ferredoxin putidaredoxin. Putidaredoxin shuttles one electron to cytochrome P-450$_{CAM}$ to reduce the heme iron from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state. Molecular oxygen binds to the heme iron and the transfer of a second electron from putidaredoxin results in oxygen-oxygen bond cleavage and substrate hydroxylation. The three dimensional structure of cytochrome P-450$_{CAM}$ has been determined and it was possible to study the shape of the active site (Poulos et al., 1987).

Cytochrome P-450$_{CAM}$ converts D-camphor into 5-exo-hydroxy camphor with remarkable regio- and stereoselectivity: only one of ten possible hydroxylation products is formed. This selectivity is due to the multiple van–der–Waals interactions between enzyme and substrate and one single hydrogen bond between the camphor carbonyl group and tyrosine-96 (Atkins & Sligar, 1988, 1990). The specific shape of the active site determines the very narrow substrate specificity of cytochrome P-450$_{CAM}$. Therefore applications in the context of biocatalysis, like the conversion of 2,2-dimetylcyclohexanone to the (*S*)-alcohol (Figure 8B), are rather rare for cytochrome P-450$_{CAM}$ (Holland, 2000).

In order to broaden the substrate specificity while retaining the regioselectivity of the hydroxylation, the exchange of single amino acids involved in substrate binding in the active site of the protein, was investigated. One of the main targets therefore was tyrosine-96. Removal of tyrosine-96 resulted in an enlarged substrate pocket and a change in substrate-enzyme interactions. By this way the substrate range for hydroxylation of functionalized cyclohexane, piperidine and piperidine derivates has been dramatically expanded (Figure 8C; Flitsch et al., 1999). The aromatic side chain (Ph), that functionalizes the

substrate, is necessary for good binding to the enzyme and it can be cleaved off after hydroxylation.

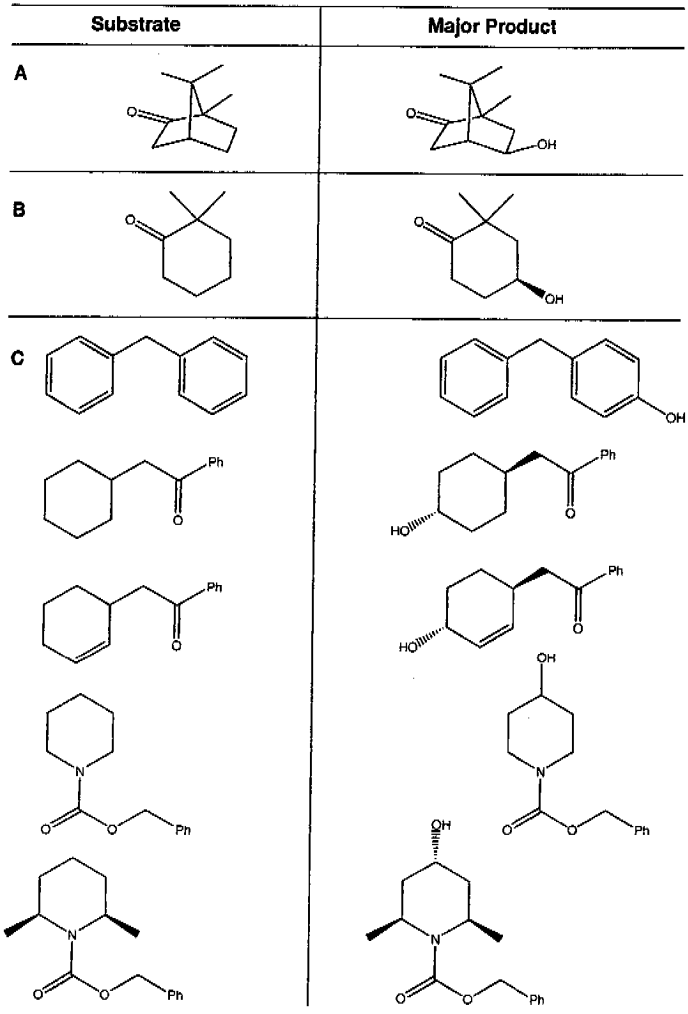


Figure 8. Hydroxylation products isolated from the incubation of substrates using (A, B) wild-type cytochrome P-450$_{CAM}$ or (C) a Y96A mutant of cytochrome P-450$_{CAM}$ from *Pseudomonas putida*. Ph denotes an aromatic side chain.

Cofactor regeneration is essential for the functionality of cytochrome P-450 systems and of first importance for practical applications. However, new tools are being investigated and mutants of cytochrome $P\text{-}450_{CAM}$ were generated by directed evolution (Zhao et al., 1998), able to hydroxylate naphthalene in the absence of cofactors thereby using hydrogen peroxide as the oxidant instead of molecular oxygen and NADH. Furthermore, these mutants showed an activity 20-fold higher than that of the native enzyme (Joo et al., 1999).

Cytochrome $P\text{-}450_{BM3}$.

Cytochrome $P\text{-}450_{BM3}$ of *Bacillus megaterium* catalyzes the hydroxylation of fatty acids such as myristic and palmitic acids at the ω-1,ω-2, and ω-3 positions; it is the only known soluble type II cytochrome P-450. Three dimensional structure determination revealed that a single polypeptide chain encompasses two functional domains: the flavoprotein NADPH reductase domain and the heme-containing cytochrome P-450 domain (Ravichandran et al., 1993). Cytochrome $P\text{-}450_{BM3}$ requires only NADPH and molecular oxygen for activity and is not dependent on additional proteins. The naturally chimeric cytochrome $P\text{-}450_{BM3}$ is considered the evolutionary most advanced P-450 monooxygenase system (Degtyarenko, 1995; Narhi & Fulco, 1986). It is used to study the soluble nature of the bacterial enzyme and its wide substrate specificity, which is usually only found in microsomal cytochromes P-450.

Synthetically useful, diastereoisomerically-defined vicinal diol derivatives of fatty acids were obtained by hydroxylation of hydroxy myristic acids by cytochrome $P\text{-}450_{BM3}$ (Figure 9A; Ahmed et al., 1999). Since several studies are being performed on this enzyme in order to understand the relevant features responsible for efficient and stereoselective hydroxylation, knowledge on the binding of the substrate as well as the shape of the active site increases (Ost et al., 2000; Truan et al., 1999). Mutagenesis of cytochrome $P\text{-}450_{BM3}$ enabled to change the regiospecificity of the enzyme, leading to a mutant that preferentially hydroxylates at the chemically less favorable ω position of laurate and myristate (Oliver et al., 1997). Control of dissolved oxygen made it possible to manipulate

the regioselectivity of the hydroxylation of the unsaturated long chain fatty acid *cis*-pentadecen-10-oic acid. Under oxygen limiting conditions it was possible to obtain hydroxylation at positions ω-1,ω-2, and ω-3, whereas various hydroxylated products were formed when dissolved oxygen was present in excess (Figure 9B; Schneider et al., 1999).

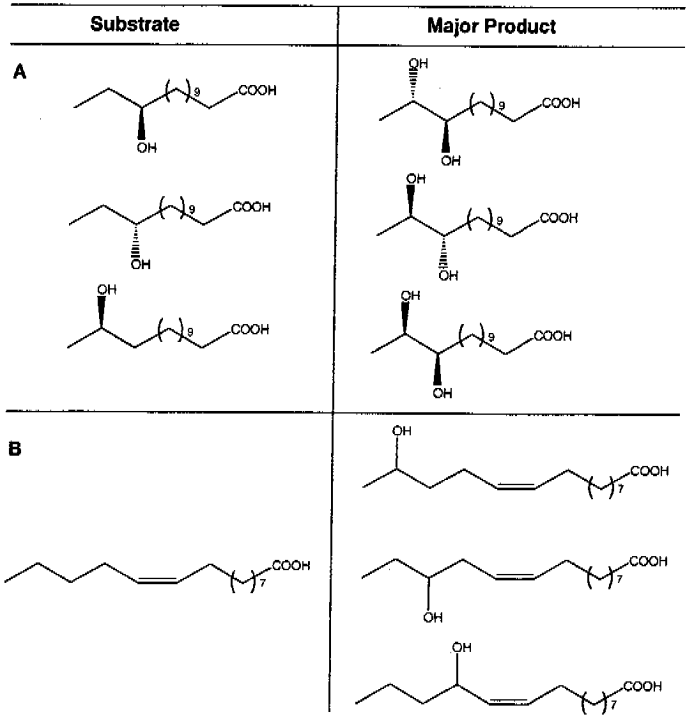


Figure 9. Hydroxylation products isolated from the incubation of (A) hydroxy-myristic acids and (B) *cis*-pentadecen-10-oic acid with cytochrome P-450$_{BM3}$ from *Bacillus megaterium*.

Several techniques are being applied to investigate interactions between the substrate and the binding site for subsequently designing mutant-enzymes with novel features. For both, cytochromes P-450$_{BM3}$ and P-450$_{CAM}$, molecular dynamics simulation revealed new insight in substrate-induced conformational changes (Lüdemann et al., 2000; Chang & Loew, 1999). This will enlarge the

possibility of predicting the consequence of deliberate exchanges of amino acids at various positions in the protein. Also, the formation of hybrids between membrane-associated cytochromes P-450 and soluble bacterial cytochromes P-450 by combinatorial approaches has been initiated (Sieber et al., 2001) and may open new possibilities for the application of cytochrome P-450 systems in biocatalysis.

2.4 Scope of this study.

The main objectives of this study were to elucidate the degradation pathway of IPA by *Pseudomonas* sp. KIE171 and to develop a procedure for the biotransformation of IPA to L-alaninol, suitable for large-scale production of L-alaninol. The hypothesis that L-alaninol is an intermediate in the degradation pathway of IPA was confirmed by mutant studies. The genes of the pathway for IPA degradation were isolated and analyzed. Results obtained from these genetic studies suggested a novel pathway for IPA catabolism.

Two approaches were chosen for the construction of strains suitable for L-alaninol production. In a first approach, genes whose products are responsible for the degradation of L-alaninol were inactivated. In a second approach, a plasmid carrying all genes necessary for the conversion of IPA to L-alaninol was expressed in *E. coli*. Yields of L-alaninol obtained with these two types of constructs were determined and compared to each other.

3. Materials and Methods

3.1 Materials.

All chemicals used were of the highest quality available and were obtained from Fluka, Sigma or Aldrich. γ-(L-glutamyl)-isopropylamide (GIPA) and γ-(L-glutamyl)-L-1-hydroxy-isopropylamide (GALO) were provided by Lonza AG. Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Restriction and DNA modifying enzymes were from Fermentas or New England Biolabs.

3.2 Bacterial strains, media and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains DH5α (GIBCO/BRL Life Technologies) and XL1-Blue (Stratagene) were used for cloning. *E. coli* BL21(DE3) (Novagen) was used as host for overexpression of proteins. *E. coli* strain S17-1 λ*pir* (Miller & Mekalanos, 1988) was used as donor strain in biparental mating experiments.

Pseudomonas sp. KIE171 was isolated from sewage sludge at Lonza AG by enrichment on a medium containing IPA as the sole carbon source (Kiener, unpublished).

E. coli strains were grown aerobically in Luria–Bertani medium (Sambrook *et al.*, 1989) at 37°C on a rotary shaker at 140-180 rpm. *Pseudomonas* sp. strain KIE171 and mutants thereof were grown aerobically at 30°C in liquid minimal medium (pH 7.0) on a rotary shaker at 140 rpm. The salt composition of the minimal medium is described in Table 2. Carbon sources were added to a final concentration of 10 or 20 mM. When required, antibiotics were used at the concentrations listed in Table 3. Solid media contained 15 g of agar per liter. Growth was monitored by measuring the optical density at 650 nm.

Table 1. Bacterial Strains and plasmids used in this study. *Pseudomonas* sp. KIE171 and its derivatives are not accessible to the public because of the patent registration. Information is available from Dr. A. Kiener, Biotechnology Department, Lonza AG, CH-3092 Visp.

Strain or plasmid	Relevant features	Reference or source
Strains		
E. coli		
DH5α	*supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	GIBCO/BRL Life Technologies
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn*10 (Tet*^{r}*)*]	Stratagene
S17-1 λpir	Tp^r Sm^r *recA thi pro hsdR*$^-$*M*$^+$ RP4:2-Tc: Mu: Km:Tn*7 λpir*	Miller & Mekalanos (1988)
BL21(DE3)	*dcm ompT hsdS* (r_B-m_B) *gal* (λ*cIts857 indI Sam7 nin5 lacUV5*-T7-1)	Novagen
MC4100	*araD139Δ(argF-lac)U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	Casadaban (1976)
Pseudomonas sp.		
KIE171 (DSM 12360)	wild type, degrading IPA	Kiener, Lonza (unpublished)
KIE171-15	IPA$^-$, L-alaninol$^+$	Kiener, Lonza (unpublished)
KIE171-B (DSM 11521)	IPA$^-$, L-alaninol$^-$	This study
KIE171-BI (DSM 11629)	IPA$^-$, L-alaninol$^-$, *ipuI*::Tn*5*	This study
KIE171-BII (DSM 13389)	IPA$^-$, L-alaninol$^-$, *ipuC*::Tn*5*	This study

KIE171-BIII (DSM 13177)	IPA$^-$, L-alaninol$^-$, *ipuI*::Tn*5*, *ipuH*::Gm^R	This study
KIE171-BIV	*ipuH*::Gm^R	This study
Pseudomonas aeruginosa PAO1	wild type	Holloway (1955)
Pseudomonas sp. HH69	wild type, degrading dibenzofuran	Fortnagel et al. (1990)
Pseudomonas fluorescens CHAO	wild type, involved in phytopathogenic fungi antagonisms	Meyer et al. (1992)
Pseudomonas olevorans GPo1	wild type	Schwartz & McCoy (1973)
Pseudomonas putida S-313	wild type, desulfonating arylsulfonates	Zürrer et al. (1987)
Pseudomonas citronellolis	wild type	DSM 50332
Sphingomonas sp. RW1	wild type, degrading dibenzo-*p*-dioxin and dibenzofuran	Wittich et al. (1992)
Bacillus subtilis BD99	*trpC2 thr-5 his*	Quirk et al. (1994)
Plasmids		
pBluescript II KS(+)	Cloning vector, Ap^R	Stratagene
pPD111	Low copy cloning vector, Cm^R	Dersch et al. (1994)
pET24b(+)	Kan^R, *lacI*^{q}, T7 promoter	Novagen
pET24a(+)	Kan^R, *lacI*^{q}, T7 promoter	Novagen
pET28a(+)	Kan^R, *lacI*^{q}, T7 promoter, N-terminal His-Tag	Novagen
pBC KS(+)	Cloning vector,Cm^R	Stratagene

pEX18Tc	Cloning vector,Tc^R	Hoang et al. (1998)
pBBR1MCS	Cm^R, broad-host-range cloning vector	Kovach et al. (1994)
pUT-miniTn*5*-Km	Ap^R, Tc^R, miniTn*5*-Km	de Lorenzo & Timmis (1994)
pUCGM	Ap^R, Gm^R; source of Gm^R cassette	Schweizer (1993)
pME4254	8 kb *Cla*I fragment in pBluescript II KS(+), obtained by transposon rescue form KIE171-BI	This study
pME4255 (DSM 13178)	4.5 kb *Not*I-*Xho*I fragment in pBluescript II KS(+), obtained by transposon rescue form KIE171-BI	This study
pME4256	*Sfi*I Δ in pME4255	This study
pME4257	20-18 kb *Not*I-*Xho*I fragment in pBluescript II KS(+), obtained by transposon rescue from Kie171-BII	This study
pME4259	22-20 kb *Xho*I fragment in pBluescript II KS(+), obtained by transposon rescue from Kie171-BII	This study
pME4265	3.5 kb *Sac*I fragment in pBluescript II KS(+), obtained by transposon rescue from Kie171-BII	This study
pME4267 (DSM 13179)	8 kb *Sac*I fragment in pPD111, obtained by transposon rescue from KIE171-BII	This study
pME4268	2.7 kb *Sma*I-*Sal*I fragment containing *ipuH* from pME4259 in pPD111	This study
pME4269	0.855 kb *Sma*I fragment containing Gm^R from pUCGM in blunted *Bam*HI site of pME4268	This study
pME4270	3.5 kb *Sma*I-*Sal*I fragment from pME4269 in pEX18Tc, *ipuH*::Gm^R	This study
pME4271	7.3 kb *Not*I-*Pvu*I fragment from pME4257 in pBC KS(+)	This study

pME4273	3.8 kb *Xho*I-*Sac*I fragment from pME4259 in pBluescript KS, Kan^R	This study
pME4274	Replacement of 3.0 kb *Bgl*II-*Sac*I fragment in pME4273 with 1 kb *Bgl*II-*Sac*I fragment from pME4277	This study
pME4275 (DSM 13180)	*Nde*I-*Hin*dIII *ipuC* insert in pET28a(+)	This study
pME4277	*Nde*I-*Hin*dIII *ipuC* insert in pET24a(+)	This study
pME4278	1.6 *Xho*I-*Pvu*II fragment from pME4274 plus 4.4 kb *Pvu*II-*Pst*I fragment from pME4271 in pBBR1MCS, '*ipuABCDEFGH*'	This study
pME4751	*Nhe*I-*Sac*I PCR fragment containing *ipuF* in pET28a(+)	This study
pME4754	0.9 kb *Xba*I-*Xho*I PCR fragment in pBBR1MCS, *ipuA*'	This study
pME4755	0.9 kb *Xba*I-*Xho*I from pME4754 + 6 kb *Xho*I-*Pst*I fragment from pME4278 in pBBR1MCS, *ipuABCDEFGH*'	This study
pME4757	pBluescript II KS(+) with blunted PCR product *ipuF*	This study

Table 2. Salt composition of minimal medium.

Salts	Concentration [mg/l]
$MgCl_2$ x 6 H_2O	720
$CaCl_2$ x 2 H_2O	26.1
$FeCl_3$ x 6 H_2O	1.44
EDTA x Na_2 x 2 H_2O	10
$FeSO_4$ x 7 H_2O	4
$ZnSO_4$ x H_2O	0.2
H_3BO_4	0.6
$CoCl_2$ x 6 H_2O	0.4
$MnCl_2$ x 4 H_2O	0.045
$CuCl_2$ x 2 H_2O	0.02
$NiCl_2$ x 6 H_2O	0.04
Na_2MoO_4 x 2 H_2O	0.06
$(NH_4)SO_4$	2000
$NaHPO_4$	2000
KH_2PO_4	1000
NaCl	2000

Table 3. Concentration of antibiotics used for selection.

Antibiotic	Concentration	
Kanamycin	50	µg/ml
Gentamycin	25	µg/ml
Tetracycline	15	µg/ml
Ampicillin	200	µg/ml
Chloramphenicol	20	µg/ml

3.3 Screening of microorganisms for the ability to degrade IPA.

The organisms *E. coli* MC4100, *Bacillus subtilis* BD99, *Sphingomonas* sp. RW1, *P. fluorescens* CHAO, *P.* sp. HH69, *P. putida* S313, *P. aeruginosa* PAO1, *P. citronellolis* DSM 50332 and *P. olevorans* Gpol were screened for the ability to utilize IPA as a carbon source. The bacteria were grown at 30 °C in 25 ml Luria–Bertani medium on a rotary shaker at 140 to 180 rpm until obvious growth had occurred, except *E. coli* MC4100 and *Bacillus subtilis* BD99, which were grown at 37°C. The cultures were then centrifuged, washed with carbon free minimal medium and used to inoculate minimal medium with 20 mM IPA to an OD_{650} of 0.01 to 0.05. Growth was followed by measuring OD_{650} during incubation for 48 h at 30°C or 37°C. As a positive control, L-glutamate or glucose were used as a carbon source. For *E. coli* it was necessary to supply thiamine and for *B. subtilis* tryptophan, threonine and histidine because they were auxotrophic for these substances.

3.4 MNNG-mutagenesis.

Chemical mutagenesis of strain KIE171 was performed with *N*-methyl-*N*'-nitro-N-nitrososguanidine (MNNG) according to the protocol of Foster (1991). The survival rate was determined (Adelberg et al., 1965) at different MNNG concentrations (0.01, 0.23, 0.33 and 0.5 µg/µl cell suspension). The cell suspension contained $2.5x10^9$ cells/ml and three different exposure times were used (5,15 and 30 min). A suspension of mutagenized cells containing approximately 50% survivors was plated on minimal medium containing 20 mM L-glutamate. Two thousand colonies were replica-plated onto minimal plates containing either 20 mM IPA or 20 mM L-alaninol as the sole carbon source. Mutants unable to grow with one or both of these carbon sources were chosen for further study.

3.5 MiniTn*5*-mutagenesis.

Mutants impaired in IPA and L-alaninol formation were also obtained by transposon mutagenesis. MiniTn*5* transposon mutagenesis (de Lorenzo & Timmis, 1994) of *Pseudomonas* sp. KIE171 was performed by biparental plate conjugation of *E. coli* S17-1λ*pir* containing plasmid pUT/miniTn*5*-Km (de Lorenzo *et al.* 1990) with the wild type strain KIE171. To isolate miniTn*5*-Km insertion mutants in KIE171, equal cell numbers of overnight cultures of *E.coli* S17-1 λpir (pUT/miniTn*5*-Km) and KIE171 were mixed and patch mated on Luria-Bertani agar at 30°C for 8 h. The cells were resuspended in 0.9% NaCl and plated on selective medium that contained 50 µg of kanamycin per ml, 10 mM L-lactate and 10 mM L-alanine. Counter selection for the donor *E. coli* S17-1 λpir was based on the absence of proline from the selective medium. Kanamycin-resistant transconjugants were obtained after incubation for one to two days at 30°C. Three thousand colonies were then replicated onto minimal media plates containing IPA, L-alaninol, L-alanine or L-lactate as carbon source and 50 µg/ ml of kanamycin. Mutants impaired in growth with one or several of these carbon sources were chosen for further study.

3.6 DNA manipulations and molecular genetic methods.

Plasmid isolation, cleavage with restriction enzymes, agarose gel electrophoresis, ligation and transformation were performed according to standard methods (Sambrook et al., 1989). Isolation of genomic DNA and of plasmid DNA was carried out using the Blood and Cell Culture DNA Maxi kit (Qiagen) and the QIAquick Gel Extraction kit (Qiagen), respectively. Southern blot analysis was performed as published (Kayser et al., 2000). PCR was carried out in a Trio Block (BioMetra). Standard reaction mixtures consisted of 50 pmol of primers, 200 nmol of dNTPs, 1.5 U *Pfu* or *Vent* polymerase or 0.1 U *Taq* DNA polymerase (Fermentas) and 1-100 ng of template in a final volume of 20 - 50 µl. For cloning purposes, *Vent* or *Pfu* polymerase were used. After

cloning of PCR products, fragments were sequenced to confirm that no point mutations had occurred.

3.7 DNA sequence analysis.

DNA sequences were determined by primer walking on both strands (Microsynth, Balgach). The oligonucleotides primers used in this work are listed in Table 15 of the appendix. Analysis of DNA and protein sequences was carried out with the Genetics Computer Group package Version 10. Similarity and information searches for protein sequences were performed in particular by using programs of SRS (http://www.expasy.ch/srs5/), Prosite (http://www.expasy.ch/prosite/), and BRENDA (http://www.brenda.uni-koeln.de/). Nucleotide sequences of *ipuABCDEFGH*, *ipuIJ*, and the 16S rRNA gene of strain KIE171-B have been deposited in the GenBank database under accession numbers AJ311159, AJ311161, and AJ11160, respectively.

3.8 Construction of mutants KIE171-BIII and KIE171-BIV.

A 7 kb *Sma*I fragment from pME4259 was digested with *Sal*I and the resulting 2.7 kb *Sma*I-*Sal*I fragment carrying *ipuH* was cloned into linearized pPD111 to give plasmid pME4268. Interruption of *ipuH* was accomplished by insertion of a 0.8 kb *Sma*I fragment, containing a gentamycin resistance gene isolated from plasmid pUCGM, in *BamH*I digested and blunted pME4268, resulting in plasmid pME4269. Subsequently, to obtain a plasmid suitable for conjugal transfer, a 3.5 kb *Sma*I-*Sal*I insert from pME4269 was cloned into the multiple cloning site of pEX18Tc, generating plasmid pME4270 (Figure 10).

E. coli S17-λpir carrying pME4270 was conjugated with mutant KIE171-BI or the wild type KIE171. Cointegrates were selected by plating on minimal media containing L-glutamate and the antibiotics tetracycline or gentamycin. Merodiploids were resolved by plating on Luria–Bertani medium containing 5% sucrose. Sucrose resistant mutants were screened for gentamycin and

tetracycline sensitivity (Figure 11). The insertion of the gentamycin resistance gene into *ipuH* was confirmed by PCR, using two specific primers (9902649 and 58-160998). The mutants obtained were KIE171 BIII, containing a double knockout of *ipuI* and *ipuH*, and KIE171 BIV, containing a single knockout in *ipuH*.

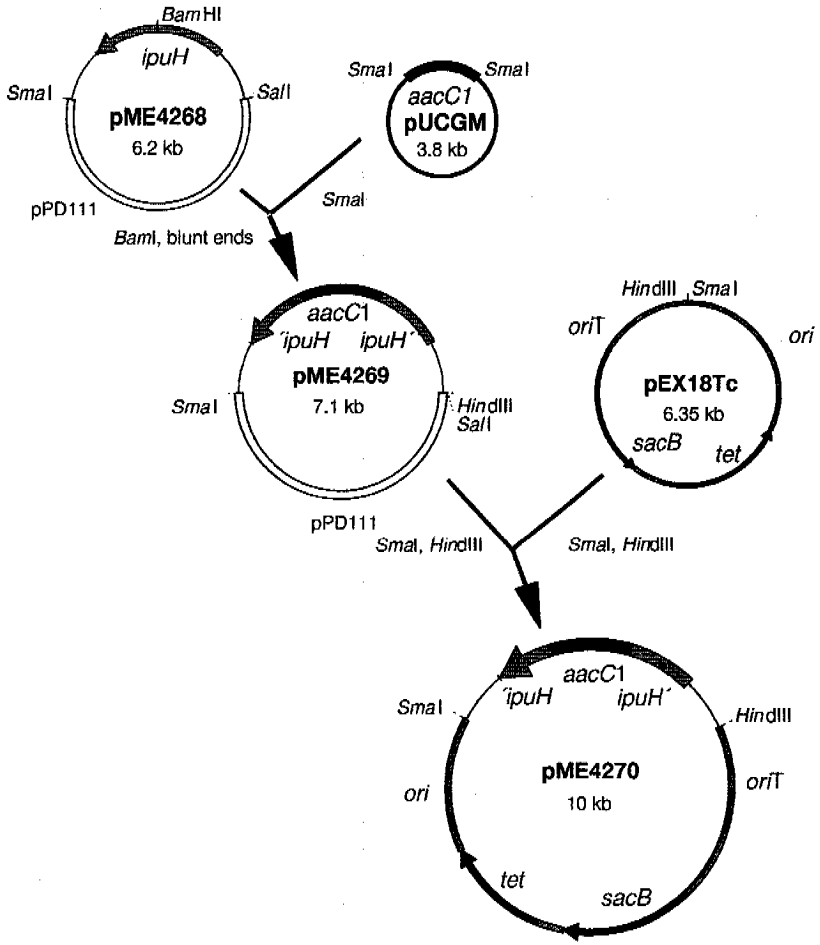


Fig. 10. Construction of a suicide plasmid for inactivation of *ipuH*.

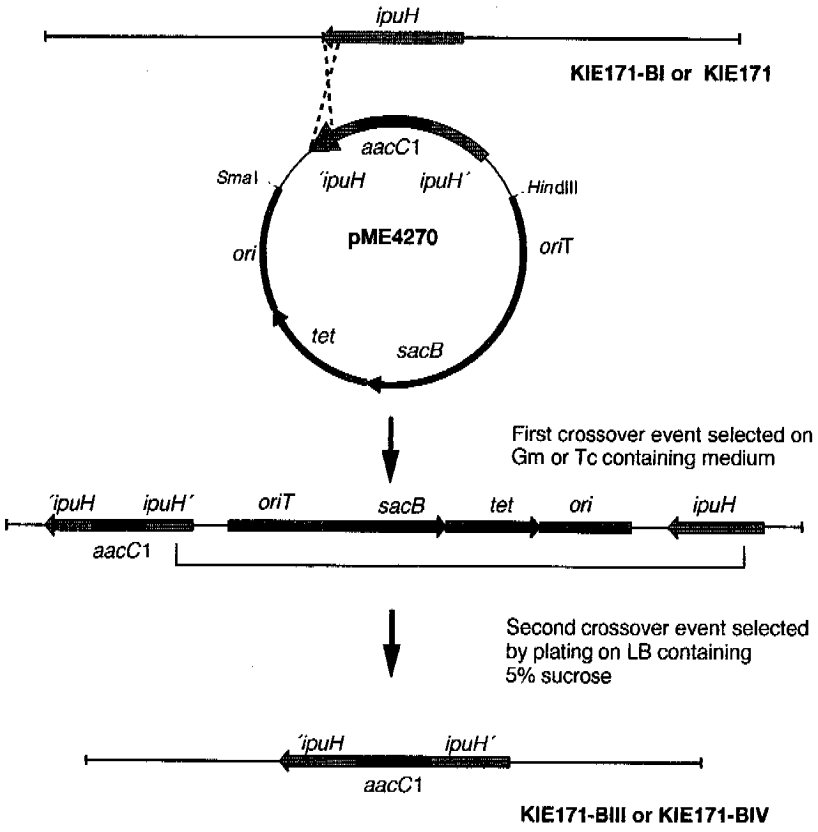


Fig. 11. Inactivation of the *ipuH* gene.

3.9 Construction of the *ipuABCDEFG* expression plasmid pME4755.

The *ipuABCDEFG* genes, which are arranged in a 6.9 kb cluster, were placed under the control of the T7 RNA polymerase promoter of the intermediate-copy vector pBBR1MCS (Figure 12). For this, a 3.8 kb *Xho*I-*Sac*I fragment from pME4259 was cloned into the *Xho*I-*Sac*I restriction sites of pBluescript II KS(+) to give plasmid pME4273. In this plasmid, *ipuC* is interrupted by a transposon insertion, which was removed by replacing the 3 kb *Bgl*II-*Sac*I fragment with the 0.95 kb *Bgl*II-*Sac*I fragment from pME4277 to give plasmid pME4274. A 7.3 kb *Not*I-*Pvu*I fragment from pME4257 was cloned in pBC KS(+), yielding plasmid pME4271. A 1.6 kb *Xho*I-*Pvu*II fragment from pME4274 and a 4.4 kb *Pvu*II-*Pst*I fragment from pME4271 were cloned together into pBBR1MCS linearized with *Xho*I-*Pst*I to give plasmid pME4278, which contained *ipuBCDEFG*. To include *ipuA* without the hypothetical *ipu* promoter, an *Xba*I site was introduced just upstream of the *ipuA* gene at bp 1292 by PCR using primer ipuA-NT and primer 60-100898 9813736. The PCR fragment was digested with *Xba*I and *Xho*I and cloned into pBBR1MCS, resulting in plasmid pME4754. The *ipuA* gene on plasmid pME4754 was sequenced to confirm that no changes had been introduced during PCR amplification. Finally, plasmid pME4755 was obtained by cloning the 0.9 kb *Xba*I-*Xho*I fragment from pME4754 into pME4278, which had been digested with *Xba*I and *Xho*I.

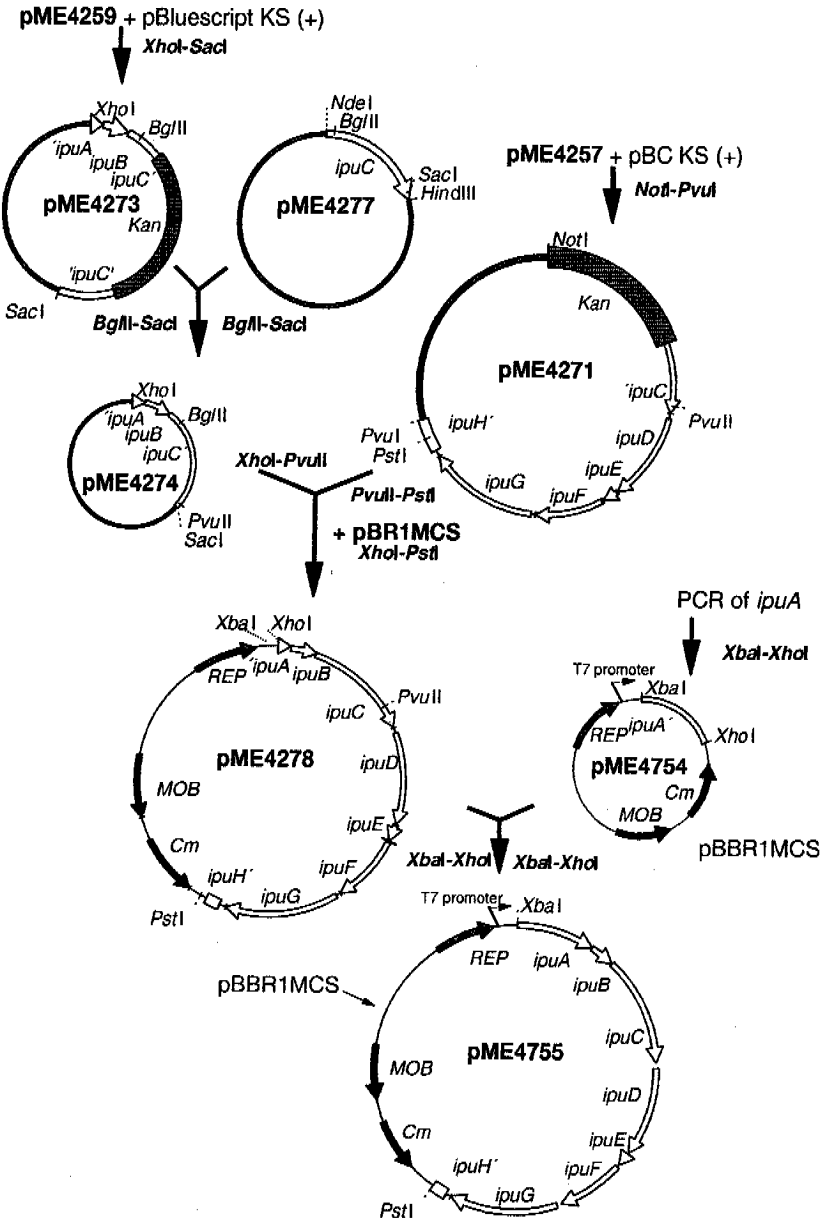


Fig. 12. Construction of the *ipuABCDEFG* expression plasmid pME4755.

3.10 Construction of an *ipuC* expression plasmid.

The *ipuC* gene was placed under the control of the T7 RNA polymerase promoter of vector pET28a(+) for the production of an N-terminal His_6-IpuC fusion protein, and under the control of the T7 RNA polymerase promoter of vector pET24a(+) for the production of wild type IpuC. For the construction of the His_6-IpuC protein, an *Nde*I site at the translation start and a *Hin*dIII site after the *ipuC* stop codon were introduced by PCR amplification using genomic DNA from wild type KIE171 as template. The oligonucleotide primers used were *ipuC*-NT (introducing a *Hin*dIII site) and *ipuC*-CT (introducing a *Nde*I site). The 1.4 kb PCR product was digested with *Nde*I and *Hin*dIII, and the fragment encoding IpuC was cloned in the expression vector pET28a(+), generating plasmid pME4275, in which *ipuC* is under the control of the T7 RNA polymerase promoter. The *ipuC* sequence of plasmid pME4275 was sequenced to confirm that no changes had been introduced during PCR amplification. Plasmid pME4277 was obtained by cloning the *Nde*I-*Hin*dIII *ipuC* insert of pME4275 into the vector pET24a(+).

3.11 Construction of an *ipuF* expression plasmid.

The *ipuF* gene was placed under the control of the T7 RNA polymerase promoter of vector pET28a(+) for the production of an N-terminal His_6-IpuF fusion protein, and of vector pET24b(+) for the production of wild type IpuF. An *Nhe*I site at the translation start and a *Sac*I site at the translation end were introduced by PCR amplification using plasmid pME4259 as a template. The oligonucleotide primers used were *ipuF*-NT (introducing an *Nhe*I restriction site) and *ipuF*-CT (introducing a *Sac*I restriction site). The 909-base pair PCR product was digested with *Nhe*I and *Sac*I, and the resulting 891-base pair fragment containing *ipuF* was cloned in the expression vector pET28a(+) generating plasmid pME4751. The *ipuF* sequence of plasmid pME4751 was sequenced to confirm that no changes had been introduced during PCR

amplification. Plasmid pME4756 was obtained by cloning the *Nhel-Sacl* insert of pME4751 containing *ipuF* into the vector pET24b(+).

3.12 Protein expression and analysis.

For the production of His_6-IpuC in *E. coli*, strain BL21(DE3) harboring the expression plasmid pME4275 was grown at 30 °C in 0.5 l Erlenmeyer flasks containing 100 ml of Luria–Bertani medium. When the culture had reached an OD_{650} of 0.6, expression was induced by addition of isopropyl thio-β-D-1-galactoside (IPTG) to a final concentration of 100 µM and the culture was incubated for another 3 h to a final OD_{650} 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 His_6-IpuF, the induction of BL21(DE3) harboring the expression plasmid pME4751 was performed at 18°C during 5 h to a final OD_{650} of 1. The same procedure was used for the production of wild type IpuF.

Cells were collected by centrifugation at 6000 *g* for 20 min at 4 °C, washed in 50 mM Tris-HCl with 2 mM EDTA at pH8 and stored at –20°C as frozen pellets or used directly for purification.

After resuspension in 4 ml of cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9 and 25 µg/ml DNase I), cells were disrupted by three passages through a French pressure cell (55 mPa, 4 °C) and cell debris were removed by centrifugation (14 000 *g*, 30 min, 4 °C). Cell-free crude extract was obtained after ultracentrifugation of the resulting supernatant (40,000 × *g* at 4°C for 60 min).

For protein purification, samples were analyzed by separation on denaturing 12% sodium dodecyl sulfate-polyacrylamide minigels (SDS-Page) by standard protocols (Ausubel et al., 1997) and stained by Coomassie Brillant Blue. The protein concentration of the supernatant was determined by using a commercial Bradford reagent (Bio-Rad) and bovine serum albumin (Sigma) as the standard. Cell-free crude extracts were either used directly or flash frozen in liquid nitrogen and stored at - 20°C. Non-denaturing polyacrylamide gel

electrophoresis (blue native PAGE), to determine the monomeric or oligomeric state of IpuF, was performed according to Schägger and von Jagow (1991).

3.13 Purification of IpuC and IpuF.

Cell-free crude extract containing His_6-IpuC was loaded onto a 2.5-ml His•Bind Resin column (Novagen), which had been activated with $NiSO_4$ and equilibrated at a flow rate of 25 ml/h with binding buffer as described by the manufacturer. The sample was washed with 25 ml of binding buffer, followed by washing with 15 ml of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Elution of the bound protein was performed with 200 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, 20% glycerol, pH 7.9. The protein solution was stored at 4 °C until further use.

His_6-IpuF was purified at 4°C in a similar manner by metal chelate affinity chromatography on a Ni-NTA agarose column (QIAGEN). The binding buffer consisted of 300 mM NaCl, 50 mM NaH_2PO_4 and 10 mM imidazole at pH 8.0. The composition of the wash buffer was 300 mM NaCl, 50mM NaH_2PO_4 and 20 mM imidazole at pH8, and the elution buffer contained 300 mM NaCl, 50 mM NaH_2PO_4 and 250 mM imidazole, pH8. His_6-IpuF was eluted with 2 ml of elution buffer and 100 µl of 87% glycerol were added for storage at -20 °C.

3.14 Assay for γ-glutamylamide synthetase (IpuC) activity.

Crude extracts and purified His_6-IpuC were assayed for γ-glutamylisopropylamide synthetase activity by measuring the substrate-dependent formation of inorganic phosphate from ATP. One unit of activity (U) is defined as the amount of enzyme forming one µmol of inorganic phosphate per minute at 25°C under standard assay conditions. Detection of inorganic phosphate was performed by a colorimetric reaction after addition of ferrous sulfate reagent and ammonium molybdate reagent. The reaction mixture (0.4 ml

final volume) contained 10 mM ATP, 10 mM substrate, 10 mM L-glutamate, 50 mM $MgCl_2$, 50mM imidazole, 3.5 mM NaCl, 0.1 mM Tris-HCl, and 25 µg of crude extract or 22.8 µg of purified His_6-IpuC. Reactions were carried out at 25°C and started by addition of His_6-IpuC to the reaction mixture.

The reaction was stopped by the addition of 900 µl of ferrous sulfate reagent (0.8%, $FeSO_4.7H_2O$ in 0.015 N H_2SO_4, pH<0) and 75 µl ammonium molybdate reagent (6.6% $(NH_4)_6Mo_7O_{24}.4H_2O$ in 7.5 N H_2SO_4, pH<0) to 100 µl reaction mixture. It was mixed vigorously and color was allowed to develop at room temperature for 2 min, after which absorbance was measured at 660 nm.

The formation of color was proportional to the amount of inorganic phosphate up to 1.5 mM and the detection limit was at about 50 µM inorganic phosphate.

3.15 Assay for γ-glutamylamide-hydrolase (IpuF) activity.

γ-Glutamylamide hydrolase activity was measured using a two-step enzymatic reaction. In the first step, His_6-IpuF or crude extract was incubated with γ-glutamylamide. In the second step, the glutamate formed in the first step was quantified using L-glutamic acid dehydrogenase and acetylpyridine NAD^+. The amount of acetylpyridine NADH formed was calculated by measuring the A_{363}.

For the first step, reactions were performed for 10 min at 30°C in a total volume of 4 ml, which contained 80 µl 0.5 M $MgCl_2$, 200 µl 0.5 M Tris-HCl at pH8, 800 µl 87% glycerol and variable amounts of the substrates. The reaction was started by addition of 96µg purified His_6-IpuF or crude extract (160 µg for wild type IpuF and 200 µg for His_6-IpuF). Fractions of 0.4 ml were collected at different times, boiled for 2 min, and centrifuged at 13000 rpm. The glutamate concentration was determined by incubation at 37°C of a reaction mixture consisting of 0.3 ml of supernatant from the first step, 50 µl 1 M KCl, 100 µl 0.5 M Tris-HCl pH 8.0, 50 µl of glutamate dehydrogenase (5 mg/ml in potassium phosphate 0.25 M pH 7.5), 5 µl 0.06 M acetylpyridine NAD^+ and 0.5 ml H_2O. The absorbance at 363 nm was measured after 30 minutes. The same protocol as described in 3.21 was used for the determination of the K_M values of IpuF for

GALO using HPLC analysis for the determination of the hydrolysis products. One unit of activity (U) is defined as the amount of enzyme forming one µmol of glutamate per minute at 30°C under standard assay conditions.

3.16 Biotransformation reactions with growing cells.

Cells were grown on minimal medium with 20 mM L-glutamate to an OD_{650} of 0.2–0.4. The biotransformation was started by the addition of 10 to 100 mM IPA or ethylamine. At regular intervals, aliquots were withdrawn and the concentrations of IPA, ethylamine, alaninol, theanine, GIPA and GALO were determined in the cell free supernatant by HPLC or GC.

3.17 Biotransformation of IPA to L-alaninol using cell suspensions.

To obtain a preculture, cells were grown overnight to stationary phase in minimal medium with L-glutamate. This preculture was used to inoculate 1 l of minimal medium with 20 mM L-glutamate as carbon source to an OD_{650} of 0.2. When the culture has reached an OD_{650} of 0.4, induction was performed with 1 mM of IPA (final concentration) and the culture was grown for another 3 to 5 h to a final OD_{650} of 0.8 to 1.0. Cells were collected by centrifugation at 6000 *g* for 20 min at room temperature, washed twice with minimal medium without carbon source to remove the IPA used for induction, and concentrated to an OD_{650} of 10 to 50 with minimal medium containing 20 mM L-glutamate. After storage for 14 h at 4°C, the biotransformation reaction was started by addition of 10 to 100 mM IPA. At regular intervals, aliquots were withdrawn and concentrations of substrate and products in the cell free supernatant were determined by HPLC or GC.

3.18 Biotransformation using a cell suspension of *E. coli* BL21(DE3) harboring plasmid pME4755.

E. coli BL21(DE3)(pME4755) was grown to stationary phase at 37 °C with constant shaking (180 rpm) in 25 ml of a medium containing 64 mM potassium phosphate (pH 7.2), 33 mM NH_4Cl_2, 1 mM $MgCl_2$, 10 mM glucose, 0.5 µM $(NH_4)_2SO_4$, 1% trace elements (Thurnheer et al., 1986) and 20 µg/ml chloramphenicol. This preculture was used to inoculate 100 ml of medium to an OD_{650} of 0.15.

When the culture had reached OD_{650} 0.4, induction was performed by addition of isopropyl thio-β-D-1-galactoside (IPTG) to a final concentration of 400 µM and the culture was grown for another 2 h to a final OD_{650} of 0.8. Cells were collected by centrifugation at 6000 *g* for 10 min at room temperature and resuspended to an OD_{650} of 50 in the medium described above without chloramphenicol. After storage for 14 h at 4°C, biotransformation was started by addition of IPA or ethylamine to 20 mM. At regular intervals, aliquots were withdrawn and the concentration of substrate and products in the cell free supernatant were determined by HPLC.

3.19 Labeling of L-alaninol with $^{18}O_2$.

Experiments were carried out in two 30-ml serum flasks, which contained 5 ml of a cell suspension with an OD_{650} of 10 or 50 in minimal medium containing 10 mM L-glutamate. Flasks were sealed with butyl rubber stoppers. In one of the flasks, the air was replaced by 100% N_2. Subsequently, the atmosphere in the flask was brought to 20 % $^{18}O_2$ by withdrawal of 5 ml of N_2 and injection of 5 ml of $^{18}O_2$ into the head space. Thus, one flask contained 20 % $^{18}O_2$ and the other flask contained 20% $^{16}O_2$ from air. The biotransformation was started by injecting IPA to a final concentration of 20 mM through the rubber seals into the cell suspension. Flasks were incubated at 30°C with constant shaking (140 rpm) during 120 hr. The cells were subsequently centrifuged and the supernatant

used for further measurements. The incorporation of $^{18}O_2$ into ^{18}O-L-alaninol was detected by HPLC/MS.

3.20 Biotransformation of isopropylamine into L-alaninol in the absence of oxygen.

The procedure for concentration of the cells to an OD_{650} of 10 was the same as described in 3.17. The experiments were carried out in two 30 ml serum flasks, which contained a suspension of induced cells (OD_{650} of 10) in 5 ml of minimal medium containing 20 mM L-glutamate. Flasks were sealed with butyl rubber stoppers as described before. A 100% N_2 atmosphere was obtained in one of the flasks by exchanging the air with N_2. A second flask, used as positive control, contained air. The biotransformation was started by injection of IPA to a final concentration of 20 mM through the rubber seals into the cell suspension. Flasks were incubated at 30°C with constant shaking (140 rpm) for 120 h. The cultures were centrifuged, and the metabolites and the substrate in the supernatant were detected by HPLC.

3.21 High-performance liquid chromatography and gas chromatography.

The disappearance of substrates and the formation of metabolites was monitored by high performance liquid chromatography (HPLC) or by gas chromatography (GC). HPLC was carried out with an Alliance HPLC system (Waters), using Millenium software. Compounds containing amino groups were derivatized with phenyl isothiocyanate (PITC), separated by HPLC and detected at 254 nm. To this end, 15 µl of cell-free supernatant were mixed with 15 µl of absolute ethanol and then treated with 140 µl of derivatization mixture (7:2:1 absolute ethanol/triethylamine/PITC). After a 10-min reaction at room temperature, the derivatized sample was lyophilized, resuspended in 500 µl of 5 mM potassium phosphate buffer (pH 6.5) and filtered (0.2 µm). Separation of derivatized compounds was performed on a Nucleosil-C18 reversed-phase

column (250x4.6 mm; particle size 7 µm) by applying a step gradient of the mobile phases A and B (A, 5 mM of potassium phosphate buffer pH 6.5; B, 80% methanol, 20% 5mM of potassium phosphate buffer pH 6.5) with a flow rate of 1 ml min^{-1}. The step gradient is listed in Table 4. Compounds eluting from the column were detected spectrophotometrically at 254 nm and identified by retention time and by co-chromatography with pure preparations.

As described in the following, L-alaninol and IPA were also detected by GC. 100µl of cell-free culture medium was injected into a Porapak P column (1800 x 2 mm, mesh 80/100, Supelco) connected to a PE8700 gas chromatograph (Perkin-Elmer) equipped with a flame ionization detector. Nitrogen was used as the carrier gas at a flow rate of 40 ml/min. The temperature was set to 180°C in the column and 250°C in the detector. Under these conditions, the retention times of IPA and L-alaninol were 0.8 min and 1.4 min, respectively, with detection limits of 4 mM and 2 mM.

Table 4. Step gradient used for HPLC analysis

Time (min)	% B	Flow rate (ml min^{-1})
0	0	0.5
4	0	0.5
11	55	1
21	55	1
22	100	1
27	100	1
28	0	1
33	0	1

3.22 Various analyses performed by outside laboratories.

Phylogenetic analysis of strain KIE171 was performed by DSMZ (Braunschweig). The enantiomeric excess of L-alaninol produced by mutant KIE171-BI was determined by Dr. A. Kiener at Lonza AG (Visp). Gas chromatography-mass spectrometry analysis and high performance liquid chromatography-mass spectrometry analysis of L-alaninol and theanine were also done by Lonza AG (Visp). ^{18}O-L-alaninol was subjected to high

performance liquid chromatography-mass spectrometry analysis at Lonza AG (Visp) and at EAWAG (Dübendorf) by Dr. H.P. Kohler.

4. Results

The prochiral compound IPA was considered a potential precursor for the microbiological preparation of the chiral building block L-alaninol. In view of this possibility, Dr. Andreas Kiener enriched organisms able to grow with IPA as the sole carbon source from wastewater of the Lonza manufacturing plant in Visp. Strain KIE171 was isolated from one of these enrichments and proved capable of utilizing IPA as the sole carbon source for growth (Dr. A. Kiener, unpublished).

4.1 *Pseudomonas* sp. strain KIE171, an organism capable of growth with IPA.

Growth properties of strain KIE171.

To find potential intermediates of the IPA degradation pathway, the utilization of different compounds by strain KIE171 was investigated. Carbon sources that supported growth were L-alaninol, L-alanine, D-alanine, L-lactate, propionic acid, aminoethanol, propane-1,2,-diol and L-glutamate, whereas D-alaninol, DL-alaninol, GIPA and acetone did not support growth (de Azevedo Wäsch, 1996). These findings indicated that L-alaninol (but not D-alaninol) could be an intermediate in the degradation pathway of IPA. KIE171 might therefore be suitable to set up a process for the biotransformation of IPA to L-alaninol.

The ability to degrade IPA is not frequent among microorganisms.

It is not known from the literature whether or not utilization of IPA by bacteria is widespread. To investigate the frequency of occurrence of IPA degradation, several bacteria were tested for utilization of IPA as the sole carbon source. The goal was to find a genetically characterized organism with the same capacity as

strain KIE171, i.e. with the ability to grow with 20 mM IPA as the sole carbon source. The organisms tested included *Escherichia coli* MC4100, *Bacillus subtilis* BD99, *Sphingomonas* sp. RW1, *Pseudomonas fluorescens* CHAO, *Pseudomonas* sp. HH69, *Pseudomonas putida* S313, *Pseudomonas aeruginosa* PAO1, *Pseudomonas olevorans* Gpol and *Pseudomonas citronellolis*. None of these strains showed growth after a period of five days. All organisms were able to grow with L-glutamate or glucose as a carbon source, therefore no further compound essential for growth was missing in the medium. The ability to degrade IPA is apparently not frequent among microorganisms.

Characteristics of strain KIE171 and derivates.

During the work with strain KIE171 and derivatives behavior typical of pseudomonads was observed. The colonies of the organism were flat, greyish, with irregular edges, and with time they tended to spread on the surface of the agar. Concentrated cell suspensions had a salmon-like color, which could indicate the presence of a pigment like pyorubrin, which is of reddish color and is also present in *Pseudomonas aeruginosa* (Kandela et al., 1997).
Liquid cultures of strain KIE171 and descending strains tended to form flocs and were very difficult to disperse. This was one of the main problems in determining the OD of growing cultures and in adjusting the OD in cell suspensions. Particularly after centrifugation cells adhered to each other, and resuspension was difficult. Colonies older than 3 days, which were stored at 4°C, sticked to the agar and formed threads when they should be restreaked.

Strain KIE171-B is most closely related to Pseudomonas citronellolis.

Characterization of strain KIE171-B (see later) was performed by the use of 16S rRNA as a phylogenetic marker molecule as well as by physiological tests by DSMZ (Braunschweig, Germany). Strain KIE171-B was classified into the rRNA similarity group-I of *Pseudomonas* on the basis of 16S rRNA analysis and the

profile of cellular fatty acids. *Pseudomonas* species from the RNA group-I belong to the γ-subdivision of proteobacteria (Palleroni, 1991; Palleroni, 1993). The 16S rRNA sequence of strain KIE171-B showed high similarity to that of *Pseudomonas citronellolis* (99 %), whereas the physiological characteristics of strain KIE171-B corresponded to results obtained with *Pseudomonas aeruginosa* (95.8 % 16S rRNA similarity to *Pseudomonas aeruginosa*). A clear attribution to a species of RNA group-I was not obtained.

4.2 Isolation of mutants converting IPA to L-alaninol.

MNNG-mutagenesis: Isolation of the L-alaninol producing strain KIE171-B.

Strain KIE171 is capable to utilize IPA as well as L-alaninol as a carbon source. The structural resemblance of both compounds makes it conceivable that L-alaninol is an intermediate in the IPA degradation pathway. However, when strain KIE171 was grown on IPA as the sole carbon source, L-alaninol was not detectable in the medium (results not shown).

In order to more closely investigate the hypothesis that L-alaninol is an intermediate in the IPA degradation pathway, enzymatic steps in the degradation of L-alaninol were mutationally blocked and the mutants obtained were examined for L-alaninol formation from IPA.

Mutants were generated by MNNG mutagenesis. Two thousand mutagenized cells were replica-plated onto minimal plates containing either 20 mM IPA or 20 mM L-alaninol as the sole carbon source. Mutants that showed reduced or zero growth with these carbon sources were selected for further study. After five rounds of screening on solid and liquid medium, one mutant was obtained that exhibited no apparent growth on IPA and L-alaninol as the sole carbon source over a time period of five days. The mutant was designated strain KIE171-B.

Biotransformation of IPA into L-alaninol by strain KIE171-B.

Strain KIE171-B was used to investigate whether production of L-alaninol from IPA was possible. Preliminary biotransformation experiments with cells of strain KIE171-B growing on 20 mM L-glutamate in the presence of 20 mM IPA resulted in the formation of L-alaninol, which was excreted in the growth medium and could be detected by HPLC (results not shown). In order to determine the amount of L-alaninol formed, the biotransformation reaction was carried out with resting cells of OD_{650} 13 in the presence of 20 mM IPA (Figure 13). After 40 hours of incubation, 8 mM L-alaninol was formed. Incubation for a longer time period resulted in degradation of L-alaninol (data not shown). Because of this breakdown, which was assumed to be due to reversion of the mutation, strain KIE171-B was not suitable for the production of L-alaninol.

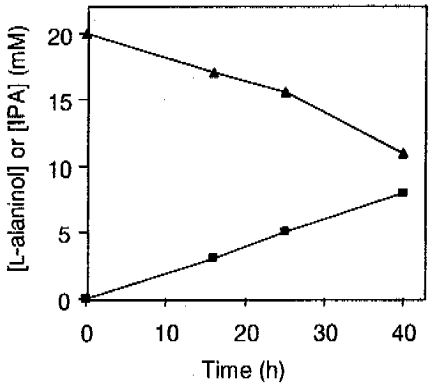


Fig. 13. Biotransformation of IPA (▲) to L-alaninol (■) with cell suspensions at OD_{650} 13 of strain KIE171-B. Biotransformation was started by the addition of 20 mM IPA. The compounds were detected by HPLC.

Since it was possible to form L-alaninol from IPA, further efforts were focused on obtaining an L-alaninol producing strain potentially suitable for large-scale production. To this end, mutants of *Pseudomonas* sp. KIE171 defective in IPA utilization were generated by miniTn*5*-Km mutagenesis (de Lorenzo & Timmis, 1994). This method has three advantages as compared to chemical

mutagenesis. First, once inserted in a target sequence, minitransposons produce complete disruption of the mutated gene resulting in non-leaky phenotypes. Further, transposon-insertion mutants are stable. Third, genetic analysis of the mutated gene region is possible, which can lead to considerable insight into the metabolic process under study.

MiniTn5-Km mutagenesis: isolation of strains deficient in IPA utilization.

Three thousand colonies resulting from cells carrying transposon insertions were isolated on minimal medium with 10 mM L-lactate and 10 mM L-alanine and 50 µg/ml kanamycin, and replicated onto minimal plates containing IPA, L-glutamate, L-lactate or L-alanine as the sole carbon source. Mutants that showed reduced or zero growth with these carbon sources were selected for further study. It was not possible to screen for mutants defective in the utilization of L-alaninol as the sole carbon source on solid medium because the wild type grew very slowly with L-alaninol. After three rounds of screening, seven mutants that showed interesting phenotypes were obtained. These seven mutants were subsequently characterized by examining their growth behavior in liquid medium with IPA, L-alaninol, L-lactate or L-alanine as the sole carbon source during a time period of five days (Table 5). All mutants grew equally well with L-lactate or L-glutamate.

Two mutants, KIE171-BI and KIE171-BII, displayed no growth with IPA or L-alaninol as the sole carbon source, but normal growth with all other carbon sources tested. Since in these two strains the mutations appeared to be located in the genes that encode IPA degradation, they were chosen for further analysis. Mutant KIE171-AII showed no growth with IPA and reduced growth with L-alaninol, whereas the other mutants showed slow growth with IPA and L-alaninol. Whereas mutant KIE171-BI transformed IPA to L-alaninol, mutant KIE171-BII failed to do so (results not shown). The strains KIE171-BI and KIE171-BII were used for genetic analysis, and strain KIE171-BI was further used for the biotransformation of IPA to L-alaninol.

Table 5. Growth in liquid culture of strain KIE171 miniTn*5*-Km insertion mutants.

Wild type or mutant	Growth* with IPA	L-alaninol	L-alanine
KIE171	+	+	+
BI	-	-	+
BII	-	-	+
AII	-	±	+
G16-25	±	±	+
F14-27	±	±	+
F11-37	+	±	±
G11-16	±	±	±

*+, OD_{650} > 0.2 after 24 hours; -, OD_{650} < 0.2 after 96 hours; ±, OD_{650} > 0.2 after 96 hours.

4.3 Isolation and analysis of the IPA utilization genes.

Minitransposon rescue: the IPA utilization gene cluster.

To investigate the nature of the genes interrupted by the minitransposons, strain KIE171BI and strain KIE171BII were further analyzed at the genetic level. The region adjacent to the minitransposon insertion site in strain KIE171-BI was isolated by making use of the kanamycin resistance gene of the minitransposon for selection of the desired clones. A 4.5 kb *Not*I-*Xho*I fragment and a 8 kb *Cla*I fragment that hybridized to a fragment of the minitransposon, were cloned into pBluescript II KS(+) from *Not*I-*Xho*I-digested genomic DNA and *Cla*I-digested genomic DNA. The resulting plasmids, pME4255 and pME4254, respectively, were used to determine the sequence of the regions flanking the minitransposon insertion in mutant strain KIE171-BI (Figure 14).

The minitransposon insertion had occurred in an *orf* that encodes a hypothetical protein with similarity to aldehyde dehydrogenases (Table 9). This open reading frame was named *ipuI*, where *ipu* stands for IPA utilization. It was concluded that the hypothetical aldehyde dehydrogenase IpuI is involved at some point in the degradation pathway for L-alaninol. Since KIE171-BI can still convert IPA

into L-alaninol, the enzyme(s) required for this step must be functional in this mutant.

Additionally, three further open reading frames where found upstream and downstream of *ipuI* on the sequenced 4.5 kb fragment. The product of the upstream open reading frame exhibited similarity (36.6% identity in a 41 aa overlap) to a recombinase (RecA of *Acinetobacter calcoaceticus*). Downstream of *ipuI*, the adjacent *orf* is coding for IpuJ, which exhibited similarity to the hypothetical protein PA5509 of unknown function in *Pseudomonas aeruginosa* (Table 6, Table 9). It was named *ipuJ* because it is probably cotranscribed with *ipuI*, although it is not clear how it could be involved in the IPA degradation pathway. A fourth *orf* is oriented in the opposite direction and exhibits similarity (91.8% identity in a 61 aa overlap) to the hypothetical protein PA1368 of unknown function in *Pseudomonas aeruginosa*. The recombinase is not expected to be involved in the IPA degradation pathway. The large non-coding region of 1340 bp between *recA* and *ipuI* is unusual.

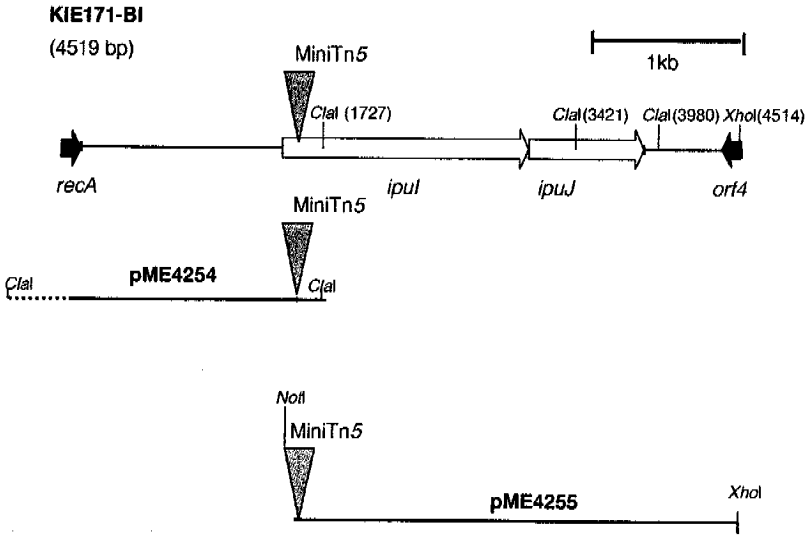


Fig. 14. Physical map of the *ipuI* gene loci. The grey triangle represents the minitransposon, which has a size of approximately 2kb. The dashed line corresponds to unsequenced DNA. The nucleotide sequence of *ipuIJ* is deposited in the GenBank database under accession number AJ311161.

Table 6. Sequence of the regions flanking the minitransposon insertion in mutant KIE171-BI. The minitransposon insertion occurred at bp 1488.

orf	*inferred function*	start base	end base	total bp	aa
recA	Recombinase	1	129*		
	Non coding region	130	1470		
ipuI	Aldehyde dehydrogenase	1471	3108	1638	546
ipuJ	unknown (similarity to *P. aeruginosa* protein PA5509)	3108	3884	777	259
	Non coding region	3885	4203		
orf4	unknown (similarity to *P. aeruginosa* protein PA1368)	4519*	4203		

* The *orf* was not completely sequenced.

Mutant KIE171-BII was unable to convert IPA into L-alaninol. The region flanking the minitransposon in KIE171-BII was cloned on three different plasmids. By using minitransposon rescue, a 18-20 kb *NotI-XhoI* fragment from *NotI-XhoI*-digested and a 20-22 kb *XhoI* fragment from *XhoI*-digested genomic DNA from KIE171-BII were cloned in pBluescript KS to give plasmids pME4257 and pME4259, respectively. A 8 kb *SacI* fragment from *SacI*-digested genomic DNA was cloned into the low copy vector pPD111, yielding plasmid pME4267, since cloning of this fragment in the high copy vector pBluescript II KS(+) was not successful. The three plasmids were used to determine the sequence of 11.3 kb of DNA (Figure 15).

The minitransposon insertion of mutant KIE171-BII had occurred in an *orf* (*ipuC*) whose product showed similarity to glutamine synthetases. This was an unexpected result because it did not fit into our model of IPA degradation. This model postulated that IPA would be stereospecifically hydroxylated by a monooxygenase in a one step reaction. Analysis of the DNA region affected by the transposon insertion now revealed the presence of a cluster of eight open reading frames, the *ipuABCDEFGH* gene cluster, flanked by two putative IS-elements of the types IS2 and IS3 (Table 7).

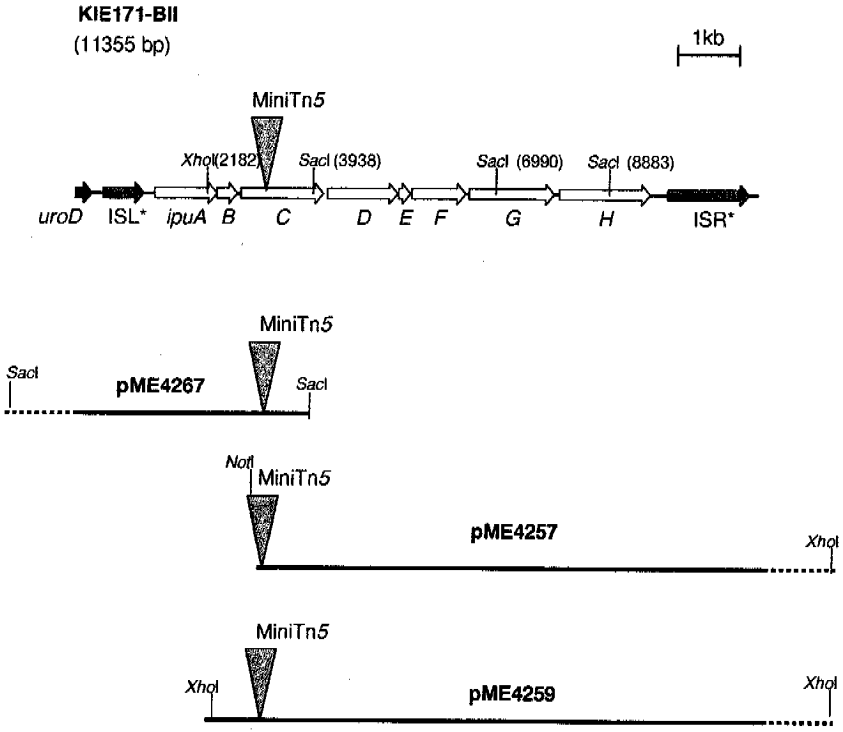


Fig. 15. Physical map of the *ipuABCDEFGH* gene locus. The grey triangle represents the minitransposon, which has a size of approximately 2 kb. The dashed line corresponds to unsequenced DNA. The nucleotide sequence of *ipuABCDEFGH* is deposited in the GenBank database under accession number AJ311159.
*: ISL and ISR showed sequence similarity to IS2 and IS3-elements of the IS3 family, the functionality was not apparent.

Table 7. Sequence of the regions flanking the minitransposon insertion in mutant KIE1717-BII. The minitransposon insertion occurred at bp 3281.

orf	*inferred function*	start base	end base	total bp	aa
uroD	uroporphyrinogen decarboxylase *	1	270		
ISL	*IS2* type** insertion sequence	≈ 470	≈1130	n.k.	
ipuA	seeTable 9	1362	2342	981	327
ipuB	seeTable 9	2342	2680	339	113
ipuC	seeTable 9	2743	4122	1380	460
ipuD	seeTable 9	4194	5354	1161	387
ipuE	seeTable 9	5371	5565	195	65
ipuF	seeTable 9	5589	6476	888	296
ipuG	seeTable 9	6533	7963	1431	477
ipuH	seeTable 9	8051	9574	1524	508
ISR	*IS3* type insertion sequence	9865	11198	1334	
	IRL	9998	10028		
	IRR	11162	11197		
	transposase ***	≈10030	≈10760		

*The open reading frame was not completely sequenced.
** No clear inverted repeats and no start of the transposase were found.
*** In the transposase more than one frame shift is present. The first frame shift is in the region of bp 10573. A start codon is missing.
n.k.: not known

Table 8. Sequence of the inverted repeats of the ISR-element

IRL	5′→3′	9998-CTGCAGGTATCACGCAGCGATTTCTATGCC-10028
IRR	3′→5′	11197-GACGTCCATAGGCCGTAGATTTAAAGAAACG-11162

The sequence was determined 470 bp upstream of the ISL-element and 172 bp downstream of the ISR-element. Inverted repeats were only found for the ISR-element (Table 8). Between the inverted repeats of the ISR-element, the sequence shows similarity to the sequence of genes coding for transposases. No clear start codon could be detected and more than one frame shift is present in this sequence. For the ISL-element neither clear inverted repeats nor a start codon for the transposase were identified (Table 7).

Upstream of the ISL-element an open reading frame was found, whose translation product exhibited similarity to uroporphyrinogen decarboxylase. This hypothetical protein is not expected to be involved in IPA degradation. Primer extension analysis performed by Dr. Jan van der Ploeg revealed that a promoter situated at bp 1324-1362 was functional.

Table 9: Comparison of the deduced amino acid sequences of the IPA utilization cluster (*ipu*) to the SWISS-PROT, TrEMBL or PIR data bases.

ORF	Size (aa)	Identity over amino acids	Accession Number (AC)	Proposed Function	Organism	Size (aa)
IpuA	327	45.2% in 325	B70015 *	Thioredoxin reductase (homologue)	*Bacillus subtilis*	332
		27.7% in 325	P80880	Thioredoxin reductase	*Bacillus subtilis*	315
		28.1% in 331	Q05741	Thioredoxin reductase	*Streptomyces clavuligerus*	321
		26.4% in 330	P52215	Thioredoxin reductase	*Streptomyces coelicolor*	321
IpuB	113	48.0% in 100	Q10839	Ferredoxin	*Streptomyces griseus*	105
		43.1% in 102	P13279	Ferredoxin	*Mycobacterium smegmatis*	106
		42.7% in 103	P00215	Ferredoxin	*Saccharopolyspora erythraea*	105
IpuC	460	33.5% in 421	P36205	Glutamine synthetase	*Thermotoga maritima*	439
		30.1% in 419	P10656	Glutamine synthetase	*Clostridium acetobutylicum*	443
		28.6% in 385	P45627	Glutamine synthetase	*Lactobacillus delbrueckii*	445
IpuD	387	30.9% in 340	P00183	Cytochrome P450-cam	*Pseudomonas putida*	414
		27.5% in 360	P26911	Cytochrome P450-soy	*Streptomyces griseus*	412
		30.1% in 375	O08469	Cytochrome P450	*Bacillus subtilis*	410

IpuE	65	32.8% in 64	P18325	Ferredoxin	*Streptomyces griseolus*	64
		40.3% in 62	P29603	Ferredoxin	*Pyrococcus furiosus*	66
		37.1% in 62	P00203	Ferredoxin	*Moorella thermoacetica*	63
IpuF	296	24.3% in 173	P04079	GMP synthetase	*Escherichia coli*	525
		25.4% in 142	P29727	GMP synthetase	*Bacillus subtilis*	718
		26.4% in 140	P44335	GMP synthetase	*Haemophilus influenzae*	523
IpuG	477	21.5% in 455	P24207	Phenylalanine-specific permease	*Escherichia coli*	456
		17.7% in 453	P46349	GABA permease	*Bacillus subtilis*	469
		23.0% in 370	Q46170	Arginine/ornithine antiporter	*Clostridium perfringens*	476
IpuH	508	42.1% in 484	O94788	Retinaldehyde-specific dehydrogenase	*Homo sapiens (Human)*	499
		43.9% in 481	P05091	Mitochondrial aldehyde dehydrogenase	*Homo sapiens (Human)*	517
		42.3% in 487	P47895	Aldehyde dehydrogenase 6	*Homo sapiens (Human)*	512
IpuI	546	29.7% in 481	P41751	Aldehyde dehydrogenase	*Aspergillus niger*	497
		30.8% in 481	P08157	Aldehyde dehydrogenase	*Emericella nidulans*	497
		29.9% in 481	P40108	Aldehyde dehydrogenase	*Cladosporium herbarum*	496
IpuJ	259	27.4% in 212	A82958*	Hypothetical protein	*Pseudomonas aeruginosa*	222
		25.8% in 198	A28199*	Na^+/K^+-exchanging ATPase alpha chain	*Gallus gallus*	1021
		27.6% in 199	B37227*	Na^+/K^+-exchanging ATPase alpha-3 chain	*Gallus gallus*	1010

* Accession Number of the PIR database

Sequence analyses of the ipu gene loci.

The amino acid sequences of the proteins potentially encoded by the eight open reading frames of the *ipuABCDEFGH* gene cluster and by the *ipuIJ* gene cluster were compared to the amino acid sequences of proteins deposited in databases (Table 9). The deduced IpuA protein consisted of 327 amino acid residues (35 kDa) and exhibited similarity to thioredoxin reductases (about 30% identity). Further investigations of the amino acid sequence revealed that IpuA does not contain the pair of redox-active cysteines typical for thioredoxin reductases, which are involved in the transfer of reducing equivalents from the FAD cofactor to the substrate (Arnér & Holmgren, 2000; Williams et al., 2000). Therefore IpuA is presumed to be a new type of reductase.

The *ipuB* gene encoded a putative 12.2 kDa ferredoxin with one [4Fe–4S] cluster and one [3Fe-4S] cluster in the C-terminal domain. Ferredoxins are water-soluble proteins, which contain at least one iron-sulfur cluster and function in two-electron transfer reactions (Harayama et al., 1991). The combination of a reductase-like protein (IpuA) and a ferredoxin (IpuB) could constitute the electron transport component of a multicomponent oxygenase system.

The protein encoded by *ipuC* was most closely related to various glutamine synthetases (30 % amino acid identity). Glutamine synthetases catalyze the ATP-dependent incorporation of ammonia into glutamate to form glutamine (Eisenberg et al., 2000; Brown et al., 1994). This enzyme regulates the assimilation of inorganic nitrogen and hence plays a key role in controlling bacterial metabolism. The amide group of glutamine is a source of nitrogen in the biosynthesis of a variety of compounds, such as tryptophan, histidine, carbamoyl phosphate, glucosamine 6-phosphate, CTP, and AMP.

The protein IpuD (44 kDa) displayed strong similarity to monooxygenases of the type cytochrome P-450. Monooxygenases catalyze hydroxylation reactions in which one atom of O_2 appears in the product and the other in H_2O. Cytochrome $P\text{-}450_{CAM}$, to which the sequence of IpuD is most related (30 % amino acid identity), is a 414–amino acid protein from *Pseudomonas putida* that catalyzes the hydroxylation of camphor into 5-exo-hydroxy-camphor (Raag & Poulos,

1991). In combination, IpuA, IpuB and IpuD could function as a three component oxygenase system.

The *ipuE* gene encoded a 7.1 kDa protein, which by analogy to related proteins is a ferredoxin with a single [3Fe-4S] cluster. It has an alanine in the position where [4Fe-4S] ferredoxins have a fourth cysteine ligand to the cluster (O'Keefe et al., 1991).

The IpuF protein (32.5 kDa) was most closely related to the hydrolase subunit of several GMP synthetases, which catalyze the final step in guanine ribonucleotide biosynthesis. They belong to the enzyme family of the amidotransferases and utilize the ammonia derived from the hydrolysis of glutamine for a subsequent amidation reaction catalyzed by the same enzyme (Raushel et al., 1999; Zalkin, 1993). The combination of IpuC, a hypothetical glutamine synthetase, and IpuF, a hypothetical glutamine hydrolase, indicates that glutamine or a derivate of glutamine is involved in the degradation of IPA.

The *ipuG* gene encoded a putative 49.9 kDa amino acid protein which has weak similarity to members of the amino acid permease family. IpuD displayed similarity (21 % identity) to PheP, the phenylalanine-specific permease of *Escherichia coli* (Pi & Pittard, 1996).

IpuH, a 54 kDa protein, and IpuI, a 57.5 kDa protein, were most closely related to aldehyde dehydrogenases (about 43.9 % and 30.8% identity, respectively). Aldehyde dehydrogenases are enzymes that oxidize a wide variety of aliphatic and aromatic aldehydes to acids (Sophos et al., 2001; Hempel et al., 1999).

The deduced IpuJ protein (28.5 kDa) exhibited similarity to a hypothetical protein (PA5509) of *Pseudomonas aeruginosa* with unknown function and to a short part (198 amino acids) of the alpha chain of Na^+/K^+ exchanging ATPases (more than 1000 amino acids long). In this region no motifs were found which would indicate a possible function of this protein.

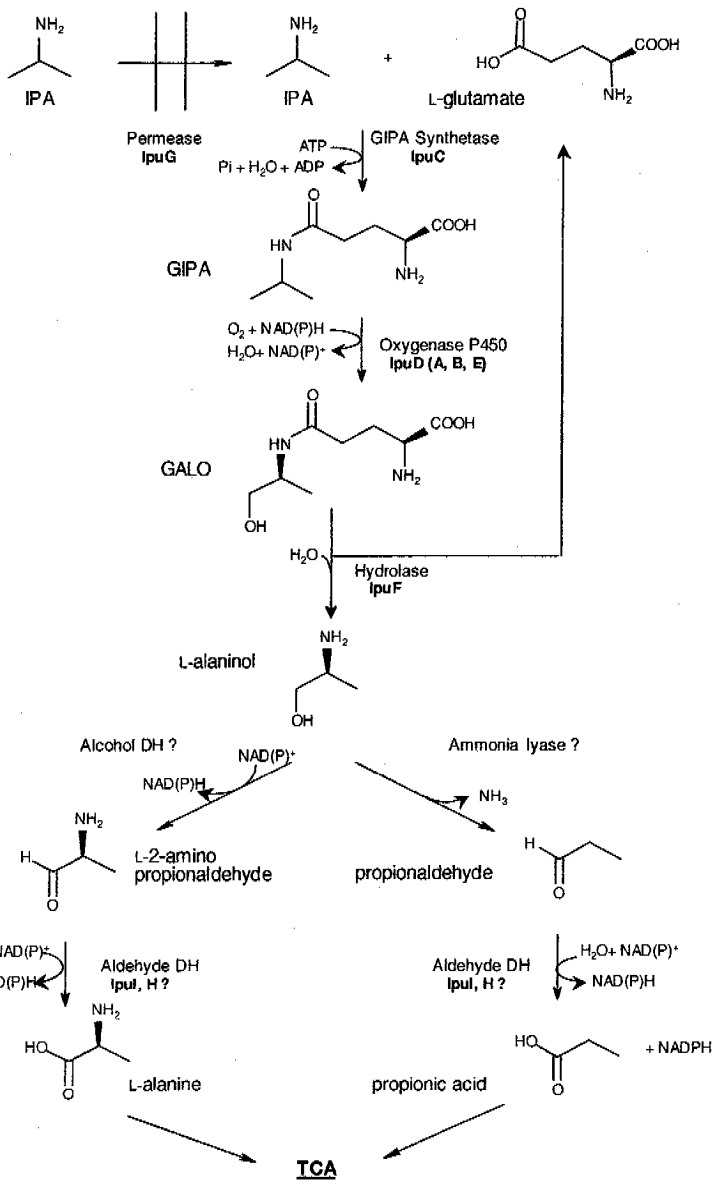


Fig. 16. Hypothetical pathway of IPA degradation by *Pseudomonas* sp. KIE171.

On the basis of these results, the pathway shown in Figure 16 is suggested for the degradation of IPA. According to this hypothetical scheme, IPA is transported through the cytoplasmic membrane by the putative permease IpuG. Once in the cell, IpuC catalyzes the formation of GIPA, in which the amino-group is protected by the formation of an amide bond. Stereospecific hydroxylation then occurs by an oxygenase system composed of IpuD, IpuA, IpuB and IpuE, generating GALO (the amide of L-glutamate and L-alaninol). The hydrolase IpuF hydrolytically cleaves the amide bond to generate L-glutamate and L-alaninol. For the further breakdown of L-alaninol, two possibilities are conceivable. L-alaninol could be oxidized by an alcohol dehydrogenase to yield L-2-aminopropionaldehyde. Alternatively, L-alaninol could be deaminated by an ammonia lyase to yield propionaldehyde. For neither possibility we have found genes that could code for the postulated enzymes. Whatever pathway is used, the aldehyde formed is probably further oxidized by the aldehyde dehydrogenases IpuI and IpuH to either L-alanine or propionic acid, compounds which can be processed by reactions of the general metabolism.

4.4 Biochemical analyses in support of the proposed pathway for IPA degradation.

Purification of His_6-IpuC.

To find evidence for the hypothetical IPA degradation pathway, two of the proteins involved, IpuC and IpuF, were purified and subjected to preliminary characterization. According to the proposed scheme, IpuC catalyzes the formation of GIPA from IPA and L-glutamate (Figure 17).

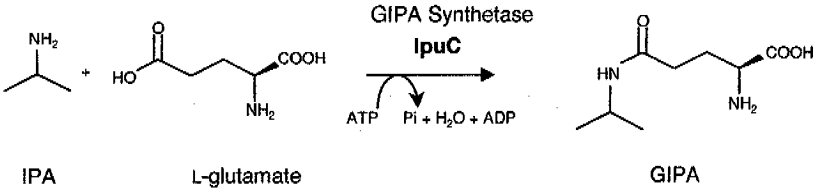


Fig. 17. Formation of GIPA form IPA and L-glutamate by IpuC.

To simplify its purification, IpuC was expressed as an N-terminally histidine-tagged fusion protein and purified in one step by metal chelate affinity chromatography (Figure 18). The expression level of IpuC was high and the molecular weight was, as expected, about 52.5 kDa. The purified His_6-IpuC protein was stable for several days when stored at 4°C in elution buffer.

To test whether the His-tag had an effect on enzymatic activity, the specific activity of crude extract containing IpuC was compared with the specific activity of crude extract containing His_6-IpuC. Both proteins represented about 40% of the total soluble protein, as determined by visual inspection of their intensities on SDS-PAGE. Crude extract containing His_6-IpuC exhibited the same level of activity as crude extract containing wild type IpuC, that is 0.67 U/mg protein and 0.75 U/mg protein, respectively (Figure 19). A crude extract prepared from *E. coli* BL21(DE3) served as negative control and exhibited a specific activity of 0.02 U/mg protein.

Consequently it was concluded that the N-terminal His_6-tag had no influence on the specific activity of IpuC. For this reason the His_6-IpuC enzyme produced from the overexpression plasmid pME4275 was used for further studies.

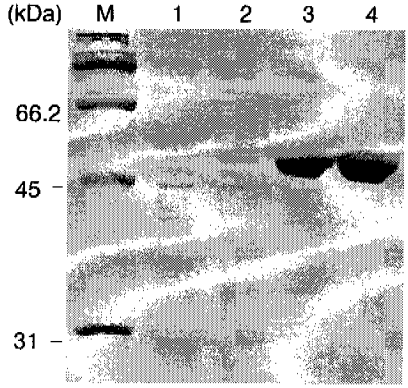


Fig. 18. Purification of His_6-IpuC from *E. coli* BL21(DE3)(pME4275). Protein samples (15 µg) were analyzed on 12% SDS-PAGE and stained with Coomassie Brilliant Blue. M, molecular weight markers (with molecular masses indicated on the left in kDa); lane 1, crude extract of induced *E. coli* BL21(DE3); lane 2, crude extract of uninduced *E. coli* BL21(DE3)(pME4275); lane 3, crude extract of induced *E. coli* BL21(DE3)(pME4275); lane 4, purified His_6-IpuC.

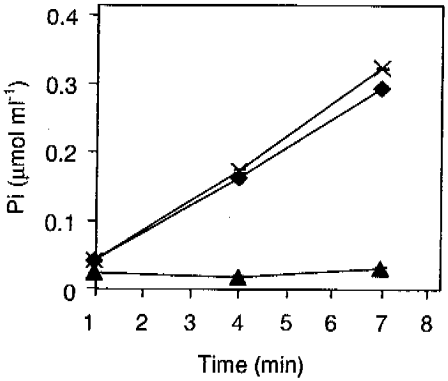


Fig. 19. Time-dependent liberation of Pi under standard assay conditions by extracts from strains *E. coli* BL21(DE3)(pME4277), *E. coli* BL21(DE3)(pME4275) and by a crude extract of the host strain *E. coli* BL21(DE3). Each vial contained 25 µg protein in a total volume of 400µl. x, wild type IpuC; ♦, His_6-IpuC; ▲, negative control.

Characterization of purified His_6-IpuC.

Since the sequence of IpuC showed similarity to the sequence of glutamine synthetases, we adapted a protocol for the biosynthetic assay for glutamine synthetase. In the assay, the formation of inorganic phosphate from ATP during the enzymatic reaction was measured colorimetrically. The presence of L-glutamate, IPA, $MgCl_2$ and purified His_6-IpuC in the reaction mixture was essential for the liberation of inorganic phosphate from ATP. Alternatively, the disappearance of the substrates and the appearance of products was analyzed by HPLC. Approximately equimolar amounts of γ-GIPA, ADP and phosphate were produced from IPA, glutamate and ATP. To examine the substrate range of IpuC, 33 potential substrates other than L-glutamate and IPA were tested (Tables 10 and 11).

The carboxylic acids presented in Table 10 were tested as potential substrates replacing L-glutamate. None of them, including D-glutamate, was a substrate for the enzyme. These results indicate that IpuC is specific for L-glutamate, whereas it accepts a large number of possible amino-alkane substrates.

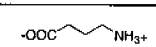
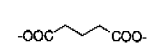
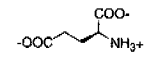
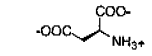
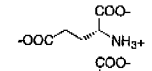
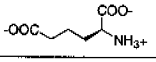
The substrates used to test the formation of the corresponding γ-glutamylamide products are listed in Table 11. For all these substances, the appearance of inorganic phosphate from ATP was measured. The sixteen substrates with which pure His_6-IpuC showed activity were amino-alkanes, amino-alcohols and amino-esters. Twelve compounds tested contained a positive or a negative charge in the side chain and were not used as substrates. The secondary amine piperazine was also not a substrate for IpuC. It is concluded that only primary amines are substrates. Further, the substrate should not have a positive or a negative charge on the side chain, whereas it can contain an ester or an alcohol group.

The formation of γ-glutamylamide compounds from the substrates methylamine (MeA), ethylamine (EtA), propylamine (PrA) and butylamine (BuA) was investigated by HPLC. The products of the biotransformation reaction were detected after 5 hours of incubation and as control the same substrates were incubated without enzyme (Figure 20). This assured that the enzymatic reaction

with His_6-IpuC was responsible for the formation of the γ-glutamylamide products.

A decrease of the substrate peaks (MeA, EtA, PrA and BuA) and of the peak corresponding to glutamate in incubation mixtures containing His_6-IpuC was observed in comparison to mixtures from which the enzyme was omitted. The peak representing ATP decreased more strongly when enzyme was present than in the controls without enzyme. The ADP peak correspondingly became larger. New peaks appeared in mixtures containing enzyme and these are thought to correspond to γ-glutamyl-methylamide (g-GmeA), γ-glutamyl-ethylamide (g-GetA), γ-glutamyl-propylamide (g-GprA) and γ-glutamyl-butylamide (g-GbuA). For γ-glutamyl-ethylamide (theanine) the presumed identity of the peak was confirmed by cochromatography with the pure compound. The progressive increase in the retention time of the peaks corresponding to the C1-C4 γ-glutamyl-amides is in agreement with the presence of an additional methylene group in each of these compounds.

Table 10. Screening carboxylic acids as substrates by measuring the liberation of inorganic phosphate (Pi) from ATP by pure His6-IpuC under standard assay conditions.

Substrate (10 mM)		Pi*
4-AMMINOBUTYRATE		-
GLUTARATE		-
L-GLUTAMATE		+
L-ASPARTATE		-
D-GLUTAMATE		-
L-2-AMINOADIPATE		-

*Definitions: + = phosphate production > 1 mM after 15 min incubation, - = phosphate production < 0.1 mM after 15 min incubation.

Table 11. Screening amines as substrates by measuring the liberation of inorganic phosphate (Pi) from ATP by pure His_6-IpuC under standard assay conditions.

Substrate (10 mM)	Pi*	Substrate	Pi*
AMMONIA	-	BUTYLAMINE	+
METHYLAMINE	+	4-AMINOBUTYRATE-METHYLESTER	+
ETHYLAMINE	+	1,4-DIAMINOBUTANE	-
ETHANOLAMINE	+	i-BUTYLAMINE	+
GLYCINE	-	s-BUTYLAMINE	+
GLYCINEMETHYLESTER	+	S-2-AMINO-1-BUTANOL	+
PROPYLAMINE	+	R-2-AMINO-1-BUTANOL	+
1-AMINO-2-PROPANOL	+	t-BUTYLAMINE	+
3-AMINO-1-PROPANOL	+	1-AMINOPENTANE	+
i-PROPYLAMINE	+	1,6-DIAMINOHEXANE	-
R-2-AMINO-1-PROPANOL	+	L-LYSINE	-
S-2-AMINO-1-PROPANOL	+	L-ARGININE	-
2-AMINO-1,3-PROPANEDIOL	+	PIPERAZINE	-
L-ALANINE	-		-
D-ALANINE	-	TAURINE	

*Definitions: + = phosphate production > 1 mM after 15 min incubation, - = phosphate production < 0.1 mM after 15 min incubation.

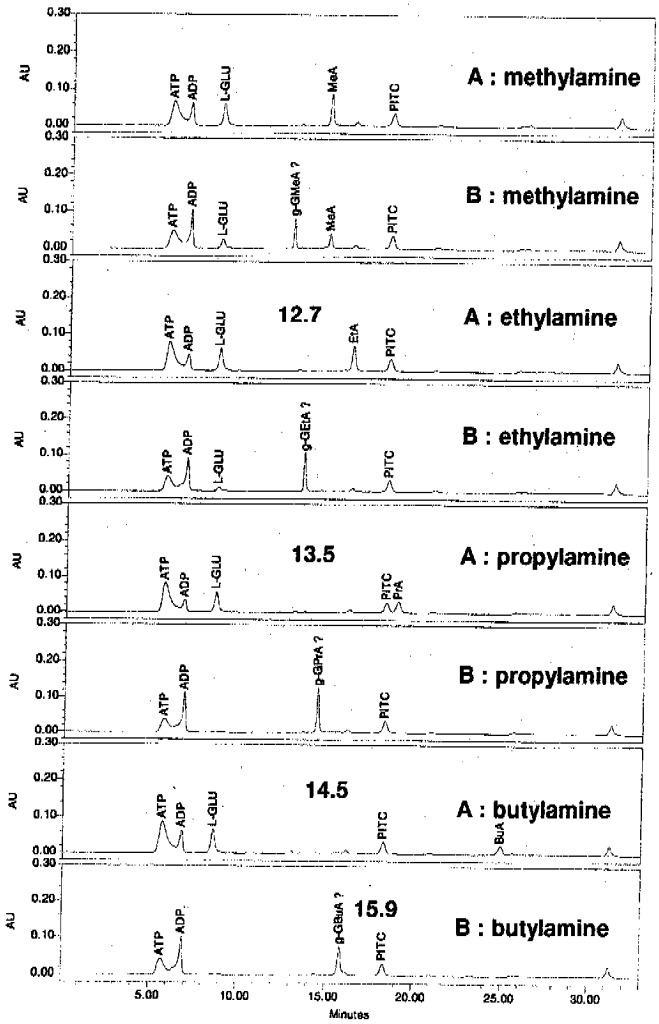


Fig. 20. Characterization by HPLC of γ-glutamylamide compounds formed by His_6-IpuC under standard assay condition. The biotransformation was performed during 5 hours. **A** = without His_6-IpuC, **B** = Incubation with His_6-IpuC, L-glutamate (L-GLU) and the following amines: methylamine (MeA), ethylamine (EtA), propylamine (PrA) and butylamine (BuA). The products formed are thought to be: γ-glutamyl-methylamide (γ-GMeA), γ-glutamyl-ethylamide (γ-GEtA), γ-glutamyl-propylamide (γ-GprA), γ-glutamyl-butylamide (γ-GbuA) and phenylisothiocyanate (PITC). ADP also appeared in the non-enzymatic reaction because of spontaneous hydrolysis of ATP.

Molecular oxygen is required for the synthesis of L-alaninol from IPA.

The second step of the proposed pathway involves a monooxygenase system composed of IpuD(ABE) (Figure 21). This system requires molecular oxygen, which can be demonstrated indirectly by determining the incorporation of labeled oxygen into the product.

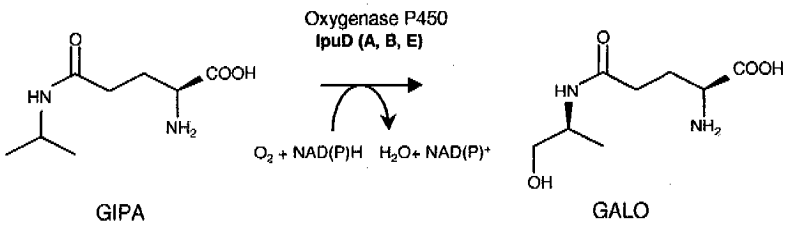


Fig. 21. Formation of GALO from GIPA by IpuD(A,B,E).

To confirm that the conversion of IPA into L-alaninol requires molecular oxygen, a biotransformation reaction was carried out with cell suspensions of strain KIE171-BIII in the presence of $^{18}O_2$. The reaction products were analyzed by high performance liquid chromatography-mass spectrometry. The fragmentation patterns from the mass spectra of supernatant obtained after incubation with $^{18}O_2$ and with $^{16}O_2$ showed that one atom of dioxygen was incorporated into L-alaninol. However, the fragmentation pattern was buried in a high background. It was therefore necessary to filter out the background in the desired mass area. The labeling experiment was performed twice. In both instances the background was high, and no clear unfiltered data were available. It has to be pointed out that even the measurement of the controls with unlabeled oxygen caused problems. Since the retention time of unlabeled L-alaninol was varied, it was difficult to reproduce the results.

A second approach was pursued to demonstrate the requirement for oxygen of the transformation reaction by performing the biotransformation of IPA in the absence of oxygen. As expected, no L-alaninol could be detected after performing the biotransformation reaction during 20 hours in the absence of oxygen (Table 12). The lack of product is a strong indication that molecular oxygen is required for the synthesis of L-alaninol.

Table 12. Dependence of L-alaninol formation from IPA by cell suspensions at OD_{650} 50 of strain KIE171-BIII on the presence of molecular oxygen.

Incubation conditions	Substrates and product after 20 hours of incubation		
	L-glutamate (mM)	L-alaninol (mM)	IPA (mM)
- O_2	20	0	20
+ O_2	0.4	4	8

Purification of His_6-IpuF.

According to the proposed pathway, IpuF catalyzes the third step in the IPA degradation pathway, the hydrolysis of GALO to L-alaninol and L-glutamate (Figure 22).

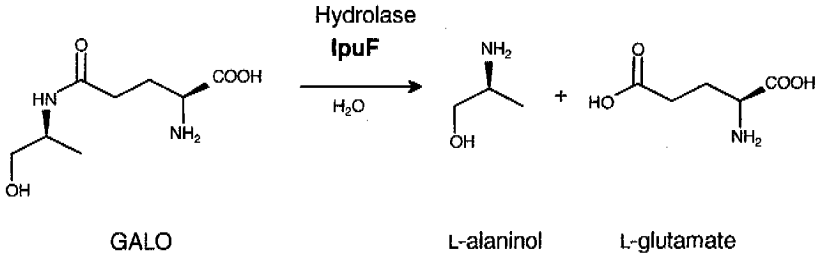


Fig. 22. Formation of L-alaninol and L-glutamate from GALO by IpuC.

Purification of IpuF was performed by metal chelate affinity chromatography as an N-terminally His_6-tagged fusion protein, expressed from pME4751 in *E. coli* BL21 (DE3). To check whether the His-tag had an effect on enzymatic activity, the specific activity of crude extract containing IpuF was compared with the specific activity of crude extract containing His_6-IpuF. Both proteins were expressed in the host *E. coli* BL21 (DE3) containing the corresponding plasmids. In both cases IpuF represented about the same amount of the total soluble protein (5-10%), as determined by visual inspection of the intensity of the corresponding band on SDS-PAGE (Figure 23). Crude extract containing His_6-IpuF exhibited about the same level of activity for GIPA as crude extract containing wild type IpuF, that is 1.35 U/mg protein and 1.44 U/mg protein, respectively. A crude extract prepared from *E. coli* BL21(DE3)(pET-28a(+)) served as negative control and exhibited no activity. We routinely used GIPA as substrate since the proposed physiological substrate, GALO, contained about 5% of L-glutamate, which perturbed the two-step enzymatic reaction. The L-glutamate background was so high that it was not possible to detect the increase of L-glutamate obtained by the reaction with His_6-IpuF.

It was concluded that the N-terminal His_6-tag had no significant effect on the specific activity of IpuF. On this account the His_6-IpuF enzyme produced from the overexpression plasmid pME4751 was purified and characterized.

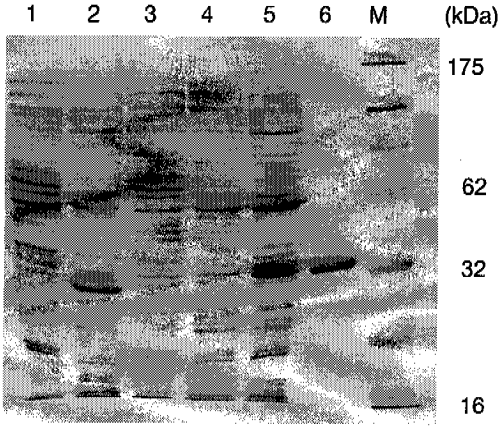


Fig. 23. Expression of IpuF from *E. coli* BL21(DE3)(pME4756) and purification of His_6-IpuF from *E. coli* BL21(DE3)(pME4751). Protein samples (20 µg) were analyzed on 12% SDS-PAGE. *M,* molecular weight markers (with molecular masses indicated on the right in kDa); lane 1, crude extract of induced *E. coli* BL21(DE3); lane 2, crude extract of induced *E. coli* BL21(DE3)(pME4756) expressing wild type IpuF (30 kDa); lane 3, crude extract of uninduced *E. coli* BL21(DE3)(pME4751) expressing His_6-IpuF (32 kDa); lane 4, crude extract of induced *E. coli* BL21(DE3)(pET-28b(+); lane 5, crude extract of induced *E. coli* BL21(DE3)(pME4751) expressing His_6-IpuF (32 kDa); lane 6, purified His_6-IpuC.

Characterization of purified His_6-IpuF.

Purified His_6-IpuF was stable upon storage at –20°C in buffer containing 20% (v/v) glycerol. It exhibited a specific activity of 13.5 U/mg of protein with GIPA as substrate at 30°C and pH 8. The activity of the enzyme dropped rapidly during incubation at 30°C with a half-life of 19.3 minutes. It was therefore not possible

to obtain a reliable temperature optimum for the enzyme. All reactions were performed at 30°C, which was the temperature at which wild type and mutant strains were grown. The subunit molecular mass, calculated from the His_6-IpuF amino acid sequence to be 34.9 kDa, was estimated by SDS-PAGE analysis as 35 kDa. The subunit molecular mass, calculated from the wild type IpuF amino acid sequence of 32.4 kDa, was estimated by SDS-PAGE analysis as 32 kDa. In order to determine the structural state of His_6-IpuF and IpuF, non-denaturing gel electrophoresis was performed. His_6-IpuF and wild type IpuF were monomers (results not shown).

Estimation of the kinetic constants and the substrate range of IpuF.

The pure enzyme His_6-IpuF showed a Michaelis-Menten-type saturation curve with respect to GIPA and GALO. Initial rates were calculated from the slope of product increase by regression analysis. Kinetic parameters were determined from double-reciprocal plots according to Lineweaver-Burk (Stryer, 1995).
The K_M of His_6-IpuF for GIPA was found to be 111 mM and the V_{max} was 13.4 U/mg (Figure 24). The contamination of GALO with about 5% L-glutamate prevented the determination of IpuF activity by the standard assay (see 3.15). The K_M of His_6-IpuF for GALO thus was estimated by measuring the glutamate liberated from the substrate by HPLC. Using this method, preliminary values of 66.2 mM for the K_M and of 1.5 U/mg for V_{max} were found for GALO (Figure 25). Preliminary determination of the K_M for GIPA with the HPLC method gave a value of 66.7 mM. This indicated that the K_M values for GIPA and GALO are in the same range.
His_6-IpuF-catalyzed hydrolysis was observed with GALO, GIPA, L-glutamine, theanine and γ-glutamyl-para-nitroanilide, but not with reduced γ-glutathione, nor with γ-glutamyl-alanine (Data not shown).

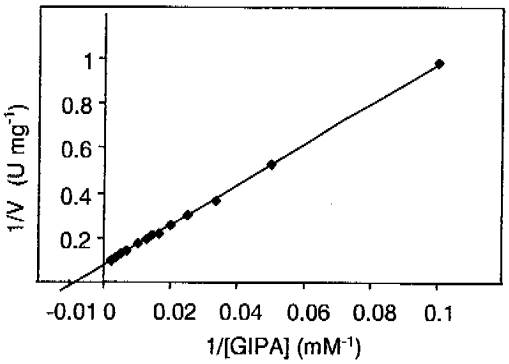


Fig. 24. Lineweaver-Burk plot for the determination of the K_M for GIPA. Enzymatic activity was determined by the standard assay.

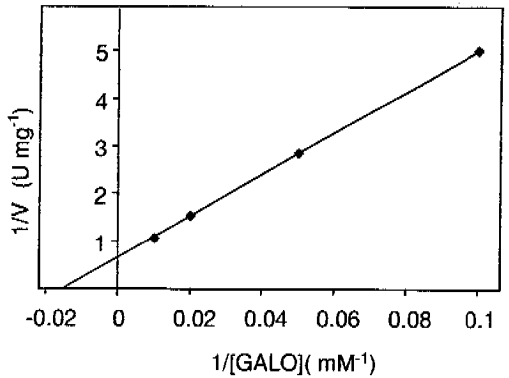


Fig. 25. Lineweaver-Burk plot for the determination of the K_M for GALO. Enzymatic activity was determined by following glutamate liberation by HPLC.

4.5 Biotransformation experiments.

Biotransformation of L-alaninol by strains KIE171-BI and KIE171-B.

Strain KIE171-BI was able to transform IPA to L-alaninol, and it was considered of potential interest for the production of L-alaninol, especially since the enantiomeric excess of the L-alaninol formed was more than 99% (Dr. A. Kiener, personal communication).

The conditions for the transformation of IPA to L-alaninol by strain KIE17-BI (*ipul*) were roughly optimized with respect to the concentration and the regime of addition of IPA and glutamate to the culture medium (Table 13 and Figure 26). Furthermore it was of interest to examine whether or not insertional inactivation of *ipul* in this strain prevented further metabolism of the L-alaninol accumulated in the medium.

Table 13. Culture conditions tested for the transformation of IPA to L-alaninol by growing cells of strain KIE171-BI. Four different biotransformation conditions were tested. 10 mM IPA and 10 mM L-glutamate were added once at time zero (A). For cultures B, C, and D, additional portions of IPA and/or L-glutamate were added after 5, 17, 29 and 41 hours of incubation. After 17 hours of incubation the cultures formed flakes, which disturbed the OD_{650} measurement.

culture	IPA (mM)	L-glutamate (mM)	max. yield* L-alaninol	Time point of max. yield* (h)	OD_{650} 0 h	17 h	41 h
A	1 x 10	1 x 10	22.5 %	17	0.22	1.6	0.8
B	1 x 10	5 x 10	35 %	29	0.22	0.75	1.6
C	5 x 10	1 x 10	3.5 %	41	0.22	0.62	0.42
D	5 x 10	5 x 10	14 %	41	0.22	0.63	0.44

* L-Alaninol yield relates to mol of L-alaninol recovered from the culture supernatant per mol of IPA added.

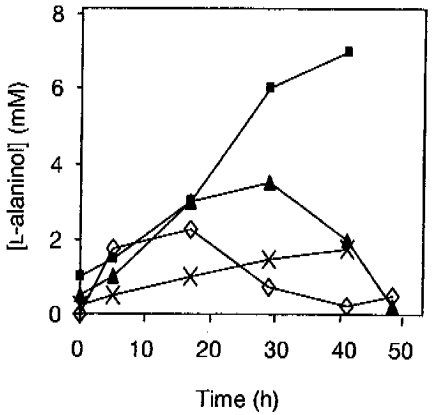


Fig. 26. Time course of L-alaninol formation by growing cultures of strain KIE171-BI. The incubation conditions of cultures A (◇), B (▲), C (x), and D (■) are defined in Table (13). At time zero the OD_{650} was 0.2. The product was detected by gas chromatography.

Three main conclusions were drawn from this experiment. First, upon prolonged incubation, L-alaninol disappeared again from the medium. This was observed with cultures A and B, where L-alaninol could not be detected anymore in the supernatant after 48 hours of incubation. In cultures C and D, IPA was repeatedly added over time and the L-alaninol degraded may have been replaced by newly formed product. Second, repeated addition of L-glutamate during the biotransformation increased the L-alaninol yield. Third, repeated addition of IPA to a final concentration of 50 mM did not lead to a proportional increase of L-alaninol yield, but disturbed the cell growth (Table 13).

The quantity of L-alaninol produced by cell suspensions (OD_{650} of 50) of strains KIE171-B and KIE171-BI was compared. In both cases L-alaninol had disappeared from the medium after 8 hours of incubation (Figure 27). These results show that strain KIE171-BI (*ipul*) is also unsuitable for the production of L-alaninol because it further metabolizes the L-alaninol accumulated in the medium. There was no difference in the biotransformation patterns of strains KIE171-B and KIE171-BI. The presumption that a reversion of the

uncharacterized mutation in strain KIE171-B is responsible for the degradation of L-alaninol thus might be wrong. An alternative explanation for the metabolism of the L-alaninol accumulated in the medium is provided by the hypothesis that in addition to IpuI, a second enzyme is involved in the degradation of L-alaninol. KIE171-BI carries a mutation in *ipuI*, whose product shows similarity to aldehyde dehydrogenases. This mutation was responsible for the ability to transiently accumulate L-alaninol from IPA. The second enzyme involved in the degradation of L-alaninol in strains KIE171-B and KIE171-BI might have a similar function as the hypothetical aldehyde dehydrogenase IpuI.

In the *ipu*-operon, the gene product of *ipuH* also shows similarity to aldehyde dehydrogenases. Assuming that the hypothetical aldehyde dehydrogenase IpuH was also involved in the degradation of L-alaninol, two approaches were followed to obtain a strain unable to further degrade L-alaninol once it has accumulated in the medium. In a first approach, I examined the effect of inactivating both *ipuI* and *ipuH* on the accumulation of L-alaninol. In the second approach, the genes required for L-alaninol formation from IPA were expressed in *E. coli*, an organism unable to degrade L-alaninol.

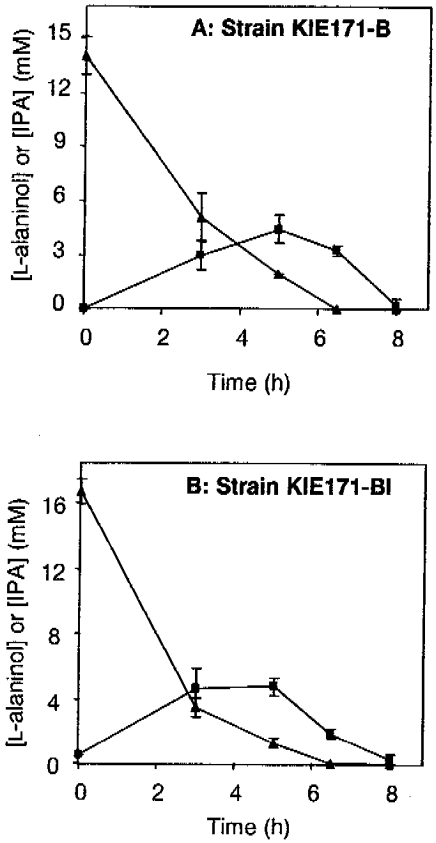


Fig. 27. Comparison of the biotransformation of IPA (▲) to L-alaninol (■) with cell suspensions at OD_{650} 50 of strain KIE171-B and strain KIE171-BI. (A) Biotransformation was started by the addition of 14 mM IPA for strain KIE171-B and (B) of 16 mM IPA for strain KIE171-BI. 20 mM L-glutamate was added at time zero. IPA and L-alaninol were determined by gas chromatography.

Biotransformation of L-alaninol by strain KIE171-BIII.

In the first approach to prevent the degradation of L-alaninol, strain KIE171-BIII (*ipuH, ipuI*) was constructed (see 3.8). It was examined whether mutational inactivation of both hypothetical aldehyde dehydrogenases (IpuH, IpuI) in this organism had an effect on the pattern of accumulation of L-alaninol in biotransformation experiments.

Like strain KIE171-BI (*ipuI*), strain KIE171-BIII was unable to grow with IPA and with L-alaninol and it converted IPA to L-alaninol. The transformation of IPA to L-alaninol by strain KIE171-BIII was followed in cultures growing with 20 mM L-glutamate as carbon source in the presence of 20 and 50 mM IPA. As shown in Figure 28, the level of L-alaninol accumulated in the medium remained stable over a period of 100 h.

The same result was obtained with cell suspensions at OD_{650} 50 of strain KIE171-BIII. In biotransformation experiments with three different concentrations of IPA (20, 50 and 100 mM) the L-alaninol formed was not degraded over a time period of 60 hours (Figure 29).

A direct comparison of the formation of L-alaninol from IPA by strains KIE171-BI and KIE-BIII under identical conditions is shown in Figure 30. It is evident that with cells of the former organism L-alaninol accumulated transiently, while with cells of the latter, the compound, once accumulated in the medium, appeared to remain stable. In conclusion, inactivation of both, the *ipuI* and *ipuH* genes, appears to prevent the breakdown of L-alaninol, whereas a single insertion in the *ipuI* gene is not sufficient for blocking the degradation of L-alaninol.

To better understand the function of *ipuH* in the degradation pathway of IPA, mutant KIE171-BIV was constructed (see 3.8). This mutant carries an insertion in *ipuH* and *ipuI* is functional. It was able to grow with 20 mM IPA as the sole carbon source. Thus, although its gene is located in the *ipu* cluster, the putative aldehyde dehydrogenase IpuI appears to be dispensable for growth with IPA.

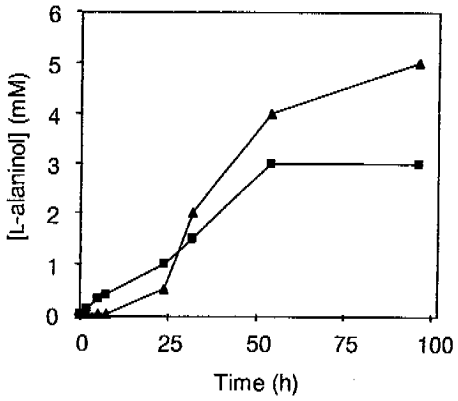


Fig. 28. Time course of L-alaninol formation by growing cultures of strain KIE171-BIII. The concentration of IPA at time zero was for culture A (▪) 20 mM and for culture B(▲) 50 mM. The maximum L-alaninol yield was 15% for culture A and 10% for culture B. The carbon source for both cultures was 20 mM L-glutamate. At time zero the OD_{650} was 0.3 and after 24h > 1.5 for both cultures. L-Alaninol was determined by HPLC.

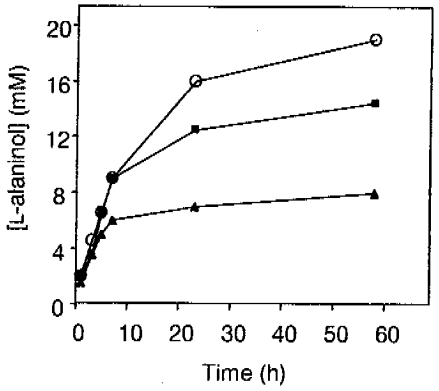


Fig. 29. Time course of L-alaninol formation by cell suspensions at OD_{650} 50 of strain KIE171-BIII. The concentration of IPA at time zero was for culture C (▲) 20 mM, for culture D (■) 50 mM and for culture E (○) 100 mM. The maximum L-alaninol yield was 40% for culture C, 29% for culture D and 19%for culture E. 25 mM L-glutamate was added at time zero as energy supply. L-Alaninol was determined by HPLC.

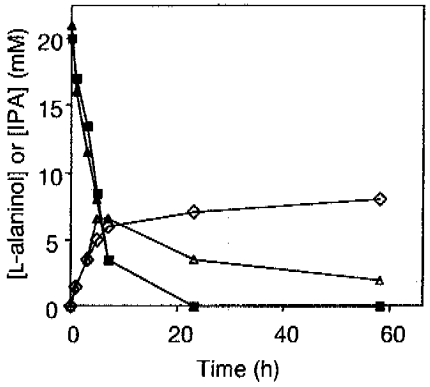


Fig. 30. Comparison of the biotransformation of 20 mM IPA to L-alaninol with cell suspensions at OD_{650} 50 of strain KIE171-BIII and strain KIE171-BI. IPA disappearance in cultures of strain KIE171-BI (▲) or strain KIE171-BIII (■), and L-alaninol formation in cultures of strain KIE171-BI (△) or strain KIE171-BIII (◇) were followed by HPLC analysis. 25 mM L-glutamate was added at time zero.

An unresolved question in all biotransformation experiments described in this work relates to the missing compounds in the balance of IPA. As described above (Figures 28, 29), the yield of L-alaninol from IPA on a mol to mol basis varied between 10% and 40 %. HPLC analysis of culture supernatants from transformation experiments with strain KIE171-BIII revealed that IPA was transformed not only to L-alaninol, but also to significant amounts of GIPA and GALO (not shown). Quantification of these additional transformation products was not reliable, but a rough estimate makes it appear likely that GIPA and GALO account for the major part of the missing products derived from IPA.

Biotransformation of L-alaninol by strain E. coli BL21(DE3) (pME4755).

In the second approach to prevent the further metabolism of L-alaninol, all genes required for L-alaninol formation were expressed in an organism, which cannot use L-alaninol. For this purpose, plasmid pME4755, which contains the *ipuABCDEFG* genes under the control of the T7 RNA polymerase promoter of

the intermediate-copy vector pBBR1MCS, was constructed (see 3.9). *E. coli* BL21(DE3) harboring the expression plasmid pME4755 was grown on 10 mM glucose and protein expression was induced by the addition of IPTG. These cells were concentrated to a suspension of OD_{650} of 50 and used for the biotransformation of IPA under the conditions used for biotransformations with cell suspensions of OD_{650} of 50 of stain KIE171-BIII. A biotransformation with *E. coli* BL21(DE3) harboring pBBR1MCS was used as negative control.

The final concentrations of substrate and product of a biotransformation with cell suspensions at OD_{650} 50 of *E. coli* BL21(DE3) (pME4755) with 15.5 mM IPA and 25 mM L-glutamate demonstrated that some L-alaninol was formed. It can be concluded that all genes necessary for the production of L-alaninol from IPA were cloned on the expression plasmid pME4755. L-Alaninol was degraded neither by the recombinant *E.coli* strain nor by strain KIE171-BIII. The latter organism was the better strain for the production of L-alaninol under the conditions examined (Figure 31).

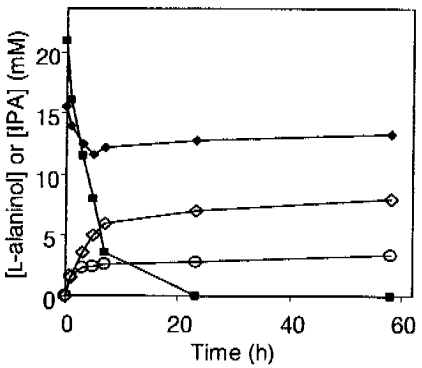


Fig. 31. Biotransformation of 20 mM and 15.5 IPA to L-alaninol with cell suspensions at OD_{650} 50 of strain KIE171-BIII and strain *E. coli* BL21(DE3)(pME4755). IPA disappearance in cultures of strain KIE171-BIII (■) or *E. coli* BL21(DE3)(pME4755) (♦), and L-alaninol formation in cultures of strain KIE171-BIII (◇) or *E. coli* BL21(DE3)(pME4755) (○) were followed by HPLC. 25 mM L-glutamate was added at time zero.

Production of theanine by strain KIE171-BIII.

A matter of particular interest was the fact that IpuC catalyzes the synthesis of a wide range of γ-glutamylamides, including theanine. Theanine is a promising compound for industry because of its high market price and its increasing commercialization in recent years. The possibility to produce theanine with whole cells was therefore studied. In a procedure similar to the transformation of IPA to L-alaninol, theanine was produced from ethylamine by strain KIE171-BIII in growing cultures as well as in cell suspensions. IPA had to be added during the cell growth to induce the expression of the Ipu-proteins, otherwise product formation was not observed (results not shown).

Preliminary experiments indicated that high yields of theanine are preferentially obtained with growing cells or with cell suspensions at a comparatively low OD_{650} of 10. In a growing cell culture with 50 mM ethylamine as substrate, 31.8 mM theanine was obtained (yield of 63%) after 24 hours, whereas the theanine yield in a cell suspension was always less (Table 14).

Table 14. Biotransformation of ethylamine to theanine with cell suspensions at different OD_{650} of strain KIE171-BIII. The product theanine was determined by HPLC. 20 mM L-glutamate was added at time zero as energy supply. By experience the margin of error for ethylamine and theanine is in the range of 20%.

KIE171-BIII	Cell suspension OD_{650} 10		Cell suspension OD_{650} 35	
Ethylamine at start time	20 mM	100 mM	20 mM	100 mM
Theanine yield after 18 (hr)	44%	9.5%	11.5%	3.1 %

5. Discussion

5.1 The IPA degradation pathway.

Although several reports on the degradation of primary amino-alkanes have appeared, the degradation of IPA has only been poorly explored (see 2.2). The IPA degradation pathway of *Pseudomonas* sp. KIE171 was deduced from the sequence of the IPA utilization genes *ipuABCDEFGHI*. It is, to our knowledge, the first degradation pathway of IPA with a chiral compound as an intermediate (Figure 16) and thus differs fundamentally from two previously proposed degradation pathways of IPA (Figure 6). This pathway is supported by experimental evidence at the level of the IpuC and IpuF enzymes, and by the identification, by HPLC analysis, of GIPA, GALO and L-alaninol as intermediates in the degradation of IPA by strain KIE171-BIII.

IpuC is a γ-glutamyl-isopropylamide synthetase.

The amino acid sequence of the first enzyme of IPA degradation, γ-glutamyl-isopropylamide synthetase IpuC (GIPA-synthetase), exhibited about 30% identity to the sequences of several glutamine synthetases. Preliminary characterization revealed that the enzyme catalyzes the condensation of IPA and L-glutamate to GIPA and that ammonium is not a substrate (see 4.4). The active site of glutamine synthetases from eukaryotes, prokaryotes (Eisenberg et al., 2000; Liaw & Eisenberg, 1994) and archaea show fifteen invariant amino acid residues involved in the ATP-dependent conversion of ammonia and glutamate to glutamine. Thirteen of these fifteen invariant amino acid residues are present in IpuC (Appendix, Figure 34). In the active site, the side chains of aspartate and glutamate residues are directly involved in catalysis and form a negatively charged "pocket" (Alibhai & Villafranca, 1994). These two highly conserved residues sustain the selectivity of glutamine synthetases for the ammonium ion. The carboxylic side chain of aspartate is proposed to be involved in catalysis by deprotonating the ammonium ion. Hence, aspartate-71

in IpuC might be responsible for the deprotonation of the charged amino-group of IPA during catalysis. The nucleophilic species generated could then react with the γ-glutamyl phosphate intermediate according to the mechanism established by Alibhai and Villafranca (1994). The carboxyl side chain of glutamate is proposed to have a catalytic function by stabilizing the transition state, leading to the formation of a tetrahedral γ-glutamyl phosphate intermediate. This glutamate residue is replaced by tryptophan at position 324 in IpuC. Tryptophan-324 might be responsible for the particular substrate range of IpuC that differs from that of glutamine synthetases. IpuC uses a broad range of amino-alkanes, amino-alcohols and amino-esters, whereas it does not use substrates containing a positive or negative charge in the side chain.

In glutamine synthetases, a conserved serine residue interacts with the glutamate residue, which is involved in the catalysis (Liaw & Eisenberg, 1994). This serine residue is also not conserved in IpuC.

Prokaryotic glutamine synthetases are regulated by reversible covalent adenylylation of the phenolic hydroxyl side chain of a specific tyrosine (Almassy et al., 1986). The adenylylated form of the enzyme is inactive and can be readily activated by deadenylylation (Jiang et al., 1998). Tyrosine-393 as well as the consensus pattern of the adenylylation site is present in IpuC, which indicates that, like in glutamine synthetases of other prokaryotes, a control of the activity of the enzyme by adenylylation may exist.

The hypothetical monooxygenase complex IpuD, and IpuA,B,E.

The second enzyme in the degradation pathway is IpuD, a hypothetical monooxygenase, whose amino acid sequence exhibited 31% identity to cytochrome P-450$_{CAM}$, the well characterized bacterial camphor hydroxylase of *Pseudomonas putida* (Poulos, 1995; Raag et al., 1993; Poulos & Raag, 1992; Raag et al., 1991, 1990; Raag & Poulos, 1989a,b; Poulos et al., 1987). I found that the oxygen atom of L-alaninol originates from molecular oxygen and that the formation of L-alaninol from IPA is strictly dependent on the presence of

oxygen (see 4.4). These observations support the notion that the hydroxylation reaction leading to L-alaninol is indeed catalyzed by a monooxygenase.

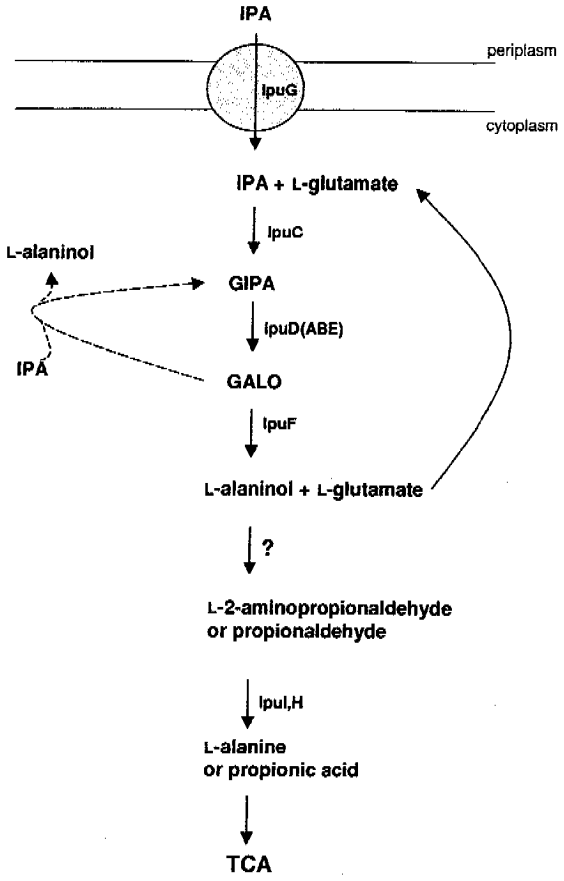


Fig. 32. The IPA degradation pathway of *Pseudomonas* sp. KIE171. The dashed lines indicate a hypothetical transferase reaction.

The presence of aspartate-236, threonine-237, glutamate-352 and of the heme-binding cysteine-343 indicates that the residues involved in the hydroxylation reaction of cytochrome P-450$_{CAM}$ are conserved in IpuD (Appendix, Figure 34). The carboxylic side chain of aspartate-236 might be implicated in the proton transfer required for oxygen-oxygen scission, according to the results obtained with cytochrome P-450$_{CAM}$ (Vidakovic et al., 1998). The heme group is most likely covalently attached through its iron to the thiolate-sulfur of cysteine-343. The residues involved in substrate binding in cytochrome P-450$_{CAM}$ are not conserved in IpuD. In cytochrome P-450$_{CAM}$ the hydroxyl group of tyrosine-96 is coupled to camphor by a single hydrogen bond, and the hydroxyl group of threonine-101 forms a hydrogen bond to one pyrrole of the heme, resulting in a small movement of camphor (Schlichting et al., 2000). In IpuD the corresponding residues are isoleucine-77 and isoleucine-82. The hydrophobic side-chain of isoleucine-77 may be involved in the fixation of the hydrophobic isopropyl-moiety of the substrate GIPA. An additional difference between cytochrome P-450$_{CAM}$ and IpuD is the presence of threonine-238 in the latter. In cytochrome P-450$_{CAM}$ this residue corresponds to valine-253.
Mutation of valine-253 of cytochrome P-450$_{CAM}$ to threonine is thought to protect cytochrome P-450$_{CAM}$ from "uncoupling" (Raag & Poulos, 1991). Substrates of similar shape as camphor (for example norcamphor and camphane) are hydroxylated by P-450$_{CAM}$ at multiple positions and promote the formation of hydrogen peroxide and/or water. This phenomenon is termed "uncoupling" and drops the efficiency of the hydroxylation. The presence of threonine-238 in IpuD may prevent "uncoupling" and may allow IpuD to hydroxylate several different compounds efficiently. An indication of the relaxed substrate specificity of IpuD might be obtained by examining the utilization of amino-alkyl compounds such as ethylamine, propylamine, butylamine as growth substrates by *Pseudomonas* sp. KIE171.

The amino acid sequence of IpuA exhibits 28% identity to various thioredoxin reductases. Sequence alignments revealed that the two active site cysteines typical for thioredoxins (Williams et al., 2000) are absent in IpuA (Appendix, Figure 34). Since the two FAD binding domains and the NAD(P) binding site are

present, IpuA seems to be a reductase, but not a "thioredoxin" reductase. The same is true for the hypothetical YumC homologue from *Bacillus subtilis* (B70015, PIR data base accession number), which does not have the active site cysteines. These observations suggest a new class of reductases.

IpuB and IpuE exhibit strong sequence identity (48% and 40%) to ferredoxins. A [4Fe-4S] and a [3Fe-4S] cluster are present in IpuB, whereas IpuE contains a single [3Fe-4S] cluster (Appendix, Figure 34). Cytochrome P-450 based monooxygenase systems contain usually only one electron transport component (Degtyarenko, 1995). This suggests that IpuB or IpuE might function with IpuA and IpuD as a three-component cytochrome P-450 based monooxygenase system, responsible for the stereospecific hydroxylation of GIPA. Further, the possibility of a four-component system comprising two ferredoxins with different redox potentials should be considered. Otherwise, the significance of a second ferredoxin in the IPA degradation pathway remains unclear.

The hydrolase IpuF.

The amino acid sequence of γ-glutamyl-2-amide-L-propanol hydrolase, IpuF, exhibited similarity to the hydrolase domain of guanosine 5'-posphate (GMP) synthetases. GMP synthetases catalyze the hydrolysis of glutamine and the ATP dependent formation of GMP from xanthosine 5'-posphate (XMP). Two different domains of GMP synthetase are implicated in these reactions. The amino-terminal domain is responsible for glutamine hydrolysis and it is named "glutamine amide transfer" (GAT) domain of type G. The carboxy-terminal domain is the actual synthetase involved in GMP synthesis (Zalkin, 1993). IpuF corresponds in sequence to the amino-terminal GAT-domain. It is 227-422 amino acids shorter than the full length GMP synthetases (Appendix, Figure 34).

Preliminary characterization of IpuF confirmed that the enzyme hydrolyses GALO (see 4.4). Further substrates for IpuF were GIPA, glutamine and

theanine. However, IpuF showed a K_M of 66.2 mM for GALO and of 111 mM for GIPA, which are both several orders of magnitude higher than the K_M values available from the literature for bacterial GMP synthetase. The K_M values for glutamine hydrolysis of GMP synthetases range from 0.2 to 1 mM (protein database BRENDA: EC 6.3.5.2).

Two active site residues, cysteine and histidine, have been shown to be involved in the hydrolysis of glutamine by the GAT-domain (Raushel et al., 1999). Both residues, cysteine-104 and histidine-200, are present in the amino acid sequence of IpuF (Appendix, Figure 34). This indicates that the mechanism for the hydrolysis of GALO may be similar to the hydrolysis of glutamine. The reaction may involve the attack of the thiolate anion of cysteine-104 on the carbonyl γ-carbon of GALO, yielding L-alaninol. An activated water molecule may subsequently hydrolyze the thioester intermediate.

The formation of GIPA requires ATP (see 4.4). In order to save energy, it would be advantageous for the organism not to hydrolyze GALO, but to transfer its glutamyl-moiety onto IPA. A hypothetical transferase domain of IpuF catalyzing the exchange of the alaninol-moiety of GALO for IPA would result in the regeneration of GIPA without investment of ATP (Figure 32). Two observations are inconsistent with a transferase function of IpuF. On the one hand IpuF hydrolyzes GIPA, the product of such a transferase reaction, and on the other hand the size of IpuF indicates the absence of the transferase function. However, it cannot be excluded that an unknown protein provides the missing transferase function.

Alcohol dehydrogenase or ethanolamine ammonia lyase.

It is not clear how the degradation of L-alaninol is initiated in *Pseudomonas* sp. KIE171. The presence of the aldehyde dehydrogenase genes *ipuI* and *ipuH* in the *ipu* gene clusters indicates that an aldehyde is formed in the degradation of L-alaninol. An alcohol dehydrogenase or an ethanolamine ammonia lyase are the two types of enzymes that may catalyze the reaction from L-alaninol to an

aldehyde, i.e. to L-2-aminopropionaldehyde or propionaldehyde (Figure 32). In the *ipu*-gene cluster no translation product could be identified, which shows similarity to an alcohol dehydrogenase or to an ethanolamine ammonia lyase.
It is thus evident that one or several enzyme(s) in *Pseudomonas* sp. KIE171, which are not present in the *ipu* gene clusters, oxidize the alcohol. One should also consider the option that an alcohol dehydrogenase, mainly involved in other metabolic processes, might accept L-alaninol as a substrate.
The degradation of L-alaninol by an ethanolamine ammonia lyase for the metabolism of L-alaninol was preliminarily investigated by Dr. Jan van der Ploeg. In *Pseudomonas aeruginosa* PAO1, two *orfs* of the ethanolamine (*eut*) operon, *eutB* and *eutC,* are present that show similarity to the large subunit and the light chain of ethanolamine ammonia lyases (Stover et al., 2000). By designing appropriate primers for ethanolamine ammonia lyases, it was possible to amplify by PCR from the chromosome of *Pseudomonas* sp. KIE171 a fragment whose sequence showed similarity to ethanolamine ammonia lyase. However, it could not be shown that the product of this hypothetical *orf* is essential for L-alaninol catabolism (van der Ploeg, unpublished). The *ipuABCDEFGH* gene cluster seems to encode the major part but not the entire IPA degradation pathway.

Aldehyde dehydrogenases IpuI and IpuH.

IpuI and IpuH exhibit 30% and 40% similarity, respectively, to NAD-dependent aldehyde dehydrogenases. In both proteins the active site motif as well as the NAD binding site motif are present (Appendix, Figure 34). These amino acids are glutamate-275 and cysteine-312 for IpuH and glutamate-314 and cysteine-348 for IpuI. A catalytic mechanism of aldehyde dehydrogenases was proposed by Hempel et al. (1999). The discovery of the *ipuI* and *ipuH* genes was decisive for the construction of appropriate strains for L-alaninol production. To produce L-alaninol from IPA and to prevent its further metabolism, both genes putatively encoding aldehyde dehydrogenases, *ipuI* and *ipuH*, had to be inactivated in *Pseudomonas* sp. KIE171.

One might also postulate that one or both of the putative aldehyde dehydrogenases catalyze the oxidation of L-alaninol to the aldehyde as well as the oxidation of the aldehyde to the corresponding acid. However, aldehyde dehydrogenases reacting with both, an aldehyde and its alcohol precursor, are not described in the literature. In contrast, alcohol dehydrogenases having dismutase activity are able to form an alcohol and an acid from the corresponding aldehyde (Höög et al., 1999; Hinson & Neal, 1972). Bifunctional enzymes, harboring an aldehyde/alcohol dehydrogenase function, are found in *E. coli* and other eubacteria (Sanchez, 1998; Goodlove et al., 1989). However, these enzymes are fusion proteins of an amino-terminal aldehyde dehydrogenase to a carboxy-terminal alcohol dehydrogenase. Evidence from sequence alignments and the predicted protein sizes are not supportive for IpuI and/or IpuH being bifunctional aldehyde/alcohol dehydrogenases.

The permease IpuG.

Since at physiological pH IPA possesses a charged amino-group, transport across the cytoplasmic membrane has to be mediated. Studies concerning urea and short chain amide uptake demonstrated the presence of a high-affinity ABC transport system in the methylamine metabolizing *Methylophilus methylotrophus* (Mills et al., 1998). Due to the similar chemical properties of IPA and urea, IPA uptake might take place using a comparable transport system. However, in the *ipu* operon no genes coding for proteins of a hypothetical primary transport system were identified, whereas the gene *ipuG,* which codes for a secondary transport system is present.

IpuG in *Pseudomonas* sp. KIE171 shows 20% amino acid sequence similarity to amino acid permeases of the amino acid–polyamine organocation (APC) superfamily and might be responsible for IPA transport. The APC superfamily of transport proteins includes members that function as solute:cation symporters and solute:solute antiporters (Jack et al., 2000; Hu & King, 1999, 1998). Two permeases similar to IpuG, the phenylalanine-specific permease (PheP) from *E. coli.* (Pi et al., 1998; Pi & Pittard, 1996; Pi et al., 1991) and the 4-aminobutyrate

permease (GabP) from *Bacillus subtilis* (Brechtel & King, 1998) have been characterized.

The ipu-operon might be located on a catabolic transposon.

The *ipuABCDEFGH*-operon is flanked by two hypothetical IS-elements (see 4.3). This suggests that it could be part of a catabolic transposon for IPA degradation. Catabolic transposons confer on microorganisms the ability to degrade various xenobiotica like chlorobiphenyl (Springael et al., 1993), *p*-toluensulfonate (Junker & Cook, 1997) or 3-chlorobenzoate (Ogawa & Miyashita, 1999). Transposons allow the horizontal spreading of degradative genes among members of microbial communities and thereby accelerate the adaptation of microorganisms to the use of new substrates (Tan, 1999; van der Meer et al., 1992). Sequence analysis of ISR and ISL that flank the *ipuABCDEFGH* operon revealed that the start codons of the genes encoding the putative transposases are missing. Further, more than one frame-shift is present in the sequences encoding the hypothetical transposases. Also, the IR of ISR could not be identified, and a duplication of nucleotides upon transposition into the target site was not found. This indicates that the genes encoding the transposases are not functional anymore. However, it cannot be excluded that the *ipuABCDEFGH* operon becomes transposable again by site-specific recombination with an element containing a functional transposase gene, as was shown for a defective transposon containing genes coding for naphthalene degradation (Tsuda & Iino, 1990). Evidence that the *ipu*-genes originate from other organisms is obtained from a comparison of the G+C content of the *ipu* operon with the G+C content typical for *Pseudomonas* sp.. Members of the genus *Pseudomonas* vary in the G+C content between 58% and 68% (Palleroni, 1993). For example, the *Pseudomonas aeruginosa* PAO1 genome has an overall G+C content of 66.6% (Stover et al., 2000). This contrasts strongly with the G+C content of the *ipu*-operon of only 51%.

Occurrence of other amine degradation pathways involving a γ-glutamylamide intermediate?

The IPA degradation pathway established in this work provides insight into the catabolism of primary amines. It is now of interest to investigate if degradative systems for primary amines that involve a γ-glutamyl synthetase, an oxygenase and a hydrolase occur also in other bacteria. Former studies on the degradation of aniline (aminobenzene) by *P. putida* (Fukumori & Saint, 1997) and by *Acinetobacter* sp. strain YAA (Fujii et al., 1997) demonstrated that genes coding for γ-glutamyl synthetase, oxygenase and hydrolase-like proteins might be involved in the degradation of aniline. However, no suggestion for their function in the degradation of aniline was provided.

The findings made in the present work strongly suggest that aniline degradation might occur by a catabolic pathway similar to the IPA degradation pathway (Figure 33). Sequence alignments of the hypothetical γ-glutamylamide synthetases TdnQ and BAA10 of the aniline degradation pathway with IpuC (Appendix, Figure 36) revealed, that the residues corresponding to tryptophan-324 of IpuC are leucines in these enzymes and not glutamates as in all known glutamine synthetases. Due to the predicted function of this residue in the active site, it might be involved in the acceptance of aniline as a substrate. The presence of dioxygenases, as well of the GAT-domain only in the hypothetical hydrolases TdnT and BAA11 (Appendix, Figure 36) strengthens the suggestion for the aniline degradation pathway.

The pattern of adjacent *orfs* coding for a hypothetical glutamine synthetase, an oxygenase and a hydrolase was not found in the completely sequenced genome of *P. aeruginosa* PAO1. However, six *orfs* were found that code for hypothetical glutamine synthetases. The strictly conserved glutamate residue, which is present in characterized glutamine synthetases, is similarly to IpuC not present in five of these *orfs* (Appendix, Figure 35). The residues are tyrosine, serine and tryptophan, as it is the case for tryptophan-324 of IpuC. Consequently these putative enzymes might be γ-glutamyl synthetases. The two *orfs* adjacent to these five hypothetical γ-glutamyl synthetases of

Pseudomonas aeruginosa PAO1 code for hypothetical GMP synthetases and/or hypothetical APC permeases.

It is not clear why IPA and possibly other amines are degraded by *Pseudomonas* sp. KIE171 via a pathway involving eight gene products and requiring the investment of ATP. Oxidative deamination of IPA by a hypothetical amine dehydrogenase (see 2.2) would yield acetone. Since *Pseudomonas* sp. KIE171 is unable to grow on acetone, deamination of IPA would lead to a potentially toxic dead-end metabolite. Deamination of IPA thus is prevented by the use of a protecting group, the γ-glutamyl-moiety.

Examples for the use of protecting groups are found in the bacterial metabolism of arginine and proline. Starting from L-glutamate, synthesis of L-proline or L-arginine depends on whether or not the amino-group of L-glutamate is protected by an acetyl-group (Leisinger, 1996; Glansdorff, 1996). *P. aeruginosa* possesses four different pathways for the catabolism of arginine, each of which serves a different physiological function. In one of these pathways, L-arginine is succinylated and N^2-succinylarginine is converted via a series of succinylated intermediates to N-succinyl-L-glutamate and finally to L-glutamate and succinate. In this example succinylation marks the intermediates of arginine catabolism and thereby allows for their discrimination from the analogous, non-succinylated intermediates of arginine biosynthesis (Haas et al., 1990).

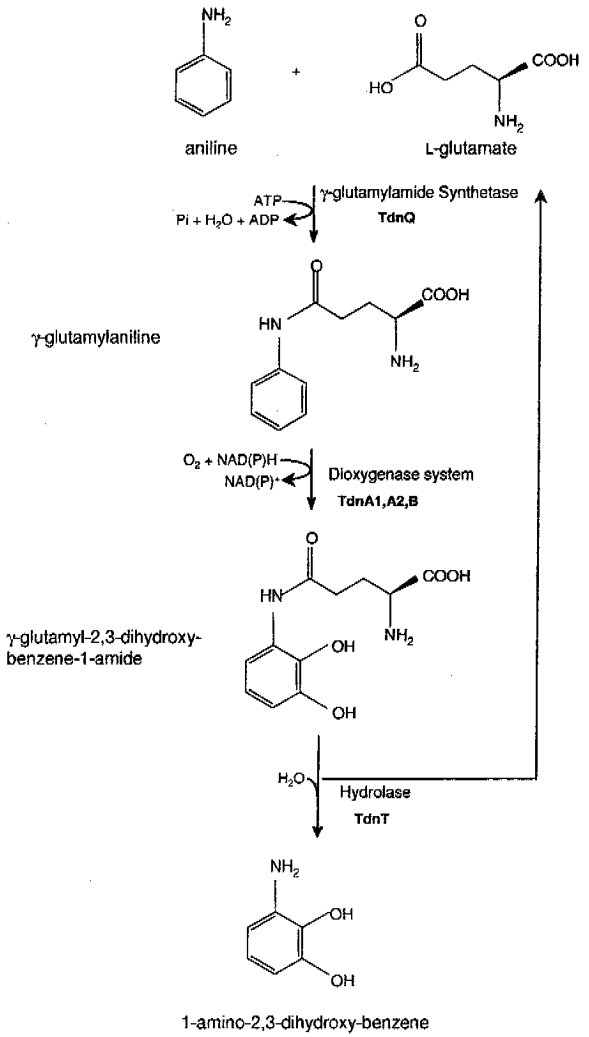


Fig. 33. Hypothetical degradation pathway of aniline in *Pseudomonas putida* UCC22(PTDN1) based on analogy to the IPA degradation pathway of *Pseudomonas* sp. KIE171. The protein functions proposed are: TdnQ, γ-glutamyl aniline synthetase; TdnA1,2 and TdnB, dioxygenase system; TdnT, hydrolase. The putative product, 1-amino-3,4-dihydroxy-benzene, is also shown.

5.2 Biotransformation: Strains and chemicals.

The present work has shown that, in order to obtain L-alaninol from IPA, it is necessary to block the degradation of L-alaninol. It appeared that two putative aldehyde dehydrogenases, IpuI and IpuH, are involved in the catabolism of L-alaninol. Two approaches were followed to prevent the degradation of L-alaninol. In a first approach, the effect of inactivating both genes, *ipuI* and *ipuH,* on the accumulation of L-alaninol was examined by construction of strain KIE171-BIII. In the second approach, the genes required for L-alaninol formation from IPA were expressed in *E. coli* BL21(DE3)(pME4755), an organism unable to degrade L-alaninol.

L-Alaninol producing strain Pseudomonas KIE 171-BIII.

Strain KIE171-BIII, in which *ipuI* and *ipuH* are inactivated, was entirely unable to metabolize L-alaninol (Figure 30). The highest yield obtained from 20 mM IPA was 40%, which corresponds to an L-alaninol concentration of 0.6 g per liter. To make the process with strain KIE171-BIII attractive for an industrial application, the yield has to be increased by a factor of about 20 (Dr. A. Kiener, personal communication). Yields in the range of 60-120 g product per liter can be obtained in industrial-scale processes for the production of L-carnitine with strain HK4 (Zimmermann et al., 1997), and similar yields have been achieved for the production of L-lysine (Lee et al., 1998) and glutamate with *Corynebacterium glutamicum* (Gourdon & Lindley, 1999). At Lonza, the production yields for heterocyclic compounds and non-proteinogenic amino acids are in the range of 9-65 g per liter (Schmid et al., 2001).
Biotransformation of IPA solutions of higher concentrations did not result in an increase of the L-alaninol yield. However, it was possible to increase the final L-alaninol concentration to 1.2 g per liter (Figure 29). In order to obtain a high concentration of the final product, we have worked with cell suspensions of OD_{650} 50 rather than with growing cells. However, with reference to the cell density, best turnover results were obtained with growing cells. The turnover for

20 mM IPA into L-alaninol in a growing cell culture of strain KIE171-BIII was 40 µM L-alaninol • OD_{650}^{-1} • h^{-1} during 50 hours. In the cell suspensions of strain KIE171-BIII it was 12 times lower for the same time period (3.2 µM L-alaninol • OD_{650}^{-1} • h^{-1}), while during the first five hours of the biotransformation the turnover was 20 µM L-alaninol • OD_{650}^{-1} • h^{-1} (Figures 28 and 29). The consumption of 20 mM IPA in a growing cell culture of wild type KIE171 was 1100 µM IPA • OD_{650}^{-1} • h^{-1}. Therefore, improvements might be obtained by optimizing the cell density when working with cell suspensions. The degradation of IPA requires energy, which is provided by L-glutamate. It is of interest to investigate whether the supply of a secondary carbon source such as gluconate, which specifically enhances the production of NADPH, increases the rate of L-alaninol production (Lee et al., 1998).

L-Alaninol producing strain E. coli BL21(DE3)(pME4755).

The IpuABCDEFG components of the IPA degradation system from *Pseudomonas* sp. KIE171 can be expressed in an active state in *E. coli* BL21(DE3). This result supports the notion that the hypothetical three-component cytochrome P-450-type based monooxgenase system IpuABD(E), the γ-glutamylisopropylamide synthetase IpuC, the hydrolase IpuF and the hypothetical permease IpuG provide all components necessary for the transformation of IPA to L-alaninol.

However, under the experimental conditions applied, the recombinant *E. coli* strain was inferior to KIE171-BIII with respect to L-alaninol formation (Figure 31). Strain KIE171-BIII produced L-alaninol constantly over 7 hours with a yield of 40%, whereas in *E. coli* L-alaninol synthesis ended after 3 hours with a final yield of only 15 % L-alaninol. This premature ending of L-alaninol synthesis in *E. coli* BL21(DE3)(pME4755) might be due to intrinsic factors that affect the performance of the enzymes. Such effects have been reported e.g. for the three-component alkane monooxygenase of *Pseudomonas oleovorans* expressed in *E. coli* (Staijen et al., 2000). Extrinsic factors such as availability of substrate and cosubstrate are also relevant for biotransformation and may affect the final yields obtained.

Chemicals produced by strain KIE171-BIII and IpuC.

A new process for the production of L-alaninol, theanine and other γ-glutamylamides was established in the present work. Theanine is the active ingredient of green tea (Hara, 2000; Graham, 1992) and it is used in the health care industry. Several benefits of theanine have been described, including relaxation, decrease of blood pressure and anti-cancer activity (Sadzuka et al., 2000 ; Sugiyama & Sadzuka, 1999; Sadzuka et al., 1996). A cost-effective procedure for the production of theanine, employing whole cells of strain KIE171 blocked in the monooxygenase IpuD, may be of industrial interest. Fifteen γ-glutamylamide compounds were synthesized in vitro with IpuC (Table 11). We expect that several other not purchasable γ-glutamylamide compounds may be produced in a similar way.

5.3 Outlook.

Further characterization of the proteins involved in the IPA degradation pathway defined in this study may provide approaches for improving the production of L-alaninol.

It is possible that the activity of IpuC, the GIPA-synthetase, is controlled similarly as in glutamine synthetases. One thus might focus on two points. First, one should investigate the influence of ammonium on the activity of IpuC. When *P. aeruginosa* is grown under conditions of nitrogen limitation, glutamine synthetases are in an unadenylylated active state, whereas an excess of ammonium triggers the conversion of the enzyme to its inactive adenylylated form (Janssen et al., 1980). Low concentrations of ammonia might have a positive effect on the activity of IpuC, thereby increasing the rate of GIPA synthesis. Further, if adenylylation is a control mechanism for IpuC, one might design a mutant of IpuC that lacks the adenylylation site.

Second, glutamine synthetases of *Salmonella typhimurium* and *E. coli* are feedback inhibited by a number of nitrogenous metabolites (Liaw et al., 1993).

IpuC might be similarly feedback-inhibited during the time course of the biotransformation by the formation of the compounds GIPA, GALO and L-alaninol.

Characterization of IpuD and further components of the monooxygenase system might be of interest. Since IpuD appears to catalyze a stereospecific hydroxylation reaction, it is of interest to investigate if IpuD hydroxylates further γ-glutamyl substrates. Studies of the other proposed components of the monooxygenase system, the putative new type of reductase IpuA and the two putative ferredoxins IpuB and IpuE, would lead to a better understanding of this new monooxygenase system. It is of interest to understand the presence of two genes encoding ferredoxins in the *ipu* gene cluster. In frame deletions of the genes encoding these proteins on plasmid pME4755 and analysis of monooxygenase activity in *E. coli* might clarify this question.

To increase the L-alaninol yield it is relevant to identify the rate-limiting step of the metabolic pathway. The high K_M of IpuF for GALO (see 4.4) indicated that IpuF might limit the turnover of GALO in the pathway. This might provoke the observed accumulation of GIPA and GALO during biotransformation of IPA. The effect of overexpressing IpuF on the rate of L-alaninol formation should therefore be investigated.

The export of L-alaninol into the medium remains unclear and might be interesting to investigate. It has been shown, that in the case of L-lysine production by *Corynebacterium glutamicum* an increase of the lysine yield was obtained by overexpression of LysE, the permease responsible for L-lysine export (Vrljic et al., 1996).

6. Appendix

Table 15. Primers used for this study.

Primer	Start base on strand	Sequence (5′ → 3′)
ipuABCDEFGH region		
	coding	
67-260599	27	TCAACATTGATGATGCGCGC
991166**	354	ACTGCAACCAACGACTTTGAG
9911949	893	GAACAGTTGGTTGGGATTG
9911954	1467	GATGGCTTGCCGCAAGTAG
9912146	1893	GTAACTGTCATTCACCGTCGA
60-301198 9821441	2635	AAGCGATCACCCAAGAGTTC
58-10698	3603	GTCGGAAGTCGGTTACAACT
58-18698	4268	TTCCACCCTTCGTAATGGGT
58-30698	4966	AACTACGCGACAATCCTGAG
58-6798	5622	AATACTGAAGCTGATCGCGC
58-150798	6170	GGGCGTGCAATACCATCCAG
58-300898	6408	CTCGATCACCTTGTTATTCC
58-110898	6460	GGTGGCTCCAGAAGTAGCAG
58-020998	6876	GGGTCGTATTGAGTGGCACG
58-090998	7377	TTGGGCACGTGATGCACGAC
58-110998	7859	GTCGTTCTACTGATCGTGGC
58-160998	8353	AGCGTTGATTCGCCTGCATC
58-230998	8738	GTGCCTGGGCTTGGTGGATC
60-051098	9259	TTGTATGGAGATTGCTCGCG
60-281098	9672	CGAATCCTTCAAACGAGAGG
60-091198	10159	ATGAGTGTGGTCTGCATGCG
60-291198	10660	CGATCTGTGCGACTACATCC
	non-coding	
9902038	662	GGTGATTGTAGAAGCGGATG
9902457	1280	CGCAGGTCCGATTCTCTATC
9902498	1781	CCTACAACTTGATCCA
9902649	2425	TCAATGTCACCGTAGGAGG
9902744	3031	GATACTGGAGATTGCCCTG
9902793	3597	TGCAGGCGCAATCTTTGTG
9902978	4242	TCGGGATTACGTGCCTCTTC
9903172	4654	ATGCCGACGAATACGGCAT
9903366**	5151	CATCCCCGACAATTCTGGAG
9903749	5536	CGTATATGTGAGAATT
9904082	6089	CTCAATACCCACGACGAG
9904179	6493	GCAATTCGTCCCTGGTGAG
9904564	6982	CTTCCGGATCTTGTTCCG
9904754	7537	ACCAGATACGGGTTGGTATC
60-150798 9811292	8147	GCGATAGAGTCGTTAA
60-100898 9813736	8531	CACCCAGCCTAATCGTGTCG
60-180898 9901374	9034	TATGGGTTGGCAGCACCGAG
67-280199 9901926	9446	ACGGTGAATGACAGTTACC

67-010299 9902358	9871	CTACTTGCGGCAAGCCATCA
67-040299 9902548	10235	CGAGCCTCTTCAAACACAGG
67-150299 9903075**	10711	TGAAAGCGCGCTCGCAA

ipuH region		
	coding	
52-090898	105	GGCTGAGCAATTCGAAGAG
52-170898	531	GATCTTAGTGAGATGGTCGC
52-230898	978	AATAGTCCTAGGCGGACGGC
56-22498	1957	TTCAAGGCTGTAACCTTGGC
56-11598**	2445	GGATGCCGATATCGAAACGG
56-17598	2884	TAAGTTCGGTCTGACCGCAA
58-110898**	3519	**GGTGGCTCCAGAA**GTAGCAG
56-120898	3981	CGATCTGCGCTACGTTGATC
	non-coding	
56-8598	297	TCAGTTGAATGACTCGGGTC
56-18598	871	AGCCTATCATCACCCTTCCA
56-130898	1991	ACGCTCTACCGAACAGCAGG
52-22498**	3441	GGTGATGT**TA**GGAGTTCTCC
52-8598	3885	ATAGCTGAAATTCTGCGCCG
52-18598	4436	TGCATTCGCAGAGGCTGTTT

Primers used to introduce restriction sites.

ipuC-NT*	AACAGGTGATACATATGAGCGAAG
ipuC-CT*	TTTGAAGCTTAGGATCTGGGCG
ipuF-NT*	GGGCCAGCTAGCATGGAAAAGCTTAG
ipuF-CT*	TCTACTGAGCTCTACTACTGCTAC
ipuA-NT*	GCCTTCTAGAATTCTTGTAGG

*The nucleotides changed to introduce a restriction site are underlined.
**Bases of the primers that differ from the sequence are shown in bold.

Fig. 34. Sequence alignments of Ipu proteins with the three most similar proteins. Alignments were performed with the program CLUSTALW (http://www2.ebi.ac.uk/cgi-bin/newclustalw.pl?). The accession numbers are, if not specified, of the Swiss-PROT database. The symbols in the consensus line stand for: "*" = identical or conserved residues in all sequences in the alignment; and ":" = conserved substitutions. The binding motifs were determined with profile scan server (http://www.isrec.isb-sib.ch/software/PFSCAN_ form.html) and are underlined.

IpuA

Sequence comparison of IpuA-like proteins. IpuA is the hypothetical reductase of *Pseudomonas* sp. KIE171 (Psp). B70015 is the accession number of the PIR database for the thioredoxin reductase YumC homologue of *Bacillus subtilis* (Bs). TrxB is thioredoxin reductase of *Streptomyces coelicolor* (Sco, accession no. P52215), of *Streptomyces clavuligerus* (Scl, accession no. Q05741) and *Bacillus subtilis* (Bs, accession no. P80880). Motifs are underlined and active site cysteines of thioredoxin reductase are on a grey background.

```
Bs    B70015: MREDTKVYDITIIGGGPVGLFTAFYGGMRQASVKIIESLPQLGGQLSALYPEKYIYDVAG : 60
Sco   TrxB  : ----SDVRNVIIIGSGPAGYTAALYTARASLKPLVFEGAVTAGGALMN---TTEVENFPG : 53
Scl   TrxB  : ----SDVRNVIIIGSGPAGYTAALYTARASLQPLVFEGAVTAGGALMN---TTDVENFPG : 53
Bs    TrxB  : --SEEKIYDVIIIGAGPAGMTAAVYTSRANLSTLMIERGIP-GGQMAN---TEDVENYPG : 54
Psp.  IpuA  : -MSNAKEYDLVIVGAGPIGLYAAYYAGFRGLKTALFDGLPQVGGQVATMYPEKLIHDVAG : 59
              :          :: *:* ** *  :* *          :::      ** :        : :  *
                         FAD (ADP-part)

Bs    B70015: FPK-IRAQELINNLKEQMAKFDQTICLEQAVESVEKQADGVFKLVQMK-KPTTLKRSCIT : 118
Sco   TrxR  : FQDGIMGPELMDNMRAQAERFGAELIPDDVVAVD--LSGEIKTVTDTAGTVHRAKAVIVT : 111
Scl   TrxB  : FRDGIMGPDLMDNMRAQAERFGAELIPDDVVSVD--LTGDIKTVTDSAGTVHRAKAVIVT : 111
Bs    TrxR  : FES-ILGPELSNKMFEHAKKFGAEYAYGDIKEV---IDGKEYKVVKAGSKEYKARAVIIA : 110
Psp.  IpuA  : FPG-IKGGDFVKNLLEQSQRQDYDLYLSELIVSLESLSDDTFSITTDRGNHYTAKAVVIA : 118
              : *   *   ::  ::  :  :        :             .:        :   ::

Bs    B70015: AGNGAFKPRKLELENAEQYEGKNLHYFVD-DLQKFAGRRVAILGGGDSAVDWALMLEPIA : 177
Sco   TrxR  : TGS---QHRKLGLPNEDALSGRGVSWCATCDGFFFKDQDIAVIGGGDTAMEEATFLSRFA : 168
Scl   TrxB  : TGS---QHRKLGLPREDALSGRGVSWCATCDGFFFKDQDIVVVGGGDTAMEEATFLSRFA : 168
Bs    TrxR  : AGA---EYKKIGVPGEKELGGRGVSYCAVCDGAFFKGKELVVVGGGDSAVEEGVYLTRFA : 167
Psp.  IpuA  : AGLGKCTPR--SLPALEGVDSPNIMHFVP-DLSVLDGNDVVIAGGGDSAVDWAIAAVPRA : 175
              : :*      :   :          :      *   :    : : ****:*::       *
                        active site cysteine       NAD(P) binding site

Bs    B70015: KEVSIIHRRDKFRAHEHSVENLHAS-KVNVLTPFVPAELIGED-KIEQLVLEEVKGDRKE : 235
Sco   TrxR  : KSVTIVHRRDTLRASKAMQERAFADPKISFVWDSEVAEVQGDQ-KLAGLKLRNVKTGELS : 227
Scl   TrxB  : KSVTIVHRRDSLRASKAMQDRAFADPKISFAWNSEVATIHGEQ-KLTGLTLRDTKTGETR : 227
Bs    TrxR  : SKVTIVHRRDKLRAQSILQARAFDNEKVDFLWNKTVKEIHEENGKVGNVTLVDTVTGEES : 227
Psp.  IpuA  : KSVTVIHRRARFRAHEASVKDMYES-GVRVVAPGEVAAYHEEN---GHEFLEIWSGDKKE : 231
              :   *:::***  :**             :             ::       *
```

```
Bs   B70015: ILEIDDLIVNYGFVSSLGPIKNWG-LDIEKNSIVVKSTMETNIEGFFAAGDICTYEGKVN : 294
Sco  TrxR  : DLPVTGLFIAIGHDPRTELFKGQLDLDPEGYLKVDAPSTRTNLTGVFGAGDVVDHTYRQA : 287
Scl  TrxB  : ELAATGLFIAVGHDPRTELFKGQLDLDDEGYLKVASPSTRTNLTGVFAAGDVVDHTYRQA : 287
Bs   TrxR  : EFKTDGVFIYIGMLPLSKPFENLGITNEEGYIETNDR-METKVEGIFAAGDIREKSLRQI : 286
Psp. IpuA  : ILNFDSFVMALGFHSDLGPMESWG-LGIEGFRIPVKPNMETNISRVFAIGDISEYPGKVR : 290
           :  :      :  *       ::       *           *::   *  **:     :
                                                    FAD (Flavin part)

Bs   B70015: LIASGFG-EAPTAVNNAKAYMDPKARVQPLHSTSLFENK : 332
Sco  TrxR  : ITAAGTGCSAAVDAEPFLAALSDEDKAEPEKTAV----- : 321
Scl  TrxB  : ITAAGTGCSAALDAERYLAALADSEQIAEPAPAV----- : 321
Bs   TrxR  : VTATGDGSIAAQSVQHYVEELQETLKTLK---------- : 315
Psp. IpuA  : LIAVGFG-EAAIAVNHAAAVISPELSVLPTHSTNEVK-- : 326
           : : * * *  *    :     :
```

IpuB

Sequence comparison of IpuB-like proteins. IpuB is the hypothetical ferredoxin of *Pseudomonas* sp. KIE171 (Psp.). Fer is ferredoxin of *Streptomyces griseus* (Sg, accession no. P13279), FdxA is ferredoxin of *Mycobacterium smegmatis* (Ms, accession no. P00215) and *Mycobacterium tuberculosis* (Mt, accession no. Q10839). Cysteines of the iron-sulfur binding region are on a grey background. A [4Fe-4S] cluster and a [3Fe-4S] cluster are present.

```
Sg   Fer   : -TYVIAQPCVDVKDKACIEECPVDCIYEGQRSLYIHPDECVDCGACEPVCPVEAIFYEDD : 59
Ms   FdxA  : -TYVIAEPCVDVKDKACIEECPVDCIYEGARMLYIHPDECVDCGACEPVCPVEAIYYEDD : 59
Mt   FdxA  : -TYVIGSECVDVMDKSCVQECPVDCIYEGARMLYINPDECVDCGACKPACRVEAIYWEGD : 59
Psp. IpuB  : MPYVISDPCIKSRDQACVDVCPVDCIYEAEGRLWIQPDECTECGACESVCPVTAESDDQD : 60
           :   ***   *:   *::*:: ********    *:*:**** :****:  * * *   : *

Sg   Fer   : TPEEWKDYYKANVEFF--------DDLGSPGGASKLGLIERDHPFVAGLPPQNA- : 105
Ms   Fer   : VPDQWSSYAQANADFF--------AELGSPGGASKVGQTDNDPQAIKDLPPQGED : 106
Mt   Fer   : LPDDQHQHLGDNAAFFHQVLPGRVAPLGSPGGAAAVGPIGVDTPLVAAIPVECP- : 113
Psp. IpuB  : LREAREFFDTVLPGCD--------GPIGSPRGAQKVGRIKSDHPRVLKIIKGVD- : 106
           :   :                      :*** **  :*    *   :  :
```

IpuC

Sequence comparison of IpuC-like proteins. IpuC is γ-glutamylisopropylamide synthetase of *Pseudomonas* sp. KIE171 (Psp.). GlnA are glutamine synthetases of *Clostridium acetobutylicum* (Ca, accession no. P10656), *Lactobacillus delbrueckii* subsp. Bulgaricus (Ld, accession no. P45627) and *Thermotoga maritima* (Tm, accession no. P36205). Motifs are underlined and active site amino acids are on a grey background. The adenylylation site is on a black background.

```
Ca  GlnA   : --MAK-YTKEDIINLVKENGVKFIRLQFTDIFGTLKN--------VAITDKQLEKALDNECM : 51
Ld  GlnA   : --MSKVITEEEIRKDVEEKNVRFLRLAFTDINGTLKN--------LEVPVSQLDDVLGNQTR : 52
Tm  GlnA   : ------MTIETIKRIIEEENVRFIRLQFTDINGTLKN--------LEITPDVFLESWEDGIM : 48
Psp.IpuC   : MSEENKKQILKVRDFIEKHNIDTIRLGAVDIDGVWRGKQVGAEYFLNKAALDGTQISNILFG : 62
           :            :   :::  :  :**   ** *  :
```

```
Ca   GlnA : -------FDGSSIDGFVRIEESDMNLRPNLDSFVIFPWR-PQQGKVARLICDVYKPDGTP : 103
Ld   GlnA : -------FDGSSIDGFVRLEESDMVLYPDLATWLVLAWTTVEEGTIGRLVCSVHNVDGTP : 105
Tm   GlnA : -------FDGSSIEGFVRIEESDMYLKPVLDTFAVLPWT-VDGAKSARVICDVYTPDGKP : 100
Psp. IpuC : WDVADHLVDGLEFTGWDSGYP-DIALIPDLSTLSLVPWQ----EKTASVLCDIQHLNGEP : 117
            :      :** ↑          *: * * * :  :  *          ::* :   :* *
                  glutamate instead of serine

Ca   GlnA : FEGDPRHVLKRANADAKELGYTMNVGP-ECEFFLFET-------DENGRATTNTQDKAGY : 155
Ld   GlnA : FEGDPRNNLKKVIAEMEEMGFSDFEIGFEAEFFLFKE-------GKNGEETTKVSDHSSY : 158
Tm   GlnA : FEGDPRYRLRRMMEKAEQLGYTPYAGP-EMEFFILPI-------NEKGEPVPEFLDHGGY : 152
Psp. IpuC : LNLSPRNLLRKAIEKAEQLGYKCYAAY-EFEFYLLNDSIASISADQWRSINPVEKSGHCY : 176
            : ::  **  *::      :::*:       * **:::          :            *

Ca   GlnA : FDLAPTDLGENARRDMTLALEEMGFEIEASHHEVAEGQNEIDFKYGDALTTADNIMTFKL : 215
Ld   GlnA : FDMASEDEGAKCRREIVETLEKLGFRVEAAHHEVGDGQQEIDFRFDNALATADKLQTFKM : 218
Tm   GlnA : FDLLPLSKVEEIRRDIAIALEKMGITVEATHHEVAPSQHEVDFRYDTFLRTADNAQTVKL : 212
Psp. IpuC : -SMLHHSSSSDIMGEVRKYMRDAGIVLEATNSEHGPGQYEINIKYDDALKAADDAIFVKN : 235
            :   :            ::   :   *: :**:: *    * *:::::.  * :**     *

Ca   GlnA : VVKSIAQRHGLHASFMPKPIFGINGSGMHVNMSLFKDG-KNAFVDENDKNGLSKVAYQFI : 274
Ld   GlnA : VVKTIARKYHLHASFMAKPVEGLAGNGMHTNMSLLKDG-KNAFYDKDGQYNLSTTALTFL : 277
Tm   GlnA : VIKTMAIFHGYHATFMPKPFYGVNGSGMHVHMSLFRGD-KNAFYDPDDPLGLSKELRYFV : 271
Psp. IpuC : GIKEIAAKHGMTATFMAKPSAEWSGSSGHVHMSLSDLAGTPVFANPENPGALSEVGYNFL : 295
            :  :* :*  :   *:** **      *   * :***        * : :    **     *:
                                  ATP-binding region

Ca   GlnA : AGLLKNIKGMAAVTNPLVNSYKRLVPGYEAPVYLAWSCKNSTALIKVPAARGAGTRVELR : 334
Ld   GlnA : NGILEHARAITCVANPTVNSYKRLIPGFEAPVYISWASRNRSPMVRIPNANEVGTRLEMR : 337
Tm   GlnA : GGILKHAKALAAVTNPTINSYKRLVPGYEAPVYISWSVGNRSALIRIPKARGKATRLEYR : 331
Psp. IpuC : AGMVALAREMSAIYLPNINSYKRTAGASWAGGNSSWGFDNRTVSHRAITSAGSAARVENR : 355
            :  *::    : ::.:  * :*****     ↑*    :*   **:   *    :    :*:* *
                                  tryptophan instead of glutamate

Ca   GlnA : CPDPSSNPYLVLACLLQAGLDGIKNNLQPPAEVEANIFAMTEQERKENGIDNLPNNLYEA : 394
Ld   GlnA : STDPTANPYLLLSACLKAGLTGIKEGKLPMAPVTSNLFEMTDDERKELGIKPLPSTLHNA : 397
Tm   GlnA : SPDPSCNIYLAFAAILAAGLDGIINKIEPPAPVEENIYEMTSERREELNIESLPGSLKEA : 391
Psp. IpuC : IPGADTNPYLVIAASLLSGLYGIENKLKPKDPILGNAYKVSPELAR-----PLAASLEEA : 410
            :       * ** ::  * :** ** :   *   :  * : :: :          *   * :*
                                                adenylylation site

Ca   GlnA : VNYMKENELAKKALGDHVYGNYVAGKAAEWDDYRTKVHDWELENYLNKY : 443
Ld   GlnA : IKAFKEDEVVKSAFSEHIVDSFLELKETEWALYTQSVSEWEVKRYFNY- : 445
Tm   GlnA : VEELKKDDVIIDALGEHIFEKFVEAAEKDWKEFSTYVTNWELQRYLYL- : 439
Psp. IpuC : AGIFRESEMARVIFPNEFVEHYAQMKVWEIKQSNSFVNNWELARYLDII : 459
            :    ::: ::    : :     :       :       * :**:  *:
```

IpuD

Sequence comparison of IpuD-like proteins. IpuD is a hypothetical Cytochrome P450 of *Pseudomonas* sp. KIE171 (Psp.). SoyC is cytochrome P450soy from *Streptomyces griseus* (Sg, accession no. P26911), CypA is P450 from *Bacillus subtilis* (Bs, accession no. O08469), and CamA is P450cam from *Pseudomonas putida* (Pp, accession no. P00183). The heme binding cysteine and active site amino acids are on a grey background, and the cysteine heme-iron ligand signature is underlined.

```
Sg    SoyC  : MTESTTDPARQNLDPTSPAPATSFPQDRGCPY------HPPAGYAPLREGRPLSRVTLFD : 54
Bs    CypA  : --MSSKEKKSVTILTESQLSSRAFKD-----------EAYEFYKELRKSQALYPLSLGA : 46
Pp    CamA  : --TTETIQSNANLAPLPPHVPEHLVFDFDMYNPSNLSAGVQEAWAVLQESNVPDLVWTRC : 58
Psp.  IpuD  : --MKDVNEVARNFDFHGEALDEIFDT----------------YSTLRNGCPVGRSENYG : 41
            :              :          :                 :  *::

Sg    SoyC  : GRPVWAVTGHALARRLLADPRLSTDRSHPDFPVPAER---FAGAQRRRVALLGVDDPEHN : 111
Bs    CypA  : LGKGWLISRYDDAIHLLKNEKLKKNYEN-VFTAKEKRPALLKNEETLTKHMLNSDPPDHN : 105
Pp    CamA  : NGGHWIATRGQLIREAYEDYRHFSSECPFIPREAGEA---------FDFIPFSMDPPEQR : 109
Psp.  IpuD  : G--FWFLTKSDDIFAAEQDPEAFSVYPSMMVPSVSEG---------IQLPPIDIDPPEHT : 90
            :     *  :          :                 :                * *::

Sg    SoyC  : TQRRMLIPTFSVKRIGALRPRIQETVDRLLDAMERQGPPAELVSAFALPVPSMVICALLG : 171
Bs    CypA  : RLRTLVQKAFTHRMILQLEDKIQHIADSLLDKVQ-PNKFMNLVDDYAFPLPIIVISEMLG : 164
Pp    CamA  : QFRALANQVVGMPVVDKLENRIQELACSLIESLRPQGQ-CNFTEDYAEPPPIRIFMLLAG : 168
Psp.  IpuD  : AYRRILLPLFTPQELKKLEQPIRDTARKLAEDFAKEGTGADASYHYSRPLPTIIFSRLAG : 150
            :   * :          :  *   *:     * :          :    :: * *  ::  : *

Sg    SoyC  : VPYADHAFFEERSQRLLRGPGADDVNRAR----DELEEYLGALIDRKRAEPGDGLLDELI : 227
Bs    CypA  : IPLEDRQKFRVWSQAIIDFSDAPERLQENDHLLGEFVEYLESLVRKKRREPAGDLISALI : 224
Pp    CamA  : LPEEDIPHLKYLTDQMTRPDGSMTFAEAK----EALYDYLIPIIEQRRQKPGTDAISIVA : 224
Psp.  IpuD  : YPEKDWPKFDKWWDDIIYER-VEKPEVANQASKDVFSYFENLLDNWKDNGESANLMDYLC : 209
            :  *  *   :    : :                      :  :   :   :        :  :

Sg    SoyC  : HRDHPDGPVDREQLVAFAVILLIAGHETTANMISLGTFTLLSHPEQLAALRAGGTSTAVV : 287
Bs    CypA  : QAESEGTQLSTEELYSMIMLLIVAGHETTVNLITNMTYALMCHHDQLEKLRQQPDLMNSA : 284
Pp    CamA  : NGQVNGRPITSDEAKRMCGLLLVGGLDTVVNFLSFSMEFLAKSPEHRQELIERPERIPAA : 284
Psp.  IpuD  : RAKIDGRPLTRDELLRYCYLLFLAGLDTTAWSIRAGLWYLANNPEDQQKLRDNPELIPLA : 269
            :          :  ::     :*:: * :*    :      *    :    *

Sg    SoyC  : VEELLRFLS-IAEGLQRLATEDMEVDGATIRKGEGVVFSTSLINRDADVFPRAETLDWDR : 346
Bs    CypA  : IEEALRFHSPVELTTIRWTAEPFILHGQEIKRKDVIIISLASANRDEKIFPNADIFDIER : 344
Pp    CamA  : CEELLRRFS--LVADGRILTSDYEFHGVQLKKGDQILLPQMLSGLDERENACPMHVDFSR : 342
Psp.  IpuD  : CEEFLRTLS-PVQVMARTCLKDTVIRGQDIKAGERVMLVFGAGNRDEEIFPNPDKIDIER : 328
            :  ** **  *        *          *  ::  : :::        *          *  *

Sg    SoyC  : PARHHLAFGFGVHQCLGQNLARAELDIAMRTLFERLPGLRLAVPAHEIRHKPGDTIQGLL : 406
Bs    CypA  : KNNRHIAFGHGNHFCLGAQLARLEAKIAISTLLRRCPNIQLKGEKKQMKWKGNFLMRALE : 404
Pp    CamA  : QKVSHTTFGHGSHLCLGQHLARREIIVTLKEWLTRIPDFSIAPGAQIQHKSG--IVSGVQ : 400
Psp.  IpuD  : QENRHLAFGGGIHRCLGSNLGRRELVVGIEEFLRAVPQFKPADPSE--------KWHGVG : 380
            :     * :** * * *** :* * *  : :   :   * :                    :
                  heme binding site

Sg    SoyC  : DLPVAW-------- : 412
Bs    CypA  : ELPISF-------- : 410
Pp    CamA  : ALPLVWDPATTKAV : 414
Psp.  IpuD  : PLKLAF-------- : 386
            :  *::
```

IpuE

Sequence comparison of IpuE-like proteins. IpuE is the hypothetical ferredoxin of *Pseudomonas* sp. KIE171 (Psp.). FdxA is ferredoxin of *Pyrococcus furiosus* (Pf, accession no. P29603), Fer is ferredoxin of *Moorella thermoacetica* (Mt, accession no. P00203), and SubB is ferredoxin of *Streptomyces griseolus* (Sg, accession no. P18325). Cysteines of the iron-sulfur binding region signature are on a grey background. A [3Fe-4S] cluster is found, and an alanine is present at the position where [4Fe-4S] has the fourth cysteine.

```
Pf    FdxA  : AWKVSVDQDTCIGDAICASLCPDVFEMNDEGKAQPKV--EVIEDEELYNCAKEAMEACPV : 58
Mt    Fer   : -MKVTVDQDLCIACGTCIDLCPSVFDWDDEGLSHVIV--DEVPEGAEDSCARESVNECPT : 57
Sg    SubB  : -MRIHVDQDKCCGAGSCVLAAPDVFDQREEDGIVVLL--DTAPPAALHDAVREAATICPA : 57
Psp.  IpuE  : -MKVTVDNNRCQGHARCVYFAPEVFEIDDEGYSRVKAGYETVP-VELQESVKKACANCPE : 58
            :   :: **:: *     *    * **:  :*         :           :::   **

Pf    FdxA  : SAITIEEA : 66
Mt    Fer   : EAIKEV-- : 63
Sg    SubB  : AAITVTD- : 64
Psp.  IpuE  : LAIKIS-- : 64
            :  **
```

IpuF

Sequence comparison of IpuF-like proteins. IpuF is a hydrolase of *Pseudomonas* sp. KIE171 (Psp.). GuaA is the GMP synthetase of *Escherichia coli* (Ec, accession no. P04079), *Haemophilus influenzae* (Hi, accession no. P44335) and *Bacillus subtilis* (Ec, accession no. P29727). Active site amino acids are on a grey background and the active site motif is underlined.

```
Ec    GuaA  : ------------MTENIHKHRILILDFGSQYTQLVARRVRELGVYCELWAWDVTEAQIR : 47
Hi    GuaA  : ------------MTNIHYHKILILDFGSQYTQLIARRVREIGVYCELWAWDVTEQQIR : 46
Bs    GuaA  : ------------MTKLVNEMILVLDFGSQYNQLITRRIREFGVYSELHPHTLTAEEIK : 46
Psp.  IpuF  : MEKLRILICDGNTEADRASFKKFVGCAPSKQFESLLKNYNSQIRTEIAFPADPGPLMTLP : 60
            :                              ::    ..*:  *:     ::     :       :

Ec    GuaA  : DFNPSGIILSGGPESTTEENS-----PRAPQYVFEAGVPVFGVCYGMQTMAMQLGGHVEA : 102
Hi    GuaA  : EFAPTGIILSGSPESTTEENS-----PRAPEYVFNAGVPVLGICYGMQTMAMQLGGLTET : 101
Bs    GuaA  : KMNPKGIILSGGPNSVYDENS-----FRCDEKIFELDIPVLGICYGMQLMTHYLGGKVEA : 101
Psp.  IpuF  : LGAYDGILITGSNSHIYEAQPGNLRQIEFAQKAFASGTPMFGVCWGMQLAVVAAGGEVLP : 120
            :      **:::*       : :          :  *    *::*:*:***      **
                                                glutamine amidotransferase class-I active site

Ec    GuaA  : SNEREFGYAQVEVVNDSALVRGIEDALTADGKPLLDVWMSHGDKVTAIPSDFITVAST-E : 161
Hi    GuaA  : SDHREFGYASVSLENSTALFANLNDNLTAS-EPKLDVWMSHGDKVTRLPENFKVTGTT-L : 159
Bs    GuaA  : ASQREYGKANIRIEGTPDLFRDLPN--------EQVVWMSHGDLVVEVPEGFTVDAATSH : 153
Psp.  IpuF  : SRVADCSCETPFATGVELTSYGSGHPMHHSRTSGFDVFSFHSDEVTRLPGGAVVTARNRN : 180
            : :   :                                 *:  * * *  ;*

Ec    GuaA  : SCPFAIMANEKRFYGVQFHPEVTHTRQGMRMLERFVRDICQCEALWTPAKIIDDAVARI : 221
Hi    GuaA  : TCPIAAMSDESRRFYGVQFHPEVTHTKKGLELLTNFVVNICGCETKWTAENIIEDAVARI : 219
Bs    GuaA  : HCPNSAMSKADKKWYGVQFHPEVRHSEYGNDLLKNFVFGVCECEGEWSMENFIEIEMQKI : 213
Psp.  IpuF  : FIQAVEIKHGRSTFWGVQYHPELSG----------------------WDQAGFLRESARSL : 219
            :       :       ::***:***:                      *    ::      :
```

```
Ec    GuaA  : REQVGDDKVILGLSGGVDSSVTAMLLHRAIGKNLTCVFVDNGLLRLNEAEQVLDMFGDHF : 281
Hi    GuaA  : KEQVGDDEVILGLSGGVDSSVVALLLHRAIGKNLHCVFVDNGLLRLHEGDQVMEMFGDKF : 279
Bs    GuaA  : RETVGDKQVLCALSGGVDSSVVAVLIHKAIGDQLTCIFVDHGLLRKGEAEGVMKTFSEGF : 273
Psp.  IpuF  : VEDGS-----YETLNHVEHAAQAISMFKAG-----AQISEENLVHFEGVDTNSFEFRP-- : 267
            :  *              *: :  *: : :*        : :  *::    :     *

Ec    GuaA  : GLNIVHVPAEDRFLSALAGENDPEAKRKIIGRVFVEVFDEEALKLEDVKWLAQGTIYPDV : 341
Hi    GuaA  : GLNITRVDAESRFLGELAGVSDPEAKRKIIGKVFVDVFDDESKKLTNVKWLAQGTIYPDV : 339
Bs    GuaA  : NMNVIKVDAKDRFLNKLKGVSDPEQKRKIIGNEFIYVFDDEADKLKGIDYLAQGTLYTDI : 333
Psp.  IpuF  : ----------LEILNWLDHLVIPTAKRRFG---------------WGGGWLQK------- : 295
            :              :*  *     *  **::                    :* :

Ec    GuaA  : IESAASATGKAHVIKSHHNVGGLPKEMKMGLVEPLKELFKDEVRKIGLELGLPYDMLYRH : 401
Hi    GuaA  : IESAASKTGKAHVIKSHHNVGGLPDYMKLGLVEPLRELFKDEVRKIGLALGLPAEMINRH : 399
Bs    GuaA  : IESG---TATAQTIKSHHNVGGLPEDMQFELIEPLNTLFKDEVRALGTELGIPDEIVWRQ : 390
Psp.  IpuF  : ------------------------------------------------------------

Ec    GuaA  : PFPGPGLGVRVLGEVKKEYCDLLRRADAIFIEELRKADLYDKVSQAFTVFLPVRSVGVMG : 461
Hi    GuaA  : PFPGPGLGVRVLGEVKKEYCDLLRRADAIFIEELRNSGWYEKTSQAFSVFLPVKSVGVMG : 459
Bs    GuaA  : PFPGPGLGIRVLGEVTEEKLEIVRESDAILREEIANHGLERDIWQYFTVLPDIRSVGVMG : 450
Psp.  IpuF  : ------------------------------------------------------------

Ec    GuaA  : DGRKYDWVVSLRAVETIDFMTAHWAHLPYDFLGRVSNRIINEVNGISRVVYDISGKPPAT : 521
Hi    GuaA  : DGRKYDWVISLRAVETIDFMTAHWAHLPYDLLGKVSNRIINEVNGISRVVYDISGKPPAT : 519
Bs    GuaA  : DARTYDYTIESRRTS-IDGMTSDWARIPWDVLEVISTRIVNEVKHINRVVYDITSKPPAT : 509
Psp.  IpuF  : ------------------------------------------------------------

Ec     GuaA : IEWE : 525
Hi     GuaA : IEWE : 523
Bs     GuaA : IEWE : 513
Psp.   IpuF : ----
```

IpuG

Sequence comparison of IpuG-like proteins. IpuG is a hypothetical permease of *Pseudomonas* sp. KIE171 (Psp.). GabP is the GABA permease of *Bacillus subtilis* (Bs, accession no. P46349), PheP is the phenylalanine-specific permease of *Escherichia coli* (Ec, accession no. P24207), and ArcD is the arginine/ornithine antiporter of *Clostridium perfringens* (Cp, accession no. Q46170).

```
Bs    GabP  : ----------MNQSQSGLKKELKTRHMTMISIAGVIGAGLFVGSGPVIHSTGP-GAVVS : 48
Ec    PheP  : MKNASTVSEDTASNQEPTLHRGLHNRHIQLIALGGAIGTGLFLGIGPAIQMAGP-AVLLG : 59
Cp    ArcD  : -----------MAEQHNEPKKLGFAQLAALIVGSMIGGGAFNLPSDMARGAGTGAVIIG : 48
Psp.  IpuG  : ----------MTSTVSAQLKGNLGPIGIALMVVATAAPLTVMVGVSPMIIGLGN-GVAAP : 49
            :                        *    : : :        :          *

Bs    GabP  : YALAGLLVIFIMRMLGEMSAVNPTSGSFSQYAHDAIGPWAGFTIGWLYWF---FWVIVIA : 105
Ec    PheP  : YGVAGIIAFLIMRQLGEMVVEEPVSGSFAHFAYKYWGPFAGFLSGWNYWV---MFVLVGM : 116
Cp    ArcD  : WIITGIGMIALAFVYQSLANRKPELTGGVYSYAKGFGEYIGFNSAWGYWLSAWIGNVSYS : 108
Psp.  IpuG  : MDAVFVGIVMLLFSVGFVSMSKYIENAGAFYAYILKG--MGRVVGLGAAS---LAVMSYT : 104
            :      :    :      :   :              *   *           :  :
```

```
Bs    GabP : IEAIAGAGIIQYWFHDIPLWLTSLILTIVLTLTNVYSVKSFGEFEYWFSLIKVVTIIAFL : 165
Ec    PheP : AELTAAGIYMQYWFPDVPTWIWAAAFFIIINAVNLVNVRLYGETEFWFALIKVLAIIGMI : 176
Cp    ArcD : VLLFGAVGYFLPMFGAGNNVASIIGASVLLWAFTLLILNGVSGAALINLITTIAKLVPIF : 168
Psp.  IpuG : LILIALEAYIGVVLSGTLSSLAGISLPWWLYTIGIVAFVGFLGYRNVELSAKVLGVALVL : 164
             :          :   :                 :   :                :  :   :

Bs    GabP : IVGFAFIFGFAPGSEPVGFSNLTGKGGFFPEGISSVLLGIVVVIFSFMGTEIVAIAAGET : 225
Ec    PheP : GFGLWLLFSGHGG-EKASIDNLWRYGGFFATGWNGLILSLAVIMFSFGGLELIGITAAEA : 235
Cp    ArcD : LFIVISLFMFNFDKFTFQFWGTSDLGGIVTQVKS----TMLVTLWCFIGIEGAVVSGRAK : 224
Psp.  IpuG : EIGVILILDLSVIGSVGASGMGTKPFEISAFLSGSPGLGILFAIFGFIGFESTVVYREEA : 224
             :       ::                :          :   :: * * *   :

Bs    GabP : SNPIESVTKATRSVVWRIIVFYVGSIAIVVALLPWNSANILESP-----FVAVLEHIGVP : 280
Ec    PheP : RDPEKSIPKAVNQVVYRILLFYIGSLVVLLALYPWVEVKSNSSP-----FVMIFHNLDSN : 290
Cp    ArcD : KQSD--VGKATVLGLIGTDIIYMLITLMALGVMDRVKLSGLENPS----MAYVLEFAVGK : 278
Psp.  IpuG : RNPERSIPTATYIAVIFITLLYFISLWCVVVGVGVDDVVGVSTKNAEGMYLDLVAKYLGH : 284
             :  :    :  *    :    ::*       :                :

Bs    GabP : AAAQIMNFIVLTAVLSCLNSGLYTTSRMLYSLAERNEAPRRFMKLSKKGVP--VQAIVAG : 338
Ec    PheP : VVASALNFVILVASLSVYNSGVYSNSRMLFGLSVQGNAPKFLTRVSRRGVP--INSLMLS : 348
Cp    ArcD : WGAVLINLGLIISLFGSLLGWALLAAEIPYVAAKDGVLPKVFAKLNKKGTP--SGSLYIT : 336
Psp.  IpuG : VMHDLAQVLLITSLFAVVISIHNIVARYKYVLGSCGVLMAQLAKVHATHSSPYVASAVQT : 344
             :       :  :: : :         :   :          : ::          : :

Bs    GabP : TFFSYIAVVMNYFSPDTVFLFLVNSSGAIALLVYLVIAVSQLKMRKKLEKTNPEALKIKM : 398
Ec    PheP : GAITSLVVLINYLLPQKAFGLLMALVVATLLLNWIMICLAHLRFRAAMRRQG-RETQFKA : 407
Cp    ArcD : NLLVQAFLILTLVVSSTYQALYSVASTAILVPYLLSAMYGFKLALTGETYDKNPTGRTRD : 396
Psp.  IpuG : FVSVSLLLAAAIMGFDPVTQIYAWGAAAGTLGYMIIVALACFSIICFFARRSESVHAWNT : 404
             :         :          :      *  :   :

Bs    GabP : WLFP--------------FLTYLTII-AICGILVSMAFIDSMRDELLLTGVITGIVLISY : 443
Ec    PheP : LLYP--------------FGNYLCIA-FLGMILLLMCTMDDMRLSAILLPVWIVFLFMAF : 452
Cp    ArcD : KIFGGIATVYAIWLCYAAELKYLLLCFILYAVGIIFFYIGKKQNNKDAEVFVGKEKILAI : 456
Psp.  IpuG : KIAP--------------ALALVGLVGFLYIAFNNLSALTGSEGYNKVNVTIVVLLIVAF : 450
             :  :                      : :   :      :  :                :::

Bs    GabP : LVFRKRKVSEKAAANPVTQQQPDILP : 469
Ec    PheP : KTLRRK-------------------- : 458
Cp    ArcD : IVLVIAIIAIVMLFNGTITI------ : 476
Psp.  IpuG : LVGSGGAVIMKMKAPRRFASILNHMS : 476
```

IpuH

Sequence comparison of IpuH-like proteins. IpuH is a hypothetical aldehyde dehydrogenase of *Pseudomonas* sp. KIE171 (Psp.). RADDH2 is the retinaldehyde-specific dehydrogenase type 2 of *Homo sapiens* (Hs, accession no. O94788), ALDH6 is the aldehyde dehydrogenase 6 of *Homo sapiens* (Hs, accession no. P47895), and ALDH2 is the mitochondrial aldehyde dehydrogenase of *Homo sapiens* (Hs, accession no. P05091). The active site amino acids are on a grey background and the motifs are underlined.

```
Hs    RADDH2: --MASLH----------------LLPSPTPNLEIKYT-KIFINNEWQNSESGRVFPVYNP : 41
Hs    ALDH6 : --MATANGAVENGQ---PDGKPPALPRPIRNLEVKFT-KIFINNEWHESKSGKKFATCNP : 54
Hs    ALDH2 : MLRAAARFGPRLGRRLLSAAATQAVPAPNQQPEVFCN-QIFINNEWHDAVSRKTFPTVNP : 59
Psp.  IpuH  : --MSGDYMVQATTN------LVGAAYWRGRADEIQFEGRCLIDGKMVEAQSGYTFDCVSP : 52
              :    :                              *:    : :*: :  :: *   *    *
```

```
Hs    RADDH2: ATGEQVCEVQEADKADIDKAVQAARLAFSLGSVWRRMDASERGRLLDKLADLVERDRAVL : 101
Hs    ALDH6 : STREQICEVEEGDKPDVDKAVEAAQVAFQRGSPWRRLDALSRGRLLHQLADLVERDRATL : 114
Hs    ALDH2 : STGEVICQVAEGDKEDVDKAVKAARAAFQLGSPWRRMDASHRGRLLNRLADLIERDRTYL : 119
Psp.  IpuH  : VDGRVLTKVSAGGEADVNRAVAAARSAFR-DRRWSGQSPVSRKRTLQAFAALIRLHRDEL : 111
              :      : :*    : *:::** **: **     *       * * *  :* *:   *  *

Hs    RADDH2: ATMESLNGGKPFLQAFYVDLQGVIKTFRYYAGWADKIHGMTIPVDGDYFTFTRHEPIGVC : 161
Hs    ALDH6 : AALETMDTGKPFLHAFFIDLEGCIRTLRYFAGWADKIQGKTIPTDDNVVCFTRHEPIGVC : 174
Hs    ALDH2 : AALETLDNGKPYVISYLVDLDMVLKCLRYYAGWADKYHGKTIPIDGDFFSYTRHEPVGVC : 179
Psp.  IpuH  : ALLETLDMGKPISASRSVDVEAVASCFEWYAEAIDKLYEQIAPTAETDLALITREPLGVV : 171
              : * :*::: ***   :  :*::      : ::*   **       *           :**:**

Hs    RADDH2: GQIIPWNFPLLMFAWKIAPALCCGNTVVIKPAEQTPLSALYMGALIKEAGFPPGVINILP : 221
Hs    ALDH6 : GAITPWNFPLLMLVWKLAPALCCGNTMVLKPAEQTPLTALYLGSLIKEAGFPPGVVNIVP : 234
Hs    ALDH2 : GQIIPWNFPLLMQAWKLGPALATGNVVVMKVAEQTPLTALYVANLIKEAGFPPGVVNIVP : 239
Psp.  IpuH  : GAIVPWNFPMLTAAWKTAPALACGNSVVLKPAEQSPLTAIRLAQLALEAGVPEGVFNVVP : 231
              : * * *****:*   **  ***  ** :*:* ***:**:*: :  *  *** * ** *::*

Hs    RADDH2: GYGPTAGAAIASHIGIDKIAFTGSTEVGKLIQEAAGRSNLKRVTLELGGKSPNIIFAD-A : 280
Hs    ALDH6 : GFGPTVGAAISSHPQINKIAFTGSTEVGKLVKEAASRSNLKRVTLELGGKNPCIVCAD-A : 293
Hs    ALDH2 : GFGPTAGAAIASHEDVDKVAFTGSTEIGRVIQVAAGSSNLKRVTLELGGKSPNIIMSD-A : 298
Psp.  IpuH  : GLGGSAGRALACHMDVDGIFFTGSTATGRLLTEYAAKSNLKRVCLELGGKSPNIILASYG : 291
              : * * : * *:: *  :: : *****  *:::   *  ****** ****** * *: :
                                NAD binding site     glutamic acid active site region

Hs    RADDH2: DLDYAVEQAHQGVFFNQGQCCTAGSRIFVEESIYEEFVRRSVERAKRRIVGSPFDPTTEQ : 340
Hs    ALDH6 : DLDLAVECAHQGVFFNQGQCCTAASRVFVEEQVYSEFVRRSVEYAKKRPVGDPFDVKTEQ : 353
Hs    ALDH2 : DMDWAVEQAHFALFFNQGQCCCAGSRTFVQEDIYDEFVERSVARAKSRVVGNPFDSKTEQ : 358
Psp.  IpuH  : DIEKAAVASAESMFNNQGEVCIAPSRLIVERSIHKRVVEIVAEVAKRRQPGDPLDPTTRM : 351
              : *:: *   :   :* ***: * * ** :*:  ::   *       ** *  * *:*  *
                                cysteine active site region

Hs    RADDH2: GPQIDKKQYNKILELIQSGVAEGAKLECGGK--GLGRKGFFIEPTVFSNVTDDMRIAKEE : 398
Hs    ALDH6 : GPQIDQKQFDKILELIESGKKEGAKLECGGS--AMEDKGLFIKPTVFSEVTDNMRIAKEE : 411
Hs    ALDH2 : GPQVDETQFKKILGYINTGKQEGAKLLCGGG--IAADRGYFIQPTVFGDVQDGMTIAKEE : 416
Psp.  IpuH  : GALVDANHADRVMGFIGRAKADGATLVAGGTRALTETGGSYVVPTVFDNVSNCMEIARDE : 411
              : *  :*  :  :::  *     :** *  **         * :: **** :* : * **::*

Hs    RADDH2: IFGPVQEILRFKTMDEVIERANNSDFGLVAAVFTNDINKALTVSSAMQAGTVWINCYNAL : 458
Hs    ALDH6 : IFGPVQPILKFKSIEEVIKRANSTDYGLTAAVFTKNLDKALKLASALESGTVWINCYNAL : 471
Hs    ALDH2 : IFGPVMQILKFKTIEEVVGRANNSTYGLAAAVFTKDLDKANYLSQALQAGTVWVNCYDVF : 476
Psp.  IpuH  : VFGPVLSVIPVANVGEAVAVANDSPYGLGAGVWTDRLSDAHKISRELRAGVVYVNCYNDC : 471
              : :****  ::    : * :  ** : :** * *:*  :  *  ::  : :* *::***:

Hs    RADDH2: NAQSPFGGFKMSGNGREMGEFGLREYSEVKTVTVKIPQKNS : 499
Hs    ALDH6 : YAQAPFGGFKMSGNGRELGEYALAEYTEVKTVTIKLGDKNP : 512
Hs    ALDH2 : GAQSPFGGYKMSGSGRELGEYGLQAYTEVKTVTVKVPQKNS : 517
Psp.  IpuH  : DITTPFGGVKQSGNGRDKSLYALDEYTELKTTWIKL----- : 507
              :    :**** * ** **:   : *  *:*:**  :*:
```

IpuI

Sequence comparison of IpuI-like proteins. IpuI is a hypothetical aldehyde dehydrogenase of *Pseudomonas* sp. KIE171 (Psp.). AldA is the aldehyde dehydrogenase *of Aspergillus niger* (An, accession no. P41751) or *Emericella nidulans* (En, accession no. P08157), and ClaH3 is the aldehyde dehydrogenase of *Cladosporium herbarum* (Ch, accession no. P40108). The active site amino acids are on a grey background and the motifs are underlined.

```
An   AldA : ------------------------------------MSDLFATITTPNGVKYEQPLG- : 21
En   AldA : ------------------------------------MSDLFTTIETP-VIKYEQPLG- : 20
Ch   ClaH : ------------------------------------MTS--VQLETPHSGKYEQPTG- : 19
Psp. IpuI : MDPGHAAQRLGVVIGGASSLWEGAYGVGTGGTASPMSGTTKIAREGNTPGSVSSWVAAQN : 60
            :                                          :       **

An   AldA : ----LFIDGEFVKGAEGKTFETINPSNEKPIVAVHEATEKDVDTAVAAARKAFEGSWRQV : 77
En   AldA : ----LFINNEFVKGVEGKTFQVINPSNEKVITSVHEATEKDVDVAVAAARAAFEGPWRQV : 76
Ch   ClaH : ----LFINNEFVKGQEGKTFDVINPSDESVITQVHEATEKDVDIAVAAARQAFEGSWRLE : 75
Psp. IpuI : PRKLILVIKTIRKETNVTTFNVISPIDGRELLVGNTSSDAEVAAALNAAETAFK-TWKLS : 119
            :    :::   : * : **: * * :   :   : ::: :*  *: **  **:  *:

An   AldA : TPSTRGRMLTKLADLFERDAEILASIEALDNGKSITMAHGDIAGAAGCLRYYGGWADKIH : 137
En   AldA : TPSERGILINKLADLMERDIDTLAAIESLDNGKAFTMAKVDLANSIGCLRYYAGWADKIH : 136
Ch   ClaH : TPENRGKLLNNLANLFEKNTDLLAAVESLDNGKATSMARVTSACASGCLRYYGGWADKIT : 135
Psp. IpuI : SKLERAQLVEALADELLKRADDLSRAVSLSIGRPAAQANETQRFKAVTLAQIEALEELGD : 179
            : :   *  ::  **: : :  : *:   ;*  *:  : *         *       :

An   AldA : GQTIDTNSETLNYTRHEPIGVCGQIIPWNFPLLMWAWKIGPAIATGNTVVIKTAEQTPLS : 197
En   AldA : GQTIDTNPETLTYTRHEPVGVCGQIIPWNFPLLMWSWKIGPAVAAGNTVVLKTAQQTPLS : 196
Ch   ClaH : GKVIDTTPDTFNYVKKEPIGVCRSDHSLELPLLMWAWKIGPAIACGNTVVLKTAEQTPLG : 195
Psp. IpuI : -ERYPSDAQVARFVRRSGQGVHLSIAPWNYPVGLLPWLIVTPILGGNTVILKHAAQTTLI : 238
            :  :   :  :   : ::   **       : *: :  * *   :  ****:: * * ** *

An   AldA : GLYAANVIKEAGIPAGVVNVISGFGRVAGSAISH--HMDIDKVAFTG------STLVGRT : 249
En   AldA : ALYAAKLIKEAPFPAGVINVISGFGRTAGAAISS--HMDIDKVAFTG------STLVGPT : 248
Ch   ClaH : GLVAASLVKEAGFPPGVINVISGFGKVAGAALSS--HMDVDKVAFTG------STVVGRT : 247
Psp. IpuI : GRIVKEAYEAIGGPAGVLQVLELGHDQVTSAIKSGFVKGVNFIGSVGGGLAVHAAAAGTL : 298
            :          :   * **::*:        :*:      .:: :   *      ::  *

An   AldA : ILQAAAKSNLKKVTLELGGKSPNIVFNDADIDNAISWANFGIFYNHGQCCCAGSRILVQE : 309
En   AldA : ILQAAAKSNLKKVTLELGGKSPNIVFDDADIDNAISWANFGIFFNHGQCCCAGSRILVQE : 308
Ch   ClaH : ILKAAASSNLKKVTLELGGKSPNIVFEDADIDNAISWVNFGIFFNHGQCCCAGSRVYVQE : 307
Psp. IpuI : TVHAAAAGTLTHVHLELGGKDPAYVRPDADIETAAAEIADGCFSNAGQSCCSVERIYLHE : 358
            :  ::***   * :* ****** *  *  ****: * :    * * * ** **:  *: ::*
            glutamic acid active site region       cysteine active site region

An   AldA : GIYDKFIARLKERALQNKVGDPFAKDTFQGPQVSQLQFDRIMEYIQHGKDAGATVAVGGE : 369
En   AldA : GIYDKFVARFKERAQKNKVGNPFEQDTFQGPQVSQLQFDRIMEYINHGKKAGATVATGGD : 368
Ch   ClaH : SIYDKFVQKFKERAQKNVVGDPFAADTFQGPQVSKVQFDRIMEYIQAGKDAGATVETGGS : 367
Psp. IpuI : AIRVPFLECFRNEMLKYKLGHPMDPATTVGPVVKASAAEFIRNQIRGAIAMGAEAYVEPA : 418
            :  *   *:  :::   :  :* *:   *  ** *     : * : *      **

An   AldA : RHG---TEGYFIQPTVFTDVTSDMKINQEEIFGPVVTVQKFKDVEDAIKIGNSTSYGLAA : 426
En   AldA : RHG---NEGYFIQPTVFTDVTSDMKIAQEEIFGPVVTIQKFKDVAEAIKIGNSTDYGLAA : 425
Ch   ClaH : RKG---DKGYFIEPTIFSNVTEDMKIVKEEIFGPVCSIAKFKTKEDAIKLGNASTYGLAA : 424
Psp. IpuI : LEFSVENASCYLAPTLLTRVAANMHIMQEETFGPVACVQTVRDDAEAISLMNDSKFGLTA : 478
            :          :: **::: *: :*:* :** ****  :   :   :** : * : :**:*
```

```
An   AldA  : GIHTKDVTTAIRVSNALRAGTVWVNSYNLIQYQVPFGGFKESGIGRELGSYALENYTQIK : 486
En   AldA  : AVHTKNVNTAIRVSNALKAGTVWINNYNMISYQAPFGGFKQSGLGRELGSYALENYTQIK : 485
Ch   ClaH  : AVHTKNLNTAIEVSNALKAGTVWVNTYNTLHHQMPFGGYKESGIGRELGEDALANYTQIK : 484
Psp. IpuI  : SVWTRDLDAGLGLVDQLDAGTVFVNRCDHADLYLPWGGQKLSGLGRGNGKEGLLGVMDVK : 538
           :  : *::: : : : : * ****:;*  :       *;** * **:**  *   *    ; *

An   AldA  : AVHYRLGDALF- : 497
En   AldA  : TVHYRLGDALFA : 497
Ch   ClaH  : TVSIRLGDALFG : 496
Psp. IpuI  : SFHLRAL----- : 545
           : :   *
```

Fig. 35. Multiple sequence alignments of six hypothetical glutamine synthetases of *Pseudomonas aeruginosa* PAO1 with γ-glutamyliso-propylamide synthetase IpuC of *Pseudomonas* sp. KIE171 (Psp.). The sequence alignment was generated with the program CLUSTAL W (http://www2.ebi.ac.uk/cgi-bin/newclustalw.pl?). The symbols in the consensus line stand for: "*" = identical or conserved residues in all sequences in the alignment and ":" = conserved substitutions. The sequence for the five hypothetical glutamine synthetases (PA0296, PA02040, PA5522, PA0298 and PA5508) of *Pseudomonas aeruginosa* (Pa) is annotated in the database of the Pseudomonas genome project (http://www.pseudomonas.com/index.html). GlnA is the glutamine synthetase of *Pseudomonas aeruginosa* PAO1 (accession no. Q9HU65). The active site amino acids are on a grey background. The adenylylation site is on a black background.

```
Pa   PAO296 : MSVPQRAVQLTEPSEFLKEHPEVQFVDLLIADMNGVVRGK-RIERNSLNKVFEKGINLPA : 59
Pa   PAO2040: MSVPLRAVQLTEPSLFLQEHPEVQFVDLLISDMNGVVRGK-RIERNSLPKVFEKGINLPA : 59
Pa   PA5522 : ----MHFADPREAREFLAAHPEVRSIELFLIDANGVPRGK-LLHRDELLAVYESGRPLPS : 55
Pa   PA0298 : -----MTTKLDQLTSWLKER-KITEVECLISDLTGIARGK-ISPTN-KFIAEKGMRLPES : 52
Pa   GlnA   : ---------MSYKSHQLIKDHDVKWVDLRFTDTKGKQQHVTMPARDALDDEFFEA----- : 46
Pa   PA5508 : ----------------MNRLQPVRLVSFVTTDLAGITRGR-SLPLATLEEQLASGCGWVP : 43
Psp. IpuC   : -MSEENKKQILKVRDFIEKH-NIDTIRLGAVDIDGVWRGKQVGAEYFLNKAALDGTQISN : 58
              :                    :   :  :     * *  :

Pa   PAO296 : SLFALDITGSTVESTGLG--LDIGDADRICYPIPGT---LSMEPWQKRPTAQLLMTMHEL : 114
Pa   PAO2040: SLFALDITGSTVESTGLG--LDIGDADRICYPIPGT---LSMEPWQKRPTAQLLMTMHEL : 114
Pa   PA5522 : TILGL--TINGDDVEETGLVWDVGDADCWTFPLPGS---LTLQPWRQSPTGQVQVSMHPE : 110
Pa   PAO298 : VLLQTVTGDYVEDDIYYD-LLDPADIDMVCRPDENA---VFLVPWAIEPTAMVIHDTFDK : 108
Pa   GlnA   : --------GKMFDGSSIAGWKGIEASDMILMPDDST---AVLDPFTEEPTLILVCDIIEP : 95
Pa   PA5508 : ----ANSSLTPQDLIDESS-PWGSHGDLRLLPDPNSRVRVEQGPDAAAPALDYLHGNLVE : 98
Psp. IpuC   : ILFGWDVADHLVDGLEFTG-WDSGYPDIALIPDLST---LSLVPWQEK-TASVLCDIQHL : 113
              :                  :     *   *  :     *     :

Pa   PAO296 : EGEPFFA-DPREVLRQVVARFTEMEL--TIVAAFELEFYLIDQE---------------N : 156
Pa   PAO2040: EGEPFFA-DPREVLRQVVARFTEMEL--TIVAAFELEFYLIDQE---------------N : 156
Pa   PA5522 : LGLPAAAGDPRHVLQRVIHSLQAECF--HPVMAVELEFYLLDRER--------------D : 154
Pa   PA0298 : LGNPIEL-SPRNILKRVLKMYADKGW--RPIVAPEMEFYLTKRS---------------D : 150
Pa   GlnA   : STMQGYERDPRNIAKRAEEYLKSTGIGDTVFVGPEPEFFIFDEVKFKSDISGSMFKIFSE : 155
Pa   PA5508 : TDGTPWPACPRSLLRAEVERYRDSGL--QVIAAFEHEFSLLGLP---------------- : 140
Psp. IpuC   : NGEPLNL-SPRNLLRKAIEKAEQLGY--KCYAAYEFEFYLLNDS---------------- : 154
              :          ** : :                 * **  :

Pa   PAO296 : VNGRPQPPRSPISGKRPQS--VQVY-SIDDLDEYVECLQDIIDGARAQGIPADAIVAESA : 213
Pa   PAO2040: VNGRPQPPRSPISGKRPQS--VQVY-SIDDLDEYVECLQDIIDGARAQGIPADAIVAESA : 213
Pa   PA5522 : SDGRPLP-ALQMNGQRPRA--TQVY-GVYELEQLQPFLDDLYAACEAQCLPARTAISEYA : 210
Pa   PA0298 : DPDYPLQAPVGRSGRQETG--RQSF-SIDAANEFDPLFEDMYDWCEAQGLDLDTLIHEEG : 207
Pa   GlnA   : QASWNTDADIESGNKGHRPGVKGGYFPVPPVDHDHEIRTAMCNALEEMGLVVEVHHHEVA : 215
Pa   PA5508 : ---------------GERP--AAAF-SLQAQRAAGQFPGWLVSALAQAGTEPEMFLPEYG : 182
Psp. IpuC   : -IASISADQWRSINPVEKS--GHCY-SMLHHSSSSDIMGEVRKYMRDAGIVLEATNSEHG : 210
              :                        :  :        :     *       *

Pa   PAO296 : PA-QFEVNLNHVNDALKACDHAVLLKRLVKNIAYDHEMDTTFMAKPYPGQAGNGLHVHIS : 272
Pa   PAO2040: PA-QFEVNLHHVADPMKACDYAVLLKRLIKNIAYDHEMDTTFMAKPYPGQAGNGLHVHIS : 272
Pa   PA5522 : PG-QVEITLQHRFDTLQAIDEGVRYKRLVKGVANRHGLQACFMAKPFADLSGSGLHLHVS : 269
Pa   PA0298 : TA-QMEINFRHG-DALDLADQILVFKRTMREAALKHNVAATFMAKPMTGEPGSAMHLHQS : 265
Pa   GlnA   : TAGQNEIGVKFN-TLVAKADEVQTLKYCVHNVADAYGKTVTFMPKPLYGDNGSGMHVHMS : 274
Pa   PA5508 : QR-QYEVTCRPA-QGVAAADRAVNVREVTREVARQMGLRTCFAPLPAPGAVTNGVHLHLS : 240
Psp. IpuC   : PG-QYEINIKYD-DALKAADDAIFVKNGIKEIAAKHGMTATFMAKPSAEWSGSSGHVHMS : 268
              :   * *:       :   *    :  :  *       *   *     :  *:* *
```

```
Pa   PAO296  : LLDKHG-NNIFTSED--PEQNAALRHAIGGVLETLPASMAFLCPNVNSYRRFGSQFYVPN  : 329
Pa   PAO2040: LLDKHG-NNIFTSED--PEQNAALRHAIGGVLETLPASMAFLCPNVNSYRRFGSQFYVPN  : 329
Pa   PA5522  : LADAAG-NNLFASED--PAGTPLLRQAIGGMKACLLESLALFCPNANSFRRFQANSYAPL  : 326
Pa   PAO298  : IVDVKTGKNIFSNAD--GTMSELFLHHIGGLQKFIPEVLPLFAPNVNSFRRFLPDTSAPV  : 323
Pa   GlnA    : ISKDCK--NTFAGEG-YAGLSETALYFIGGIIKHGKALNGFTNPSTNSYKRLVPGFEAPV  : 331
Pa   PA5508  : LQHADG-SPLLYEPGRPNDLSELGEHWAAGVLAHLPALCALTAPTAASYLRLKPHHWSAA  : 299
Psp.IpuC    : LSDLAG-TPVFANPENPGALSEVGYNFLAGMVALAREMSAIYLPNINSYKRTAGASWAGG  : 327
              : :        :                      *:         :  *   *: *     *
tryptophan, tyrosine and serine instead of glutamate for five Pa proteins

Pa   PAO296  : APSWGLDNRTVALRVP-TGSPDAV------RLEHRVAGADANPYLLLAAVLAGVHHGLTN  : 382
Pa   PAO2040: APSWGLDNRTVALRVP-TGSPDAV------RLEHRVAGADANPYLLLASVLAGVHHGLTN  : 382
Pa   PA5522  : APTWGINNRTVSLRVP-GGPASSR------HIEHRICGADANPYLAAAALLAAVRLGIRE  : 379
Pa   PAO298  : NVEWGEENRTVGLRVP-DSSPENR------RVENRLAGADANPYLALAASLLCGYIGMVE  : 376
Pa   GlnA    : NLAYSARNRSASIRIPYVSSPKAR------RIEARFPDPAANPYLAFAALLMAGLDGIQN  : 385
Pa   PA5508  : YACLGLRNREAALRICPVVSVGGKPLGKQYNLEFRPMDATTCPHLAMAAVLIAGRLGIER  : 359
Psp.IpuC    : NSSWGFDNRTVSHRAI-TSAGSAA------RVENRIPGADTNPYLVIAASLLSGLYGIEN  : 380
              :          **    *                 :* *     : *:*  *: *      *:

Pa   PAO296  : KVEPGAPIEGNSYEQ--------MEPSLPNNLRDALRELDESEIMAKYIDP------KYI  : 428
Pa   PAO2040: KVEPGAPIEGNSYEQ--------LEPSLPNNLRDALRELDDSEILAKYIDP------KYI  : 428
Pa   PA5522  : RLDPGAPITGNGYAQ--------ATQALPSDWLTALRALEGSAWAREALGE------DFL  : 425
Pa   PAO298  : GIKPSAQVKGRGYER--------RNLRLPLTIEAALERMENCKPLEQYLGS------KFI  : 422
Pa   GlnA    : KIHPGDAADKNLYDLPPE-----EAKEIPQVCGSLKEALEELDKGRAFLTKGGVFTDEFI  : 440
Pa   PA5508  : RLPLRALADVDPHGLSDFERQARGIQALPATLGDALDCLQRDEALCAELPK------PLL  : 413
Psp.IpuC    : KLKPKDPILGNAYKVSPE-----LARPLAASLEEAAGIFRESEMARVIFPN------EFV  : 429
              : :          :                :          :          :         :
              adenylylation site class-I

Pa   PAO296  : DIFVACKESELEEFEHSISDLEYNWYLHTV : 458
Pa   PAO2040: DIFVACKESELEEFEYSISDLEYNWYLHTV : 458
Pa   PA5522  : KIYLAIKQAEYRAFMGEVGEQDWRWYLNQA : 455
Pa   PAO298  : SGYVAVKRAEHENFKRVISSWEREFLLLSV : 452
Pa   GlnA    : DAYIELKSEEEIKVRTFVHPLEYDLYYSV- : 469
Pa   PA5508  : DTYLAMKRHELALTAGLSDDDLCRHYAELY : 443
Psp.IpuC    : EHYAQMKVWEIKQSNSFVNNWELARYLDII : 459
              :   :  *  *
```

Fig. 36. Multiple sequence alignments of hypothetical glutamine synthetase TdnQ and BAA10, as well as the hypothetical GMP synthetase like proteins TdnT and BAA11 involved in aniline degradation of *Pseudomonas putida* UCC22(PTDN1) and *Acinetobacter* sp. strain YAA with γ-glutamyl-isopropylamide synthetase IpuC or the hydrolase IpuF of *Pseudomonas* sp. KIE171 (Psp.). The sequence alignment was performed with the program CLUSTAL W (http://www2.ebi.ac.uk/cgi-bin/newclustalw.pl?). The symbols in the consensus line stand for: "*" = identical or conserved residues in all sequences in the alignment and ":" = conserved substitutions. BAA10 (BAA13010) is the hypothetical glutamine synthetase of *Acinetobacter* sp. YAA (Asp., accession no. D86080), and TdnQ is the hypothetical glutamine synthetase of *Pseudomonas putida* UCC22(PTDN1) (Pp, accession no. P95529). BAA11 (BAA13011) is the hypothetical GMP synthetase of *Acinetobacter* sp. YAA (Asp., accession no. D86080), and TdnT is the hypothetical GMP synthetase of *Pseudomonas putida* UCC22(PTDN1) (Pp, accession no. P95529).

Glutamine synthetase like proteins

```
Asp. BAA10: MSEKLDFITKNNLWTDKQRDAADKVLAEIDSLGLEMIRLSWADQYGLLRGKALSVAA-LKA : 60
Pp   TdnQ : MSGK--FIEKHGIWSDTQKAAAADVLNKIEKAGLQMVRLSWPDQYGLLRGKMLSVAA-LRS : 58
Psp. IpuC : ------------MSEENKKQILKVRDFIEKHNIDTIRLGAVDIDGVWRGKQVGAEYFLNK : 48
          :               :: ::     *   *:   :: :**   *  *: *** :     *

Asp. BAA10: AFSEGSEVTMAPFSFNLVSEWVFNPFTAGGGFGIDEFDELGGVPSVVMVPDPTTFKVLPW : 120
Pp   TdnQ : AFASGSEITMAPFFFDTASAIVFNPFSA---DGGLGSAELAGSPNVVMVPDPTTFRILPW : 115
Psp. IpuC : AALDGTQISNILFGWDVADHLV---------DGLEFTGWDSGYPDIALIPDLSTLSLVPW : 99
          : *   *::::   * ::     *                    * * : ::** :*: ::**

Asp. BAA10: ADKTGWMLADLHWKSGEPFPLCPRGIMKKAVKSLSDEGYLFKCGIELEWYLTKIVDRSLS : 180
Pp   TdnQ : ADRTGWMLADLYMTSGRPFALSPRAILKKALVEMQDLGYDYQAGLEVEWYLTRIVDPCLE : 175
Psp. IpuC : QEKTASVLCDIQHLNGEPLNLSPRNLLRKAIEKAEQLGYKCYAAYEFEFYLLNDSIASIS : 159
          :  ::*  :* *:    * *: * ** :::**:    : **      * *:**       :

Asp. BAA10: PESLGAPGVQPDAIQVQPVAQ-GYSYLLEYHLDQVDDIMSKVRKGLLELNLPLRSIEDEL : 239
Pp   TdnQ : PETLGGPGTPAAPPKVMPVAK-GYSYLLENHLDEVEPIMAEVRQHLLALGMPLRSIEDEW : 234
Psp. IpuC : ADQWRS----------INPVEKSGHCY-SMLHHSSSSDIMGEVRKYMRDAGIVLEATNSEH : 209
          :  :               : ** : *  *    *       ** :**: :    : * : : *

Asp. BAA10: APSQMETTFDVMEGLEAADAALLIKSAIKQICSRHGYHATFMCKPAINGFSVASGWHMHQ : 299
Pp   TdnQ : APSQMETTFDVMPGLDVADTMVLFRNAVKQVCRRRGYLASFMCKPAIQGF-LASGWHLHQ : 293
Psp. IpuC : GPGQYEINIKYDDALKAADDAIFVKNGIKEIAAKHGMTATFMAKPSAEWS--GSSGHVHM : 267
          :  * * *  :      *  **  :: :  :*:: ::*  *:** **:  :     *  *:*

Asp. BAA10: SLVDKDTRKNLFIPSEGEVVSPLGRAYAGGLLANGSAASSFTTPTVNGYRRRQPHSLAPD : 359
Pp   TdnQ : SLTARDSGANAFIPQPGEALSALGRSYVGGLLEHACAASSFTTPTINGYRRRRPYSLAPD : 353
Psp. IpuC : SLSDLAGTPVFANPENPGALSEVGYNFLAGMVALAREMSAIYLPNINSYKRTAGASWAGG : 327
          : **             *     :* :*  :  *::       *::  * :* *:*    *:*
                                        tryptophan or leucine instead of glutamate

Asp. BAA10: RRAWAKENKAAMVEVISATGDPASRIENRIGEPGANPYLYMASQIVSGLDGIKIKRDP-G : 418
Pp   TdnQ : RVTWAKDNRAAMAEVISAPGDPASRVENRIGEPAANPYLYLASQVFSGIDGIRRQLDP-G : 412
Psp. IpuC : NSSWGFDNRTVSHEAITSAG-SAARVENRIPGADTNPYLVIAASLLSGLYGIENKLKPKD : 386
          :  :*  :*::   * *:: *  *:*:**** :**** :*: : **: **  :  *

Asp. BAA10: GLQGAPVGAQ---VPMLPTALAEALDALEHDSELFRSCFGDTFIKYWLQLRRSEWARFLD : 475
Pp   TdnQ : PLQETPTAGD---VTILPHNLSEALEVLET-SKFFREAFGEEFIRYWMHLRRSEWKRFVD : 468
Psp. IpuC : PILGNAAKVSPELARPLAASLEEAAGIFRE-SEMARVIFPNEFVEHYAQMKVWEIK---- : 441
          :  :   *          *   * **   :   *:: *  * : *: :: :::  *
                adenylylation site class-I
```

```
Asp. BAA10: AEGAEAAEPTGAVTQWEQKEYFNLL : 500
Pp   TdnQ : AEGQVDFSGD-PVTNWEHREYFELF : 492
Psp. IpuC : -------QSNSFVNNWELARYLDII : 459
          :             * :**   *::::
```

GMP synthase like proteins

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Asp. BAA11: --MSKRFALLWCS-EEE-RFDYREEMVNAFKTE-------------NSDWEVIS-AFTDL : 42
Pp   TdnT : MTAMKKYAVIWCS-DASGDLELQEKMISAFGRE-------------NEEWEIIQPAENNF : 46
Psp. IpuF : ---MEKLRILICDGNTEADRASFKKFVGCAPSKQFESLLKNYNSQIRTEIAFPADPGPLM : 57
          :      ::  :: *  :         ::::     :                :       :

Asp. BAA11: NKIIDNYDGFVISGSEYSVNADKEK--FSGLFEFIR-AVHKKEKPIVGICFGCQSLAVAL : 99
Pp   TdnT : LERVFDYSGHVISGSPKSVIDDAQTPLVSNLLAFLRGAAQRGEGPVVGLCFGAQAIAAAL : 106
Psp. IpuF : TLPLGAYDGILITGSNSHIYEAQPG--NLRQIEFAQKAFASG-TPMFGVCWGMQLAVVAA : 114
          :    :  * * :*:**   :           : * : *      *: *:*:* *   *
                                        glutamine amidotransferase class-I active site

Asp. BAA11: GGEVGLN------PSREFRFGTDELTFQNGLNKHVG--TSEERVRLIESHGECVIRRPLG : 151
Pp   TdnT : GGEVGRN------PSGRFKLGVDRLEWSNEAQVLFGPQVGAGPTVLVQSHGECVTTLPPG : 160
Psp. IpuF : GGEVLPSRVADCSCETPFATGVELTSYGSGHPMHHS---RTSGFDVFSFHSDEVTRLPGG : 171
          :  ****              *  * :   :                    :   * : *   * *

Asp. BAA11: STLLARSD--STAVEIFAVGPYAVGIQGHPEISKKTLEQDFLRVHLEDGNLQEDEVRRFH : 209
Pp   TdnT : GVQLASSQ--TIPHEVFLVNGQFLGIQGHPEVDRQFLQQKFMAYHR--ALFDDDEWVGVQ : 216
Psp. IpuF : AVVTARNRNFIQAVEIKHGRSTFWGVQYHPELS-GWDQAGFLRESAR-SLVEDGSYETLN : 229
          :      *          *:         *:* ***:     :  *:          ::      :

Asp. BAA11: AELSGYQPPQAIRQLVKATLHKQINFQNLVGDV--------------------------- : 242
Pp   TdnT : QESQQDLDPERVIALGRRLLDAGR-LPATPQDISALPA---------------------- : 253
Psp. IpuF : HVEHAAQAISMFKAGAQISEENLVHFEGVDTNSFEFRPLEILNWLDHLVIPTAKRRFGWG : 289
          :                    :        :     :

Asp. BAA11: ------
Pp   TdnT : ------
Psp. IpuF : GGWLQK : 295
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PATENT APPLICATIONS AND PUBLICATIONS

- de Azevedo Wäsch, S.I., van der Ploeg, J.R., Leisinger, T., Kiener, A., Heinzmann, K., Gilligan, T. (1999). Process for the production of L-alaninol and γ-glutamylisopropylamide and a microorganism strain for the genus *Pseudomonas.* World Patent Application, WO99/07199 A 19990218.

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