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Regulation of *Pseudomonas putida* genes involved in the metabolism of acidic amino acids

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Dedicated to my parents
as a token of gratitude

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am

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1. Introduction

1.1 Amino acid metabolism: general overview

Bacteria can utilize a wide range of nitrogen compounds as sole sources of cellular nitrogen. These range from simple inorganic compounds such as dinitrogen (N_2) and nitrate (NO_3^-) to complex compounds including amino acids. For many bacteria, including the enteric group, ammonium ions are the preferred nitrogen source. However, they frequently have to utilize alternate nitrogen sources such as amino acids and, to accomplish this, they are capable of activating the necessary pathways. The synthesis, and in some cases the activity of these enzymes is tightly regulated in concert with the availability of the respective substrates. The metabolic pathways can be divided into two classes: pathways necessary for utilization of nitrogen from the extracellular medium and biosynthetic pathways for intracellular production of nitrogen-containing compounds. The coordinated expression of the enzymes of nitrogen metabolism is primarily dependent on intracellular nitrogen pool.

In all organisms, amino acids are involved in a wide variety of cellular processes. Amino acids not only constitute the building blocks for protein synthesis but also serve as precursors of important metabolites such as lipids, carbohydrates, vitamins, and nucleotides. Most nitrogen atoms found in macromolecules are initially derived from the amino acids glutamate (Glu) and glutamine (Gln). The amino groups of both compounds are utilized for the production of other amino acids, and the amide group of Gln is used directly for the synthesis of purines, pyrimidines and various other compounds.

Our current knowledge of amino acid metabolism in bacteria and its regulation is mainly based on research with enterobacteria, notably *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae,* whereas very little is known about the modes of amino acid utilization in other microorganisms such as the pseudomonads. The pseudomonads are a large group of the γ-proteobacteria that are engaged in a variety of metabolic activities including degradation of biogenic and xenobiotic pollutants (Timmis, 2002). In addition, *Pseudomonas* strains play a significant role as biocontrol agents in plant protection (Lee and Cooksey, 2000; Walsh *et al*., 2001) and plant growth promotion (see below). In the present work we, therefore, focussed on the metabolism of the acidic amino acids (Asp, Glu) and their amides (Asn, Gln) and its regulation in the *Pseudomonas putida* strain KT2440.

1.2 Pathways for ammonia assimilation in enteric bacteria

Ammonia is the energetically least expensive nitrogenous substrate to process as it can be directly incorporated into glutamine (Gln) and glutamate (Glu), the key nitrogen donors for biosynthetic reactions. The assimilation of NH_4^+ proceeds by either of two pathways which both yield Glu as the main product (cf. Fig. 1.1).

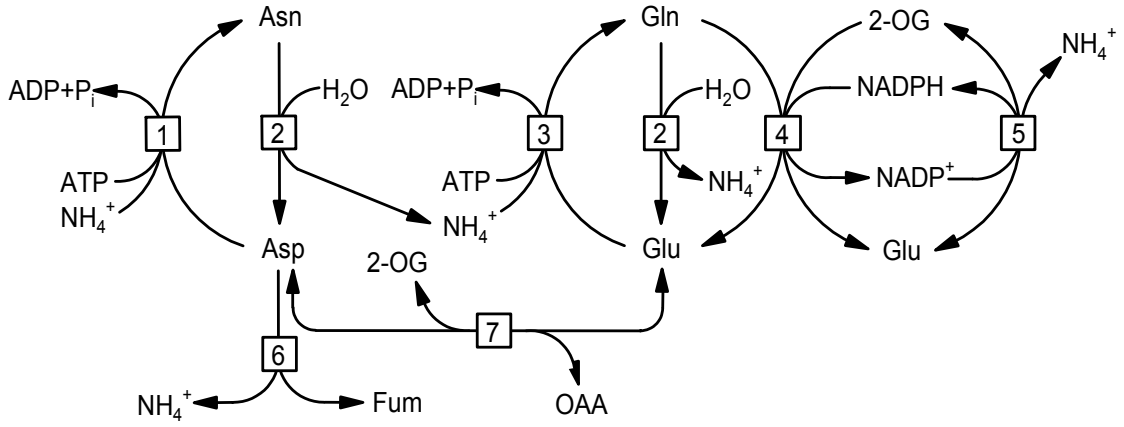


Figure 1.1: Enzymes involved in the metabolism of acidic amino acids and their amides. The enzymes involved are: asparagine synthetase [1], glutaminase/asparaginase [2], glutamine synthetase (GS) [3], glutamate synthase (GOGAT) [4], glutamate dehydrogenase (GDH) [5], aspartase [6] and aspartate transaminase [7]. Asn- asparagine, Asp- aspartate, Fum- fumarate, OAA- oxaloacetate, 2-OG -2-oxoglutarate,

1.2.1 Glutamate dehydrogenase pathway

Glutamate dehydrogenase (GDH, enzyme 5) catalyzes the reductive amination of 2-oxoglutarate to glutamate with NADPH as the reducing agent. ATP is not required.

$$\text{2-Oxoglutarate} + NH_3 + \text{NAD(P)H} + H^+ \rightarrow \text{Glu} + \text{NAD(P)}^+$$

This pathway is the preferred one when the ammonium concentration in the medium is high.

1.2.2 GS/GOGAT pathway

This pathway is also ubiquitous in bacteria and is active when ammonium levels are low. Glutamine synthetase (GS, enzyme 3) has a much lower K_m (~0.1 mM) for ammonia than

GDH (~2 mM in *E. coli*). GS first converts glutamate and ammonia in an ATP-dependent reaction to glutamine. Glutamate synthase (GOGAT, enzyme 4) then transfers the amido group from glutamine to 2-oxoglutarate yielding two molecules of glutamate.

$$\text{Glu} + NH_3 + \text{ATP} \text{ —} GS \rightarrow \text{Gln} + \text{ADP} + P_i$$

$$\alpha\text{-Ketoglutarate} + \text{Gln} + \text{NADPH} \text{ —} GOGAT \rightarrow 2\ \text{Glu} + NADP^+$$

Genetic evidence indicated that GS is a highly regulated enzyme at both the transcriptional and the post-translational level. In enteric bacteria, GS is reversibly modified by the bifunctional enzyme adenylyltransferase (ATase) in response to nitrogen availability (see below). Adenylylation and deadenylylation regulate the catalytic activity of GS. When the intracellular nitrogen level is sufficient, ATase catalyzes the transfer of AMP from ATP to the subunits of GS which progressively inactivates the enzyme. Conversely, when the intracellular nitrogen level is low, the adenylyl group is removed from GS and the enzyme becomes active again. In *E. coli,* two protein components, P_I and P_{II}, are involved in the adenylylation and deadenylylation process. The P_I fraction contained an ATase whose ability to adenylylate or deadenylylate GS was specified by the P_{II} protein and by the concentrations of P_I, ATP, UTP, Gln and 2-ketoglutarate.

Structural analyses of GOGAT revealed that the enzyme is a heterodimer, whose larger glutaminase subunits and smaller transaminase subunits are encoded by *gltB* and *gltD* genes, respectively. GOGAT is essential for the derepression of the Ntr response in many bacteria (see section 1.4) as it removes Gln which represses the Ntr system.

1.3 Enzymes of amino acids utilization

In ammonia-limited conditions, cells utilize many alternative nitrogen sources such as nitrate, urea and amino acids (Magasanik, 1996). As already mentioned, *E. coli* and other enterobacteria derive all their nitrogen from Glu or Gln (Reitzer, 1996a, 1996b). Nevertheless, Glu and Gln are inferior to NH_4^+ in supporting growth of enteric bacteria. In *Pseudomonas* the situation is different. Several strains of *P. fluorescens* and *P. putida* rapidly grow on acidic amino acids and their amides, even when these are supplied as the sole source of carbon and nitrogen (Hüser *et al*., 1999; Klöppner, 1999).

1.3.1 Asparagine synthetase

The reactions catalyzed by asparagine synthetase (Fig. 1.1, enzyme 1) use either Gln or ammonia as a nitrogen source to convert of Asp to Asn. Two families of asparagine synthetases have been found. Members of the AsnA family which occur in *E. coli* and *Klebsiella aerogenes* (Humbert and Simoni, 1980; Reitzer and Magasanik, 1982) use only ammonia as the amino group donor. The other group is the AsnB family, members of which were found in both prokaryotes and eukaryotes (Hughes *et al*., 1997; Scofield *et al*., 1990). These enzymes use both Gln and ammonia as the nitrogen donor, but Gln is the preferred one.

1.3.2 Glutaminase/asparaginase

Enzymes that catalyze the hydrolysis of Gln and Asn are widely distributed in microorganisms. One such group of amidohydrolases called glutaminase/asparaginases convert asparagine and/or glutamine to their respective dicarboxylates, aspartate and glutamate (Fig. 1.1, enzyme 2). According to their subcellular localization and kinetic properties, there are two major subgroups (Class I and Class II). Class I asparaginases are constitutive cytoplasmic enzymes with a marked preference for L-Asn. By contrast, class II enzymes, encoded by the *ans*B gene, are located in the periplasm and show a wider specificity for L-Asn and L-Gln as well as for their D-isomers (Cedar and Schwartz, 1967; Kovelenko *et al*., 1977; Derst *et al*., 2000). The role of asparaginases has been studied extensively in Gram-negative bacteria such as *E. coli* (Cedar and Schwartz, 1967), *Salmonella enterica* (Jennings *et al*., 1993), *Erwinia chrysanthemi* (Gilbert *et al*., 1986), and *Vibrio proteus* (Sinha *et al*., 1991) and also in some Gram-positive organisms such as *Bacillus subtilis* (Atkinson and Fischer, 1991) and *Staphylococcus aureus* (Rozalska and Mikucki, 1992).

E. coli contains both the periplasmic (type II) and the cytosolic (type I) asparaginase isoenzyme. Type II asparaginases from *E. coli* and *E. chrysanthemi* have received considerable attention as they are used in the treatment of leukemias. Malignant transformed haematopoietic cells are sometimes unable to synthesize sufficient Asn for their own metabolism, so that the asparaginase-induced depletion of Asn in serum impairs function of the transformed cells and eventually causes their death (Roberts, 1976; Jacob *et al*., 1996; Müller and Boos, 1998).

In *Pseudomonas*, the acidic amino acids (Asp, Glu) and their amides (Asn, Gln) strongly and specifically induce the periplasmic glutaminase/asparaginase isoenzyme (PGA,

Klöppner, 1999; Hüser *et al*., 1999). However, the physiological roles of class II glutaminases/ asparaginases and their regulation in *P. putida* KT2440 at the molecular level are not well understood.

1.3.3 Aspartase

This enzyme (Fig. 1.1, enzyme 6) is also referred to as aspartate ammonia lyase. It plays an important role in amino acid metabolism by reversibly converting the product of glutaminase/asparaginase, L-Asp, to fumarate and ammonium ion. Thus it feeds the carbon skeleton of Asp into the tricarboxylic acid cycle.

1.3.4 Aspartate transaminase

This enzyme (Fig. 1.1, enzyme 7), also known as aspartate aminotransferase or glutamate-oxaloacetate transaminase, catalyzes the formation of oxaloacetic acid and glutamic acid from aspartic acid and 2-oxoglutarate.

1.4 Nitrogen control by the Ntr system

In many natural environments inhabited by prokaryotes, ammonia is not present at sufficient concentrations. In such cases, bacteria opt to utilize a wide range of alternate nitrogen sources. In ammonia limiting conditions, cells synthesize proteins that transport and degrade nitrogenous compounds, and assimilate the ammonia produced. There is increasing evidence indicating that in most, if not all, bacteria the expression of genes involved in nitrogen assimilation and catabolism is controlled by a global nitrogen regulatory (Ntr) system. The main components of the system, which has been most extensively studied in *E. coli*, are shown in an overview in Fig. 1.2.

1.4.1 Components

The Ntr system basically consists of four proteins 1) uridylyltransferase/uridylyl removing enzyme (UT/UR) 2) the *gln*B encoded P_{II} protein and 3+4) the proteins of a two-component regulatory system, NtrB and NtrC. The activated NtrC protein stimulates expression of about 100 genes and thus initiates the Ntr response (Reitzer, 2003).

All functional proteins of the Ntr system can be interconnected between two different states. UT/UR controls the state of the P_{II} protein in response to the nitrogen status of the cell. The unmodified form of P_{II} inactivates both glutamine synthetase (GS) and the

NtrB/NtrC system. This, in turn, prevents the expression of Ntr-related proteins. The uridinylated form of P_{II} is no longer capable of inhibiting NtrB/NtrC and thus allows initiation of the Ntr response.

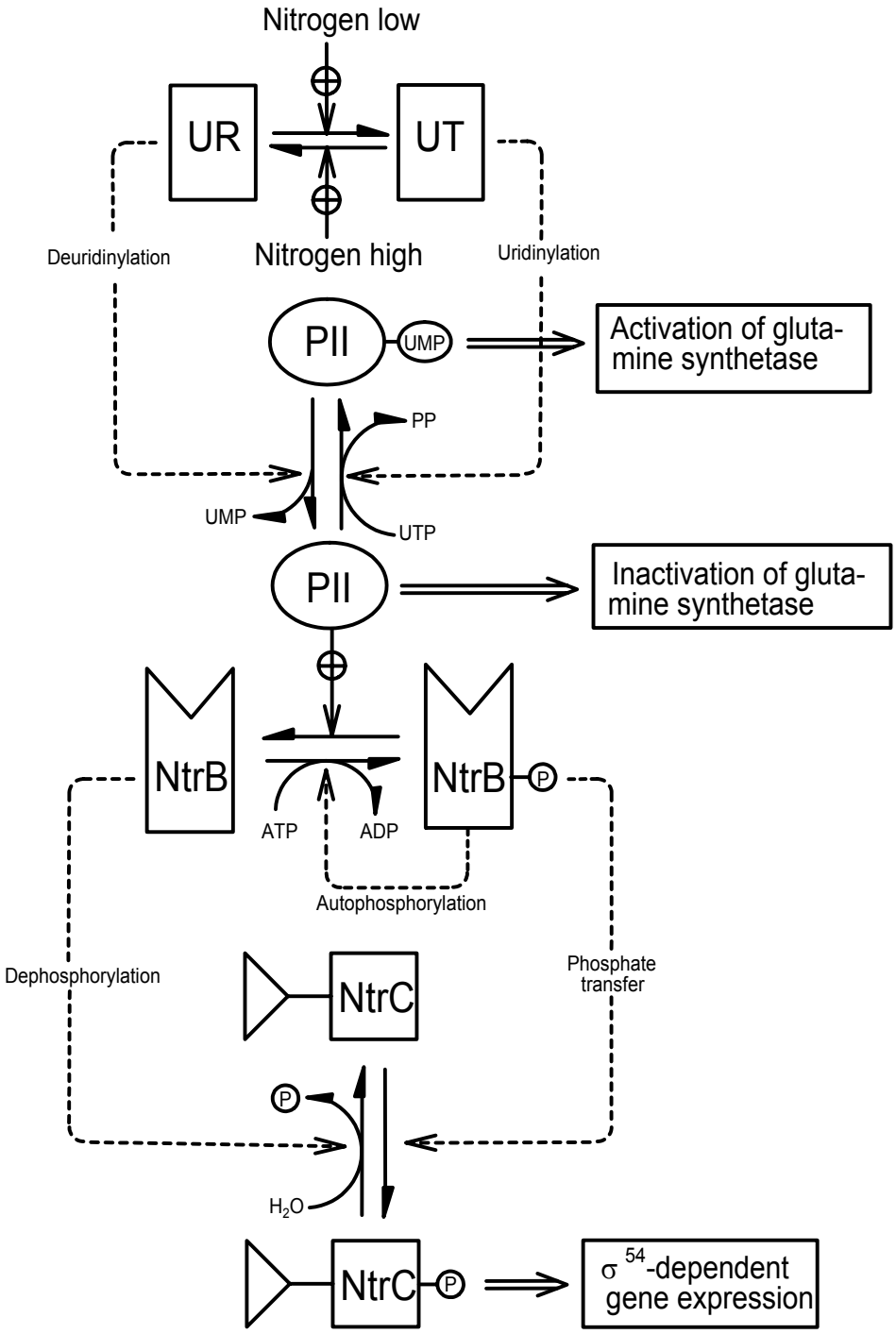


Figure 1.2: The Ntr system in *Escherichia coli*

1.4.2 Uridylyltransferase-uridylyl removing enzyme (UT/UR)

The primary sensor of the nitrogen status is the UT/UR bifunctional enzyme. Its effect on the P_{II} protein mainly depends on the [glutamine]/[2-oxoglutarate] ratio. When the cells are nitrogen-limited, the [Gln]/[2-OG] ratio is low which, in turn, stimulates UTase to covalently modify P_{II} by transferring an uridylyl monophosphate (UMP) group to a specific tyrosine residue of the protein at the expense of UTP. In conditions of good nitrogen supply, i. e. when the [Gln]/[2-OG] ratio is high, the uridylyl-removing action of UT/UR is promoted, resulting in the formation of free P_{II}. 2-OG probably induces a conformational change in P_{II} that allows uridylylation, while binding of glutamine to UT/UR results in a conformation that preferentially deuridylylates P_{II}.

A major function of P_{II} is to control the activity of glutamine synthetase (GS). More than 30 years ago it was recognized that GS activity is mainly regulated by enzyme-catalyzed adenylation/deadenylation (Magnum *et al*., 1973; Adler *et al*., 1975) which, in turn, is controlled by the state of P_{II}. Adenylation and deadenylation of GS are brought about by a bifunctional adenylyltransferase-adenylyl removing enzyme (AT/AR) similar to UT/UR. The two forms of P_{II} interact with AT/AR and control its catalytic activity, i. e.

$$P_{II}(UMP)_4 \longrightarrow AR \uparrow \longrightarrow \text{deadenylated (more active) GS}$$
$$P_{II} \longrightarrow AT \uparrow \longrightarrow \text{adenylated (less active) GS}$$

1.4.3 Control of the NtrB/NtrC system by P_{II}

NtrB (also referred to as NR_{II}) and NtrC (NR_I) are members of a so-called two-component regulatory system (such systems are discussed in more detail below). In the absence of P_{II}, NtrB acts as a protein kinase. It transfers a phosphate residue from ATP to one of its own histidine residues (autophosphorylation) and from there to an aspartate residue of NtrC. (Keener and Kustu, 1988; Weiss and Magasanik, 1988; Weiss *et al*., 1991). NtrC, the so-called response regulator of the system, i. e. a transcription factor that, in its phosphorylated form, activates the σ^{54}-dependent transcription of genes. Binding of free P_{II} to NtrB stimulates the phosphatase activity of the latter and thus leads to transcriptionally inactive NtrC. In summary, the modification of P_{II} in response to the cellular nitrogen status provides the intracellular switch that regulates the phosphatase and kinase activities of NtrB and hence the transcriptional activity of NtrC.

1.4.4 Genes regulated by the Ntr system

As already mentioned, in enteric bacteria a large number of genes (at least 100) are transcriptionally regulated by NtrBC (Zimmer *et al*., 2000). These include the *ntrBC* operon itself, *glnA* which encodes glutamine synthetase and many genes that code for transport systems. So, the Ntr system of *E. coli* controls the transport of Gln (*glnHPQ*), Glu (*gltJKL*), arginine (*argT*), histidine (*hisJQMP*), putrescine (*potFGHI*, *ydcSTUV*), peptides (*oppABCDF, dppABCDF*) and of other N-containing compounds. The genes required for nitrate and nitrite assimilation (*nasFEDCBA*), the nitrogen fixation regulatory genes *nifLA* of *K. pneumoniae*; and the nitrogen regulation gene (*nac*) of *K. aerogenes* also depend on the Ntr system for expression.

To date, very little is known about the function of the Ntr system in *Pseudomonas*. It was superficially characterized in *P. aeruginosa* and *P. putida*. Several mutants of *P. aeruginosa* were isolated because of their inability to assimilate poor nitrogen sources, and a number of these were shown to have pleiotropic phenotypes with respect to nitrogen utilization. Eberl *et al*. (2000) reported that in *P. putida* KT2442 mutations in the *gltB* gene, encoding a major subunit of GOGAT affects the biosynthesis of the enzyme which result in Ntr^- phenotype i.e. the inability to utilize a number of amino acids as sources of nitrogen. The GOGAT deficient mutants failed to grow on nitrite, urea, low levels of ammonium (below 1 mM) and some amino acids. In addition, the GOGAT mutant was severely impaired in the ability to survive prolonged incubation in nitrogen-free medium that only 0.001% of the initial populations remained viable. These results clearly indicates that a mutation in *gltB* gene give rise to a nitrogen-sensitive mutants.

1.5 Two Component Systems

Two-component regulatory systems play an important role in the adaptation of bacterial cells to the environmental signals such as nutrient availability, oxygen tension or osmolarity (Dunny and Winans, 1999). The basic biochemical events of two-component signal transduction were first established by Ninfa and Magasanik (1986) for the nitrogen regulatory system in *E. coli* (see section 1.4). In its basic form, a two-component system consists of a pair of proteins: a sensor kinase (histidine protein kinase, HPK) and a cognate response regulator (RR). Most, but not all, HPKs are associated with the plasma membrane, usually via one or two membrane-spanning sequences and they typically contain extracellular sensory input modules fused to the protein kinase catalytic module.

This arrangement enables HPKs to detect environmental signals. Such signals trigger autophosphorylation of the transmitter module in the histidine kinase domain of HPKs at a specific histidine residue (H). The sensor HPK then regulates the activity of a cytoplasmic RR by transferring its histidine-bound phosphate to an aspartate residue (D). Response regulators consist of a "receiver domain" that contains the aspartate-phosphorylation site and a "output domain", a DNA-binding module whereby the RR functions as a transcription factor (Dunny and Winans, 1999; Hoch and Silhavy, 1995).

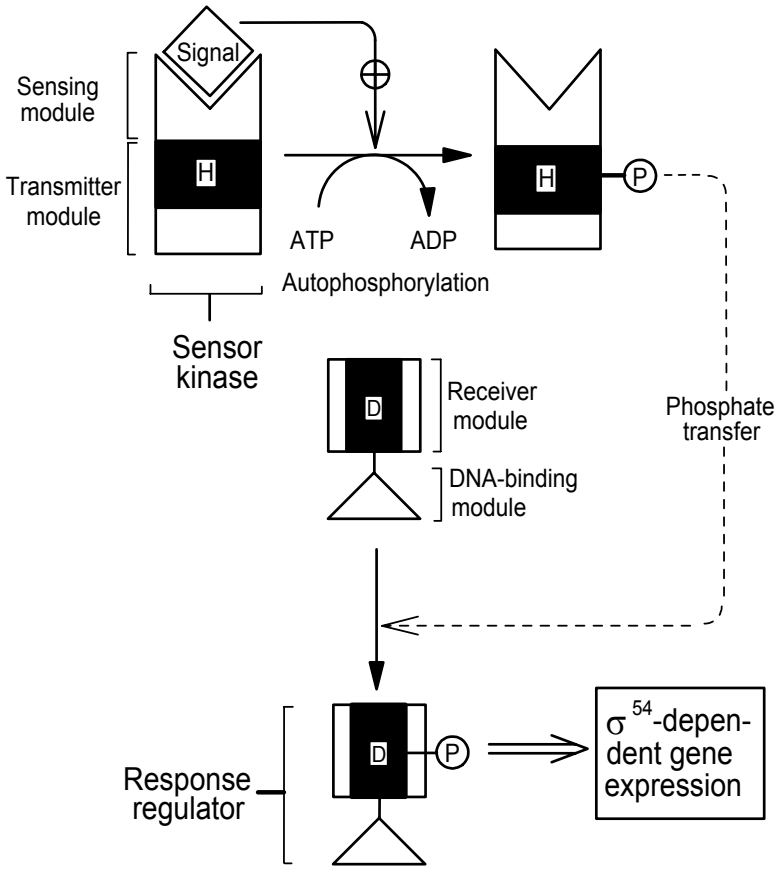


Figure 1.3: Components and functioning of a two-component system

The phosphorylated form of the response regulator then induces the expression of the relevant genes necessary for adaptation to the changed environmental status. Well known two-component systems include NtrB-NtrC (controlling nitrogen assimilation, see above) and PhoR-PhoB (required for phosphate assimilation).

The *P. aeruginosa* genome contains more than 500 genes that encode either transcriptional regulators or two-component regulatory system proteins. Recently, Nishijyo *et al*. (2001) identified in *P. aeruginosa* PAO1 a novel two-component system, CbrA-CbrB, which belongs to the NtrB-NtrC family. This system controls several specific pathways and modulates the catabolism of various natural substrates in response to the carbon/nitrogen ratio. In addition, it controls the expression of catabolic pathways, specifying the major route of arginine degradation. CbrA and CbrB negative mutants of strain PAO1 were unable to utilize several amino acids such as arginine, histidine and proline as sole source of carbon and nitrogen (Nishijyo *et al*., 2001). By comparing their sequences with a known components of regulatory systems, CbrA and CbrB were identified as a sensor/histidine kinase and its cognate response regulator, respectively. The N-terminal half (490 residues) of CbrA appeared to be a sensor membrane domain, whereas the C-terminal part showed 34% sequence identity with NtrB of *E.coli* and other kinases of the NtrB family. The CbrB protein was ~45% identical to the response regulators of the NtrC family. Studies on the expression of catabolic pathways of arginine and histidine indicated that the σ^{54}-RNA polymerase holoenzyme is absolutely essential for the expression of the respective enzymes, with CbrB acting as a transcriptional activator. As the *P. aeruginosa* PAO1 genome contains all genes encoding the nitrogen regulatory proteins (Stover *et al*., 2000), it is conceivable that a signal transduction system similar to Ntr also operates in *Pseudomonas*. However, the signals that determine the activity of the CbrA-CbrB system in *P. aeruginosa* are still largely unknown.

1.6 Sigma factors in bacterial gene expression

Sigma factors associated with RNA polymerase are involved in specific binding to DNA and thus play an important role in regulation of differential gene expression (Helmann and Chamberlin, 1988). There are several sigma factors present in bacteria. *E. coli* has seven σ subunits, and each constitutes a distinct function. σ^{70} associated with RNA polymerase initiates transcription of house keeping genes and some nonessential genes which are induced under certain conditions. σ^{S} is considered as a general stress factor since it is mainly associated with a variety of growth-impairing stresses such as nutrient limitation, high osmolarity, oxidative stress and high temperature. σ^{32} and σ^{E} are also associated with

stress. σ^{32} is required for the response to damage of cytoplasmic proteins, which is most commonly associated with heat shock, and σ^{E} controls the response to extracytoplasmic or extreme heat stress. σ^{54} encoded by the *rpoN* gene was first discovered during an analysis of GS and nitrogen assimilation in enteric bacteria (Hirschman *et al*., 1985). It is an important factor involved in nitrogen assimilation. It is also involved in a variety of other processes such as carbon source utilization, certain fermentation pathways, flagellar synthesis, and bacterial virulence. σ^{54}-dependent transcription has several distinct features. σ^{70}-like factors associated with core RNA polymerase (E) forms a open promoter complex. In contrast, $E\sigma^{54}$ catalyzes strand separation only with the help of a distinct class of transcriptional activators. Due to this unique property, transcription can be turned off completely. The σ^{54}-dependent activators bind to sites that are effective regardless of distance and orientation. The activators interacts with $E\sigma^{54}$ from these binding sites. This interaction sometime requires DNA bending proteins such as integration host factor (IHF).

1.6.1 σ^{54}-dependent promoters

σ^{54}-dependent genes are controlled through modulation of the activator's ATPase activity. σ^{54}-dependent activators contain a regulator domain that controls ATPase activity by several mechanisms like phosphorylation, interaction with ligand, or interaction with regulatory proteins. σ^{54}-dependent promoters contain an easily recognizable site for $E\sigma^{54}$ since their expression absolutely requires σ^{54}. In *E. coli*, all the known σ^{54}-dependent promoters are located outside the structural genes. The average size of the intergenic region that contain a known σ^{54}-dependent promoter is about 200-250 bases. The distance from the 3' end of the $E\sigma^{54}$ binding site to the nucleotides coding for the initiation codon is, on average, 50 bases.

1.6.2 σ^{54}-dependent genes of nitrogen metabolism

Many σ^{54}-dependent genes are involved in nitrogen assimilation. These genes specify GS, the regulators NR_I, several transport systems, and a few catabolic operons. Glu and Gln are the major intracellular nitrogen donors, and they provide about 75 and 25% of the cell's nitrogen, respectively (Reitzer and Schneider, 2001). Ammonia assimilation and the GS activity is known to be dependent on σ^{54} factor. The pathways of ammonia assimilation and control of GS activity is already discussed in section 1.2.

1.7 Transport of Nitrogenous Compounds

The transport of nitrogen-containing nutrients into the cell is the initial and thus a very important process in bacterial nitrogen metabolism.

1.7.1 Ammonium transport

There is considerable evidence for the rapid diffusion of free ammonia across cytoplasmic membranes. In most of the prokaryotic species, transport of ammonium ions (NH_4^+) occurs by an active transport system (Amt). In most cases, Amt activity is repressed in the presence of high extracellular ammonium concentrations. Several studies indicated that Amt expression is controlled by the Ntr system (Jayakumar *et al*., 1986). In *E. coli*, a peripheral membrane-associated protein (AmtA) is mainly responsible for ammonium transport.

1.7.2 Nitrate transport

P_{II}-like proteins play a role in nitrate utilization or uptake in many organisms. *GlnB* mutants of *Bacillus subtilis* and *Rhizobium leguminosarum* failed to utilize nitrate as a sole nitrogen source, indicating that GlnB is involved in nitrate utilization (Amar *et al*., 1994; Wray, *et al*., 1994). Like ammonium uptake, nitrate assimilation is controlled by the Ntr system. A *glnB* mutant of *Azospirillum brasilense* excreted ammonium when the cells were grown in presence of nitrate. It has been proposed that the observed effect could be due to deregulation of the nitrate assimilation pathway, with the consequent accumulation of intracellular ammonium leading to ammonia excretion (Liang, *et al*., 1993). A *P. putida* KT2442 *nasB*$^-$ mutant (devoid of nitrate reductase) was also shown to be highly responsive to ammonia deprivation (Eberl *et al*., 2000). These authors also demonstrated that expression of *nasB* gene is dependent on the presence of a functional *gltB* gene.

1.7.3 Amino acid transport

Transport of an extracellular amino acids into the cell is an important step in their utilization as a sources of cellular nitrogen and for various other metabolic processes such as protein and nucleotide biosynthesis. The ABC (ATP binding cassette) transporter is one of the active transport systems of the cell, which is widespread in archea, eubacteria, and eukaryotes. It is also known as the periplasmic binding-dependent transport system in

Gram-negative bacteria and the binding-lipoprotein-dependent transport system in Gram-positive bacteria. The transporters consist of two integral membrane proteins (permeases), two peripheral membrane proteins that bind and hydrolyze ATP, and a periplasmic substrate-binding protein. In *E. coli* more than half of the genes activated by nitrogen limitation code for transport systems. Many transport systems for the amino acids were shown to be dependent on σ^{54} factor.

Glutamine transport has been studied extensively in *E. coli*. Gln transport system requires a GlnH, a high-affinity Gln-specific binding protein in the periplasm. The *glnHPQ* operon specifies GlnH and two other membrane proteins, which interact with GlnH. Expression of *glnHPQ* requires nitrogen limitation. Nitrogen limitation increases the production of *glnHPQ* transcripts five- to nine fold. *E. coli* contain five transport systems for glutamate and aspartate. Nitrogen limitation induces a periplasmic protein gltI that binds to both Glu and Asp.

E. coli and other Gram-negative bacteria digest peptides intracellularly after their passage through the outer membrane and transport via periplasmic binding protein-dependent transport systems. Nitrogen limitation induces the expression of *dppABCDE* and *oppABCDE*. The products of these operons are the major peptide transporters in *E. coli*. The first step in peptide transport is passage through the outer membrane, and nitrogen limitation results in 25-fold higher transcription of *ompF*, which codes for an outer membrane channel.

Very little is known about the regulation of amino acid transport in *Pseudomonas*. Considering this fact, it would be of great importance to study such systems in more detail.

1.8 Plant growth-promoting rhizobacteria

In recent years, the interest in the interactions between plant roots and soil organisms has been growing at a rapid pace. Plants are known to establish two types of symbiotic relationship.

1) plant-bacterial symbiosis as see with rhizobia and bradyrhizobia and 2) plant-fungal symbiosis. Plant-bacterial symbiosis result in the provision of nitrogen to the plant via fixing of atmospheric nitrogen by the symbiont, while plant-fungal symbiosis supplies the plants with phosphates. In return, the plants supplies both type of organisms with carbon compounds for their nutrition.

In addition to symbiotic microorganisms many other bacteria inhabit the rhizosphere (the root surface and the surrounding soil areas). Some of these non-symbiotic bacteria also have the potential to improve crop yields. These so-called plant growth-promoting rhizobacteria (PGPR) which belong in diverse genera such as *Pseudomonas* and *Bacillus*, enhance plant growth either directly through the synthesis of phytohormones (giberellin and indole acetic acid) or indirectly by the production of antibiotics that control pathogenic fungi and competing bacteria (Bloemberg *et al*., 2001). Additionally, PGPR increase availability of compounds such as nitrate, phosphate, sulfate, carbon dioxide and water for use by the roots.

Due to these effects, PGPR are of great interest for sustainable crop protection and have, therefore, drawn much attention. However, their use in the field often failed because potential PGPR were unable to colonize the rhizosphere of inoculated plants and to survive in this environment. Colonization of roots by introduced bacteria is an important step in the interaction between beneficial bacteria and their host plants. In recent years, several groups have initiated projects with the aim to elucidate the interactions that mediate root colonization. In most of these studies, fluorescent pseudomonads are used which inhabit the rhizospheres of most crop plants. Pseudomonads are free-living saprophytic organisms in soil or water where they play an important role in decomposition, biodegradation, and in the carbon and nitrogen cycles. Because of this lifestyle, pseudomonads are characterized by great metabolic diversity. Consequently, they are also important in bioremediation i.e. the microbial degradation or inactivation of hazardous chemicals in the environment, and in biofertilization i.e. the process in which microorganisms increase the availability of nutrients (Lugtenberg *et al*., 1991).

1.9 Root colonization by bacteria

One of the most relevant aspects is the process of bacterial establishment in the rhizosphere, since an effective biocontrol depends on the efficiency or root colonization (Chin-A-Woeng *et al*., 2000). To date, only a few bacterial traits involved in rhizosphere colonization have been identified. It was reported that immotile *Pseudomonas* mutants were impaired in colonization (de Weger *et al*., 1987). Mutants of *P. fluorescens* defective in the synthesis of the O-antigen of lipopolysaccharide (LPS) were also less effective in root colonization. Further characterization revealed that mutants lacking the O-antigen of LPS have a decreased growth rate compared to their parental strains (Dekkers *et al*.,

1998b). The ability to synthesize amino acids and vitamin B_1 was also shown to be essential for colonization. Simons *et al*. (1996) reported that genes involved in the synthesis of amino acids and vitamin B_1 are essential for establishment in the rhizosphere. In one of the mutants, mutation in the *nuo*4 gene encoding a subunit of NADH: ubiquinone oxidoreductase resulted in impaired root colonization. This enzyme is involved in the generation of the proton motive force used for the synthesis of ATP, active transport of various nutrients and ATP-dependent rotation of the flagella. The action of a member of the λ integrase family of site-specific recombinases was found to be essential for colonization (Dekkers *et al*., 1998a). Site-specific recombinases have been implicated in the production and regulation of fimbriae in *E. coli*, the production of two different forms of LPS in *Francisella tularensis*, antigenic variation of surface lipoprotein antigens in *Mycoplasma bovis* and the production of two flagellin genes in *Salmonella typhimurium*. This suggest that lacking the ability for DNA rearrangements can affect one or more traits already described to be important for root colonization.

Attraction of PGPR by the host is probably a key process for the initiation of mutualistic plant-bacterial interactions. Bacterial chemotaxis towards different nutrients known to be present in root exudates has been demonstrated (Vande Broek *et al*.,1995). Non-flagellated and non-chemotactic mutants of *Azospirillum brasilense* showed reduced ability to colonize wheat roots (Vande Broek *et al*., 1998). Although the question of bacterial motility in soil is still under debate, a series of experiments performed with *P. fluorescens* and *A. brasilense*, pointed out the existance of a directed motion of bacterial cells towards wheat roots in soil (Bashan, 1986). However, this motion was heavily influenced by soil composition and humidity. The role of motility in attachment and colonization has been examined in detail by Turnbull *et al*. (2000). Motility seems to be important for competitive root colonization by *P. fluorescens* (Turnbull *et al*., 2001), as well as for the attachment of *P. putida* to wheat roots under conditions of nutrient limitation.

1.10 The role of root exudates in plant-bacterial interactions

Many plants release a large fraction of their assimilates into the rhizosphere. It is now well established that such root exudates play a central role in the communication between root bacteria and their plant hosts (De Weger *et al*., 1995). Apparently, many plants have gained the ability to exert control over the rhizosphere community through the release of compounds that can enhance beneficial associations while limiting deleterious interactions.

In parallel, microbes can increase their survival within the rhizosphere by establishing a spectrum of relationships with the plant that can be exerted in response to root exudation. Indeed, root exudates were shown to induce in rhizosphere bacteria the expression of specific genes, some of which appear to be involved in the utilization of exudate components (van Overbeek and van Elsas, 1995; Lee and Cooksey, 2000). Exuded organic compounds such as citrate and malate also play an important role in mobilization of phosphorous, complexation of iron and solubilization of zinc and manganese.

1.11 Components of root exudates

Root exudate components generally have been categorized into three classes: low-molecular weight, high-molecular weight and volatile compounds (Fig. 1.4). Low-molecular weight compounds represent the main portion of exudates and mainly consist of sugars, amino acids, organic acids, vitamins and various secondary metabolites. High-molecular weight compounds consist of mucilage and proteins, while carbon dioxide, certain secondary metabolites, low-molecular weight alcohols and aldehydes constitute volatiles (Nelson, 1991; Fan *et al*., 1997).

Different plant species contain many common constituents of each of these categories but their amounts and time of release may vary. Several factors such as temperature, light, age, soil type and moisture have been shown to affect the nature and the timing of exudate release (Rovira, 1969).

Although sugars account for most of the organic matter in exudates, there is no evidence indicating that they play a major role in plant-bacterial interactions. Lugtenberg *et al*. (1999) could not find a significant contribution of sugars to tomato root colonization by a well-studied *Pseudomonas* biocontrol strain. In addition to monosaccharides, exudates contain organic acids such as succinate, malate, acetate, and pyruvate (Waschutza *et al*., 1992) and significant amount of amino acids.

The predominant amino acids in root exudates are the acidic amino acids Asp and Glu and their amides Asn and Gln. In barley-root exudates, the acidic amino acids and their amides account for almost 50% of all amino acids (Barber and Gunn, 1974). Similar results were obtained with other plants (Boulter *et al*., 1966; Shepherd and Davis, 1994). In the corn rhizosphere, amino acids afford as much as 220 mg nitrogen/kg of soil dry weight, while nitrate and ammonia together account for less than 60 mg/kg (Jones and Darrah, 1993).

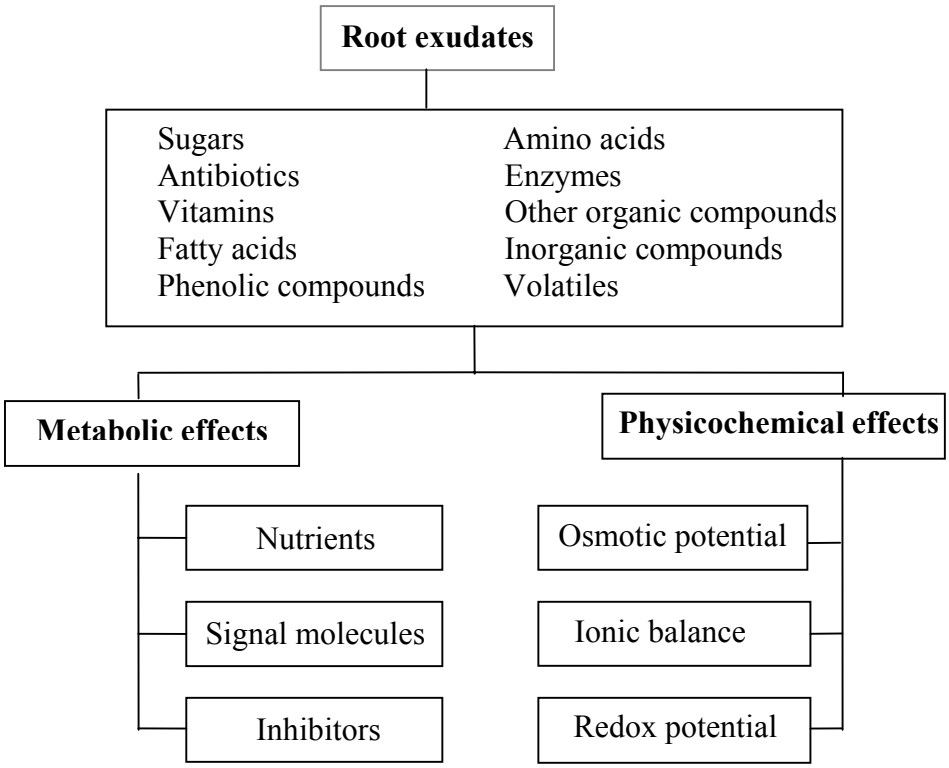


Figure 1.4: Functional roles of root exudates

In addition to acidic amino acids and their amides, proline is one of the most abundant amino acid present in corn root exudate (Vilchez *et al*., 2000b). Moreover, it was shown that root exudates can induce bacterial enzymes involved in the metabolism of proline (Vilchez *et al*., 2000a, 2000b**)** or lysine (Espinosa-Urgel and Ramos, 2001). Certain *put* genes which encode enzymes of proline catabolism are specifically induced by corn-root exudates. The *put*P gene product is involved in the uptake of proline to the cytoplasm of the cell, and the *put*A gene product, a multifunctional protein catalyzes the formation of Glu from proline. This gene was found to be adjacent but divergent from the *putP* gene. In *P. putida,* the PutP protein is predominantly localized as an integral inner-membrane protein that belongs to the family of Na^+ substrate symporters (Blohn von *et al*., 1997). Both *putA* and *putP* are regulated at the transcriptional level, with proline acting as an inducer.

1.12 Choice of organism

Among the numerous known strains of *Pseudomonas*, *P. putida* KT2440 is known as an especially efficient root colonizer. *P. putida* KT2440, a derivative of the soil isolate mt-2 (Franklin *et al*., 1981), colonizes the root system of a number of different plants,

establishing and persisting in the rhizosphere at a relatively high population density (Molina *et al*., 2000). While *Pseudomonas putida* KT2440 is non-pathogenic, other pseudomonads can cause disease. For example, *P. aeruginosa* is an important opportunistic pathogen *and P. syringae* is a plant pathogen. In addition *P. putida* KT2440 is very easy to handle and has the ability to utilize a wide range of carbon and nitrogen sources. Recently, the full genome sequence of *P. putida* KT2440 became available (Nelson *et al*., 2002) which greatly facilitates the identification and manipulation of relevant genes.

1.13 Aims and objectives of this study

The aims of the present study were to

- further characterize the role of acidic amino acids and their amides in nitrogen metabolism of *P. putida* KT2440
- identify differentially expressed proteins during growth on various combinations of carbon and nitrogen sources
- characterize genes involved in the utilization of amino acids
- identify regulatory systems that control utilization of acidic amino acids and their amides

2. Materials

2.1 Microorganisms

Stocks of all microorganisms were prepared in sterile 40% glycerol. Routinely used stocks were kept at -20 ^{0}C whereas, for longer use stocks were kept at -80 ^{0}C.

Microorganism	Genotype	Source
E. coli		
BL21	*ompT hsdS*(r_B– m_B–) *dcm*+ Tet^r *gal* λ	Stratagene
CodonPlus(DE3)-RIL	(DE3) *endA* Hte *[argU ileY leuW Cam^r]*	
DH5α^{TM}	*F$^-$ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 deoRrecA1 endA1 hsdR17 ((r_k^- m_k^+)phoA supE44 λ$^-$ thi-1 gyr A96 rel A1*	Life Technology
HB101	*supE44hsdS20$^-$(r_b^- m_b^-) recA13 ara-14 proA21acY1 gaIK2 rpsL20*	Amersham Pharmacia
TG1	*supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (r_k^- m_k^-)[F' traD36 proAB lacI^{q}ZΔM15]*	Stratagene
XL1 Blue	*recA1 endA1 gyr A96 thi-1 hsdR17 sup E44lac[F' proA BlacIZΔM15 Tn10(Ter^r)]*	Stratagene
E. coli S-17 pOT182		Merriman, 1993
E. coli S-17		Simon *et al*., 1983
Pseudomonas		
P. putida	Wildtype; ATCC 12633	DSM
P. putida KT2440	*mt-2hsdR1 (r^- m^+)*	Bagdasarian *et al*.,1982
P. putida KT2440 rpoN$^-$	*rpoN* mutant	Köhler *et al*., 1989
aauS$^-$	aauS$^-$ derivative of KT2440	This work
aauR$^-$	aauR$^-$ derivative of KT2440	This work
ansB$^-$	ansB$^-$ derivative of KT2440	This work
gltB$^-$	gltB$^-$ derivative of KT2440	This work
Tn-SM2	gltB:: Tn5-OT182	This work
Tn-SM3	gltB:: Tn5-OT182	This work
Tn-SM6	gltB:: Tn5-OT182	This work
Tn-SM9	gltB:: Tn5-OT182	This work
Tn-SM15	gltB:: Tn5-OT182	This work
Tn-SM27	gltB:: Tn5-OT182	This work
Tn-SM29	gltB:: Tn5-OT182	This work
Tn-SM30	gltB:: Tn5-OT182	This work
Tn-SM31	gltB:: Tn5-OT182	This work
Tn-SM33	gltB:: Tn5-OT182	This work

2.2 Antibiotics

Antibiotics were first filter sterilized and then added to the medium when the temperature of the medium reached 50 ^{o}C.

Antibiotic	Stock (mg/ml)	Final Concentration (µg/ml)
Ampicillin	100	100
Carbenicillin	50	300
Chloramphenicol	100	50
Gentamycine	10	15
Kanamycin	10	25 and 30
Tetracycline	10	25

2.3 Plasmids

Plasmid	Characteristic(s)	Source
pGEX-6P-3	Expression vector	Amersham BioScience
pJQ200	Cloning vector	Quandt and Hynes, 1993
pK18	Cloning vector	Pridmore, 1987
pOT182	Self cloning promoter probe Vector	Merriman and Lamont,1993
Tn5-OT182	Derivative of pSUP102(Gm):: Tn5-B21	Merriman and Lamont, 1993

2.4 Oligonucleotides

All synthetic nucleotides were made available from MWG BioTech (Ebersberg).

2.4.1 Oligonucleotide primers for gene expression

Primer name	Nucleotide sequence	Restriction Site
ABCFor	5'-CACATCATGGTCATGCCTTC-3'	-----
ABCRev	5'-ACCTGACCATCACCGAGAAC-3'	-----
ansBFor	5'-CTGTCCTGGGTCTTGGTCAT-3'	-----
ansBRev	5'-GTATGGCTATGGCAACGTCA-3'	-----
AspFor	5'-GGTTGATTTCGGTCAGCAGT-3'	-----

AspRev	5'-ACCTGCACCCTAACAACGAC-3'	-----
FumaFor	5'-ATACGGCCAGTACCCACGTA-3'	-----
FumaRev	5'-GTAGCTGCTTGACTGCACCA-3'	-----
LyaseFor	5'-GGTTGATTTCGGTCAGCAGT-3'	-----
LyaseRev	5'-ACCTGCACCCTAACAACGAC-3'	-----
PorFor	5'-AGACCCGCATGCTGTATTTC-3'	-----
PorRev	5'-ACTGGTCACCCACTTTCAGC-3'	-----
RhoFor	5'-ATCCTGCTGGACTCGATCAC-3'	-----
RhoRev	5'-GAGCGGTTGATGTTGATGG-3'	-----

2.4.2 Oligonucleotide primers for transposon mutant sequencing

Primer Name	Nucleotide sequence	Restriction Site
OT182For	5'-GATCCTGGAAAACGGGAAAG-3'	-----
OT182Rev	5'-ACATGGAAGTCAGATCCTGG-3'	-----
pOT182For	5'-CGACGGGATCCATAATTTTT-3'	-----
pOT182Rev	5'-CGTTACCATGTTAGGAGGTC-3'	-----

2.4.3 Oligonucleotide primers for gene replacement

Primer Name	Nucleotide sequence	Restriction site
aauSFor	5'-CGCggatccCGAATAC**C**CTTGAAGGCCTGA-3'	*Bam*HI
aauSRev	5'-CCCAAGCTTTCAGTTTTTCCACACCATCG-3'	*Hind*III
aauRFor	5'-CGCggatccGCCTGGTCGA**A**CGTGGTACG-3'	*Bam*HI
aauRRev	5'-CCCaagcttGATGTCTTCACGGCGCTCAC-3'	*Hind*III
ansBFor	5'-GAGGCTAAGCGAGGAAATGA-3'	--------
ansBRev	5'-GTAGCCAGCCGAAACTGAAG-3'	--------
ansBLT	5'-ATGAATGCCGCACTGAAAAC-3'	--------
ansBRT	5'-ACGACCCAGTCGTTCTTGTC-3'	--------
ansB5Rev	5'-GCGCTTGGGGCGAAGGTT-3'	--------
gltBFor	5'-CGCggatccCGCA**A**ACATCTTCCAGGAGT-3'	*Bam*HI
gltBRev	5'-AActgcagACCAGCGTGGTGTATTCCTT-3'	*Pst*I
gltBpK18For	5'-CACAGGAAACAGCTATGACCA-3'	--------

gltBpK18Rev	5'-ACCAGCGTGGTGTATTCCTT-3'	--------
gltBP3For	5'-ATTTCACACAGGAAACAG-3'	--------
gltBP4Rev	5'-CTCCAGCGGCTCGACCTG-3'	--------
KTABCFor	5'-CGCggatccTGGTCGATGGC**A**ACTTCGATT-3'	*Bam*HI
KTABCRev	5'-CCCaagcttTGCTGCCCTTGTCCATGAAG-3'	*Hind*III

2.4.4 Oligonucleotide primers for protein overexpression

Primer Name	Nucleotide sequence	Restriction site
aauR1For	5'-CGCggatccATGAACCAAGCGCCTCTTAC-3'	*Bam*HI
aauR2Rev	5'-CCGgaattcTCAGGCGAGGCCGTATTTTTTC-3'	*Eco*RI

Lower case and underlined = Restriction site introduced

Highlighted and underlined = Nucleotide base introduced

2.5 DNA and RNA Markers

DNA and RNA markers were kept at -20 ^{0}C for longer use. Routinely used markers were kept at 4 $^\circ$C for up to six months.

Marker	Obtained from
λ*BstE*II	peqlab Biotechnology GmbH, Erlangen
1 kb DNA- Ladder	peqlab Biotechnology GmbH, Erlangen
100 bp Ladder	peqlab Biotechnology GmbH, Erlangen
Mass Ruler^{TM} DNA Ladder, Low Range	Fermentas, St.Leon- Rot
RNA Ladder High Range	Fermentas, St.Leon-Rot
Prestain Protein Marker	New England BioLabs, Beverly

2.6 Kits

All components of DIG DNA Labeling and Detection kit and RT-PCR kit were stored at -20 ^{0}C and buffer P1 from QIAprep Spin Plasmid kit was stored at 4 ^{0}C.

DIG DNA Labeling and Detection kit	Boehringer Mannheim, Mannheim
DNeasy Tissue Kit	Qiagen, Hilden
QIAamp Tissue Kit	Qiagen, Hilden
QIAprep Spin Plasmid Kit	Qiagen, Hilden
QIAquick Gel Extraction Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QIAquick Nucleotide Removal Kit	Qiagen, Hilden
RNeasy Mini Kit	Qiagen, Hilden
RT-PCR Kit	Invitrogen Life Technologies, Eggenstein

2.7 Enzymes and Chemicals

Chemicals (usually of analytical grade) and enzymes were supplied by the Promega (Mannheim), Sigma (Steinheim), Merck (Darmstadt), Serva (Heidelburg), Roth (Karlsruhe), Roche (Mannheim), Peqlab Biotechnology (Erlangen), Life Technology (Eggestein), Amersham Pharmacia Biotech (Freiburg), Fermentas (St. Leon-Rot), New England BioLabs (Beverly), and Fluka (Buchs).

2.7.1 Enzymes

All enzymes except glutamate dehydrogenase were stored at -20 ^{0}C for prolonged use. Glutamate dehydrogenase was stored at 4 ^{0}C.

Enzyme	Source/Type	Obtained from
Alkaline Phosphatase	Calf intestine alkaline Phosphatase (CIP)	New England BioLabs, Beverly
DNA polymerase	PfuTurbo-Polymerase	Stratagene, Heidelburg
	Taq- Polymerase	Roche, Mannheim
Glutamate dehydrogenase		Sigma, Steinheim
Restriction enzymes		Boehringer Mannheim; Roche, Mannheim; New England BioLabs, Beverly
Reverse Transcriptase SupreScript ^{TM} II		Life Technologies, Eggenstein

RNase OUT Recombinant	Life Technologies, Eggenstein
RNase H	Life Technologies, Eggenstein
RQ1 DNase	Promega, Mannheim
T4-DNA ligase	Fermentas, St. Leon-Rot; New England BioLabs, Beverly

2.7.2 Chemicals

Acrylamide, N, N'-methylene bisacrylamide (30 %, 0.8%)	Roth, Kerlsruhe
Agarose	Sigma, Steinheim
α-cyano-3-hydroxycinnamic acid	Sigma, Steinheim
Ammoniumpersulfate (APS)	Merck, Dermstadt
Bromophenol Blue	Roth, Karlsruhe
Dithiothreitol (DTT)	Sigma, Steinheim
Formaldehyde	Sigma, Steinheim
Glycerol	Sigma, Steinheim
Glycine	Sigma, Steinheim
Immobilized pH gradient strips	Amersham Pharmacia, Freiburg
Iodoacetamide	Sigma, Steinheim
Pharmalytes 3-10	Pharmacia Biotech, Freiburg
PhastGel Blue R	Pharmacia Biotech, Freiburg
Saturated phenol	Roth, Karlsruhe
Serva Blue G	Serva, Heidelburg
Silver nitrate	Roth, Karlsruhe
Sodium dodecylsulphate (SDS)	Merck, Darmstadt
Sodium thiosulphate	Sigma-Aldrich, Steinheim
N,N,N',N'-Tetramethylendiamine (TEMED)	Merck, Darmstadt
Thiourea	Sigma-Aldrich, Steinheim
Trypsin	Promega, Mannheim
Urea	Sigma-Aldrich, Steinheim

2.8 Instruments

2.8.1 Bacterial growth

Apparatus	Model	Manufacturer
Autoclave	VST40/60S	Zirbus GmbH, Osterode
Incubation shaker	Novotron AK 82	Bottmingen, Switzerland
	G25	New Brunswick Scientific, Nürtingen
Heat incuabtion cupboard	FT550	Heraeus, Hanau
Sterile bench	D624 RF	Schirp Reinraumtechnik

2.8.2 Centrifuges

Apparatus	Model	Manufacturer
Centrifuge	Biofuge fresco	Heraeus, Hanau
	Centrifuge 5415	Eppendorf, Hamburg
	J2-21	Beckmann
	Minifuge RF	Heraeus Sepatech, Hanau
	Suprafuge 22	Heraeus Sepatech

2.8.3 Photometers

Apparatus	Model	Manufacturer
Spectrophotometer	UV/Vis 551	Perkin-Elmer, Überlingen
	Ultrospec 3000	BiotechPharmacia, England
	U-2000	Hitachi, Tokyo

2.8.4 Electrophoresis

Apparatus	Model	Manufacturer
2-D Gel electrophoresis	Hoefer SE 600	Perkin Elmer, Life Sciences
Digital Camera	D120 Zoom digital Camera	Kodak
Isoelectric focussing	MultiphorII	Pharmacia, Freiburg
Easy-Cast electrophoresis	B2	Woburn, USA
Power supply	Power-supply 3000/150	Pharmacia, Freiburg
	Consort E452	AGS GmbH, Heidelburg
Video- Scanner	Mitsubishi Video copy processor with thermoprinter	Mitsubishi

2.9 Membranes and special materials

	Obtained from
Cellulose acetate filter (0.2 µm)	Sartorius, Göttingen
Hybond^{TM}-N^{+}	Amersham Pharmacia biotech, Freiburg
Membrane filter (0.2 µm)	Schleicher& Schüll, Dassel
Millex®-GS (0.22 µm)	Millipore, Bedford, USA
Millex®-HA (0.45 µm)	Millipore,Bedford, USA
96-Well-Plates, Sterile	Greiner GmbH; Frickenhausen
24-Well-Plates, Sterile	Greiner GmbH; Frickenhausen

2.10 HPLC Analysis

Acetonitrile	Riedel-deHaen, Seelze
Amino acid standard	Sigma, Steinheim
Methanol	J. T. Baker,
Phenylisothiocyanate (PITC)	Sigma, Steinheim
Pyridine, Sodium acetate	Merck, Darmstadt
Triethylamine	Sigma, Steinheim

2.11 Other apparatus

Apparatus	Model	Manufacturer
Analytical balance		Sartorius
	AB54	Mettler Toledo, Gießen
DNA Thermocycler	T Personal	Biometra, Goettingen
	Cetus	Perkin Elmer, Langen
Heat bath	F3	Haak, Karlsruhe
Heating block	Thermostat 5320	Eppendorf, Hamburg
HPLC	D-7500	Hitachi, Japan
Microplate reader	Reader 3550 UV with software-kinetic collector	Biorad, München
pH meter	Model U2000	Orion-Colora, Lorch
Micropipettes		Gilson, France
Sonicator	Sonorex RK-103	Bandelin, Berlin
Speed-Vac Concentrator		Savant,Martinsried, Germany
Ultrasonicator	Sonoplus GM70	Bandelin, Berlin

2.12 Computer programs and Internet-Links

Clustal W alignment	http://www.igbmc.u-strasbg.fr/BioInfo/clustaw/Top.html
2-D Gel analysis	MelanieII®, BioRad
Compute pI	http://scansite.mit.edu/cgi-bin/calcpi
Emboss Transeq	http://www.ebi.ac.uk/emboss/transeq
ExPASy-Tools	http://www.us.expasy.org/tools/
Multialign interface	http://prodes.toulouse.inra.fr/multialin/multialin.html
Sequence blast	http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html
Peptide mass	http://www.expasy.org/tools/peptide-mass.htm
Pseudomonas database	http://pseudomonas.bit.uq.edu.au/gene_browser.phtml
Pseudomonas genome	http://www.pseudomonas.com/
PromScan Promoter Scanning	http://www.promscan.uklinux.net/

Protein Identification	MS-Fit
Protein Machine	http://www.ebi.ac.uk/~tommaso/translate.html
PowerPoint	Microsoft
Sigmaplot	Jandel Scientific
The Institute for Genomic Research	http://www.tigr.org
Windows	Microsoft
Word	Microsoft

3. Methods

3.1 Safety

All potentially harmful operations were carried out in restricted areas. Media, solutions, and instruments required for experiments with bacterial cultures were autoclaved before use. Temperature-sensitive solutions were sterilized by filtration through 0.4 μm membranes. All bacterial waste was decontaminated by autoclaving for 30 min at 121 ^{o}C or higher before disposal. Waste containing ethidium bromide was first passed through the column packed with activated charcoal and then disposed off in a special container. All dangerous chemicals were handled using safety glasses and gloves.

3.2 Bacterial growth

3.2.1 Storage and revival of bacterial cultures

100 μl of frozen cells from 40 % glycerol stock was inoculated into 5 ml of LB medium (Luria-Bertani medium, Sambrook *et al*., 1989). *E. coli* and *Pseudomonas* cultures were grown overnight with shaking at 37 ^{o}C and 30 ^{o}C, respectively. A loopfull of culture was streaked on LB plates and plates were incubated overnight at respective temperatures. The colonies formed could be used up to 1 month when kept at 4 ^{o}C. For longer storage, a fresh culture of exponentially growing cells was mixed with sterile 40% glycerol in an 1:1 ratio (v/v) and stored at –80 ^{o}C.

LB medium	
NaCl	10 g/L
Tryptone	10 g/L
Beef extract	5 g/L

3.2.2 Cultivation

Pseudomonas putida KT2440 and mutants were grown overnight in M9^{+}-medium at 30 ^{o}C with shaking. The cells were spun down at 6,000 rpm for 10 min. The pellet was washed in 3 ml of M9^{-}-medium and centrifuged again at 6,000 rpm for 5 min. The pellet was resus-

pended in $M9^-$-medium. Cell density was measured as A_{600}. The resulting cell suspensions were diluted appropriately with $M9^-$-medium so that nearly equal number of cells were present in an inoculum.

M9 medium (Sambrook *et al*., 1989)

a) $M9^+$-medium

Na_2HPO_4, anhydrous	6.78 g/l
KH_2PO_4	3.0 g/l
NaCl	0.5 g/l
NH_4Cl	1.0
* 20% glucose, filter sterilized	20 ml
* 1M $MgSO_4$	2 ml
* 1M $CaCl_2$	100 µl
	pH = 7.4

b) $M9^-$-Solution

Na_2HPO_4, anhydrous	6.78 g/l
KH_2PO_4	3.0 g/l
NaCl	0.5 g/l
*1M $MgSO_4$	2 ml
* 1M $CaCl_2$	100 µl
	pH = 7.4

* Sterilized separately and cooled to room temperature before adding into M9 medium.

Carbon and nitrogen source(s)	Stock solution	Final concentration
α-Ketoglutarate§	100 mM	10 mM
Aspartate	100 mM	10 mM
Asparagine	100 mM	10 mM
Fumarate§	100 mM	10 mM
Glutamate	1 M	10 mM
Glutamine	100 mM	10 mM
Saccharose§	100 mM	10 mM
Succinate	100 mM	10 mM

* All filter sterilized
§ Neutralized with 1 N NaOH

In order to check the effect of different carbon and nitrogen sources on growth and enzyme activities of wild-type and mutants, equal volumes of inoculum were added to tubes containing $M9^-$-medium supplimented with different carbon and nitrogen sources.

Samples were removed after different time intervals and checked for growth (A_{600}) and enzyme activities. *E. coli* cells were grown in LB medium under identical conditions except that cells were incubated at 37 ^{o}C.

3.3 Preparation and transformation of competent cells

3.3.1 Preparation of competent *E. coli* cells (Hanahan *et al*., 1983)

A single colony of *E.coli* cells was picked up using a sterile toothpick and incubated in 5 ml of LB medium with shaking at 37 ^{o}C overnight. 30 ml of LB medium was inoculated with 0.3 ml overnight culture and was allowed to grow till $OD_{595\ nm}$ reached to 0.4-0.5. Bacterial cells were transferred to a sterile, ice-cold 50-ml polypropylene tube and the cells were recovered by centrifugation at 3500 rpm for 10 min at 4 ^{o}C. The medium was decanted from the cell pellet and the tube was allowed to stand in an inverted position on a pad of paper towel for 1 min to drain away the last traces of medium. The pellet was resuspended by swirling in 20 ml sterile, ice-cold 0.1 M $CaCl_2$ solution and incubated in ice for 30 min. Cells were spun down at 3500 rpm for 5 min at 4 ^{o}C. The pellet was resuspended in 5 ml of 0.1 M $CaCl_2$ and again kept in ice for 30 min. The treated cells were directly used for transformation or were dispensed in aliquots and frozen at -80 ^{o}C.

3.3.2 Transformation of competent host cells

10 µl of ligation mixture was added to 100 µl of competent *E. coli* cells. The contents of the tube was mixed by swirling gently and the tube was stored in ice for 30 min. The cells were heat-shocked at 42 ^{o}C for 60 s and the tube was rapidly transferred to an ice bath where the cells were allowed to chill for 5 min. 400 µl of LB medium was added to each tube. The cultures were shaken at 37 ^{o}C, 130 rpm for 1 h. Transformed competent cells were transferred onto LB agar plates containing appropriate antibiotic. The plates were stored at room temperature until the liquid had been absorbed. Plates were inverted and incubated at 37 ^{o}C.

3.3.3 Preparation of electro-competent *Pseudomonas* cells

A single colony of *Pseudomonas* from a fresh agar plate was inoculated into a flask containing 5 ml of LB medium. The culture was incubated overnight at 30 ^{o}C on a rotary

shaker (220 rpm). 0.5 ml of this overnight culture was inoculated in 50 ml LB medium. The flask was incubated at 30 ^{o}C with agitation (220 rpm) until OD_{595nm} of the growing culture reached to 0.6. The flask was transferred to an ice-water bath for 30 min. The culture was transferred to ice-cold centrifuge bottles. The cells were harvested by centrifugation at 3500 rpm for 10 min at 4 ^{o}C. The supernatant was discarded and cells were resuspended in 20 ml sterile ice-cold 10% glycerol. Cells were harvested by centrifugation at 3500 rpm for 5 min at 4 ^{o}C. The resulting pellet was resuspended in 20 ml sterile ice-cold 10% glycerol and again centrifuged under same conditions. Finally the cells were resuspended in 5 ml ice-cold 10% glycerol.

3.3.4 Electroporation of competent *Pseudomonas* cells

Electroporation is an efficient means of transferring macromolecules such as DNA into bacteria. It is the process of applying high-voltage electric field pulses of short duration to create temprory pores in the membrane of cells. These pores are generally large enough to allow macromolecules to diffuse into the cell. Upon removal of an electric field and a period of recovery, these pores are resealed and the DNA is replicated within the cell.

90 µl of the freshly made electrocompetent cells were pipetted into ice-cold 1.5 ml microfuge tube. The cells were placed on ice, together with an appropriate number of electroporation cuvettes. 10 µl of the DNA was added to each microfuge tube and the tubes were incubated in ice for 5 min. The DNA/cell mixture was pipetted into an ice-cold 1 mm electroporation cuvette. The solution was tapped to ensure that the suspension of bacteria and DNA sits at the bottom of the cuvette. Moisture from outside of the cuvette was dried. The cuvette was placed in a electroporation devise. A pulse of electricity at 1.8 kV was delivered to the cells. Immediately after the pulsing, the electroporation cuvette was removed from electroporation devise and 400 µl LB medium was added to the cuvette. After this, cells were transferred to a polypropylene tube and was incubated with gentle rotation (~130 rpm) for 3 h at 37 ^{o}C. Electroporated cells were spread onto LB agar medium containing appropriate antibiotic. The plates were stored at room temperature until the liquid had been absorbed. The plates were inverted and incubated at 30 ^{o}C.

3.4 Motility and chemotaxis assays

Transposon mutants were examined for their motility phenotypes by transferring fresh individual colonies to Petri dishes containing a 0.3% $M9^-$-agar supplimented with 10 mM amino acids and tetracycline. These motility plates were incubated for 1 to 3 days at 30 ^oC, and the motility phenotype of each mutant was assessed. Motile strains swarm through the semi-solid agar away from the site of inoculation, resulting in a larger area of growth than for nonmotile strains. Chemotaxis towards amino acids was examined on 'swarm plates'. Sterile filter paper discs were soaked in amino acids (Asn Asp, Gln, Glu, 10 mM each) and transferred to the plates. Fresh bacterial colonies were inoculated at a suitable distance from soaked filter paper discs, incubated at 30 ^oC for 24 to 36 h, and examined for growth towards amino acids.

3.5 Survival in nitrogen limiting conditions

Nitrogen starvation regimens were set up after harvesting an overnight grown culture of transposon mutants, *gltB* mutant and wild-type strain. Cells were washed twice with $M9^-$-medium followed by resuspension in $M9^+$-medium depleted of ammonium. Growth and starvation of cells were carried out at 30 ^oC and the cell mass of the cultures was measured spectrophotometrically as the optical density at 600 nm (OD_{600}). Starvation survival of nitrogen depleted cultures was monitored by determination of viable counts by plating 0.1-ml samples of different dilutions on LB plates.

3.6 Isolation of bacterial DNA

3.6.1 Isolation of genomic DNA by the DNA Mini Kit (Qiagen)

The QIAamp DNA minikit (250) from QIAGEN was used for the preparation of DNA. Bacterial cells were grown overnight in 5 ml of appropriate medium at optimum temperature. 1.5 ml of the culture was transferred into a microfuge tube and centrifuged at a maximum speed for 30 s at 4 ^oC to isolate the cell pellet. The pelleted bacterial cells were suspended in 180 µl ATL buffer. After that, 20 µl proteinase K was added and mixed with the cell pellet by vortexing. The mixture was incubated at 56 ^oC for 10 min. The samples were vortexed occasionally during incubation to disperse the contents. After incubation, the sample was centrifuged briefly to remove the drops from the inside of the lid.

Reagents	
ATL	Cell lysis buffer
Proteinase K	DNase inhibitor
AL	Lysis buffer
Absolute ethanol	Precipitation
AW1	Wash buffer 1
AW2	Wash buffer 2
AE	Elution buffer

To attain a RNA-free genomic DNA, 20 µl RNase A (20 mg/ml) was added to the sample and mixed thoroughly by gentle vortexing. Next, 200 µl buffer AL was added to the sample, mixed again by pulse vortexing for 15 s and incubated at 70 ^{o}C for 10 min. 200 µl of absolute ethanol was added to the lysate and mixed by gentle inversion to precipitate cell components. A QIAamp spin column was placed in a microcentrifuge tube and the mixture including precipitate was transferred to the column. After centrifugation for 1 min at 6,000 x g, the fluid in the spin column was drained into the microcentrifuge tube, while the DNA from the supernatant was bound to the column material. The column was then washed by adding 500 µl buffer AW1 without wetting the rim and again centrifuged for 1 min. The flow-through was discarded and the column was placed in a clean 2-ml collection tube. The column was washed another time with 500 µl buffer AW2 as above. To eliminate any chance of possible buffer AW2 carryover, the column was placed in a fresh microcentrifuge tube and centrifuged again for 1 min. Finally, the bound DNA was eluted with 50 µl buffer AE.

3.6.2 Phenol-chloroform extraction

150 µl phenol saturated with TE buffer was added to the cell suspension and the mixture was dispersed homogeneously by shaking for 5 min. The bacterial lysate was centrifuged at maximum speed for 30 s to separate the two phases. 200 µl of the upper aqueous phase of the emulsion was carefully transferred into a fresh microfuge tube and the protein-containing interphase was discarded. 150 µl absolute chloroform was added to the aqueous phase, shaken gently to mix the content and centrifuged at maximum speed for 30 s. 200 µl of the aqueous phase was transferred into a new microfuge tube. This step was repeated twice.

3.6.3 DNA precipitation

1/20 volume (10 µl) 5 M NaCl and 2 volumes of absolute ethanol were added to the DNA solution. The solution was mixed by vortexing and then allowed to stand for 2 min at room temperature. The precipitated DNA was collected by centrifugation at maximum speed for 10 min at 4 ^{o}C in a microfuge. The supernatant was discarded and the tube was allowed to stand in an inverted position to drain away the fluid. DNA was washed by adding two volumes of 70% ethanol and the tube was inverted several times. The DNA was recovered by centrifugation at maximum speed for 5 min at 4 ^{o}C. The tube was kept open at room temperature until the ethanol had evaporated completely. DNA was dissolved in 200 µl TE buffer (pH 8.0) and mixed by gentle shaking. DNA was stored at 4 ^{o}C for further use.

3.7 Isolation of Plasmid DNA

The QIAprep-spin Plasmid Kit (250) from QIAGEN was used for the preparation of double-stranded DNA from overnight cultures of *Pseudomonas* and *E. coli* in LB medium. These cultures were centrifuged at 4 ^{o}C for 30 s at 13,000 rpm to isolate the cell pellet.

Reagents	
P1	Cell suspension buffer
P2	Cell lysis buffer
N3	Neutralization buffer
PB	Wash buffer
PE	Wash buffer
EB (elution buffer)	10 mM Tris-Cl, pH 8.5

The pelleted bacterial cells were resuspended in 250 µl buffer P1. After that, 250 µl SDS-containing lysis buffer P2 was added and mixed with the cell pellets by inverting 4-6 times. The mixtures were incubated at room temperature for 5 min. 350 µl neutralization buffer N3 was added to the lysate and mixed by gentle inversion to precipitate the cell components. The precipitate was removed by centrifugation at 4 ^{o}C for 10 min at 13,000 rpm and the supernatant was discarded. A QIAprep-spin column was placed in a microcentrifuge tube and the supernatant was applied to the column. After centrifugation

for 1 min, the fluid in the spin column was decanted, while the plasmid DNA from the supernatant was bound to the column material. The column was then washed by adding 500 µl PB buffer and again centrifuged for 1 min. The flow-through was discarded. The column was washed a second time with 750 µl PE buffer as above. To avoid possible carryover of ethanol containing PE buffer which may inhibit subsequent enzymatic reactions, the column was again kept in a new microcentrifuge tube and centrifuged for additional 1 min. Finally, the bound DNA was eluted with 50 µl EB buffer by centrifugation.

3. 8 Agarose gel electrophoresis (Maniatis *et al*., 1982)

In agarose gels DNA fragments can be separated from each other according to their size. The electrophoretic mobility of DNA fragments is inversly related to their length, as each nucleotide in a nucleic acid molecule carries a single negative charge.

Reagents	Concentration
Gel solution	0.8-1.7% (w/v) in 1X TAE
Ethidium bromide stock	10 mg/ml
Gel loading dye solution	0.25 % (w/v) bromophenol blue in H_2O
	0.25% (w/v) xylenecyanol FF in H_2O
	30% (v/v) glycerol in H_2O
Electrode buffer	1X TAE

TAE-buffer (50 X), per liter	
Tris	242 g
Acetic acid	57.1 ml
0.5 M EDTA	100 ml, pH= 8.0

Running buffer (10X), per liter

Tris	30 g
Glycine	144 g
10% SDS	500 ml

DNA bands can be detected by staining with ethidium bromide which fluoresces under ultraviolet light when bound to DNA.

The gel matrix was prepared as follows: 0.8-1.7% (w/v) agarose was dissolved in 1X TAE buffer by boiling. 8 µl ethidium bromide (10 mg/ml) was added to this gel solution and poured into the corresponding gel apparatus and the gel was allowed to solidify at room temperature. To load a DNA sample, wells of 4 mm size were prepared by placing a comb into the liquid agarose. After solidification, 500 ml electrode buffer was poured into the apparatus to cover the gel surface. 10 µl of DNA solution was mixed with 2 µl of 6X

dye solution and placed into the wells of the gel. After that, the gel was run at 80 V until the blue dye was 1 cm away from the bottom of the gel. The DNA bands in the gel were then visualized on a transilluminator using UV light (365 nm). To determine the size of DNA, a standard λ-DNA marker (300 bp to 4,000 bp size) was run with sample. The gels were photographed by using KODAK DC120 camera. For isolation of DNA from the agarose gel, the desired DNA band was cut from the gel by using a clean scalpel and kept in a microcentrifuge tube for the following DNA extraction.

3.9 DNA extraction from agarose gels

After staining, DNA-fragments were visualized under UV-light at 365nm. DNA fragments were excised with a clean, sharp scalpel. The gel slice was weighed in a microfuge tube.

Reagents	
Buffer QG	Dissolving buffer
Buffer PE	Washing buffer
Buffer EB (elution buffer)	10 mM Tris-Cl, pH 8.5

Three volumes buffer QG was added to one volume of the gel. The tubes were incubated at 50 ^{o}C for 10 min. To dissolve the gel completely, the mixture was vortexed every 2-3 min during incubation. If the colour of the mixture was violet, 10 µl 3 M sodium acetate, pH 5.0 was added to the reaction mixture. To increase the yield of DNA fragments, one gel volume isopropanol was added to the sample and mixed well. QIAquick spin columns were placed in a 2-ml collection tube. To bind DNA, the sample was applied to the QIAquick column, and centrifuged for 1 min at 10,000 rpm. The flow-through was discarded and 0.5 ml buffer QG was added to QIAquick column and again centrifuged for 1 min at 10,000 rpm. The column was washed by adding 0.75 ml buffer PE to the column and centrifuged for 1 min at 10,000 rpm. Residual ethanol from buffer PE was completely removed by centrifuging the column for an additional 1 min. The column was placed in a new 1.5 ml microfuge tube. The DNA was eluted by adding 50 µl buffer EB to the center of the QIAquick membrane. To increase DNA concentration, the column was allowed to stand for 1 min at room temperature, and then centrifuged for 1 min at 13,000 rpm. For further use, the DNA was stored at -20 ^{o}C.

3.10 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) uses multiple cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences.
In a sterile 0.5-ml microfuge tube the following components were added

10X amplification buffer	5 µl
2 mM dNTPs	10 µl
Forward primer (100 pm)	1µl
Reverse Primer (100 pm)	1 µl
Taq DNA polymerase/PfuTurbo Polymerase	1-2 Units
Template DNA	200-400 ng
Total Volume	50 µl

The use of thermostable polymerase from *Thermus aquaticus* (Saiki *et al*., 1988) is the enzyme of choice for increased efficiency of PCR. The enzyme is highly processive 5'-3' DNA polymerase that lacks 3'-5' exonuclease activity. The enzyme exhibits highest

activity at a pH of around 9 and temperature around 75 ^{o}C. Taq DNA polymerase activity is stable against prolonged incubations at elevated temperatures (95 ^{o}C). Another enzyme of choice to obtain high-fidelity PCR is PfuTurbo DNA polymerase. PfuTurbo DNA polymerase amplifies complex genomic DNA targets upto 10 kb and vector targets upto 19 kb in length. To obtain the desired products in high yield, oligonucleotide primers complementary to 5'- and 3'- end of the amplification fragment were used.

The nucleic acids were amplified using the denaturation, annealing, and polymerization times and temperatures as listed below.

Cycle Number	Denaturation	Annealing	Polymerization
First 25-30 cycles	1-5 min at 94-95 ^{o}C	30-40 s at 45-68 ^{o}C	1 min at 72 ^{o}C
Last Cycle			5-10 min at 72 ^{o}C

5-10 µl of a sample from test reaction mixture was withdrawn and analyzed by electrophoresis through an agarose gel. Control reaction and an appropriate size DNA markers were also included with test reactions. Gels were stained with ethidium bromide to visualize DNA bands.

3.11 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR combines cDNA synthesis from RNA templates with PCR to provide a rapid, sensitive method for analyzing gene expression. RT-PCR is used to detect or quantify the expression of messages, often from small amounts of RNA. In addition, the technique is used to analyze differential gene expression or to clone cDNAs without constructing a cDNA library. RT-PCR is more sensitive and easier to perform than other RNA analysis techniques, including Northern blot, RNase protection assays, *in situ* hybridization, and S1 nuclease assays. The RT reaction can be primed with random primers, oligo(dT), or a gene-specific primer (GSP) using a reverse transcriptase (see Fig 3.1).

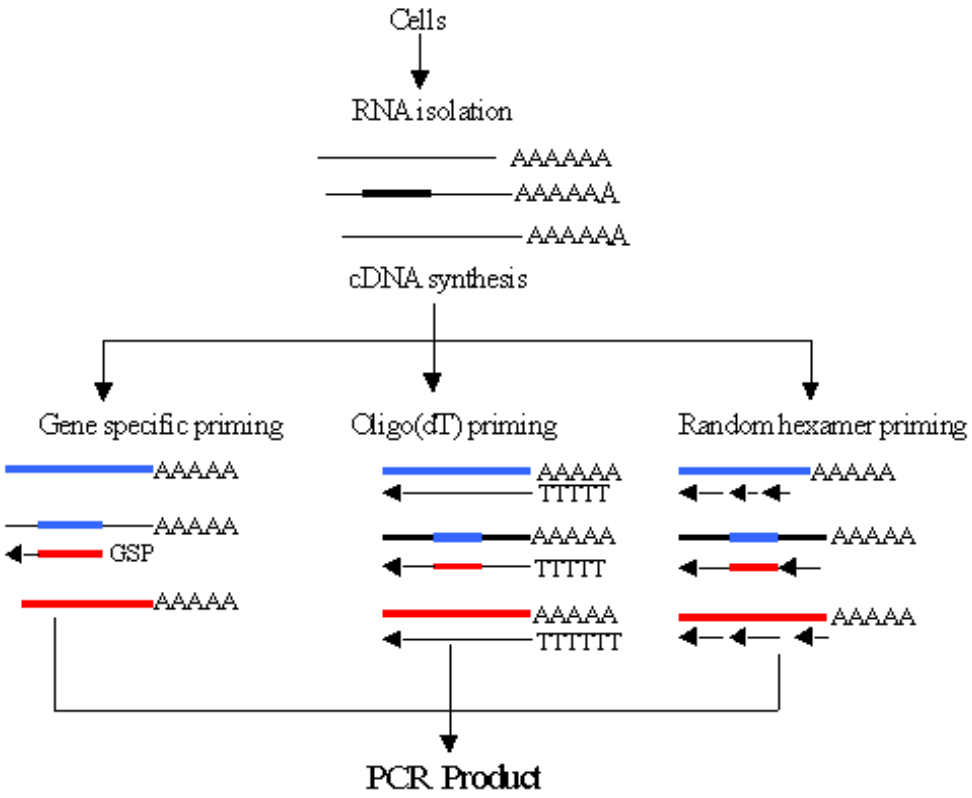


Figure 3.1: Principle of RT-PCR

3.11.1 Isolation of total RNA

Bacterial cells were harvested by centrifugation at 6,000 rpm for 5 min at 4 ^{o}C. The supernatant was discarded, and all remaining medium was removed carefully by aspiration.

Reagents	
TE buffer	0.1 M Tris, 0.01 M EDTA, pH 8.0
Lysozyme in TE buffer	400 µg/ml
β-Mercaptoethanol	Reducing agent
Buffer RTL	Resuspension buffer
Ethanol (96%)	Precipitation
RW1	Wash buffer 1
Buffer RPE	Wash buffer 2
RNase-free water	Elution

The bacterial pellet was loosened by flicking the bottom of the tube and resuspended in 100 µl lysozyme-containing TE buffer by vortexing and incubated at room temperature for 5 min. 350 µl RTL buffer was added to the sample, mixed thoroughly by vortexing. 250 µl ethanol (96%) was added to the lysate and mixed thoroughly by gently pipetting the mixture several times. The sample including precipitate was applied to the column and centrifuged for 20 s at 10,000 rpm. 700 µl buffer RW1 was added to the column, and centrifuged for 20 s at 10,000 rpm to wash the column. To wash the column, 500 µl buffer RPE was pipetted onto the column and centrifuged for 20 s at 10,000 rpm. To dry the RNeasy silica-gel membrane, another 500 µl buffer RPE was added to the column and centrifuged for 2 min at 10,000 rpm. To eliminate any chances of buffer RPE carryover, the column was placed in a new 2 ml collection tube and centrifuged at 13,000 rpm for 1 min. To elute RNA, 40 µl RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and centrifuged for 1 min at 10,000 rpm.

3.11.2 First-strand (cDNA) synthesis

The Superscript RT-PCR system is designed for the sensitive and reproducible detection and analysis of RNA molecules in a two-step process. cDNA synthesis was performed in the first step by using total RNA primed with oligo(dT). *P. putida* KT2440 was grown in $M9^-$-medium containing 22 mM glucose and 19 mM NH_4Cl; 10 mM glutamate till optical density (OD_{600nm}) reached to 0.3-0.5. Total RNA was isolated as described in section 3.11.1. Residual DNA was removed by treating total RNA with RNAse free DNAse. DN-Ase (1 U/µl) was added to total RNA and incubated at 37 ^oC for 30 min. The reaction was terminated by adding 1 µl RQ1 DNase stop solution. The reaction mixture was incubat- ed at 65 ^oC for 10 min to inactivate the DNase. The reverse transcription was carried out on the same day of RNA isolation. Reverse transcription reaction was carried out in a 20 µl reaction volume. In a 1.5 ml tube, 1 µg RNA, 0.1 mM dNTP, 50 pmol oligo(dT) primer were mixed and the volume was adjusted to 10 µl with DPEC treated water. RNA and primer were denatured by incubating the reaction mixture at 65 ^oC for 5 min and immedia-tely kept on ice for 1 min. A master reaction mixture containing 1X RT buffer, 5 mM $MgCl_2$, 20 mM dithiothreitol, 2 U RNaseOUT Recombinant RNase inhibitor was prepared. 9 µl of master reaction mixture was added to RNA/Primer mixture, mixed gently and incubated at 42 ^oC for 2 min. 0.25 U SuperscriptII was added to the prewarmed reaction mixture. Samples were transferred to a thermal cycler preheated to 42 ^oC and incubated for

90 min. The reaction was terminated by incubating at 70 ^{o}C for 15 min and immediately chilled on ice. 1µl RNase H (2 U/µl) was added and incubated at 37 ^{o}C for 20 min. cDNA synthesis reactions were used for PCR or for longer use were stored at -20 ^{o}C.

3.11.3 Polymerase chain reaction

PCR was carried out in a 50 µl reaction mixture, using 2 µl of the RT reaction as template for PfuTurbo DNA polymerase.

Cycle(s)	Temp (^{o}C)	Time (min)	Temp (^{o}C)	Time (min)	Temp (^{o}C)	Time (min)
1	94	2				
26	94	1	54	0.5	72	4
1					72	5

To a thin walled PCR tube, cDNA template, 1X buffer, 0.5 U PfuTubro DNA polymerase, 100 pmol of each primers (section 2.4.1), 0.2 mM dNTP were added. DNA was amplified for 26 cycles with a Perkin Elmer Cetus thermo cycler using the above PCR sequence. Control set without polymerase enzyme was also carried out separately for monitoring the performance of the system. 10 µl of RT-PCR products were then subjected to electrophoresis in a 1.5% agarose gel visualized by staining with ethidium bromide. Gels were photographed by Zoom digital camera and analyzed after scanning.

3.12 DNA hydrolysis with restriction endonucleases

Restriction endonucleases are enzymes that cleave the sugar-phosphate backbone of both strands of DNA while leaving a phosphate group on the 5' ends and a hydroxyl group on the 3' ends. Restriction endonucleases create double-strand breaks at specific recognition sequences within DNA molecules. Some enzymes cut at precisely opposite sites in the two strands of DNA, thus generating blunt ends without overhangs. Other enzymes cut asymmetrically and generate 5' or 3' overhangs which are called sticky or cohesive ends. One unit is defined as the amount of enzyme required to digest 1 µg of λ-DNA in 1 h at 37 ^{o}C. For restriction digestion, reaction mixtures containing 0.1 to 1 µg DNA, 20 U restriction enzyme and 1/10 volume of 10X reaction buffer were incubated at 37 ^{o}C for 2-5 h. The total volume of the reaction was adjusted to 20 µl with dH_2O.

3.13 Dephosphorylation of 5'-phosphate groups

Alkaline phosphatase catalyzes the removal of 5'-phosphate groups from DNA and RNA. Since calf intestinal phosphatase (CIP) treated fragments lack the 5'-phosphoryl termini required by T4-DNA-ligase, they can not self ligate. One unit of CIP is defined as the amount of enzyme that hydrolyzes 1 µmol of p-nitrophenylphosphate to p-nitrophenol in 1 min at 37 ^{o}C. Restriction-digested vector DNA (0.1-1 µg) was suspended in 1X reaction buffer. 0.5 U/µg CIP was added to the vector DNA. The reaction mixture was incubated at 37 ^{o}C for 15 min. Before starting the ligation reaction, the dephosphorylated DNA was purified by Phenol-chloroform method as described in section 3.6.2.

3.14 DNA-ligation

T4 DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA. For the ligation reaction, 50-200 ng vector-DNA with 200-1,000 ng DNA-fragment, 1/10 volume 10X reaction buffer and 2µl T4-DNA ligase (1U/µl) were mixed gently in a microfuge tube. Before adding reaction buffer and T4-DNA ligase, the vector DNA and the DNA fragment were heated at 65 ^{o}C for 3 min and then immediately cooled on ice for 3 min. The reaction mixture was incubated for 3-5 h at room temperature.

3.15 Knock-out of the *ansB* gene

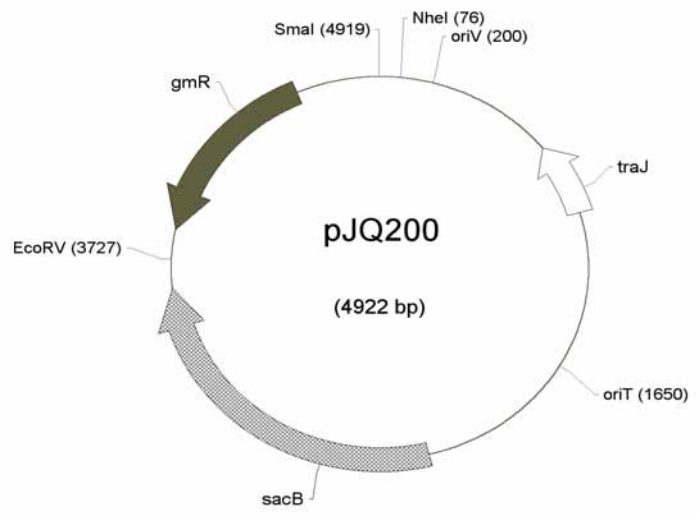


Figure 3.2: Plasmid vector pJQ200. Gm^{R}, Gentamycine resistant gene; oriT, origin of replication, *sacB*, sucrose sensitive gene (Quandt and Hynes, 1993).

3.15.1 Insertion of a kanamycin cassette into the *ansB* gene

A 2389-bp fragment harboring the entire *ansB* gene was amplified by PCR from *P. putida* KT2440 DNA using PfuTurbo DNA polymerase and gene-specific primers ansB For:5'-GAGGCTAAGCGAGGAAATGA-3' and ansB Rev: 5'-GTAGCCAGCCGAAACTGAA-G-3'. The amplified product was cloned into plasmid pJQ200-mp18 **(**Quandt and Hynes, 1993, see fig. 3.2) cut with *Bam*HI and *Sal*I. A *Sal*I fragment containing a kanamycin resistance cassette was cut from the pUC4K vector (Amersham BioSciences). A single *Sal*I site of the *ansB* gene (at 623 nucleotide base position) was then used to insert a *Sal*I fragment containing the kanamycin resistance cassette of pUC4K, yielding pJQKansB.

3.15.2 Construction of *ansB* mutant and plasmid conjugation

A defined *P. putida* KT2440 *ansB* mutant was constructed by diparental mating on cellulose acetate filters using *E. coli* S-17 as donor strain. pJQansB recombinant vector was first delivered into *E. coli* S-17. Transformed cells were selected on LB plates supplemented with kanamycin. The vector carrying a defective *ansB* gene was then delivered into *P. putida* KT2440 via conjugation with *E. coli* S-17 (harboring pJQansB) by using a membrane filter mating technique as described in section 3.17. Gentamycin-resistant (Gm^r) and kanamycin-resistant (Kn^r) transconjugants were identified after 24-36 h incubation at 30 ^oC. *P. putida* KT2440 and *E. coli* S-17 (harboring pJQansB) were also grown separately under the same conditions as a control. To eliminate donor cells, the colonies were propagated two to three times on the same medium by replica plate method as described in section 3.18. To check PGA activity, transconjugant colonies were grown in $M9^-$-medium containing 10 mM glutamate; 22 mM glucose and 19 mM NH_4Cl. Colonies with less or no enzyme activity compared to *P. putida* KT2440 were selected. *P. putida* KT2440 was also grown with mutants under the same conditions, as a positive control.

3.15.3 Genetic analysis

To confirm insertion of the inactivated *ansB* into the host genome, DNA was isolated from the transconjugants and subjected to PCR using the primer pair ansBLT: 5'-ATGAATG-CCGCACTGAAAAC-3' and ansBRT: 5'-ACGACCCAGTCGTTCTTGTC-3'. The PCR sequence used was: 94 ^oC, 3 min; 94 ^oC, 30 s; 54 ^oC, 30 s; 72 ^oC, 2 min, and 72 ^oC for 5 min and was amplified for 25 cycles. Amplified *ansB* was electrophoresed with standard marker in 1% agarose gel.

3.16 Transposon mutagenesis

The transposon (Tn) was first discovered in the 1940s by Barbara McClintock, even before the structure of DNA had been elucidated. Transposons are segments of DNA that can move around to different positions in the genome of a single cell. These mobile segments of DNA are sometimes called "jumping genes". Transposons contain one or more genes, conferring phenotypic characteristics to the bacterial strain that possess them. These genes may confer resistance to an antibiotic, heavy metal or provide a metabolic function. Each transposable element is occasionally activated to move to another DNA site in the cell (or between plasmid and chromosome), by a process called transposition, catalysed by its own site-specific recombination enzyme, transposases. The transposition event can occur in a number of ways; by replication of the molecule and insertion of the copy either randomly or at some preferred site (replicative transposition); or the whole transposon element can be cut out of the DNA and inserted into a new site, whilst the previous site is resealed (non-replicative transposition). Chromosome mutagenesis and gene transfer are often promoted by the movement of transposable DNA elements. The transposition frequency can be modulated both by mobile element-encoded factors and by various host factors. Transposon Tn5 has been used extensively for the genetic analysis of Gram-negative bacteria (Berg, 1989). Tn5-OT182 is derived from Tn5. This Tn inserts almost at random in target DNA, in addition it functions in a wide range of Gram-negative bacteria and is very well characterised both physically and genetically.

3.16.1 Applications of transposon mutagenesis

It is molecular research that has led to transposons becoming such a powerful tool for the genetic analysis of bacteria (Berg and Groissman, 1989). One of the main genetic values of transposons comes in the form of a technique called 'transposon mutagenesis'. This approach provides complete disruption of the mutated gene. The use of Tn5 and Tn10-derived transposons has been succesfully utilized by a number of research groups (Renzikoff *et al*., 1993). The bacterial transposable element Tn5 consists of two inverted elements, IS50R and IS50L. These flank a central region that codes for several antibiotic resistance genes. IS50R encodes two proteins, a transposase and an inhibitor of transposition. Transposon mutagenesis system allows the analysis of biochemical and regulatory pathways in bacteria (Kraiss *et al*., 1998).

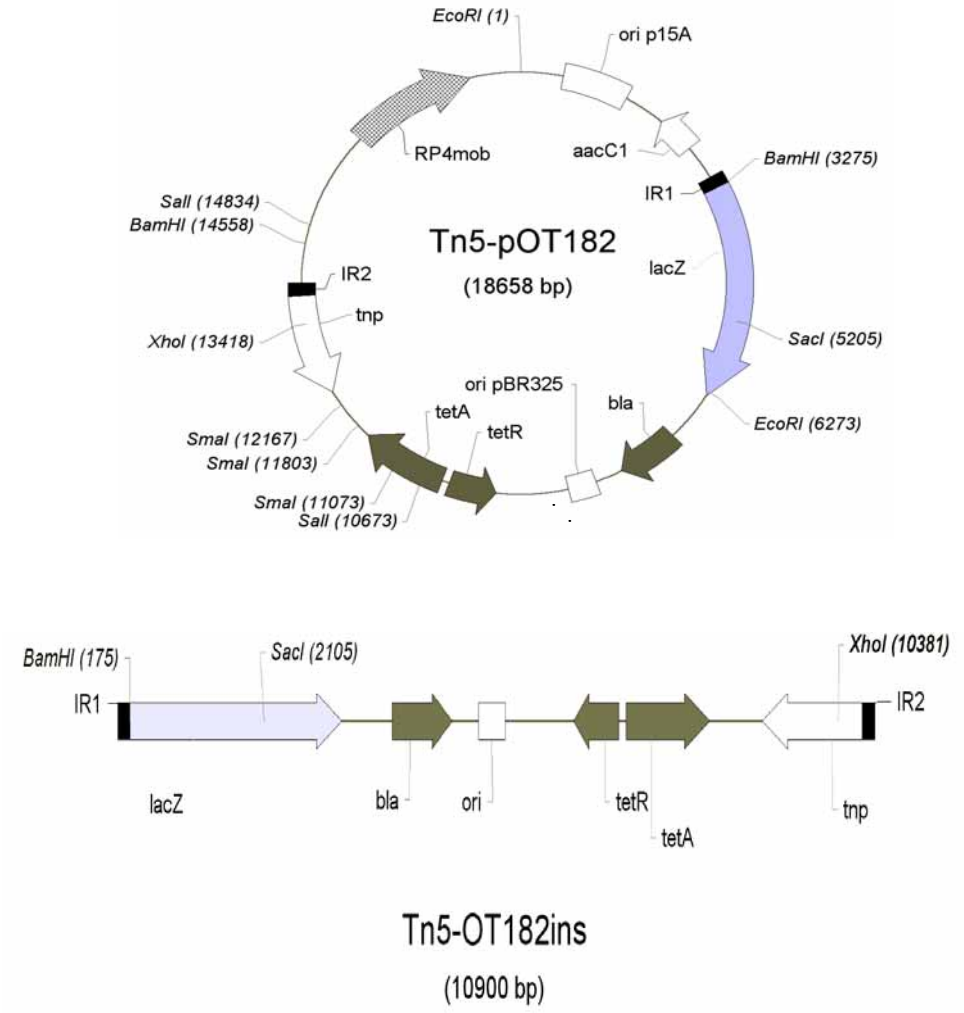


Figure 3.3: Transposon carrier plasmid pOT182 (Merriman and Lamont, 1993). ori1= p15A origin of replication; aacC1 = gentamycin-acetyltransferase I-gene; *LacZ* - β-galactosidase gene; *bla* = β-lactamase gene; ori2 = pBR322 replication origin; *tetR* = tet-regulator gene; tnp = transposase; IR = inverted repeat.

3.17 Transposon mutagenesis and plasmid conjugation

Transposon mutagenesis with the self-cloning promoter probe vecto Tn5-OT182 (Merriman and Lamont, 1993) carrying tetracycline and carbenicillin resistance markers was performed by diparental mating. Tn5-OT182 was delivered to *P. putida* KT2440 via conjugation with *E. coli* S17-1 (pOT182) using a membrane filter mating technique. The donor strain (*E. coli* S17-1 harboring pOT182) was grown overnight in 5 ml of LB broth containing tetracycline at 37 ^{o}C for 16 to 18 h with shaking (230 rpm). The recipient strain (*P. putida* KT2440) was also grown under these conditions at 30 ^{o}C without antibiotic selection. 200 µl of each saturated culture was added to 5 ml of fresh LB medium with

appropriate antibiotic and was allowed to grow to an OD_{595} of 0.5. The donor and recipient strains were mixed in the ratio of 1:1, 1:5, and 5:2. Control assays, using donor and recipient alone, were also performed. The cellulose acetate filter papers (0.2 mm-pore size, Sartorious) were washed with 2 ml of sterile 0.85% NaCl. After that, filter papers were placed on LB plates and incubated for 1h in a 37 ^{o}C incubator. 100 µl aliquots from each mixture were spread on prewarmed filter papers placed on LB plates without antibiotic and the plates were incubated overnight at 30 ^{o}C. Filter papers were washed with 1 ml of sterile $M9^{-}$-medium and diluted appropriately with the same medium. 100 µl of diluted culture was spread on $M9^{-}$-medium plates supplemented with 10 mM glutamate; 22 mM glucose and 19 mM NH_4Cl containing tetracycline and carbenicillin. Tetracycline-resistant (Tc^{r}) and carbenicillin-resistant (Cb^{r}) transconjugants were identified after 24 to 36 h incubation at 30 ^{o}C. Transconjugants were transferred 2-3 times onto the same medium by replica plating in order to eliminate the donor cells. The resulting transconjugants were individually assayed for PGA activity (Section 3.24.1). Those with strongly reduced or missing activity were selected for further studies.

3.18 Replica plating

This is a technique in which the pattern of bacterial colonies on a culture plate is copied using sterile membranes. It is mainly used for identifying and selecting mutant colonies of a microorganism. The microorganisms are cultured on a plate of agar, then a replica is made by blotting the colonies with a membrane and blotting the membrane on another plate. This results in all colonies from the first plate being in identical locations on the second plate. The second plate either contains some substances which kills off, or selectively permits the growth of the mutant of interest.

Plates to be replicated were marked with a line perpendicular to the edge for orientation. Sterile circular block covered with velvet was pressed gently onto the plate. Then the copied colonies were blotted on a new LB plate containing selective antibiotic or substa- nces. The plates were incubated for 12-15 h at 30 ^{o}C or until the colonies regrow.

3.19 Self-cloning

For 'Self cloning', approximately 2 µg of DNA from transposon mutants was digested with *Xho*I. The resulting fragments were ligated with T4 DNA ligase and used to transform *E. coli* DH5α. Plasmid DNA was isolated from Tc^{r} clones, purified on QIAprep spin

columns, and sequenced using the primers pOT182For: 5'-CGACGGGATCCATAAT-TTTT-3' and pOT182Rev : 5'-CGTTACCATGTTAGGAGGTC-3'.

3.20 Southern blotting

Southern blotting, invented by Ed Southern in 1975, is a technique to detect specific sequences in complex populations of DNA fragments. DNA fragments generated by restriction digestion are separated by fragment size class by agarose gel electrophoresis and then transferred onto nitrocellulose filters for hybridization with specific probes.

3.20.1 Restriction digestion and purification of pOT182 probe

pOT182 vector was digested with *Xho*I restriction enzyme

10X Reaction buffer	2 µL
Bovine serum albumin	0.2 µL
*Xho*I (0.25 U)	1 µL
Vector plasmid/DNA (400 ng)	10 µL
dH_2O	6.8 µL

After restriction digestion, the plasmid DNA was purified by using Phenol-choloroform extraction method as described in section 3.6.2 and finally dissolved in 10 µl dH_2O.

3.20.2 DIG labeling of pOT182 probe DNA

The DNA was denatured by heating in a boiling water bath for 10 min and quickly chilled in an ice bath for 5 min followed by a brief centrifugation. To the freshly denatured probe, 2 µl hexanucleotide mix, 2 µl dNTP-labeling mix and 1 µl Klenow enzyme were added. The components of the tube were mixed completely and incubated overnight at 37 ^{o}C. The reaction was stopped by adding 2 µl EDTA and 2.5 µl LiCl. The DNA was precipitated by adding chilled 75 µl absolute ethanol and incubated at -80 ^{o}C for 30 min. The sample was centrifuged at 13,000 rpm, for 20 min at 4 ^{o}C. The supernatant was discarded and the DNA was washed by adding 150 µl 70% ethanol and again incubated at -80 ^{o}C for 15 min. The precipitated DNA was centrifuged at 13,000 rpm, for 10 min at 4 ^{o}C. The supernatant was discarded and the DNA was dissolved in 50 µl TE buffer with gentle swirling. 12.5 µl of

labelled probe was added into 10 ml hybridization buffer and kept in a boiling water bath for 10 min. The probe was rapidly cooled in a ice-bath for 10 min.

EDTA	0.2 M, pH 8.0
Lithium chloride (LiCl)	4 M
Ethanol	70% and 96 %
TE buffer	0.1 M Tris, 0.01 M EDTA, pH 8.0
Hybridization buffer	100 ml 5X SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS, 1% blocking reagent

3.20.3 Capillary transfer of DNA from agarose gels to membranes

The genomic DNA of transposon mutants was digested with *Xho*I and *Eco*RI restriction enzymes. After digestion, samples were run through 0.8% agarose gel. The gels were stained with ethidium bromide. A transparent ruler was placed alongside the gel and was photographed by Kodak digital camera. After fractionation of the DNA by gel electrophoresis, the gels were transferred to a clean glass plate. The unused areas of the gel including the section of the gel above the wells were trimmed away using a razor blade. The DNA was denatured by soaking the gel in 250 ml of denaturation solution for 30 min at room temperature with constant gentle agitation on a rotary platform. The gel was rinsed briefly in deionized water, and then neutralized it by soaking in 250 ml neutralization solution for 30 min at room temperature with constant gentle agitation. By using a fresh scalpel a piece of nylon membrane ~1mm larger than the gel in each dimension was cut.

Membrane was immersed in 200 ml 10X SSC for 5 min. A piece of thick blotting paper was placed on a glass plate in such a way that the ends of the blotting paper were drapped over the edges of the plate. The dish was filled with 10X SSC until the level of the liquid reached almost to the top of the support. The gel from the solution was removed and inverted it so that its underside is facing upward. The top of the gel was wetted with 10X SSC. The wet membrane was placed on the top of the gel so that the cut corners were aligned with the gel. Two pieces of thick blotting paper were wetted in 10X SSC and placed them on top of the wet membrane. A stack of paper towels were placed on the blotting papers. A glass plate was kept on top of the stack and weighed it down with a

~400 g weight. In between paper towels were replaced before the entire stack becoming wet with transfer buffer. The DNA was allowed to transfer for ~16 h.

Denaturation solution	1.5 NaCl, 0.5 M NaOH
Neutralization solution	1 M Tris-HCl (pH 7.4), 1.5 M NaCl
10X SSC	87.65 g NaCl, 44.1 g Sodium citrate
	1 L dH_2O, pH 7.0

The gel was peeled from the membrane and discarded. The membrane was soaked in 250 ml 2X SSC for 2 min at room temperature then removed from the solution and excess fluid was allowed to drain away. The membrane was placed flat on a paper towel, sandwiched between two sheets of dry blotting paper and baked for 2 h at 80 ^{o}C.

3.20.4 Hybridization of a labelled probe with DNA fragments

The DNA-containing membrane was floated on the surface of a tray containing 2X SSC until it became thoroughly wetted from beneath. The wet membrane was incubated in 10 ml of 1X prehybridization buffer for 1 h at 68 ^{o}C. Before use, the labelled probe was denatured by heating for 10 min at 100 ^{o}C and was chilled rapidly in ice water bath. Denatured probe was added to fresh prehybridization solution and the solution was delivered into the bag which was incubated overnight at 68 ^{o}C. The hybridization solution was poured off into a polypropylene tube and stored at -20 ^{o}C for further use. The membrane was removed from the bag and immediately submerged in a tray containing 50 ml 2X SSC and 0.1% SDS for 5 min at room temperature with gentle agitation on a slowly rotating platform. The rinse solution was replaced with 0.2X SSC and 0.1% SDS and incubated at 68 ^{o}C for 15 min two times.

3.20.5 Detection of hybridized probe and DNA fragment

The membrane was washed briefly in a 20 ml of wash buffer and then incubated in 20 ml of blocking solution for 30 min. During incubation, the tray was rotated gently after every 2 min to spread blocking solution evenly over membrane. 1:1,000 diluted antibody solution was added to the blocking solution and spread evenly over the membrane. The membrane was incubated in antibody solution for 30 min with gentle shaking after every 2 min. Then membrane was washed twice in 20 ml of washing buffer for 15 min at room temperature.

The membrane was equilibrated in detection buffer for 2-3 min. After this, membrane was incubated in 2 ml freshly prepared color substrate solution in an appropriate container in the dark and developed still desired spot intensities were achieved.

Solution	Composition	Use
Washing buffer	0.1 M maleic acid, 0.15 M NaCl; pH 7.5 ; 0.3% (v/v) Tween 20	Remove unbound antibody
Maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl; pH adjusted to 7.5 with solid NaOH	Dilute blocking solution
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5	Adjust pH to 9.5
Blocking stock solution (10x)	10% (w/v) blocking reagent in maleic acid buffer	Preparation of blocking solution
Antibody solution	Anti-Digoxigenin	Binding to the DIG-labeled probe
Colour-substrate solution	40 µl of NBT/BCIP + 2 ml detection buffer	Visualize antibody-binding

3.21 Gene Replacement

Gene targeting by homologous recombination is a genetic tool that permits modification of cellular genes in a precise and predetermined fashion. Although homologous integration of transfected DNA into the genome was considered to be an extremely rare event in other organisms, techniques for this process have been established for several model systems. Targeting knockout of genes involves first designing and construction of an appropriate targeting vector in which the gene of interest has been disrupted with a positive selectable marker. The second step involves the introduction of the targeting vector into a bacterial culture followed by selection for those cells in which the internal positive selectable marker has become integrated into the genome of the bacteria.

3.21.1 *GltB* gene replacement

The pK18 vector was used for targeted knock-out of an intact *gltB* gene with a denatured *gltB* gene. pK18 (Pridmore, 1987) is a small multi-copy kanamycin-resistance plasmid, co-ntaining the pUC lacZα-complementation peptide and the pUC18 and pUC19 multiple cloning site. The plasmids and their derivatives allow simple and rapid transfer of inserts from one replicon to another.

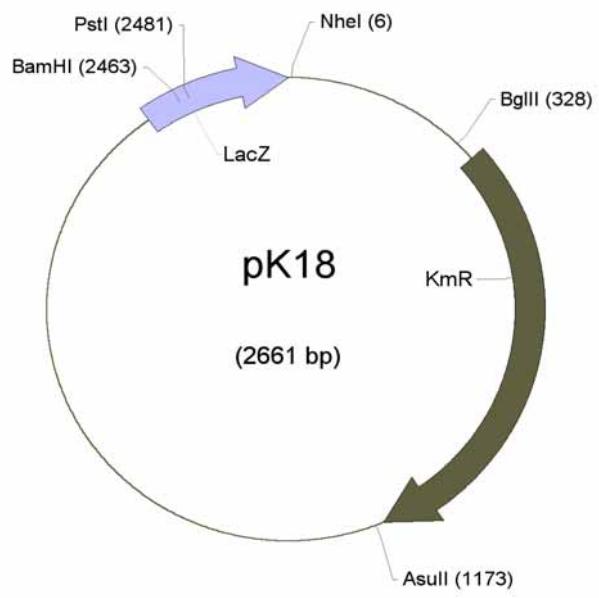


Figure 3.4: Map of the pK plasmid. *lacZ* and Km^R gene are represented by arrows showing the direction of transcription.

3.21.2 Amplification and cloning of *gltB*

An 834 bp fragment of *gltB* gene was amplified from genomic DNA of *P. putida* KT2440 using the gene-specific primers gltBFor: 5'-CGCggatccCGCA**A**ACATCTTCCAGGA- GT-3' (*Bam*HI restriction site introduced into the primer is underlined, highlighted nucleotide was introduced to generate a frameshift) and gltBRev: 5'-AActgcagACCAG- CGTGGTGTATTCCTT-3' (*Pst*I restriction site introduced into the primer is underlined).

The amplified product thus generated was purified by phenol-chloroform method as described in section 3.6.2. Both amplified *gltB* fragment and pK18 vector were digested with *BamH*I and *Pst*I. The digested pK18 vector was then dephosphorylated with calf intestinal alkaline phosphatase. The dephosphorylated vector and digested *gltB* fragment were again purified by phenol-chloroform extraction method. The PCR product generated in this way was cloned into the compatible *Bam*HI-*Pst*I site of pK18 vector and then

transformed in *E. coli* HB101. The transformed cells were plated out on LB medium containing kanamycin and the plates were incubated overnight at 37 ^{o}C. The plasmid DNA of the transformant cells was isolated by using Miniprep plasmid isolation kit. The presence of *gltB* gene in pK18 vector was confirmed by DNA sequencing with M13 universal primer and by restriction digestion analysis with *Bam*HI and *Pst*I. After that, recombinant pK18 vector was electroporated into the electrocompetent *P. putida* KT2440, and the plasmid integrants were selected on LB medium containing kanamycin. Integration of denatured *gltB* fragment into the KT2440 genomic DNA was confirmed by PCR using the following primers gltBP3 For: 5'-ATTTCACACAGGAAACAG-3' and gltBP4Rev: 5'-CTCCAGCGGCTCGACCTG-3'. The forward primer was derived from pK18 vector sequence (starts from nt 2410), whereas the reverse primer was designed from *gltB* gene sequence (starts from nt 2880).

3.21.3 Knock-out of the *aauS* (sensor kinase) and *aauR* (response regulator) genes

A *aauR* gene fragment was amplified from genomic DNA of *P. putida* KT2440 using the gene-specific primers aauRFor: 5'-CGCggatccGCCTGGTCGA**A**CGTGGTACG-3' (the *Bam*HI restriction site introduced into the primer is underlined whereas the highlighted nucleotide was introduced to generate a frameshift) and aauRRev: 5'- CCCaagcttGATGT-CTTCACGGCGCTCAC-3' (*Hind*III restriction site is underlined). PCR amplification was performed with an automated thermocycler by using the following program.

Cycle(s)	T (^{o}C)	Time	T (^{o}C)	Time	T (^{o}C)	Time
1	95	5 min				
28	95	30 s	55	40 s	72	1 min
1					72	5 min

The amplified product was purified by phenol-chloroform method as described in section 3.6.2. The amplified *aauR* fragment and pK18 vector were digested with *Bam*HI and *Hind*III restriction enzymes. The vector was then dephosphorylated with calf intestinal alkaline phosphatase. The dephosphorylated vector and the digested amplified product

were again purified by phenol-chloroform extraction method. The PCR product generated in this way was cloned into the compatible *Bam*HI and *Hind*III site of pK18 vector and then transformed in *E. coli* HB101. The presence of the *aauR* gene fragment in pK18 vector was confirmed by restriction digestion analysis with *Bam*HI and *Hind*III. After that, the recombinant pK18 vector was electroporated into electro-competent *P. putida* KT2440 cells.

Similarly, the *aauS* (sensor kinase) gene was inactivated using the primers aauSFor: 5'CGCggatccCGAATAC**C**CTTGAAGGCCTGA-3' (*Bam*HI site underlined, the highlighted nucleotide introduced into the primer to generate a frameshift) and aauSRev: 5'-CCC aagcttTCAGTTTTTCCACACCATCG-3' (*Hind*III site underlined).

3.22 Overexpression of the AauR protein

3.22.1 Expression system

The Glutathione S-transferase (GST) gene fusion system (Amersham Biosciences) is mainly used for the expression, purification, and detection of fusion proteins produced in *E. coli*. This system allows high-level expression of genes as fusions with *Schistosoma japonicum* GST. Expression in *E. coli* yields proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. The pGEX-6P-3 PreScission Protease vector offer the most efficient method for cleavage and purification of GST fusion protein.

3.22.2 Amplification and cloning of the *aauR* gene

A complete, in frame *aauR* gene was amplified by using aauR1For: 5'-CGCggatccATG-AACCAAGCGCCTCTTAC-3' (*Bam*HI site underlined) and aauR2Rev 5'-CCGgaattcT-CAGGCGAGGCCGTATTTTTTC-3' (*Eco*RI site underlined) primers. The resulting PCR product was purified by using the PCR purification kit. The pGEX vector and the insert DNA were digested sequentially by *Eco*RI and *Bam*HI restriction enzymes. The insert was then ligated into the dephosphorylated vector. The ligation mixture was transformed into the competent DH5α cells and the transformed cells were spread on ampicillin containing LB plates. Positive clones were selected and the insertion was verified by restriction digestion analysis (*Rsa*I) and PCR by using aauR1For and aauR2 Rev primers.

3.22.3 Transformation into the expression host

Plasmid DNA was isolated from the positive clones and transformed into the competent *E. coli* BL21 (DE3) RIL strain (Stratagene). The transformed and untransformed cells were spread on LB plates supplemented with ampicillin and chloramphenicol.

3.22.4 Screening

Screeing was performed to verify that the insert is in the proper orientation and the correct junctions are present such that the reading frame is maintained. Plasmid DNA from the Cmp^r-Amp^r clones was isolated by QIAPrep Mini Kit and sequenced by pGEX5' and pGEX3' sequencing primers.

3.22.5 Bacterial growth and expression of fusion protein

The ability of clones to express the fusion protein was evaluated by optimizing growth and expression conditions. A single colony of *E. coli* harboring a recombinant pGEX plasmid was grown overnight in 50 ml LB medium supplimented with ampicillin and chloramphenicol at 37 ^oC. A 5 ml overnight culture was inoculated into the fresh 500 ml of the same medium pre-warmed to the growth conditions. The culture was allowed to grow with aeration to an A_{600} of 0.7. IPTG to a final concentration of 1 mM was added to the growing culture and incubation continued for an additional 6 h. The culture was transferred, centrifuged at 6,000 rpm for 10 min at 4 ^oC to sediment the cells. Supernatant was discarded and the drained pellet was kept on ice. An aliquot of the culture was analyzed for protein expression by SDS-PAGE.

3.22.6 Purification of the recombinant AauR-GST fusion protein

The cell pellet was completely suspended in ice-cold 20 ml GST-PBS buffer. The cells were disrupted by sonication in a 100 ml beaker for 7 min (15 s pulse, 15 s pause).

PBS buffer	140 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	1.8 mM KH_2PO_4, pH 7.3
Elution buffer	50 mM Tris-HCl
	10 mM glutathione reduced, pH 8.0

The resulting homogenate was centrifuged at 12,000 rpm for 20 min at 4 ^{o}C. The supernatant was removed and the pellet was solubilized in 5 ml of 8 M urea and stirred for 30 min at 4 ^{o}C. The resulting suspension was centrifuged at 12,000 rpm for 15 min at 4 ^{o}C. The supernatant was dialyzed against 1 L PBS buffer for 1 h. This step was repeated twice.

The AauR protein was purified on a 1 ml GSTrap FF column with glutathione as the ligand (Amersham BioSciences). The column was equilibrated with 5 volumes of PBS buffer at 0.2 ml/min flow rate. Clear supernatant was loaded onto the column at the same flow rate. The column was washed with 10 bed volumes of PBS at an 1 ml/min flow rate. Bound proteins were eluted by adding 3.0 ml of elution buffer. The eluant was collected in clean tubes (1 ml fractions) and checked for purity by SDS-PAGE.

3.22.7 Removal of the GST tag by enzymatic cleavage

The fusion protein was treated with PreScission Protease (Amersham Biosciences) whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX plasmids. PreScission Protease is a fusion protein of GST and human rhinovirus 3C protease which specifically recognizes the amino acid sequence Leu-Glu-Val-Leu-Phe-Gln-↓Gly-Pro, cleaving between Gln and Gly.

PreScission cleavage buffer:	50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA 1 mM dithiothreitol, pH 7.0
Binding buffer:	1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3

The column was equilibrated with five volumes of binding buffer. The eluted protein sample was loaded on to the eqilibrated column. The column was then washed with 10 column volumes of binding buffer and then with 10 column volumes of PreScission cleavage buffer. The PreScission protease mixture was prepared freshly by adding 80 µl (160 units) PreScission Protease to 920 µl of PreScission cleavage buffer at 4 ^{o}C. The mixture was loaded onto the column. The column was incubated overnight at 4 ^{o}C. Finally, the column was eluted with PreScission cleavage buffer. Fractions of 500 µl were collected in clean Eppendorf cups.

3.22.8 SDS-PAGE (Laemmli, 1970)

By this technique, proteins are separated on the basis of their mass under denaturing conditions. SDS, an anionic detergent, disrupts noncovalent intercations in native proteins. Mercaptoehtanol reduces the disulphide bonds of the proteins.

Reagents

Acrylamide solution:	30 % acrylamide, 0.8 % bisacrylamide
Separation gel buffer:	1.5 M Tris-HCl, pH 8.8
Stacking gel buffer:	0.5 M Tris-HCl, pH 6.8
Loading buffer:	0.1 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 3 % mercaptoethanol, 10% (v/v) glycerol, 0.01 % (w/v) bromophenol blue
10 X running buffer:	250 mM Tris, 1.9 M glycine, 1 % (w/v) SDS

Composition of the gel solution

	Separation gel solution (12% acrylamide)	Stacking gel (4.5% acrylamide)
Acrylamide solution	4,000 µl	650 µl
Separation gel buffer	2500 µl	------
Stacking gel buffer	-----	1250 µl
10 % (w/v) SDS	100 µl	50 µl
Water	3350 µl	3050 µl
10 % (w/v) APS	50 µl	25 µl
TEMED	25 µl	10 µl

On the average, one SDS molecule binds for every two amino acids. Therefore, the complex of SDS with the denatured protein carries a large net negative charge and migrates towards the anode. The velocity of migration of SDS-protein complexes is inversely proportional to the mass of the protein.

The cell pellet was suspended in PBS buffer, pH 7.6. The resulting suspension was mixed with loading buffer and incubated at 95 ^{o}C for 3 min for denaturation. The samples were then loaded into the wells under the running buffer with a microsyringe. Separation of the proteins was carried out at the 30 V for 30 min. Once proteins entered into the separation gel the current was set at 200 V and allowed to run for additional 45 min. Finally, the proteins in the gel were visualized by staining with Coomassie blue.

3.22.9 Coomassie staining

Fixation solution	20% methanol, 1 ml 85% phosphoric acid
Staining solution	45% (v/v) methanol, 10%(v/v) glacial acetic acid, 0.1% (w/v) Serva A Blue
De-staining solution	5% ethanol, 7% acetic acid

After electrophoresis, the separating gel was immersed in the fixation solution for 1 h at room temperature with gentle shaking. The gel was then kept in staining solution for atleast 1 h. After that, gel was rinsed with water, destained by destaining solution for 1 h. The destaining solution was changed 2 times during the process. Before drying the gel, it was washed again with water and then dried at 70 ^{o}C under vaccum.

3.23 Primer extension

Primer extension is mainly used to map the 5' termini of the mRNA. In this technique, poly(A)$^{+}$ RNA is first hybridized with a single-stranded oligodeoxynucleotide primer, radiolabelled at its 5' terminus, which is complimentary to the target RNA. The primer is then extended by using reverse transcriptase enzyme. The resulting cDNA is complimentary to the RNA template and is equal in length to the distance between the 5' end of the primer and the 5' terminus of the RNA.

3.23.1 Preparation of the Oligonucleotide probe

The reaction mixture was incubated for 1 h at 37 ^{o}C. The kinase reaction was stopped by heating the reaction mixture at 65 ^{o}C for 5 min. After this, 80 µL dH_2O was added to the

reaction tube. G-25 column was prepared for loading by centrifuging it at 2700 rpm for 2 min after breaking the bottom seal. 100 µL of the labelled primer was loaded onto the column and centrifuged again for 2 min at 2700 rpm. The oligonucleotide primer was labelled in a reaction containing:

Oligonucleotide primer (200 pmol)	1 µL
10X polynucleotide kinase buffer	2 µL
Polynucleotide kinase	1.5 µL
[γ-^{32}P] ATP	5 µL
ddH_2O	11.5 µL

3.23.2 Hybridization and extension of the oligonucleotide primer

To 10 µL of the RNA following components were added

10mM dNTP mix	1 µL
5X Strand buffer	4 µL
Labelled primer	2 µL
DTT (0.1M)	2 µL

The oligonucleotide/RNA mixtures was placed at 37 ^{o}C for 2 min for annealing. Then, 1 µL of MLV-reverse transcriptase was added to the reaction mixture and mixed properly. The tubes were incubated at 37 ^{o}C for 1 h. The primer extension reaction was terminated by adding 20 µL of the stop solution. Before loading onto the gel, the samples were heated at 70 ^{o}C for 5 min.

Stop solution:	80% formamide; 10% EDTA, pH 8.0; 1 mg/ml xylene cyanol; 1 mg/ml bromophenol blue

3.23.3 Preparation of the sequencing gel and electrophoresis

Sequencing gel was prepared by mixing the components listed below and allowed to solidify for 45 min. After solidification of the gel, it was kept in gel chamber. The gel

chamber was filled with 0.5 X TBE (Tris base-5.4 g/L, boric acid-2.7 g/L, EDTA-0.46 g/L) and runned for 10 min at 3000 V before loading the samples.
Sequencing gel composition:

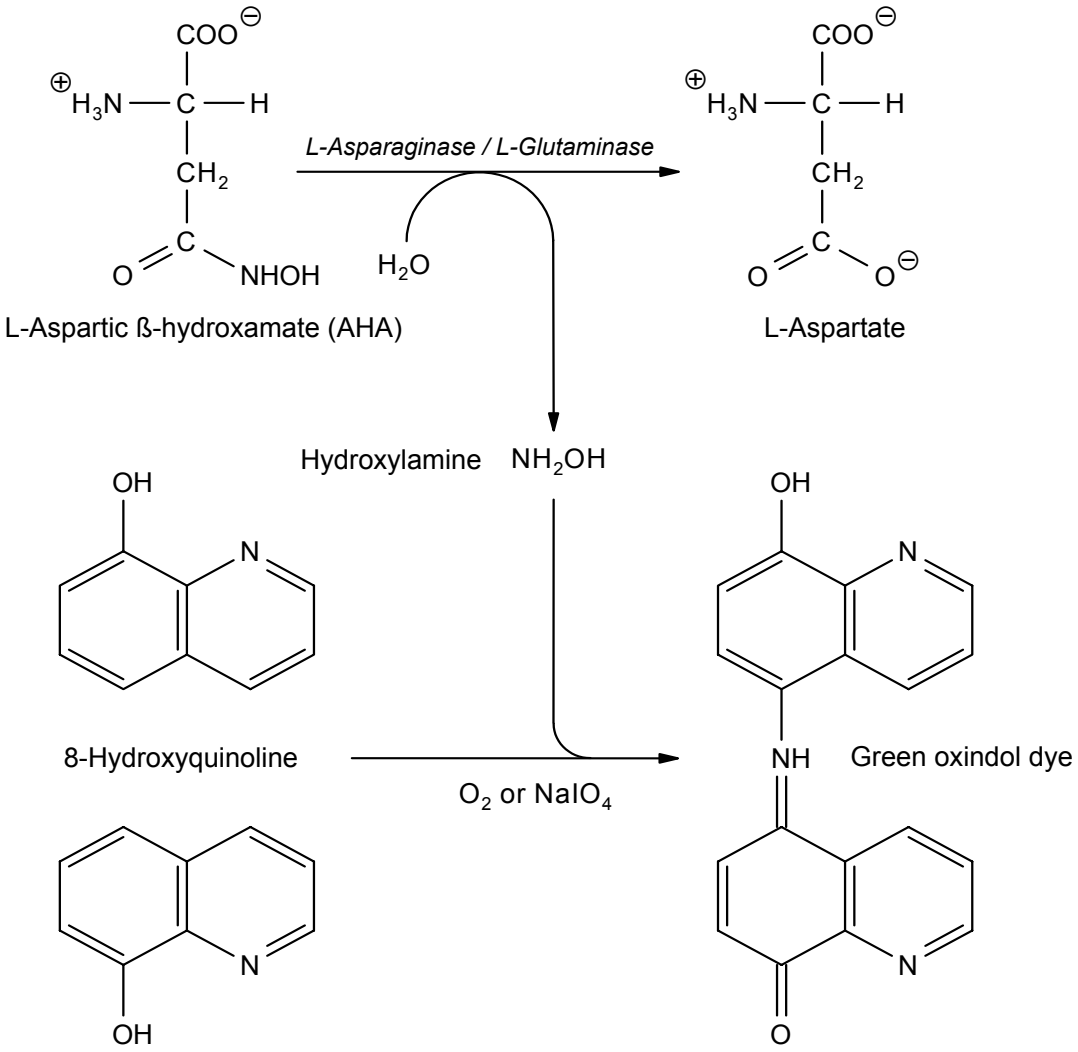
Urea	21 g
5X TBE	5 ml
Rotiphorex40	7.4 ml
TEMED	30 µL
10% APS	400 µL
dH_2O	37.5 ml

3-4 µL of samples were loaded onto the gel and runned for approximately 1.5 h till the bromophenol blue reached 1 mm away from the bottom of the gel. Gels were dried at 60 ^{o}C for 1.5 h. Finally gels were autoradiographed overnight and scanned.

3.24 Enzyme Assays

3.24.1 Assay of glutaminase/asparaginase with L-aspartic acid β-hydroxamate (L-AHA)

In this work, an asparagine analog L-aspartic acid β-hydroxamate (L-AHA) was used as the substrate for glutaminase/asparaginase. The assay is based on the reaction of hydroxylamine liberated from L-AHA with 8-hydroxyquinoline at high pH. The resulting green oxindol dye has an absorption coefficient of about 1.75 x 10^4 M^{-1} cm^{-1} at 705 nm and is thus detectable with high sensitivity. One unit of asparaginase activity is the amount of enzyme that catalyses the hydrolysis of 1 µmol substrate in 1 min at 25 ^{o}C. 10 µl enzyme solution was added to 30 µl of buffered 1 mM L-AHA and incubated for up to 60 min at room temperature. The reaction was stopped by addition of 240 µl of stop solution. The absorption was measured at 655 nm in an ELISA-Reader.



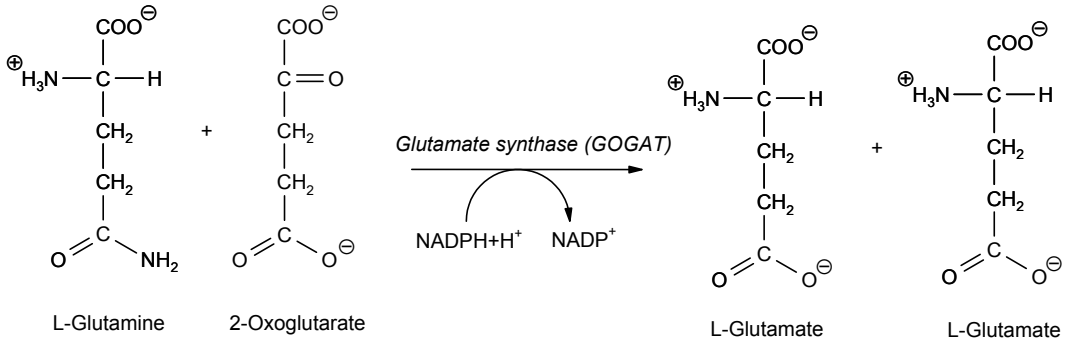
Glutaminase/asüpataginase assay

Reagents	Concentration
Substrate solution	1 mM AHA in 50 mM MOPS (pH= 7.0)
Na_2CO_3	1 M in dH_2O
Chromogen	1% (w/v) Hydroxyquinoline in Dimethylsulfoxide
Oxidant	1% (w/v) $NaIO_4$ in dH_2O
Stop solution	8 ml Na_2CO_3 + 1 ml Chromogen + 200 µl Oxidant

3.24.2 Glutamate-Synthase (GOGAT) Activity

Glutamate-Synthase activity was estimated according to Meister and Meers (Meister, 1985; Meers *et al*., 1970) by following the decrease of NADPH absorption. NAD(P)H has

an absorption coefficient of 6220 $L \cdot mol^{-1} \cdot cm^{-1}$ at 340 nm. The enzyme activity was measured in a microtiter plate in a total volume of 300 µl.



Reagents	Concentration
L-Glutamine	10 mM in 100 mM Tris-HCl, pH = 7.8
NAD(P)H	0.35 mM in dH_2O, prepared freshly
α-Ketoglutarate	5 mM in dH_2O, neutralized with 0.1 N NaOH

260 µl L-Gln, 10 µl α-ketoglutarate, 10 µl NADPH, and 20 µl of enzyme solution were added to a microtiter plate. Blanks were prepared in the same way as test, except that distilled water was added instead of enzyme solution. Absorbance was measured every 3 min at 340 nm.

3.25 Protein estimation

Bacterial cells were disrupted by sonication (4 × 15 s). After each cycle, the cells were cooled on ice for 15 s. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4 ^{o}C. Protein concentrations of the samples were measured by the Bradford method and the BCA method (Bradford, 1976, Friedenaur and Beilet, 1989).

3.25.1 Bradford method

100 µl of appropriately diluted protein sample was mixed with 5 ml Bradford reagent and incubated at room temperature for 20 min. Absorption was measured at 595 nm against a blank (100 µl H_2O, 5 ml Bradford reagent). Different concentrations of bovine serum albumin were used for the standard curve.

Reagents	Concentration
Bradford reagent	0.01% (w/v) Serva Blue G, 5% (v/v) ethanol, 10% (v/v) phosphoric acid

3.25.2 BCA (Bicinchonic acid) Method

Reagents	Concentration
Bovine serum albumin stock	2.0 mg/ml
Working reagent	50 parts of Reagent A + 1 part of Reagent B

To the 0.1 ml of appropriately diluted protein samples, 2.0 ml of working reagent was added. The tubes were mixed properly to ensure a homogeneous mixture and incubated at 37 ^{o}C for 30 min. After cooling to room temperature absorption at 562 nm was measured against a water as reference. For standard curves, BSA concentrations ranging between 25 to 200 µg/ml were used.

3.26 Proteomics

"Proteomics" is the large-scale screening of the proteins of a cell, organism or biological fluid, a process which requires stringently controlled steps of sample preparation, 2-D electrophoresis, image detection and analysis, spot identification, and database search. The expression of a particular protein cannot be directly related to its mRNA level because protein maturation and degradation are dynamic processes that can dramatically alter the final amount of active protein. In order to be able to correlate mRNA levels with protein expression, there should be a systematic method for separating and visualizing the protein components of a cell.

3.27 Two-dimensional (2D) electrophoresis

The core technology of proteomics is 2-D polyacrylamide gel electrophoresis (2D-PAGE). Two-dimensional electrophoresis was first introduced by O'Farrell (O'Farrell, 1975) and Klose (Klose, 1975). At present, there is no other technique capable of simultaneously resolving thousands of proteins in one separation procedure. 2D-PAGE sorts proteins

according to two independent properties in two discrete steps. The first dimension, isoelectric focussing (IEF) separates proteins according to their isoelectric points, i.e. by migrating to a point in the gel where the pH causes the net charge on the protein to become neutral. The replacement of classical first-dimension ampholyte based pH gradients with well-defined immobilized pH gradients has resulted in higher resolution, higher protein loading capacity, improved reproducibility, and an extended basic pH limit for 2D-PAGE. The second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights. Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample. Thousand of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtained.

3.27.1 Sample preparation

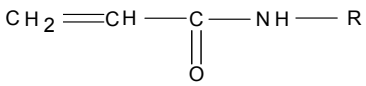
Pseudomonas putida KT2440 was grown in flasks containing M9$^-$- medium with 22 mM glucose and 19 mM NH_4Cl; 10 mM glutamate; 10 mM glutamate and 10 mM fumarate as C and N sources (cf. section 3.2.2). The samples were prepared freshly. Bacteria were harvested by centrifugation. After washing with M9 salt solution, the pellet was suspended in 2 ml TE/PMSF buffer, pH 7.5. The cells were disrupted by sonication for 1 min (15 x 4) at a low temperature and with minimum heat generation. The resulting homogenate was centrifuged for 10 min at 10,000. The supernatant was transferred into a fresh Eppendorf tube. The supernatant was again spun twice at 14,000 rpm for 30 min. All steps were carried out at 4 ^oC.

TE/PMSF buffer	
Tris-HCl	10 mM, pH= 8.0
EDTA	1 mM prepared in 0.1 N NaOH
Phenylmethylsulfonyl fluoride (PMSF)	0.3 mg/ ml in ethanol

3.27.2 Protein precipitation

Five volumes of 100 % ice-cold acetone was added to the one volume of protein extract. The proteins were allowed to precipitate overnight at -20 ^oC. Proteins were pelleted at 0 ^oC, at 6,000 rpm for 10 min. The residual acetone was removed by air drying.

3.27.3 First-dimension (Isoelectric focussing)



R= weakly acidic or basic buffering group

The first-dimension separation procedure involves protein solubilization, Immobilized pH Gradient (IPG) strip rehydration, sample application, and isoelectric focussing. The immobilized pH gradient is created by covalently incorporating a gradient of acidic and basic buffering groups into a polyacrylamide gel at the time it is cast.

For IEF, proteins were solubilized in a rehydration solution such that the end volume 100 µl contains 100 µg of protein. Subsequently, 320 µl of rehydration solution was added to the solubilized protein samples and mixed well to ensure a homogeneous solution. Samples were delivered slowly to the center of the slot of the DryStrip reswelling rehydration tray in such a way that no air bubble was present in the samples. The protective cover from IPG strips (18 cm NL with a linear pI gradient from 3.0 to 10.0) was removed and strips were positioned with the gel side down and the pointed end against the sloped end of the slot. Each IPG strip was overlayed with 3 ml of low viscosity paraffin oil and then the Reswelling tray was covered with lid. The strips were allowed to rehydrate at room temperature for 24 h, removed from the Reswelling tray and rinsed briefly in a stream of deionized water.

Step(s)	V	mA	W	Vh
1	500	1	5	1,000
2	500	1	5	2,000
3	3,500	1	5	10,000
4	3,500	1	5	35,000

Rehydration solution	
Urea	1.92 g
Thiourea	0.61 g
CHAPS	80 mg
Pharmalytes 3-10	52.5 µl
Dithiothreitol (DTT)	17.5 mg
Bromophenol blue	Trace
dH_2O	3 ml

The strips were kept on its edge on a damp filter paper for several seconds to drain excess moisture. The strips were placed in the grooves of a MultiphorII Immobiline DryStrip tray in such a way that the acidic (pointed) end was near the anode and the blunt end was near the cathode. The moistened electrode strips were placed across the cathodic and anodic ends of the aligned IPG strips. Each electrode was aligned over the electrode strip, ensuring that the marked side corresponded to the side of the tray giving electrical contact. Finally, the strips were covered with paraffin oil. During isoelectric focussing temperature was maintained at 20 ^{o}C. Isoelectric focussing was carried out by using the above voltage/time profile.

3.27.4 Second dimension (SDS-PAGE)

After IEF, the second-dimension separation was performed. SDS-PAGE consists of four steps: (1) preparing the second-dimension gel, (2) equilibrating the IPG strip(s) in SDS buffer, (3) placing the equilibrated IPG strip on the SDS gel, and (4) electrophoresis. The technique is performed in polyacrylamide gels containing sodium dodecyl sulphate (SDS), an anionic detergent that denatures proteins by wrapping around the polypeptide backbone in a ratio of approximately 1.4 g SDS per g protein.

3.27.5 IPG strip equilibration

The equilibration step saturates the IPG strips with the SDS buffer system required for the second-dimension separation.

Equilibration buffer for 5 gels

Ingredients	Solution A	Solution B
0.5 M Tris-HCl, pH 6.8	2.5 ml	2.5
Urea	9 g	9 g
87% Glycerol	8.6 ml	8.6 ml
10 % SDS	10 ml	10 ml
DTT	87.5 mg	-
Iodoacetamide	-	1.125 g
Bromophenol blue	-	Trace

IPG strips were kept individually in channels. The strips were equilibrated with 5 ml of equilibration solution A for 15 min at room temperature with continuous rocking. After this, the equilibration solution was decanted and 5 ml of equilibration solution B was added to each strip. The strips were equilibrated for 15 min at room temperature with continuous rocking. After equilibration, the IPG strips were placed on filter paper moistened with dH_2O. To drain the excess solution, the IPG strips were gently blotted with moistened filter paper.

3.27.6 Preparing SDS slab gels (Laemmli, 1970)

In the second dimension proteins were separated in the Investigator™ System (Perkin Elmer, Life Sciences, Cambridge, UK).

Components	Separation gel, per 6 gels	Stacking gel, per 6 gels
40 % acrylamide	167.7 ml	5.4 ml
2% bis-acrylamide	89.5 ml	2.2 ml
1.5 M Tris (pH= 8.8)	136.2 ml	------
4X upper buffer, pH = 6.8	-------	15 ml
10 % SDS	5.74 ml	------
dH_2O	150 ml	36.9 ml
40 % APS	350 µl	55 µl
TEMED	275 µl	33 µl

4X Upper buffer, pH 6.8	
Tris-HCl	0.5 M, pH = 6.8
SDS	0.4%

A homogeneous single percentage SDS gel containing 12.5% total acrylamide was prepared and poured in between two plates which were separated by spacers. The gel was immediately overlayed with a thin layer of 1 ml of deionized water and allowed to polymerize at room temperature for 2-3 h. After ensuring that polymerization was even, the overlay was removed. After this, each gel cassette was overlayed with 5 ml of stacking gel and again overlayed with 1 ml deionized water. The gels were allowed to polymerize for 30-60 min at room temperature.

The equilibrated IPG strips were positioned between the plates on the surface of the second-dimension gel. 1 ml of melted 1% agarose (prepared in 1X upper buffer) was poured on the top surface of the slab gel such that no air bubble was introduced into the agarose. Immediately the IPG strips were embedded in agarose and then gently pushed down with a ruler so that the entire lower edge of the IPG strips was in contact with the top surface of the slab gel. The Investigator unit was filled with running buffer, temperature was set to 20 ^{o}C and the safety lid was placed on the top of the system. The current was supplied at 400 V, 400 mA and 12 W. The gels were allowed to run until bromophenol blue was 1-2 mm away from the bottom of the gel. The gels were removed and visualized by silver and Coomassie staining.

3.27.7 Silver staining (Bloom *et al*., 1987)

Silver staining is the most sensitive, nonradioactive staining method. It requires a high purity reagents and precise timing for reproducible, high-quality results. All staining and developer solutions were prepared freshly. After the run, the proteins were fixed by incubating the gels in fixation solution with gentle agitation on a shaking platform for at least 1 h at room temperature. The gel slabs were rinsed with 50% ethanol (3 changes, 20 min per change) with gentle agitation . The gels were sensitized with a sensitization solution for 1 min. The solution was discarded and the gel slabs were quickly rinsed with 3 changes of dH2O (20 s each).

Fixation solution:		
Ingredient(s)	**Volume**	**Final Concentration**
Ethanol	500 ml	50 %
Acetic acid	120 ml	12 %
37% formaldehyde*	0.5 ml	0.05 %
dH_2O	380 ml	
Sensitization:		
Ingredient(s)	**Volume**	**Final Concentration**
$Na_2S_2O_3.5H_2O$*	0.2 g	0.02 %
dH_2O	1000 ml	
Staining solution:		
Ingredient(s)	**Volume**	**Final Concentration**
Silver nitrate*	2 g	0.2 % (w/v)
37% formaldehyde*	0.75 ml	0.075 %
dH_2O	1000 ml	
Development solution:		
Ingredient(s)	**Volume**	**Final Concentration**
Na_2CO_3	60 g	6 %
$Na_2S_2O_3.5H_2O$*	4 mg	0.0004 %
37% formaldehyde*	0.5 ml	0.05 %
dH_2O	1000 ml	
Stop solution:		
Ingredient(s)	**Volume**	**Final Concentration**
Glycine	15 g	1 %
dH_2O	1500 ml	
*Reagents were added immediately prior to use		

Staining solution was added to the gel and gently agitated for 20 min at room temperature. The silver nitrate solution was discarded, the gels were quickly rinsed with distilled water (2 changes, 20 s per change) and transferred to the developing solution and gently agitated with a rocking motion until the bands reached to the desired intensity (usually 1 min.). To terminate the developing reaction, the gels were immediately transferred to stop solution

and incubated with gentle shaking for 30 min. After this, they were washed overnight in distilled water with gentle agitation.

Silver stained 2D-gel images were first scanned and analyzed by using MelanieIII® (BioRad) software package.

3.27.8 Coomassie staining

Stock Solution (0.2%): 1 tablet of PhastGel Blue R was dissolved in 80 ml of distilled water and stirred for 5 to 10 min. To this, 120 ml of methanol was added and stirred until all of the dye is dissolved.
Final Solution (0.02%): One part of stock solution was mixed with 9 parts of methanol: acetic acid:distilled water (3:1:6).

Gels intended for MALDI-TOF analysis were stained by PhastGel Blue R. Each gel was incubated with 200 ml of final solution overnight in a shaking condition and then washed in distilled water for several hours till background staining of the gels disappeared. Distilled water was changed after every 30 min.

3.28 Mass Spectrometry

The basic requirements of proteome analysis are: a wide dynamic detection range, high-confidence protein identification and protein quantification. Mass spectrometry (MS) can fulfill all these requirements. During the past decade, Matrix-assisted laser desorption ion- ization-time-of-flight mass spectrometry (MALDI-TOF MS) has proven to be one of the most succesful ionization methods for the mass spectrometric analysis and investigation of large molecules such as proteins.

In this technique, a matrix and sample (analyte) is irradiated by a nanosecond laser pulse. A laser beam, serves as the desorption and ionization source in MALDI. The matrix plays a key role by absorbing the laser light energy, which prevents unwanted fragmentation of analyte, and enables vaporization of the illuminated substrate. A rapidly expanding matrix plume carries some of the analyte into vaccum with it and aids the sample ionization process. Nitrogen lasers operating at 337 nm (a wavelength that is well absorbed by most matrices) are the most common illumination source.

Once the sample molecules are vaporized and ionized, they are transferred and accelerated in an electric field in a time-of-flight tube. In the flight tube, the molecules are separated from the matrix ions. During the flight in the tube, different molecules are separated according to their mass to charge ratios (m/z) and reach the detector at different times. In this way each molecule yields a distinct signal. This method is used for detection of molecules with molecular masses between 400 and 350,000 Da.

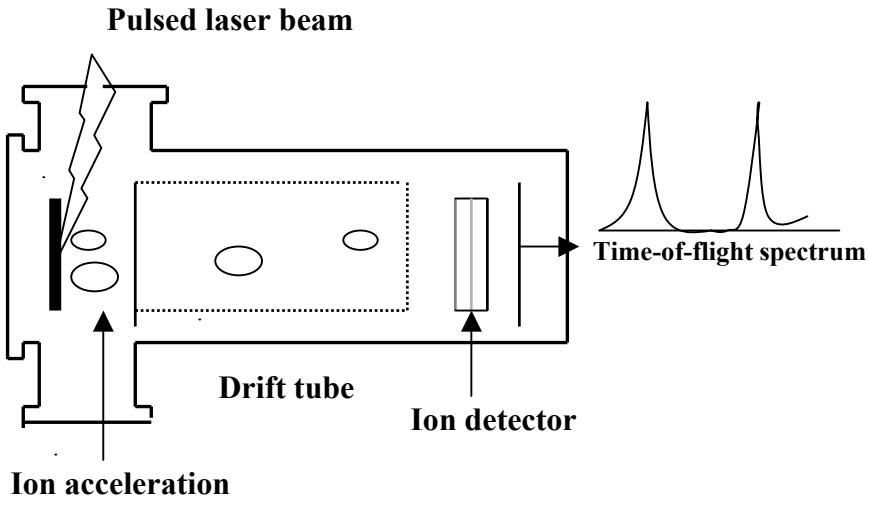


Figure 3.5: Scheme of simple time-of-flight mass spectrometer with a pulsed laser source

This is a very sensitive method, which allows the detection of low (10^{-15} to 10^{-18} mole) quantities of sample with an accuracy of 0.1-0.01%. Protein identification by this technique has the advantage of short measuring time and negligible sample consumption.

3.28.1 Preparation of protein samples for MALDI-TOF

Samples for MALDI-TOF analysis need to meet certain requirements for obtaining useful spectra. The total amount of sample needed for MALDI analysis depends on the sample type. For small molecular weight peptides (1,000 Da or less) the minimum amount is 16 pmol/µl . For a mass of 66,000 Da, the mimimum amount needed for analysis is 160 pmol/µl . Therefore, the larger the molecular mass is, the more the sample needed. Protein spots of interest from stained 2D gels were excised using a clean, sharp scalpel. For larger spots, the excised part of the gel was chopped into pieces and transferred into a clean Eppendorf tube.

Solutions:	
Trypsin digest 1:	100 mM Tris in 50 % acetonitrile, pH 8.5
Trypsin digest 2:	100 mM Tris in 10 % acetonitrile, pH 8.1
Trypsin digest 3:	100 mM Tris, 1 mM $CaCl_2$ in 10 % acetonitrile, pH 8.1
Enzyme solution:	Trypsin (Promega); 1 µg/5 µL in reconstitution buffer
Stop and elution solution:	2 % trifluoroacetic acid (TFA), 75 % acetonitrile
Solution 6:	1 %TFA, 30 % acetonitrile
α-Cyano-3-hydroxy- :	30 µg α-Cyano + 500 µL Acetonitrile, vortex for
cinnamic acid	5 min, add 500 µL H2O, vortex 5 min,
	centrifuge at 5000 rpm for 1 min.

Peptides were extracted according to Otto *et al*. (1996). The excised spots were covered with 500 µl of Trypsin-digest 1 and incubated at 30 ^{o}C for 20 min with shaking. This step was repeated until the colour disappeared. To restore spot size, the supernatant was discarded and the spots were mixed with 500 µl Trypsin-digest 2 and incubated at 30 ^{o}C for 30 min with continuous shaking. The supernatant was removed and the gel slices were dried in the Speedvac to 20-40% of the original volume. Enzyme solutions were prepared freshly by suspending trypsin in 200 µl resuspension buffer and incubated at room temperature for 15 min with continuous shaking. 1.4 ml of Trypsin-digest 3 was added to the enzyme solution and mixed well. 30 µl of this solution (Trypsin and Trypsin-digest 3) was added to the gel slice and incubated overnight at 37 ^{o}C on a shaker. Peptides were extracted by adding 100 µl of stop and elution solution and incubated overnight at room temperature with continuous shaking. The supernatant was transferred to a new Eppendorf tube and dried in a Speedvac to a residual volume of 1-2 µl. Concentrated peptides were resuspended in 10 µl of solution 6. For MALDI-TOF analysis, 0.5 µl of desalted peptide solution was mixed with 0.5 µl α-Cyano-3-hydroxy-cinnamic acid matrix, pipetted on a metal target plate and allowed to crystallize at room temperature. Crystallized samples were subjected to MALDI-TOF analysis. Peptide masses were determined by Dr. Völker (MPI for Terrestrial Microbiology, Marburg) in the positive ion reflector mode in a Voyager DE RP mass spectrometer with internal calibration. Peptide mass finger-prints were compared to databases using the MS-Fit program (http://prospector.ucsf.edu). The searches considered oxidation of methionine, pyroglutamic acid formation at the N terminal of glutamine

and modification of cysteine by carbamidomethylation as well as partial cleavage leaving one internal cleavage site. Spots that could not be identified by the above method were further analyzed by MALDI-Post Source Decay (PSD) sequencing (performed by Protagen AG, Bochum, Germany).

3.28.2 Desalting of protein samples (Millipore Inc.)

ZipTip^{TM} is a 10 µl pipette tip with a 0.5 µl bed of resin fixed at its end. It is intended for concentrating, desalting, and removing detergents from biological samples (< 40 kd) for MALDI MS, or other analytical techniques. ZipTip$_{C18}$ contains C18 spherical silica (15 µm, 200 Å pore size) in a 0.6 µl bed volume.

Wetting buffer:	50 % acetonitrile in water
Equilibration buffer:	0.1 % TFA in water
Elution buffer:	Matrix in 50 % acetonitrile

The tip was first prewetted by depressing the plunger to a dead stop using the maximum volume setting of 10 µl. Tip was aspirated and dispensed twice with wetting buffer. The tip was equilibrated for binding by washing it twice with the equilibration buffer. After equilibrating the tips, peptides and proteins were aspirated and dispensed 5 to 10 cycles by fully depressing the pipette plunger to a dead stop. Tip was washed by aspirating and dispensing two cycles of 0.1% trifluoroacetic acid (TFA). The peptides were eluted by aspirating and dispensing 2 to 4 µl of elution buffer.

3.29 Determination of intracellular levels of glutamine and glutamate

For the assay of intracellular Glu and Gln, the *P. putida* KT2440 and *gltB* mutant were grown in M9^{-}-medium with 22 mM glucose and 19 mM NH_4Cl. Samples were removed after 24 and 96 h. Cells were washed, suspended in 4 ml 80% ethanol, and incubated at 90 °C for 15 min. An aliquot of sample was removed for protein estimation. After centrifugation at 14,000 x g, the clear supernatant was evaporated to dryness and then dissolved in 50 mM N-ethylmorpholine, pH 8.0 (a volatile buffer system), and divided in two equal parts. After treating one of them with 100 U of *E. coli* asparaginase (Medac, Wedel) for 1 h to convert Gln to Glu, both samples were taken to dryness once again and dissolved in N-

ethylmorpholine buffer as above. The Glu content of the resulting solutions was determined by an enzymatic assay based on glutamate dehydrogenase. In the wells of a microtiter plate 220 µl buffer was mixed with 20 µl of the Glu-containing sample and 20 µl glutamate dehydrogenase (Sigma, 1600 U/ml). After starting the reaction with 20 µl 10 mM NAD^+, the formation of NADH was followed at 340 nm until completion. The total absorption change obtained in this way (corrected with appropriate blanks) is proportional to the amount of Glu initially present.

Reagents	Concentration
N-ethylmorpholine, pH 8.0	50 mM
*GDH enzyme	8 U/ mg protein
NAD^+	20 mg/ml in H_2O

* GDH stock solution was diluted in 40 % glycerol

For protein estimation, the cells were disrupted by sonication (15 s x 4) and immediately cooled in ice. Protein content was estimated by Bradford method (see section 3. 25.1).

3.30 HPLC analysis of amino acid uptake

Precolumn derivatization with phenylisothiocyanate (PITC) followed by reversed-phase HPLC is an increasingly popular method for quantitative amino acid analysis. The method offers the advantages of sensitivity in picomole range, short analysis time, and quantitation of all amino acids at a single wavelength. The system (Merck-Hitachi, Darmstadt) consisted of L-7100 pumps, an UV-Vis detector, AS-2000A autosampler, D-7500 integrator and a L-7612 solvent degasser.

3.30.1 Derivatization of amino acids

Precolumn derivatization with phenylisothiocyanate (PITC) was carried out as described by Mora *et al*. (1988). The tubes were prepared for analysis by washing them with detergent and water, briefly soaking in nitric acid. Tubes were then rinsed several times with distilled water, and finally dried at 80 ^oC before use. Samples and standard glutamine and glutamate were derivatized by PITC.

The *ansB* knock-out mutant and *P. putida* KT2440 were grown in $M9^-$-medium containing Gln and Glu (10 mM each) as sole C and N sources. Samples were removed at

different time intervals. Amount of amino acids present in the medium was analyzed by HPLC. 100 µl from each sample was concentrated in the cleaned tube under vaccum. 50 µl coupling buffer was added to the concentrated samples, mixed homogeneously and then dried under vaccum. For derivatization, 190 µl coupling buffer and 10 µl PITC were added to the vaccum dried samples and were allowed to stand at room temperature for 30 min. After incubation samples were again dried under vaccum. Finally derivatized samples were dissolved in 95 µl buffer A and 5 µl acetonitrile.

3.30.2 Chromatography

Coupling buffer: 35% H_2O, 30% Acetonitrile, 25% Pyridine, 10% Triethylamine

Buffer A : 50 mM Sodium acetate, 2.75% Triethylamine (pH 6.4, adjusted with phosphoric acid)

Buffer B: 50% buffer A, 40% Acetonitrile, 10% Methanol.

Gradient used:

Time (min)	0	25	25.1	35	35.1	45
Buffer A (%)	98	98	0	0	98	98
Buffer B (%)	2	2	100	100	2	2

The resulting PITC-amino acid derivatives were separated and analyzed using a Lichrospher® 60 RP select B column (5 µm particle size; Merck, Darmstadt) at 50 ^oC and a flow rate of 1.0 ml/min in a Merck-Hitachi LabChrom gradient system. Whenever chromatography was delayed after derivatization of a sample, the PITC-amino acids were stored at -80 ^oC in a screw-capped bottles. Absorbance of the eluate was monitored at 254 nm.

4. Results

4.1 Selection of strain

As already discussed in the introduction, *P. putida* KT2440 has become a model organism for studies of the interactions between plants and rhizosphere bacteria. Moreover, the full genomic DNA sequence of this organism is now available (Nelson *et al*., 2002). Previous experiments had established that *P. putida* KT2440 is able to utilize a wide range of amino acids as sources of carbon and/or nitrogen (Klöppner, 1999; Hüser *et al.*, 1999). Mainly for these reasons, *P. putida* KT2440 was selected for the present work which focuses on the metabolism of the acidic amino acids in pseudomonads and its regulation.

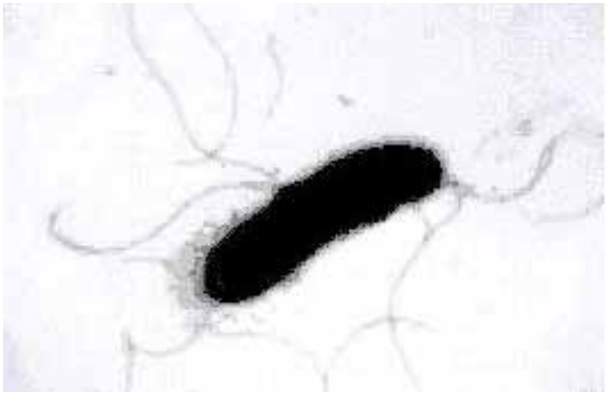


Figure 4.1: *Pseudomonas putida* cell

4.2 Growth of *P. putida* KT2440 on amino acids

Growth of different *Pseudomonas* strains was checked on different amino acids as carbon and nitrogen sources. All examined *Pseudomonas* strains were capable of utilizing the acidic amino acids (Asp and Glu) and their amides (Asn and Gln) as growth substrates (see Table 4.1). Doubling times on 10 mM Asn or Gln were about 50 min and thus in the same range as those during growth on NH_4^+/glucose. Among the other amino acids, Pro and Ala also supported growth of strain KT2440, whereas Lys and Leu were not utilized.

Amino acids	*P. fluorescens* ATCC 13525 *)		*P. fluorescens* Pf-5 *)		*P. putida* ATCC 12633 *)		*P. putida* KT2440	
	N	C + N	N	C + N	N	C+ N	N	C+ N
Asp	++	++	++	++	+++	++	++	++
Asn	+++	+++	+++	+++	+++	++	+++	+++
Glu	++	+	++	+	+	+	++	++
Gln	+++	+	+++	++	+++	+	+++	++
Ala	++	++	+++	++	++	++	n. d.	+
Ser	++	++	++	+	+++	++	+	+
Pro	+	(+)	++	+	–	+	++	++
Lys	–	–	–	–	–	–	–	–
Arg	++	+	++	++	++	++	n.d.	n.d.
Leu	(+)	(+)	+	+	–	(+)	–	–

Table 4.1: Growth of *Pseudomonas* strains on different amino acids. The cells were pre-grown on M9$^-$- medium containing NH_4^+/glucose overnight and then transferred to M9$^-$-medium supplemented with 22 mM glucose plus 10 mM amino acids (N), or 10 mM amino acids as the sole source of carbon and nitrogen (C + N). Relative growth rates were estimated as an increase in OD at 595 nm between 3 h and 5 h after transfer to fresh medium. +, 0.05-0.1; ++, 0.1-0.2; +++, >0.2. *) Data from Klöppner (1999).

4.3 Regulation of PGA expression by amino acids

Earlier work from our group had established that the expression of periplasmic glutaminase/ asparaginase (PGA) of *P. fluorescence* ATCC13525 and *P. putida* ATCC 12633 is strongly and specifically enhanced by acidic amino acids and their amides (Klöppner, 1999; Hüser *et al*., 1999). In the present work a similar induction pattern was observed with *P. putida* KT2440. For these experiments, the cells were pre-grown overnight on NH_4^+/glucose and then transferred to media containing amino acids as the sole source of carbon and nitrogen. As shown in Fig. 4.2, all four amino acids led to an about 20-fold increase of PGA activity within 12 h, while proline supported rapid growth but had no effect on PGA activity. All other amino acids tested also failed to induce PGA activity (data not shown).

As shown by Fig. 4.2, the time courses of PGA induction by Asn and Asp on one hand, or Gln and Glu on the other, were almost the same, however, PGA induction by Asp and Asn was delayed by about 6 h. Therefore, maximum PGA activity during growth on these amino acids was only seen after 12 h. Asn, Pro, and NH_4^+/Glc led to immediate growth, while growth on Glu and Gln only started after a delay of about the same duration as observed for PGA induction (Fig. 4.2).

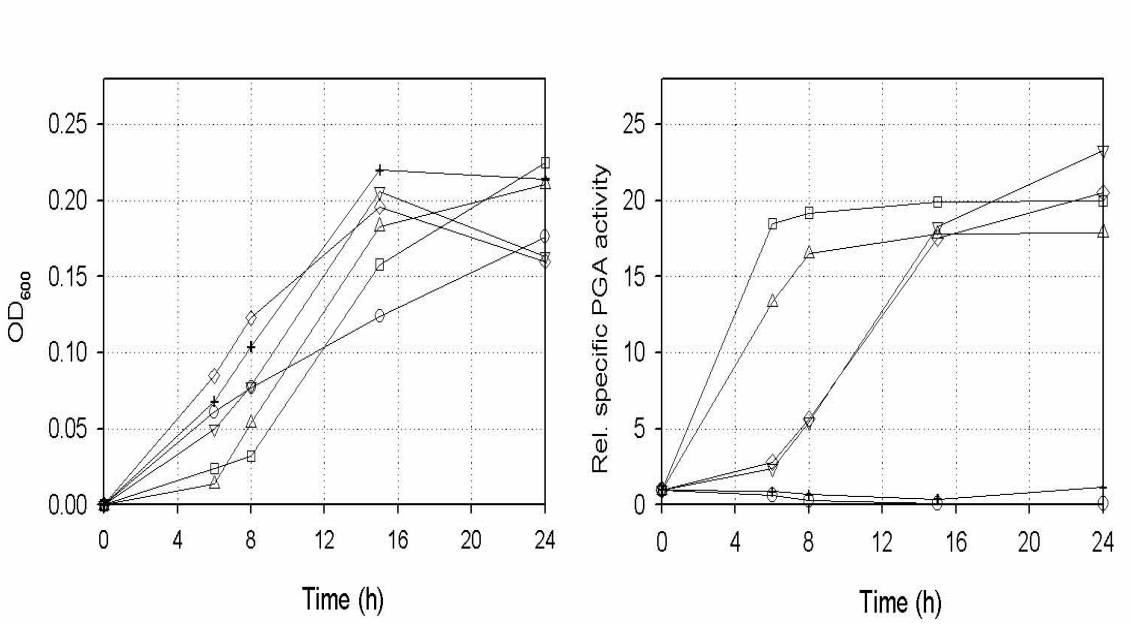


Figure 4.2: Kinetics of PGA induction in *P. putida* KT2440 by Asn (-✧-), Asp (–∇–), Gln (-□-), Glu (–□ –), proline (+), NH_4^+/glucose (–O–). The cells were pre-grown in NH_4^+/glucose minimal medium overnight and then transferred to the same medium, or to minimal medium containing 10 mM amino acids as the sole source of carbon and nitrogen. Growth was monitored as an increase in optical density at 600 nm vs. time after transfer to fresh medium.

Klöppner (1999) as well as Hüser *et al*. (1999) reported that the expression of PGA in *P. fluorescens* ATCC 13525 and *P. putida* ATCC 12633 was subject to carbon catabolite repression by good carbon sources.

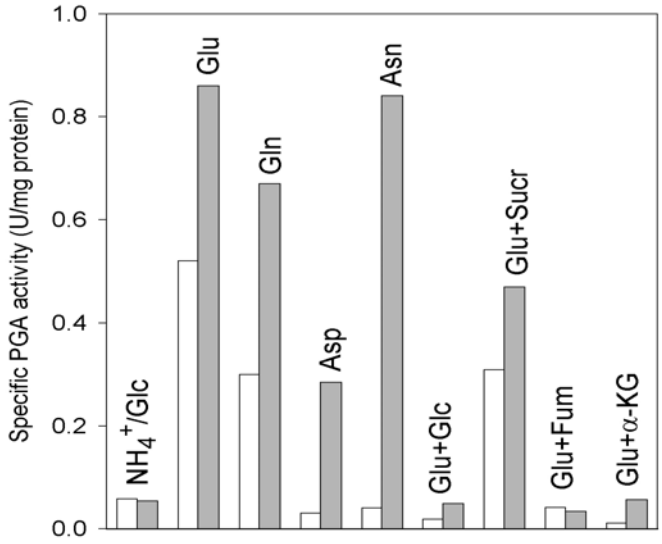


Figure 4.3: Regulation of PGA expression in *P. putida* KT2440. Specific PGA activities (in U/mg protein) were measured 6 h (open bars) and 24 h (filled bars) after transfer from NH_4^+/glucose to $M9^-$ medium supplemented with different carbon and nitrogen sources, i. e. (from left to right) NH_4^+/glucose,10 mM each of Glu, Gln, Asp, and Asn; 10 mM Glu +22 mM glucose (Glu+Glc), 10 mM Glu +10 mM sucrose (Glu+Sucr), 10 mM Glu +10 mM fumarate (Glu+Fum), or 10 mM Glu +10 mM α-ketoglutarate (Glu+α-KG).

A similar behaviour was seen with *P. putida* KT2440 (Fig. 4. 3). Glucose and intermediates of the citric acid cycle (fumarate, 2-oxoglutarate) almost completely suppressed the inductive effect of Glu on PGA induction, while sucrose which is not metabolized by *P. putida* KT2440, was much less effective as a repressor.

4.4 Role of PGA in Gln utilization by *P. putida* KT2440

In order to further characterize the role of periplasmic PGA in the assimilation of Asn and Gln, we constructed a *P. putida* KT2440 mutant where the PGA-encoding *ansB* gene was inactivated by a targeted disruption. The gene was amplified from KT2440 genomic DNA, cloned into pJQ200, inactivated by insertion of a kanamycin resistance cassette**,** and then re-introduced into the KT2440 genome by homologous recombination (see sections 3.15.1 and 3.15.2). The transconjugants obtained in this way were analyzed for PGA activity and growth rates on amino acids.

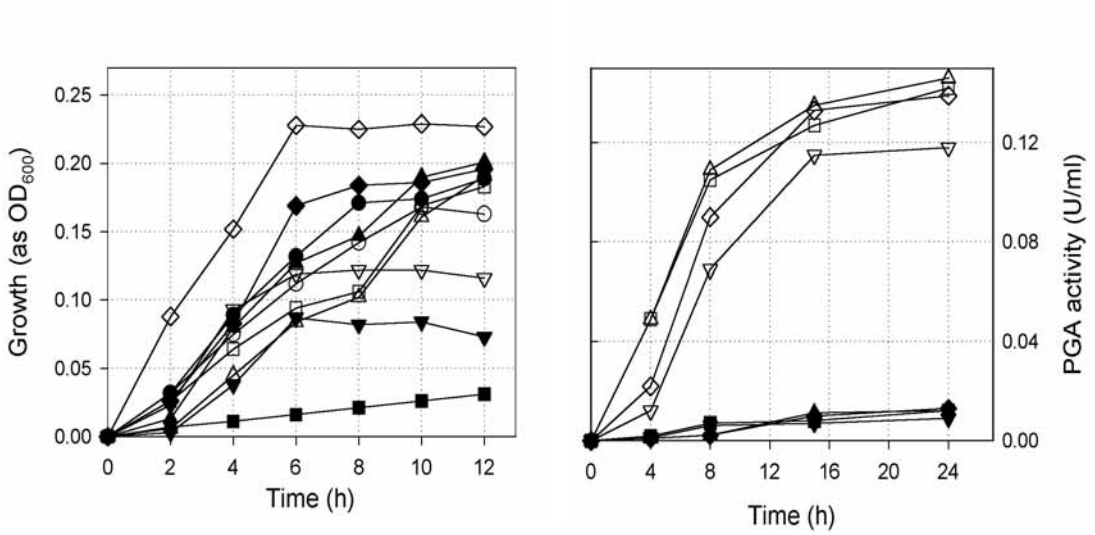


Figure 4.4: Growth and PGA activity of *ansB* mutant (closed symbols) and *P. putida* KT2440 (open symbols) on Asn (-◆-,-◇-), Asp (-▽-,-▼-), Gln (-■-, -□-), Glu (-Δ-,-▲-), and Glucose/NH_4^+ (-●-, -O-). Cells were pre-grown overnight in NH_4^+/glucose minimal medium (NH_4^+/Glc), washed and transferred to a) the same medium or b) $M9^-$ media containing 10 mM each of Asn, Asp, Gln or Glu as sole source of C and N.

As expected, the transconjugants were unable to catalyze the hydrolysis of AHA when grown on Gln, Glu and Asp. However, some activity was seen during growth on Asn, as

shown in Fig. 4.4. When amino acids were provided as the sole source of carbon and nitrogen, Gln was the only amino acid that did not support rapid growth of the mutant.

4. 5 Amino acid utilization by an *ansB* disruption mutant

As mentioned above, PGA is located in the periplasm of the cells. Thus, the inability of the *ansB*- disruption mutant to grow on Gln might be due to the fact that Gln cannot be taken up but, in order to be utilized, has to be hydrolyzed to Glu first which then enters the cell.

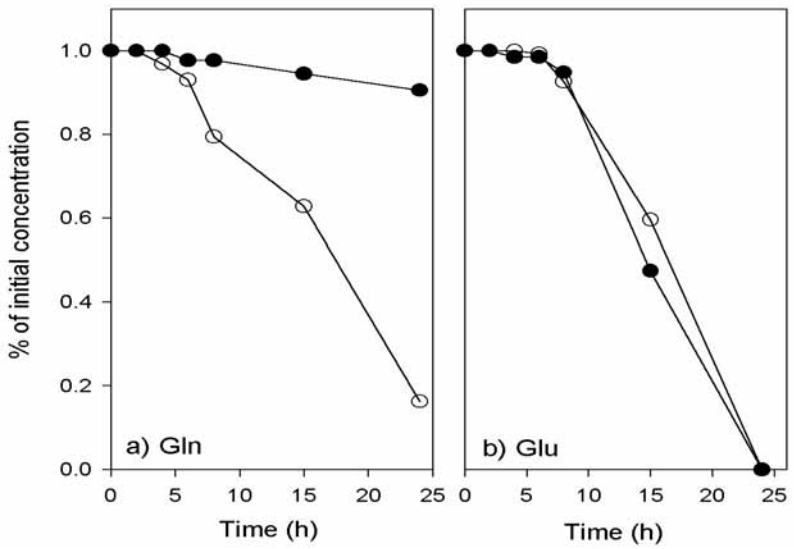


Figure 4.5: Uptake of Gln and Glu by *P. putida* KT2440 wild type (open symbols) and the *ans*B dis- ruption mutant (closed symbols). The cells were pre-grown on NH_4^+/glucose minimal medium and then transferred to M9$^-$- media supplemented with a) 5 mM Gln and b) 5 mM Glu as the sole source of carbon and nitrogen. The concentrations of Glu and Gln in the medium (determined by quantitative amino acid analysis, see section 3.30) are plotted vs. incubation time.

To examine this possibility, we used quantitative amino acid analysis to follow the disappearance of Gln and Glu from media inoculated with *P. putida* KT2440 (wild type) and the *ansB*- disruption mutant, respectively. As shown in Fig. 4.5a, the ability of the *ansB*- mutant to consume Gln was severely impaired. By contrast, the rate of utilization of Glu was unaffected in the mutant (Fig. 4.5b). The consumption of both amino acids (Gln and Glu) did not start immediately but after a lag phase of 6-8 h. This indicates that not only PGA but also other proteins required for utilization of Glu and Gln have to be induced after transfer from NH_4^+/glucose to amino acids.

4.6 Identification of further amino acid-induced proteins

In former studies on the effects of amino acids on gene expression in *Pseudomonas*, PGA was used as an indicator enzyme mainly because its activity is easy to measure with intact cells. Using 2D gel electrophoresis, Klöppner (1999) already shown that PGA of *P. fluorescens* is differentially expressed in the absence and presence of amino acids like Asn and Asp. In addition, she identified a second protein induced by acidic amino acids, i. e. a putative Gln- binding protein associated with an ABC transporter (Klöppner, 1999).

With the aim to identify additional proteins that are induced or repressed by acidic amino acids and/or their amides, we performed further 2D-PAGE experiments with *P. putida* KT2440 extracts prepared at 0, 2, 4 and 6 h after transfer of cells from overnight cultures on NH_4^+/glucose to a) the same medium (Glc/NH_4^+, Fig. 4.6) or b) to M9$^-$-medium with 10 mM Glu as the sole source of carbon and nitrogen (Fig. 4.7) or c) to M9$^-$- media containing Glu + fumarate as sources of carbon and nitrogen (Fig. 4.8). To facilitate comparison, a series of several gels with different samples from the same experiment were run simultaneously. Major protein spots that appeared unique or were significantly upregulated under these conditions are marked by circles, whereas the corresponding spots downregulated under these conditions are indicated by dashed circles.

A group of at least 9 major protein spots (Pp1-Pp9, Pp stands for *P. putida*) were coordinately induced by Glu as compared to their levels in cells grown on NH_4^+/glucose (Figs. 4.6 and 4.7). The intensity of the respective spots gradually increased with incubation time from 0 to 6 h. Spots Pp1, Pp3/Pp4, Pp5, Pp6 and Pp9 were already present at low intensities immediately after transfer, whereas Pp2, Pp7 and Pp8 only started to appear after 2 h (see Fig. 4.7). Spots Pp10-Pp13 responded to different carbon and nitrogen sources in a similar fashion. As compared to growth on Glu, their expression was strongly upregulated during growth on NH_4^+/ glucose (compare Fig. 4.6 and Fig. 4.7).

Figure 4.6: Two-dimensional electrophoresis maps (pH 3-10) of soluble proteins differentially expressed by *P. putida* KT2440 during growth on NH_4^+/glucose minimal medium (NH_4^+/Glc). Selected proteins upregulated during growth in this medium (Pp10-Pp13) are marked by circles. Proteins which were upregulated in Glu but downregulated in NH_4^+/Glc are highlighted by dashed circles.

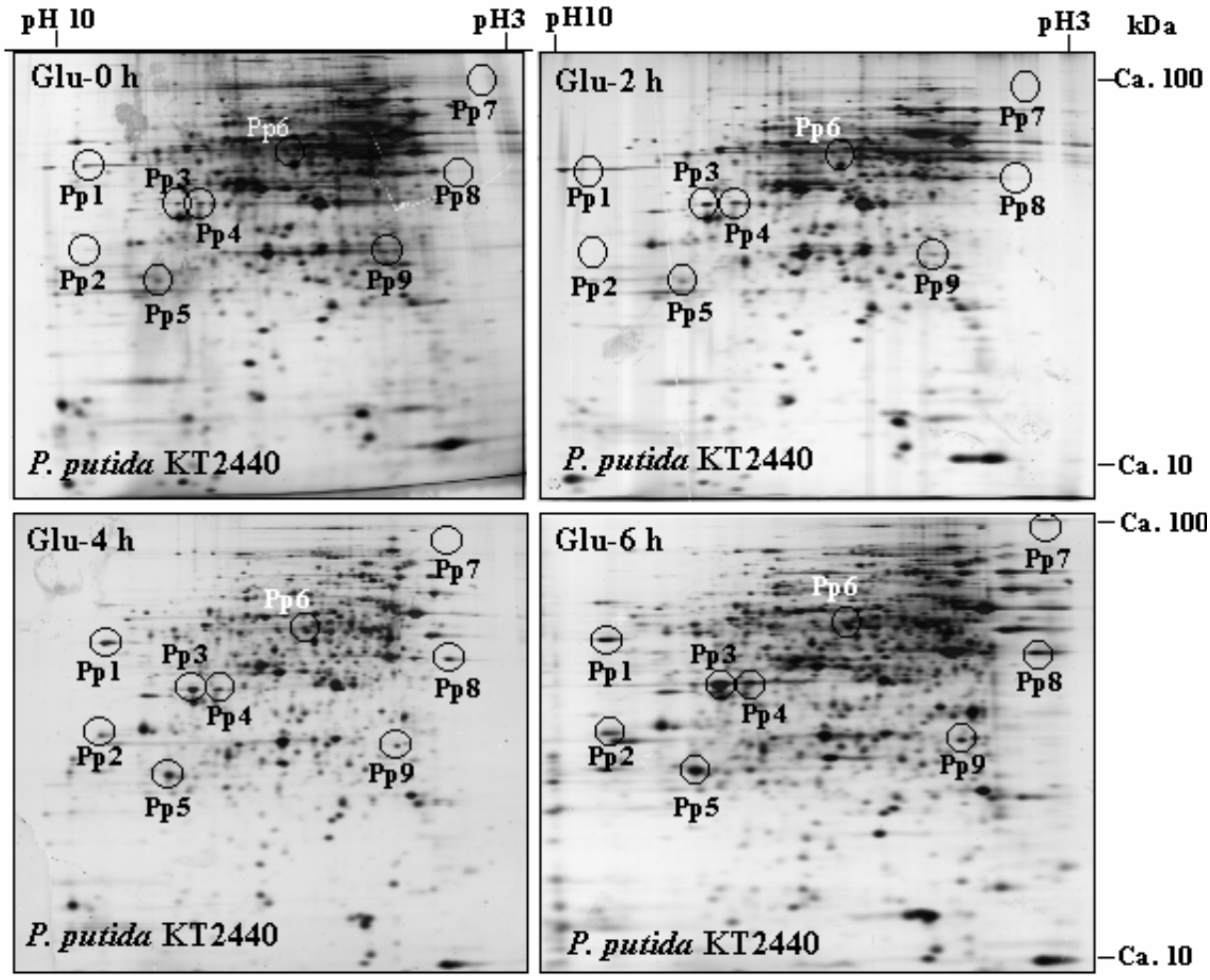


Figure 4.7: Two-dimensional electrophoresis maps (pH 3-10) of soluble proteins expressed by *P. putida* KT2440 during growth on M9^{-}-medium supplemented with 10 mM Glu as the sole source of C and N. Selected proteins that were upregulated during growth on Glu (Pp1-Pp9) are marked by circles.

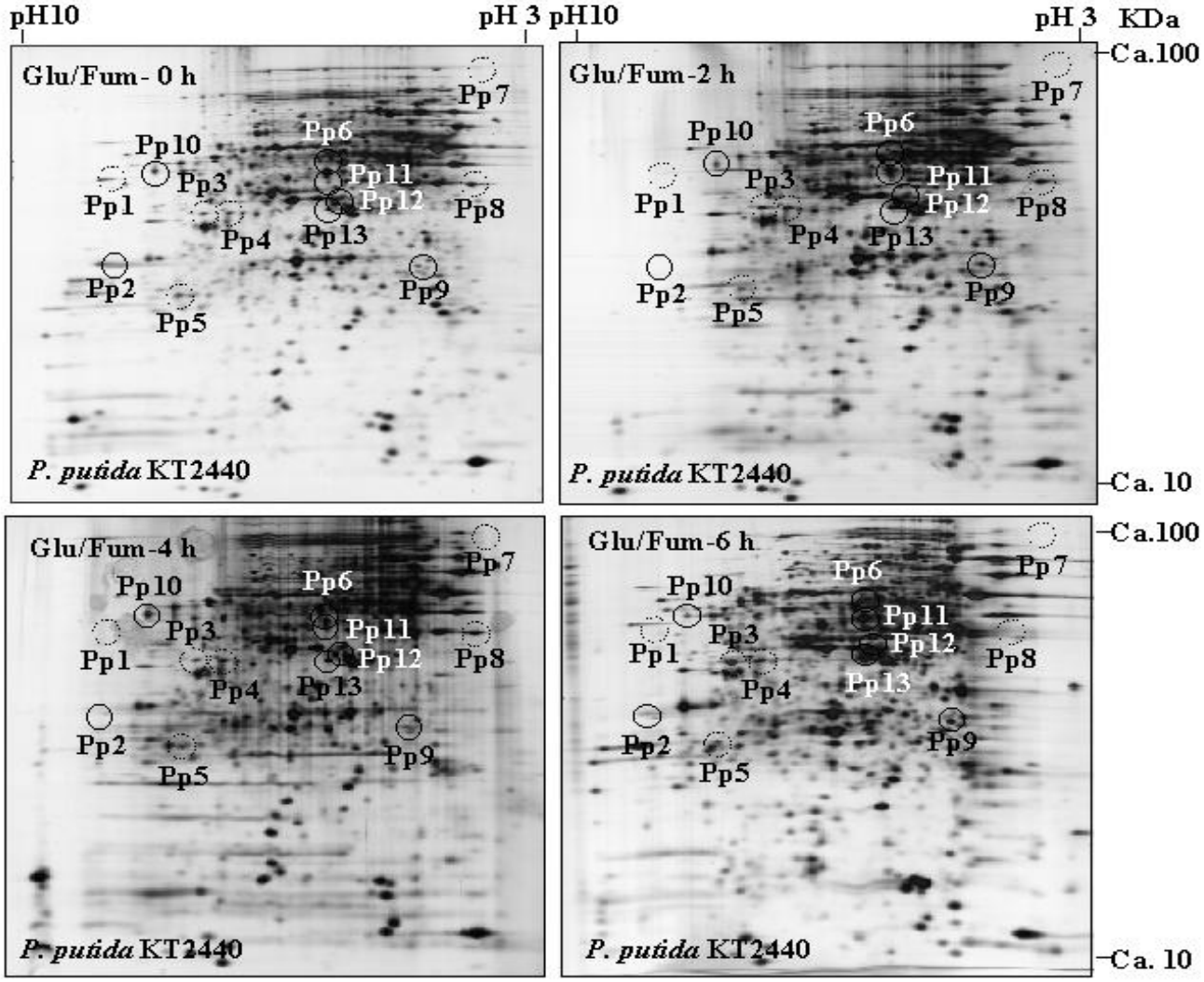


Figure 4.8: Two-dimensional electrophoresis maps (pH 3-10) of soluble proteins differentially expressed by *P. putida* KT2440 during growth on M9-medium containing 10 mM each Glu and fumarate as the source of C and N. Selected proteins unaffected during growth on Glu and fumarate are marked by circles. Proteins which were upregulated in Glu but downregulated in Glu and fumarate are highlighted by dashed circles.

As shown in figure 4.6, Pp11-Pp13 were consistently present in all extracts with increasing intensities, while Pp10 only appeared after 2 h of growth on NH_4^+/glucose.

All Glu-induced proteins except Pp2, Pp9 and, in part, Pp6 were partially or totally repressed in the fumarate-containing media. On the other hand, all four NH_4^+/glucose induced proteins (Pp10-Pp13) were unaffected by the presence of fumarate (Fig. 4.8). These results are also summarized in table 4.3 (see below).

For identification, a total of 13 spots (Pp1–Pp13) differentially expressed in both the conditions (Glu, NH_4^+/Glucose) were selected, cut from several gels and pooled. The gel pieces were then digested with trypsin *in situ* and desalted as described in sections 3.28.1 and 3.28.2. The unfractionated mixtures of tryptic peptides were then analyzed by MALDI-TOF or MALDI-PSD mass spectroscopy, in order to obtain as many fragmentation spectra of proteolytic peptides as possible. In 11 out of 13 cases, proteins could be identified with a high degree of confidence. Only spots Pp2 and Pp7 did not produce MS spectra of sufficient quality to allow their identification. All other spots were identified and assigned to proteins deduced from the *P. putida* KT2440 genome.

Three proteins, Pp3/Pp4, Pp11 and Pp12 were identified by MALDI-TOF, i. e. solely based on peptide masses with reference to the genome of *P. putida* KT2440 (http://www.tigr.org). For unequivocal identification of proteins, several parameters such as modification of cysteine by carbamidomethylation and oxidation of methionine were considered. The MS data was directly used as input for the Mascot™ and MS-Fit™ programs, which search protein sequence databases to identify any sequence that generates a theoretical pattern of peptide masses matching the experimentally measured one. The experimental mass values were then compared with calculated peptide mass or fragment ion mass values, obtained by applying carbamidomethylation cleavage rule to the entries in sequence database. By using an appropriate scoring algorithm, the closest match or matches were identified.

The other proteins were identified by the MALDI-PSD technique. It was of particular importance to obtain a significant degree of amino acid sequence coverage in order to confirm the correctness of the identified proteins. The basis for the identification of some of the peptides are given in appendix 8.2. The protein sequences of the respective genes were derived from the *P. putida* KT2440 genome (Nelson *et al*., 2002).

Protein Pp1 was identified as transcription termination factor, which is the product of the *rho* gene. Being based on 12 matching peptides, the identification of this protein is unequivocal. As already mentioned, spots Pp2 and Pp7 did not produce MS spectra of sufficient quality to allow their identification. Both Pp3 and Pp4 were identified as periplasmic glutaminase/ asparaginase (PGA) with 11 peptide mass matches. Protein Pp5 was identified as ATP-binding protein of an ABC transporter. Although only two peptides were matching, these showed strong sequence similarity to ATP-binding proteins from several bacterial transporters. Protein Pp6 encoded aspartate ammonia-lyase (aspartase) which is the product of *aspA* gene in *P. putida* KT2440. Here, 7 peptide masses matched the fragmentation patterns for aspartase. Pp8 was identified as an outer membrane protein, which is the product of *oprD* gene. Pp9, a member of the Glu induced proteins, was identified as putative carboxyphosphonoenol pyruvate-phosphonomutase. Although, both Pp8 and Pp9 were identified based on only 2 and 3 peptide matches, respectively, they showed strong similarities with peptide sequences from other organisms.

Four proteins were upregulated when *P. putida* KT2440 was grown in the presence of NH_4^+/glucose. Pp10, encoded by a *gabT* gene, was identified as 2,4-diaminobutyrate 2-oxoglutarate transaminase, with 4 peptide mass similarities. Among the 4 induced proteins, Pp11 was identified as fumarase protein by MALDI-TOF technique. This protein a product of the *fumC* gene was identified with 7 strong peptide mass matches. Pp12 and Pp13 were identified as members of the ABC transporters with 6 and 2 peptide mass similarities, respectively. Until, in *P. putida* KT2440, sugar ABC transporter sugar binding protein (Pp12) is not assigned to any gene product. Putrescine ABC transporter putrescine binding protein (Pp13) is encoded by a *potF* gene.

The data that emerged from mass spectrometric analysis of the identified spots is summarized in table 4.2. The table lists the names of the encoding genes, their known or putative functions, isoelectric points, and molecular masses calculated from the genome data. The isoelectric points of the identified proteins were calculated using Expasy's "Compute pI" program (http://scansite.mit.edu/cgi-bin/calcpi). For calculating the protein masses, the "Peptide Mass" program http://www.expasy.org/tools/peptide-mass.html) was used. The calculated pI (IEP_{calc}) and molecular masses ($Mass_{calc}$) of all proteins were roughly matching the pI values estimated from the 2D gels.

a) Induced during growth on Glutamate

Protein Spot	Identified as	Identified by	Locus	IEP_{calc}	$Mass_{calc}$ kDa
Pp1	Transcription termination factor	PSD (12)	*rho* (PP5214)	7.7	47.0
Pp2	Not identified				
Pp3 **Pp4**	Periplasmic Glutami-nase/asparaginase	PM (11)	*ansB* (PP2453)	6.6	36.1*
Pp5	ABC transporter ATP-binding protein	PSD (2)	? (PP1068)	8.2	28.1
Pp6	Aspartase ammonia-lyase (Aspartase)	PSD (7)	*aspA* (PP5338)	5.7	51.5
Pp7	Not identified				
Pp8	Outer membrane porin D	PSD (2)	*oprD* (PP1206)	4.8	46.1*
Pp9	Carboxyphosphonoe-nolpyruvatephosph-onomutase (putative)	PSD (3)	? (PP1389)	5.1	31.8

b) Induced during growth on NH_4^+/glucose

Protein Spot	Identified as	Identified by	Locus	IEP_{calc}	$Mass_{calc}$ kDa
Pp10	2,4-Diaminobutyrate 2-oxoglutarate transaminase	PSD (4)	*gabT* (PP4223)	6.5	48.8
Pp11	Fumarase	PM (7)	*fumC* (PP0944)	5.7	48
Pp12	Sugar ABC transporter, sugar binding protein	PM (6)	(PP1015)	5.7	45.4
Pp13	Putrescine ABC transporter, putres-cine binding protein	PSD (2)	*potF* (PP5181)	6.1	40.1

Table 4.2: Characteristics of differentially expressed protein spots in *P. putida* KT2440. PM (n) - identified from peptide masses, PSD (n)- Identified by MALDI-PDS, n- number of matching peptides.

The Glu-induced proteins (Pp1-Pp9) have a wide range of pI values (4.8-8.2) and molecular masses ranging from 31 to 52 kDa. On the other hand, the NH4+/glucose induced proteins had a narrower range of pI values (5.7-6.5) and molecular masses between 40 and 49 kDa.

Protein Spot	Expressed in rpoN$^-$	Repressed by fumarate
Induced during growth on glutamate		
Pp1	-	+
Pp2	-	-
Pp3 Pp4	-	+
Pp5	-	+
Pp6	+	(-)
Pp7	-	+
Pp8	-	+
Pp9	-	-
Induced during growth on glucose + NH_4^+		
Pp10	(+)	(-)
Pp11	+	-
Pp12	+	-
Pp13	+	-

Table 4.3: Expression profile of differentially expressed proteins in the *rpoN*$^-$ mutant and repression by fumarate. +, repressed by fumarate and expressed in *rpoN* mutant; (-), weakly repressed; -, not repressed by fumarate and absent in *rpoN* mutant.

4.7 Dependence of Glu-inducible gene expression on σ^{54}

For the proteins identified here we also analyzed the dependency of their induction/repression on the presence of the alternate sigma factor σ^{54} (RpoN). Sigma factors play an important role in binding of RNA polymerase to DNA and thus are important elements in controlling differential gene expression. The alternate sigma-factor σ^{54} (σ^N) encoded by an *rpoN* is required for the expression of genes involved in the

utilization of various nitrogen and carbon sources as well as diverse other functions (Merrick, 1993; Reitzer and Schneider, 2001). Therefore, a comparative proteome analysis of the wild type KT2440 and its isogenic *RpoN*- mutant was performed to investigate the *RpoN*-dependency of the changes in the protein profile discussed above. As described previously (Köhler *et al*., 1989), an *RpoN*- mutant of strain KT2440 was unable to grow when amino acids are given as sole source of carbon and nitrogen. *P. putida* KT2440 and the *RpoN*- mutant were pre-grown overnight in NH_4^+/glucose minimal medium, washed and transferred to the M9$^-$-media containing Glu as sole carbon and nitrogen source. Cell extracts were prepared 6 h after transfer and were subjected to 2D electrophoresis as described in earlier sections. Data obtained in this experiment is summarized in Fig. 4.9 and Table 4.4. They clearly show that the expression of most, if not all, of the Glu-inducible proteins is almost completely abolished in the *RpoN*- strain, supporting the notion that their induction following transfer from glucose/ NH_4Cl medium to Glu medium requires σ^{54}. The only exception was spot Pp6 (aspartase) which appeared to be expressed more strongly in the mutant.

Protein	**Potential σ^{54} binding site**
Pp1 Rho termination factor	G**GC**CACgttttTT**GC**T
Pp3/4 Glutaminase/asparaginase)	G**GG**CTGCTACACA**GC**T
Pp5 ATP-binding protein	T**GG**TACgcgctTT**GC**A
Pp6 Aspartate ammonia lyase	T**GG**CACggtgcTT**GG**C
Pp8 Outer membrane porin D	C**GG**CACgacatCT**GC**A
Pp9 Phosphoenolpyruvate mutase	C**GG**TGCgcacgTT**GC**G
Consensus sequence	T**GG**CACGnnnnTT**GC**T

Table 4.4: Putative σ^{54} recognition sites of Glu-responsive genes in *P. putida* KT2440 (see text).

In order to support this conclusion, i.e. that the Glu-responsive proteins identified in the present study are indeed dependent on σ^{54} for expression, the *P. putida* KT2440 genome was analyzed to localize the potential σ^{54} recognition sequences in the upstream regions of the respective genes using the program PROMSCAN (http://www.promscan.uklinux.net; (Studholme *et al*., 2000). σ^{54} recognition sequences are typically located at –12/-24 relative to the start of transcription. In the consensus sequence (see Table 4.4) a GG and a GC pair, both separated by 10 nucleotides, are strictly conserved (Buck *et al*., 2000). For all of the Glu-responsive genes identified here, sequences with the expected properties were found at a reasonable distance (i.e. within 200 bp) from the respective translation start sites. Of course, for an unequivocal identification of these sites it would be necessary to locate the transcription start site in each case.

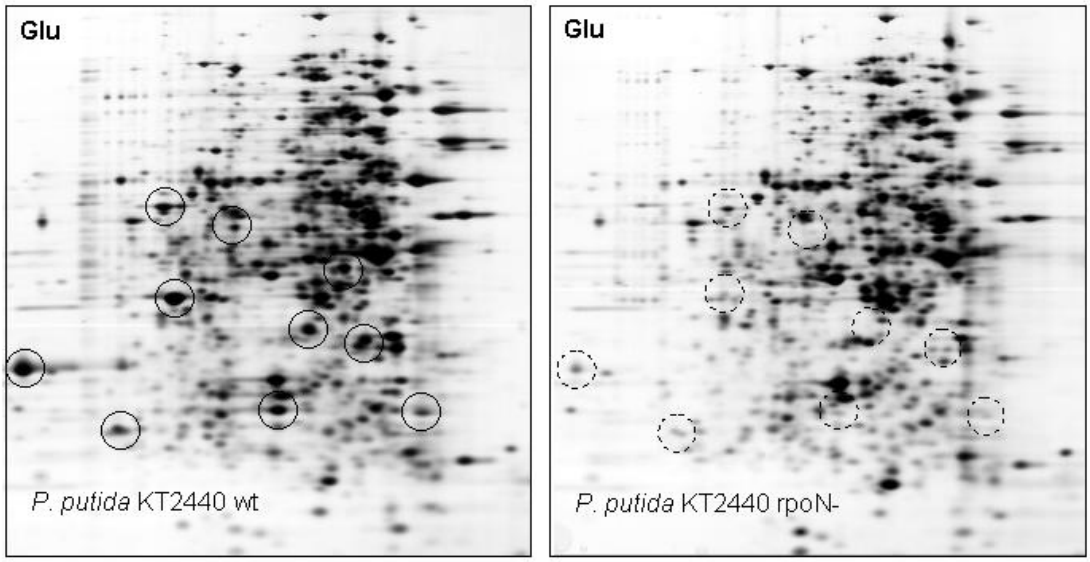


Figure 4.9: The effect of Glu on protein expression in *P. putida* KT2440 wild type (wt, left panel) and a mutant defective in the alternate sigma factor σ^{54} (rpoN-, right panel). Spots upregulated by Glu in the wild type are marked by solid circles. Corresponding spots in the mutant strain are highlighted by dashed circles.

At present, this has only been performed for the *ansB* gene of *P. putida* KT2440. By the primer extension technique the transcription start site (TS) of this gene has been identified. It is located 105 bp upstream of the translation start (TL). As expected from the results described above, a σ^{54} binding motif (G**GG**CTGCTACACA**GC**T) is present at the correct distance (i.e. -12/-24) with respect to the transcription start. In addition, a typical ribosome binding site (RBS) sequence was found 8 bp upstream of the translation start site (Fig. 4.10).

Figure 4.10: Transcription start site and σ^{54} binding motif of the *P. putida* KT2440 *ansB* gene. TS, transcription start site; RBS, ribosome binding site; TL, translation start site.

4.8 Analysis of Glu-induced gene expression by RT-PCR

To ascertain whether the effects of Glu observed on the protein level are indeed taking place on the level of transcription, a semi-quantitative reverse transcription-PCR (RT-PCR) was performed to examine the levels of mRNA for several proteins differentially expressed in the cells grown on M9$^-$-medium containing NH_4^+ plus glucose and 10 mM Glu (as described in section 3.11.). RNA isolation and RT-PCR for the genes listed in section 2.4.1 was carried out on the same day. The RNA was transcribed into cDNA by reverse transcriptase and the resulting cDNA was amplified by PCR using PfuTurbo DNA polymerase. The primers used (see section 2.4.1) were derived from the KT2440 genome. Although the procedure used here did not quantify proteins by using internal standards, the observed amounts of RT-PCR amplificates in the absence and presence of Glu (see Fig. 4. 11) were consistent with the proteomics data described above.

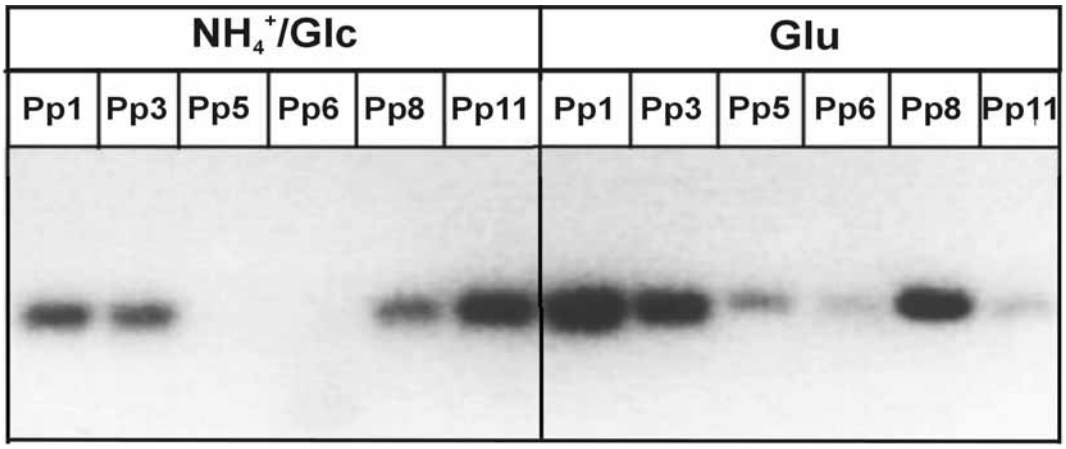


Figure 4.11: RT-PCR analysis of gene expression in *P. putida* KT2440. Pp1, transcription termination factor Rho; Pp3, PGA; Pp5, ABC transporter ATP-binding protein; Pp6, aspartase; Pp8, outer membrane porin D; Pp11, fumarase.

The Glu-responsive proteins previously identified in 2D gels (Table 4.2) seemed to be up-regulated on the transcriptional level as well. The RT-PCR products corresponding to the ABC-transporter ATP-binding protein (Pp5) and aspartase (Pp6) were not seen during growth on NH_4^+/Glc but readily detected during growth on Glu. The mRNAs for PGA (Pp3), the outer membrane porin D (Pp8), and the Rho termination factor (Pp1) were also detected in the absence of Glu but greatly increased when the amino acid was present. The formation of fumarase mRNA (Pp11), on the other hand, was strongly repressed by Glu. However, as only one pair of primers per gene was used and also did not include a Glu-independent control gene in the RT-PCR experiments, the results shown in Fig. 4.11 are still qualitative rather than quantitative.

4.9 Transposon mutagenesis to identify factors affecting PGA expression

Several different approaches are available to identify bacterial genes associated with a certain function or phenotype. The most common one is complementation: A genomic library is prepared from the species of interest using a vector that can be transferred to other bacteria. The gene(s) of interest is(are) then identified by mobilizing the library into a recipient strain which is defective in the respective function with selection for donor clones that complement the defect.

An alternative approach is to mutagenize the organism with a transposon that randomly inserts itself into the host genome at a single or only a few sites, followed by selection for transconjugants that have lost the function of interest. This technique is called transposon mutagenesis. Genes functionally involved in this function or property can then be identified by locating the position of the transposon in the genome. Again, this can be achieved by several different strategies. One of these is termed "self-cloning". It uses a transposon that can be replicated in a cloning host without the need to join it to a cloning vector. The transposon together with a neighbouring segment of the disrupted gene is cut out from the genome using an appropriate restriction enzyme and then transformed into and cloned in a suitable host such as *E. coli*. The disrupted gene is then identified by sequencing using a primer complementary to the terminal sequence of the transposon.

In the present work, we looked for genes that are necessary for the Glu-dependent expression of PGA in *P. putida* KT2440. PGA was selected because its activity can easily be detected with small amounts of cells such as individual colonies. Our hope was to find regulatory proteins, transcription factors or other functional proteins involved in the regulation of PGA expression. The transposon employed was the "self-cloning" transposon vector Tn5-pOT182 (Merriman and Lamont, 1993). Tn5-pOT182 contains a promoterless *lacZ* reporter gene which can be used to monitor transcription of the gene into which the transposon has been inserted. pOT182 was first transformed into *E.coli* strain S17 (Simon *et al*., 1983) which contains a chromosomally-integrated derivative of RP4, which is capable of causing conjugative transfer of mob-containing plasmids such as pOT182 into a wide range of Gram-negative bacteria (Simon *et al*., 1983). pOT182 was introduced into KT2440 from strain S17 by conjugation as described in section 3.17. Transconjugants resistant against tetracycline (Tc^R) and carbenicillin (Cb^R) were selected on plates containing $M9^-$-medium supplemented with 10 mM Glu; 22 mM glucose and

19 mM NH_4^+. Approximately 1,500 Tc^RCb^R transconjugants were obtained. Of these clones, about 400 were individually assayed for PGA activity as described in section 3.24.1 in the presence of Glu as the inducer. A total of 50 transconjugants were identified that exhibited low or negligible PGA activity in Glu-containing medium as compared to the wild-type.

Time →	0 h		2 h		4 h		6 h		24 h	
	N/Glc	Glu	N/Glc	Glu	N/Glc	Glu	N/Glc	Glu	N/Glc	Glu
Tn-SM3	0	0	0	0	0	0.2	3	0.2	13	60
Tn-SM6	0	0	0	0	0	2	2	3	13	16
Tn-SM9	0	0	0	0	0	0.5	4	2	4	15
Tn-SM29	0	0	0	0	0	0	0	0	0	0
Tn-SM31	0	0	0	0	0	0	0	0	0	12
Tn-SM33	0	0	0	0	0	0	0	0	0	0
WT	0	0	13	23	25	72	32	88	51	18

Table 4.5: PGA activities (mU/ml) of some representative transposon mutants at different times after transfer to $M9^-$-medium supplemented with 22 mM glucose plus 19 mM NH_4Cl (N/Glc) or 10 mM Glu (Glu). Before the experiment, the cells were pre-grown overnight in $M9^-$- medium containing NH_4^+/glucose. Tn-SM3-Tn-SM33: transconjugants obtained by Tn5-pOT182 mutagenesis; WT: wild type.

When using transposon mutagenesis, one has to make sure that the clones obtained are, in fact, derived from the recipient strain (here *P. putida* KT2440) rather than from the donor (here *E. coli* S-17). In our case this was straightforward, as all of the isolated clones produced a strong green fluorescence when grown in liquid media – a property typical of fluorescent pseudomonads such as *P. putida* but not seen with *E. coli*. About 50 of these transconjugants (Tn-SM1-Tn-SM50) were randomly selected and assayed in detail for their growth properties and the time course of PGA expression. Most of these clones exhibited very little PGA activity for the initial 6 h but an activity comparable to wild-type cells after 15-24 h, i.e. PGA expression was delayed but not abolished. The

development of PGA activity (mU/ml) of some representative Tn mutants with time during growth on Glu and NH_4^+/ glucose is shown in table 4.5.

The main goal of this study was to identify mutants rendered defective in Glu-induced PGA expression by random transposon mutagenesis. Transconjugants such as Tn-SM6 or Tn-SM9 showed delayed PGA expression were, therefore, not taken into account for further studies.

4.10 Self-cloning and sequence analysis

As mentioned above, Tn5-OT182 after digesting with an appropriate restriction enzyme, allows replication of flanking DNA sequence in *E. coli* without the need to join to a cloning vector. DNA sequences flanking the transposon in 11 transconjugants were isolated by self cloning (Merriman and Lamont, 1993). To verify insertion of the transposon, chromosomal DNA from the transconjugants was digested with *Xho*I. The resulting fragments were ligated by using T4 DNA ligase and then transformed into *E. coli* DH5α. Tc^r clones were selected. Primers specific to the right and left end of Tn5-OT182 were used to perform sequencing on each of the plasmids. The resulting sequences (about 300 bp in length) were checked against the database using an alignment search tool BLASTN.

None of the transconjugants examined in detail contained the transposon in the PGA-encoding *ansB* gene or in a gene with sequence homology to bacterial regulator proteins or transcription factors. Surprisingly, in 7 of the 11 sequenced mutants the transposon had inserted into the *gltB* gene (PP5067 in *P. putida* KT2440) which encodes the large subunit of glutamate synthase (GOGAT). In three transconjugants (in the following referred to as Tn-SM2, Tn-SM15, and Tn-SM30) the site of insertion of the transposon within *gltB* was exactly the same, being located 135 bp away from the translation start site. Fig. 4.12 shows a partial alignment of one of these clones (Tn-SM2) with the *P. putida* KT2440 *gltB* gene. A schematic map of the corresponding region of the *P. putida* KT2440 genome is depicted in Fig. 4.13. In one transconjugant, the transposon had interrupted a gene that, by sequence similarity, encodes a DNA helicase. In this mutant the transposon had integrated 53 bp away from the translation start site of the gene. In another case the transposon was localized in a gene that probably encodes a carbamoyltransferase of unknown function. These latter strains have not been further characterized so far.

The unexpected finding that the inactivation of *gltB* led to the loss of PGA activity raised the question whether the observed phenotype might be, in fact, not due to *gltB* disruption but a consequence of insertion of the transposon into additional genes. Therefore, Southern blot experiments were performed using fragments obtained by digestion with *Xho*I and

```
         101                                                    150
Tn-SM2   [.....Tn5-pOT182 vector sequence.....]C GGCGGCATCAATGC
  gltB   CTGCCATGCA  GGCGCTGACA TGCATGACCC ACCGC GGCGGCATCAATGC
                                                G  G  I  N  A

         151                                                    200
Tn-SM2   CGACGGCAAG ACCGGTGACG GTTGCGGTCT GCTCATGCAG AAGCCCGATC
  gltB   CGACGGCAAG ACCGGTGACG GTTGCGGTCT GCTCATGCAG AAGCCCGATC
          D  G  K   T  G  D   G  C  G  L   L  M  Q   K  P  D

         201                                                    250
Tn-SM2   AATTCCTGNG TGCCATGGCC CAGGAGCACT TCGCTGTCGA GCTGCCCAAG
  gltB   AATTCCTGCG TGCCATGGCC CAGGAGCACT TCGCTGTCGA GCTGCCCAAG
         Q  F  L  R   A  M  A   Q  E  H   F  A  V  E   L  P  K

         251                                                    300
Tn-SM2   CAGTACGCCG TCGGCATGGT GTTCTTCAAC CAGGACCCGG TCAAAGCCGA
  gltB   CAGTACGCCG TCGGCATGGT GTTCTTCAAC CAGGACCCGG TCAAAGCCGA
          Q  Y  A    V G  M  V   F  F  N   Q  D  P    V K  A E

         301                                                    350
Tn-SM2   AGCTGCCCGC GCCAACATGG ACCGCGAGAT CCTCGCCGCT GGCCAGA···
  gltB   AGCTGCCCGC GCCAACATGG ACCGCGAGAT CCTCGCCGCT GGCCTGA···
         A  A  R   A  N  M   D  R  E  I   L  A  A   G  L  K
```

Figure 4.12: Partial alignment of the sequence adjacent to the transposon in transconjugant Tn-SM2 and the *P. putida* KT2440 *gltB* gene. Nucleotides common to both the sequences are highlighted by bold face. The corresponding amino acid sequence of *gltB* is indicated below.

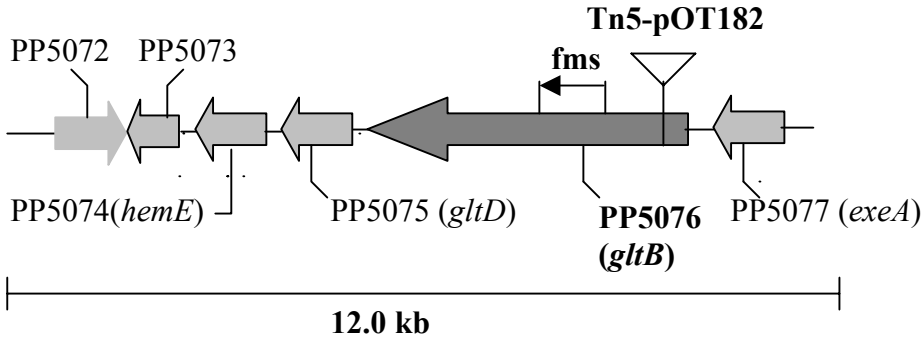


Figure 4.13: Genetic organization of the *gltB/gltD* region of *P. putida* KT2440. *gltB* (PP5067, 4446 bp) and *gltD* (5075, 1416 bp) encode the subunits of glutamate synthase. Tn5-pOT182: site of insertion of the transposon in transconjugants Tn-SM2, Tn-SM15, and Tn-SM30 (see text); fms: 834 bp region used to construct a defined *gltB*- disruption mutant by a frame shift mutation (see section 3.21.2)

*Eco*RI, respectively, and labelled pOT182 as the probe. With all three transconjugants (Tn-SM2, Tn-SM15, and Tn-SM30) the gels showed only one band after cutting with either one of the enzymes, indicating that each harboured a single copy of the transposon in an unique position (Fig. 4.14).

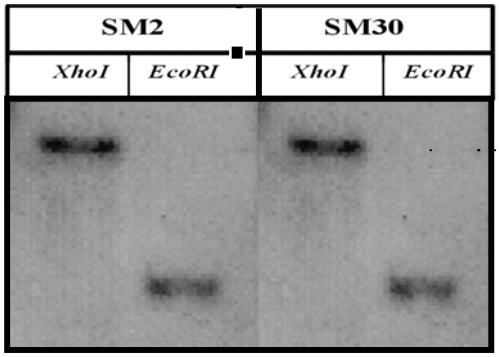


Figure 4.14: Selectivity of Tn5-pOT182 insertion. Genomic DNA of transconjugants Tn-SM2 and Tn-SM30 was digested with *XhoI* and *EcoRI* and the fragments hybridized with a digoxigenin-labelled pOT182 probe as described in section 3.20

4.11 Characterization of *gltB*-negative transposon mutants

As expected, the three Tn mutants did not exhibit detectable glutamate synthase (GOGAT) activity (data not shown). Next, we checked the ability of strains Tn-SM2, Tn-SM15 and Tn-SM30 to utilize various sources of carbon and nitrogen including NH_4^+/glucose, amino acids (Asn, Asp, Gln, Glu) alone and amino acids combined with NH_4^+ and glucose. Both wild type cells and mutants grew well in $M9^-$-medium supplemented with glucose and ammonia. By contrast, the ability of the Tn mutants to utilize amino acids as sole source of carbon and nitrogen was severely impaired. Tn-SM2 and Tn-SM30 failed to grow in all above mentioned amino acids. On the other hand, Tn-SM15 exhibited slow growth in Asn and Gln starting after 15 h. The effect of above four amino acids on PGA activity was also measured. With wild-type *P. putida* KT2440 growing on Asp, Glu or the corresponding amides, PGA expression was fully induced within 6-15 h, depending on the type of amino acid used, while the Tn mutants did not exhibit PGA activity at all.

	Asparagine			Aspartate		
	Asn	Asn +Glc	Asn+NH_4^+	Asp	Asp +Glc	Asp+NH_4^+
Wild type	++	++++	+++	++	+++	+++
Tn-SM2	(+)	+++	-	-	(+)	-
Tn-SM15	+	+++	+	-	+	-
Tn-SM30	-	+++	-	-	+	-
gltB- mutant	-	+++	-	-	++	-

	Glutamine			Glutamate			
	Gln	Gln +Glc	Gln+NH_4^+	Glu	Glu +Glc	Glu+NH_4^+	NH_4^++Glc
Wild type	+++	+++	+++	+++	+++	+++	+++
Tn-SM2	-	++	-	-	+	-	+++
Tn-SM15	-	+++	-	-	+	-	+++
Tn-SM30	-	+++	-	-	+	-	+++
gltB- mutant	-	+++	-	-	++	-	++

Table 4.6: Utilization of various carbon and nitrogen sources by *P. putida* KT2440 and different mutant strains. Growth was assessed by measuring OD_{450} after 12 h in M9$^-$ medium supplemented with 10 mM amino acids, 22 mM glucose and 19 mM NH_4^+. -, $O.D._{450}$ < 0.05; (+),$O.D._{450}$ 0.05-0.1; +, $O.D._{450}$ 0.1-0.2; ++, $O.D._{450}$ 0.2-0.4, +++, 0.4-0.6; ++++, $O.D._{450}$ >0.6.

While the Tn mutants studied did not grow on amino acids alone, glucose in combination with amino acids supported rapid growth of the mutants. On the other hand, amino acids supplied together with NH_4^+ as a nitrogen source did not allow growth of the mutants. This indicates that it is the utilization of amino acids as carbon source that is abolished by disruption of the *gltB* gene.

4.12 Motility and Chemotaxis

In addition to growth and PGA activity, the Tn mutants were assayed for their motility phenotype and chemotaxis towards amino acids. Motility was checked on "swarming plates" which allow detection of swarming growth on a given medium. This assay was performed either on normal Petri dishes (cf. Fig. 4.15) or in microtiter plates (Fig. 4.16). The latter method allowed the screening of a large number of strains under identical conditions. The approach is illustrated by Figs. 4.15 and 4.16. *P. putida* KT2440 wild type (left) showed a high motility when grown on amino acids. By contrast an isogenic *rpoN*-mutant was non-motile (right). This effect was already described by Köhler *et al*. (1989).

Most of the mutants (including Tn-SM2, Tn-SM15 and Tn-SM30) were entirely non-motile (see Table 4.7); only a few showed an attenuated motility (Fig. 4.16). The *gltB* insertion mutants were also examined for their chemotactic response towards Asn, Asp, Gln, and Glu (10 mM each) as described in section 3.4. The chemotactic response was considered positive when the length of the flare of growth towards the test compound was distinct.

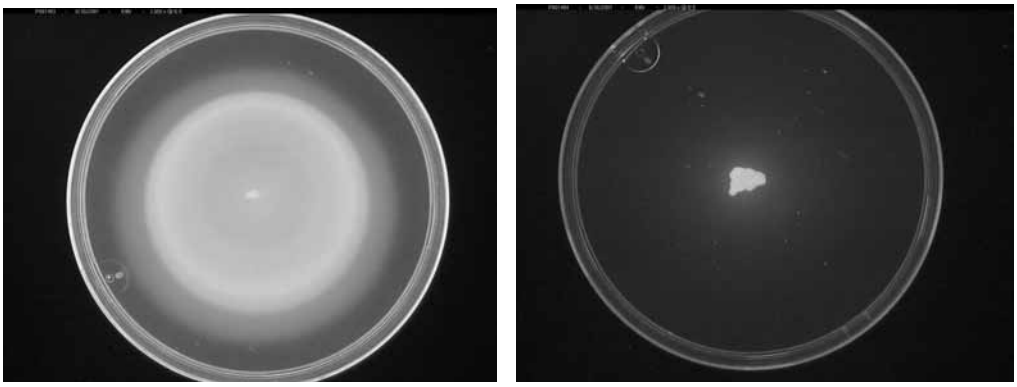


Figure 4.15: Motility of the a) *P. putida* KT2440 wild type (left panel) and b) *P. putida rpoN* mutant (right panel) in M9- medium supplemented with Asn.

Wild-type cells showed a strong chemotactic response towards the above mentioned amino acids. Tn-SM2 and Tn-SM15 failed to respond to any of the amino acids, while Tn-SM30 showed weak chemotactic response towards asparagine only (Table 4.7).

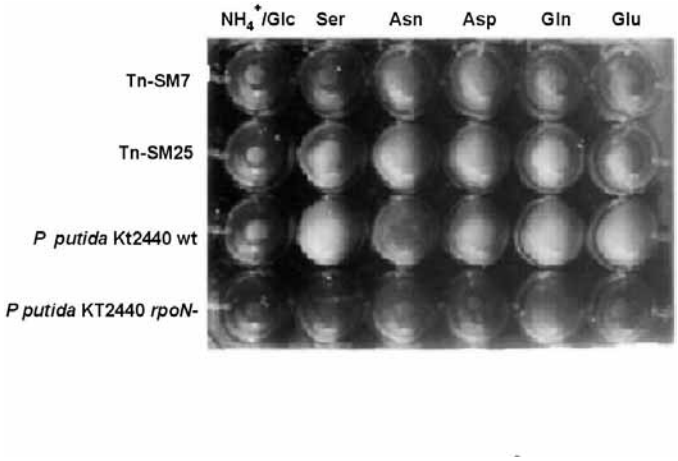


Figure 4.16: Swarming growth of two representative transposon mutants, *P. putida* KT2440 and *rpoN*-mutant in M9$^-$-medium containing 19 mM NH_4Cl plus 22 mM glucose and 10 mM each serine, Asn, Asp, Gln and Glu. Fresh colonies were spot inoculated in a 96-well microtiter plate containing a 0.3 % M9 agar supplemented with above amino acids and tetracycline. The plates were incubated at 30 ^{0}C for 1 to 3 days.

Mutants	Asparagine		Aspartate		Glutamine		Glutamate	
	M	C	M	C	M	C	M	C
Tn-SM2	-	-	-	-	-	-	-	-
Tn-SM15	-	-	-	-	-	-	-	-
Tn-SM30	-	(+)	-	-	-	-	-	-
rpoN	-	-	-	-	-	-	-	-
Wild-type	+	+	+	+	+	+	+	+

Table 4.7: Motility (M) and Chemotaxis (C) of transposon mutants in 10 mM amino acids. -, negative; +, positive; (+), weak. The assays were performed as described in section 3.4.

4.13 Properties of a targeted *gltB*- disruption mutant

Although the results of Southern blot experiments shown in Fig. 4.14 indicated that Tn5-pOT182 had inserted into the KT2440 genome at a single site, they could not completely exclude the possibility that the transposon had also interrupted a second gene. To ascertain that the phenotype observed with strains Tn-SM2, Tn-SM15 and Tn-SM30 was only due to the inactivation of *gltB* gene, a targeted *gltB*- disruption mutant was constructed by homologous recombination. As described in section 3.21.2, the mutant was constructed by i) amplifying a 834 bp fragment of the *gltB* gene from KT2440 genomic DNA and inactivating it by introduction of a frame shift mutation, ii) cloning the nonfunctional *gltB* gene into pK18, and iii) re-introducing the nonfunctional *gltB* gene into the *P. putida* KT2440 genome by homologous recombination. Integration of inactivated *gltB* gene into the KT2440 genome was verified by PCR using suitable primers (gltBP3For and gltBP4Rev, see section 3.21.2). The *gltB*- disruption mutant obtained in this way showed a behaviour very similar to that of transposon mutants Tn-SM2, Tn-SM15, and Tn-SM30. This is illustrated by Fig. 4.17, which compares the growth curves and PGA induction kinetics of wild type cells and the g*ltB*- mutant during growth on amino acids as the sole source of carbon and nitrogen. None of the amino acids tested supported rapid growth or led to PGA expression (the strong increase of OD_{450} after 12-15 h especially with Asn as the nutrient is not due to cell growth but due to the formation of the fluorescent dye which is typical of *P. putida*). As shown by Table 4.6, the behaviour of the targeted *gltB* disruption mutant on other combinations of carbon and nitrogen sources was also comparable to that of the three strains obtained by random mutagenesis, i. e. the *gltB* mutant was able to utilize amino acids only when supplied together with glucose.

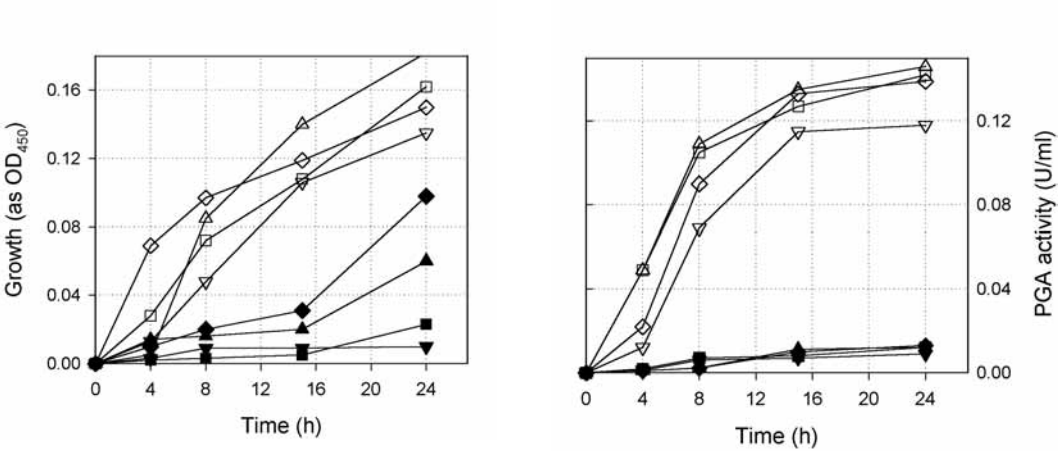


Figure 4.17: Growth and PGA activity of a *gltB* mutant (close symbols) and *P. putida* KT2440 (open symbols) in M9$^-$-medium supplemented with Asn (-◆-,-✧-), Asp (-▼-, -▽-), Gln (-■-, -□-), and Glu (-▲-, -Δ-) as the only source of carbon and nitrogen.

4.14 Survival of the *gltB*-mutant in conditions of nitrogen starvation

A study by Eberl *et al*. (2001) on a *P. putida* strain with a defective *gltB* gene showed that survival of such cells under conditions of nitrogen starvation was greatly impaired. In order to see whether this was also the case with our GOGAT-deficient mutants, we performed a similar survival experiment (Fig. 4.18).

In agreement with the data of Eberl *et al*., *P. putida* KT2440 remained fully viable for at least 24 days of nitrogen starvation. In contrast, the mutants were severely impaired in the ability to survive prolonged incubation in nitrogen-free medium. After 5-7 days of incubation in nitrogen-free medium, the cell counts dropped steeply, and after 24 days of nitrogen starvation no viable colonies of the mutants were found any more.

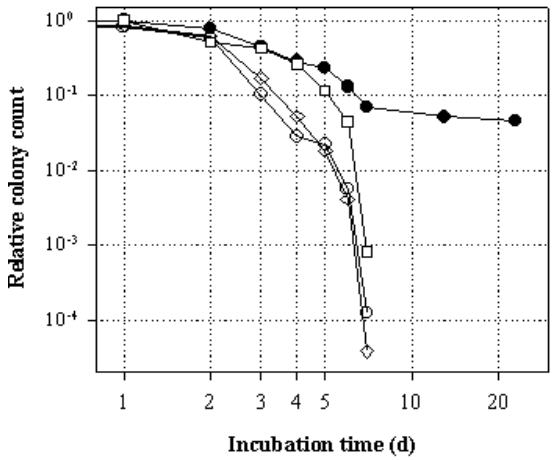


Figure 4.18: Starvation survival (viable cell count) of *gltB*- (-o-), Tn-SM2 (-✧-), Tn-SM30 (-□-) and *P. putida* KT2440 wild type (-●-) in nitrogen depleted media. The experiment was performed as descr- ibed in section 3.5.

4.15 Differential protein expression in *gltB*- Tn mutants

In order to check the effect of inactivation of *gltB* gene on the level of protein expression, 2D-PAGE experiments were performed with cell extracts prepared after transfer from NH_4^+/glucose to media containing Glu as sole source of C and N. Fig. 4.19 compares the protein expression patterns observed in this way with *P. putida* KT2440 wild type (left panel) and Tn-SM2 (right panel) after 6 h of growth on Glu. Major spots upregulated in the wild-type cells under this condition are marked by solid circles. Most of these proteins were not up-regulated in Tn-SM2 are indicated by the dashed circles in Fig. 4.19b. Similar results were obtained with Tn-SM15 and Tn-SM30 as well.

Figure 4.20 compares 2D-gels obtained with extracts of the targeted *gltB* disruption mutant on NH_4^+/glucose (left panel) and Glu (right panel). The expression patterns obtained are virtually identical, indicating that not only induction of PGA but also the inductive effect of Glu (cf. Fig. 4.7) on other proteins was abolished by the inactivation of *gltB* as well.

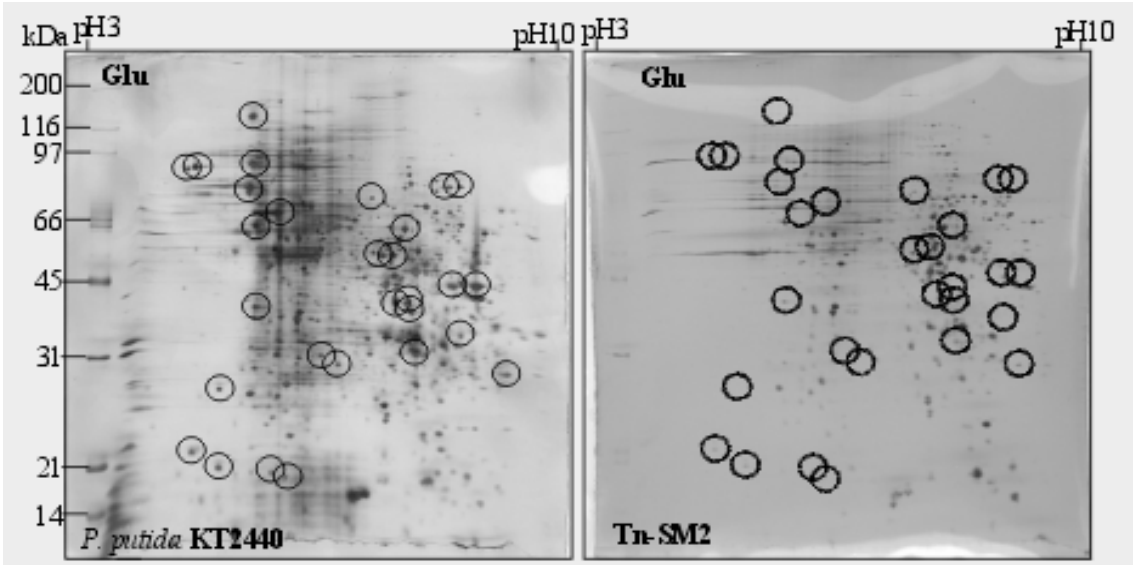


Figure 4.19: Two-dimensional electrophoresis gels (pH 3-10) of soluble proteins of *P. putida* KT2440 and transconjugant Tn-SM2 during growth on M9$^-$-medium containing 10 mM Glu as the sole source of C and N. Spots up-regulated by Glu in the wild type are marked by solid circles. The position of the corresponding spots in the mutant strain are highlighted by circles (right panel).

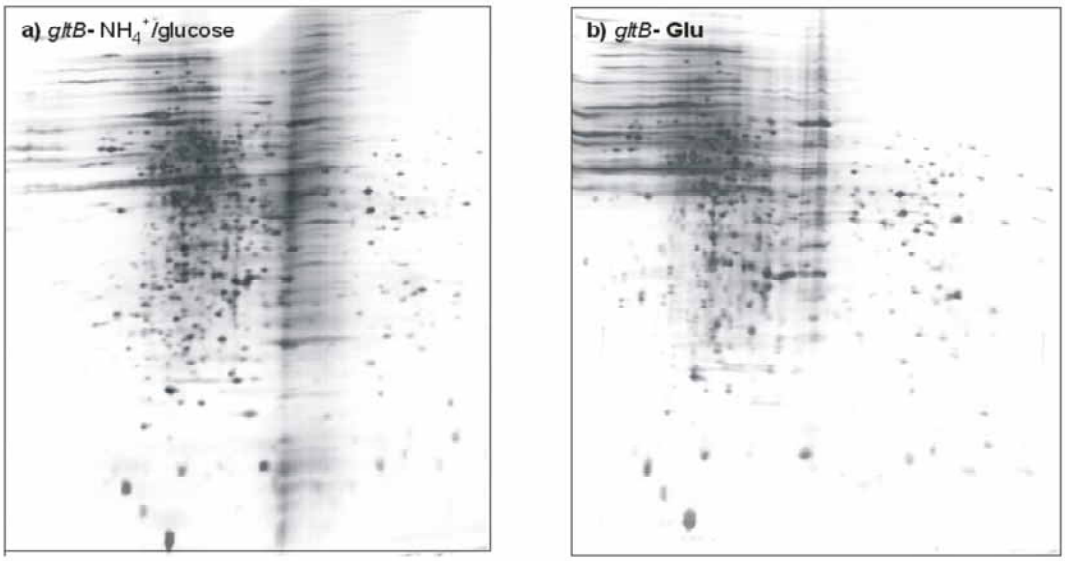


Figure 4.20: Two-dimensional electrophoresis of soluble proteins expressed by the *gltB*- mutant of *P. putida* KT2440 6 h after transfer from M9$^-$-medium supplemented with glucose/NH_4^+ to the same medium (left panel) or to M9$^-$-medium containing 10 mM glutamate (right panel).

4.16 Determination of the intracellular pools of Glu and Gln

As discussed in the introduction, glutamate synthase (GOGAT), one subunit of which is encoded by *gltB*, converts Gln and 2-oxoglutarate to two molecules of Glu. Thus, inactivation of *gltB* is likely to affect the intracellular concentrations of Gln and Glu. As no experimental data on this issue have been presented in the literature, we compared the intracellular levels of Glu and Gln in wild-type cells and *gltB*-mutant growing on different carbon and nitrogen sources. Cell extracts containing the amino acid fraction were prepared by heating the cell pellet in ethanol, and the concentrations of Glu and Gln determined by an enzymatic assay as described in section 3.29.

The levels of Glu and Gln in the wild type cells and the *gltB*-disruption mutant were monitored on NH_4^+/glucose for several days. In the *gltB*-mutant, both Gln and Glu accumulated to high concentrations (several μmol per mg of cellular protein) while no accumulation was seen in the wild type cells (Fig. 4.21). Similar results were obtained with cells kept on Glu and glucose as an additional carbon source (data not shown). In the absence of an external N source no detectable amounts of Glu or Gln were found.

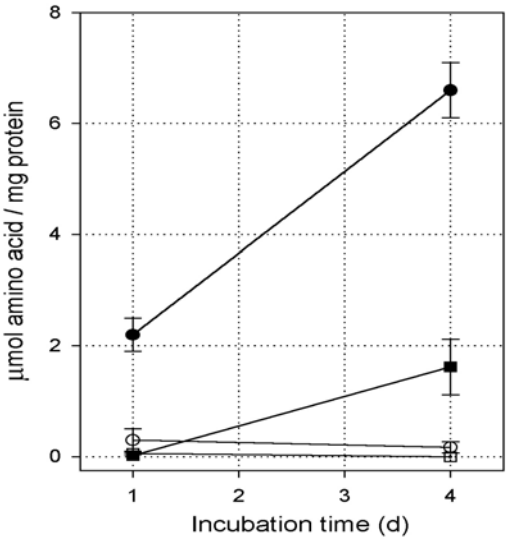


Figure 4.21: Intracellular concentrations of Glu (-o-, -●-) and Gln (-□- , -■-) (in µmol per mg of cellular protein) as a function of time after transfer to M9 medium containing 19 mM NH_4^+ and 22 mM glucose. The figure compares data for wild type *P. putida* KT2440 (open symbols) and a *gltB* disruption mutant (filled symbols).

4.17 Identification of a two-component system involved in the regulation of glutamate metabolism

In the proteomics experiments described in section 4.6 we found that during growth of *P. putida* KT2440 on Glu- among other proteins - two components of an ABC transporter are induced. They are encoded by the *P. putida* KT2440 genes PP1068 and PP1071 and correspond to the ATP-binding subunit of the transporter (PP1068) and the periplasmic binding protein associated with it (1071). Immediately adjacent to this group of genes are two further genes which, by sequence homology, encode a so-called two-component system (see section 1.5). Although the neighbourhood of these two systems could be only accidential, we speculated that the system encoded by PP1066/67 might have a function in amino acid metabolism. This speculation was supported by a comparison with the genomes of *P. aeruginosa* PAO1 and *P. fluorescens* SBW25 (see Fig. 4.22). On the basis of sequence similarity, PP1068-1071 correspond to the *P. aeruginosa* genes PA1339-1342. This probable operon in *P. aeruginosa* is followed (in upstream direction) by 4 additional genes that all are connected with glutamate and/or aspartate metabolism: PA1338 encodes a γ-glutamyltransferase, PA1337 (*ansB*) encodes PGA, and the subsequent genes PA1335/1336, which encode a two-component system, show a high sequence similarity to PP1066/1067.

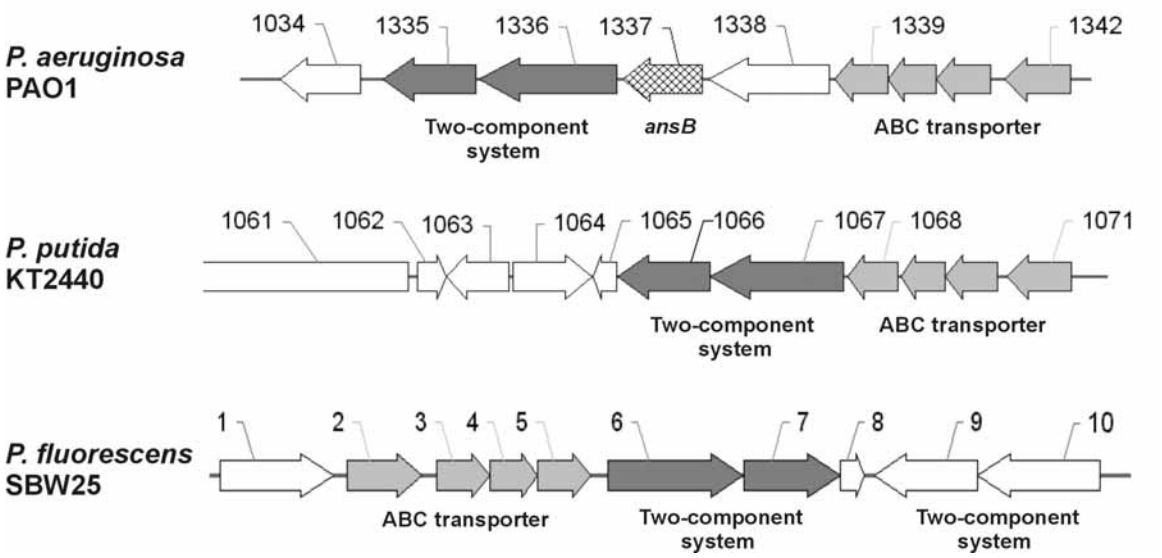


Figure 4.22: Comparison of selected regions in three *Pseudomonas* genomes (see text)

In the unfinished genome of *P. fluorescence* SBW25 (in progress at the Sanger Centre, U.K), the ABC transporter-encoding genes with the highest similarity to PP1068-1071 (genes 2-5) are directly followed by genes 6 and 7 which are very similar to PP1066/1067. This suggested that PP1066/1067 might also be functionally involved in the metabolism of acidic amino acids and their amides. We tested this hypothesis by inactivating PP1066 and PP1067 by the same method already used for the disruption of the *gltB* gene (see section 4.13) and examining the properties of the resulting mutants. As will be shown below, the results indicate that the two component system encoded by PP1066 and PP1067 is in fact involved in the control of the metabolism of the acidic amino acids. Therefore, we termed the system encoded by these two genes "aau" which stands for "**a**cidic **a**mino acid **u**tilization". As judged by sequence homology, the *aau* system involves a sensor kinase (aauS-PP1067) and a regulator protein (aauR-PP1066). In the annotated *P. putida* KT2440 genome (Nelson *et al*., 2002) the protein products of the system are annotated as "sensor histidine kinase" and "σ^{54}-dependent response regulator", respectively, of unknown function.

4.18 Characteristics of *aauS* and *aauR* disruption mutants

In order to characterize the role of the *aau* system in the regulation of the utilization of acidic amino acids (Asp, Glu) and their amides (Asn, Gln), mutants were generated by homologous recombination as described in section 3.21.3, in which *aau*S and *aauR* were disrupted. As usual, these mutants (*aauR*- and *aauS*-) were compared with the parental strain with respect to their growth properties and the induction of PGA and other Glu-responsive proteins.

As shown by Fig. 4.23, growth of *aauR*- on Asn and Asp was normal, while growth on Gln as sole source of carbon and nitrogen was slightly and growth on Glu was strongly impaired. The levels of PGA activity were very low in the mutant. Only growth on Asn led to significant activities after 10-12 h. These findings indicate that a functional *aau*R is indeed required for the expression of genes involved in the utilization of Gln and Glu. Table 4.8 shows a more detailed summary of the growth properties and PGA activities of the *aauR*- and *aauS*- disruption mutants on various combinations of carbon and nitrogen sources.

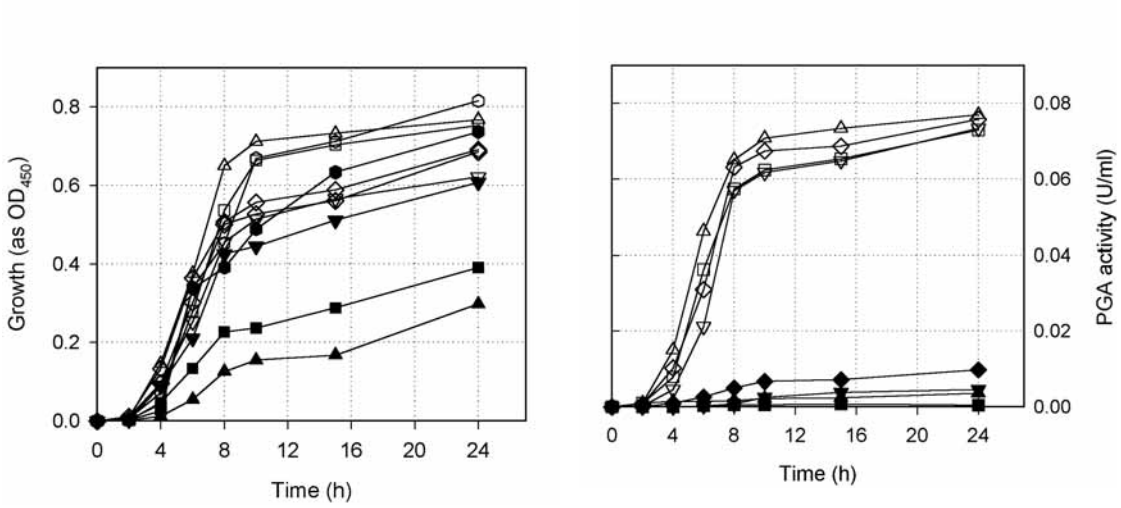


Figure 4.23: Growth and PGA induction of the *aauR*- mutant (close symbols) and *P. putida* KT2440 (open symbols) on glucose/NH_4^+ (-●-, -o-) or M9 containing Asn (-◆-,-✧-), Asp (-▼-, -∇-), Gln (-■-, -□-), or Glu (-▲-, -Δ-), each at 10 mM.

Although, there were some differences between both strains, the *aau*S- mutant showed a similar growth behaviour and PGA induction pattern to the *aau*R- mutant. However, it exhibited longer doubling time during growth on all four amino acids when supplied as sole source of C and N. In comparison to the other three amino acids, growth was most

strongly retarded in Glu-containing medium. This was not only the case when Glu was combined with succinate, but also when it was supplied as the sole source of C and N.

	aauS-				***aauR-***			
Additional C- source →	**none**		**Glucose**	**Succinate**	**none**		**Glucose**	**Succinate**
C/N-source ↓	Growth (% of wt)	PGA activity (% of wt)	Growth (% of wt)	Growth (% of wt)	Growth (% of wt)	PGA activity % of wt	Growth % of wt	Growth % of wt
Asn	109	12	58	96	95	10	99	96
Asp	30	<1	58	66	90	5	70	66
Gln	16	<1	49	70	41	1	80	70
Glu	7	<1	51	44	23	2	40	44
NH_4^+	n.d.	n.d.	91	98	n.d.	n.d.	100	81

Table 4.8: Relative percentage growth and PGA activities of *aauR*- and *aauS*- mutants after 12 h of growth in different combinations of carbon and nitrogen sources. Cells were grown overnight in NH_4^+/glucose minimal medium, washed and transferred to $M9^-$-media containing Asn, Asp, Gln, Glu, (10 mM each), 10 mM amino acids and 22 mM glucose, or 10 mM amino acids plus 10 mM succinate. Growth (as OD_{450}) and PGA activity are given as % of the respective values obtained with wild type cells under the same conditions.

In order to check whether *aau*S- and *aau*R- mutants are able to utilize amino acids as nitrogen source, $M9^-$-media with the above four amino acids was supplemented with glucose and succinate as additional carbon source. Succinate was chosen because in *Rhizobium leguminosarum* and *Sinorhizobium meliloti* succinate is a good inducer of *dct* system which is responsible for the transport of dicarboxylic acid (Ledebur *et al.,* 1990; Jording *et al*., 1992). Both *aauR*- and *aauS*- showed rapid growth on succinate-containing media (see table 4.8), suggesting that the *aau* system is different from *dct*, i.e. it is not required for the utilization of succinate. Compared to wild-type, *aau*R- mutant showed slightly slower growth on succinate and Glu. A somewhat different situation was observed with the *aauS*- mutant. Here, in addition to media containing Glu and succinate, growth was also retarded in Asp and succinate-containing medium.

4.19 Differential protein expression in wild type and the *aauR*- mutant

In order to examine the effect of inactivation of a response regulator *aauR* on protein expression, crude protein extracts from *P. putida* KT2440 wild type cells and the *aau*R-mutant were analyzed by 2-D gel electrophoresis 6 h after transfer from NH_4^+/glucose to $M9^-$/glutamate (Fig. 4.24)**.** In the *aauR*- mutant at least 23 protein spots (R1-R23) were repressed and 4 protein spots (I1-I4) were up-regulated. Most of the differentially expressed proteins are located in the neutral-to-acidic pH range.

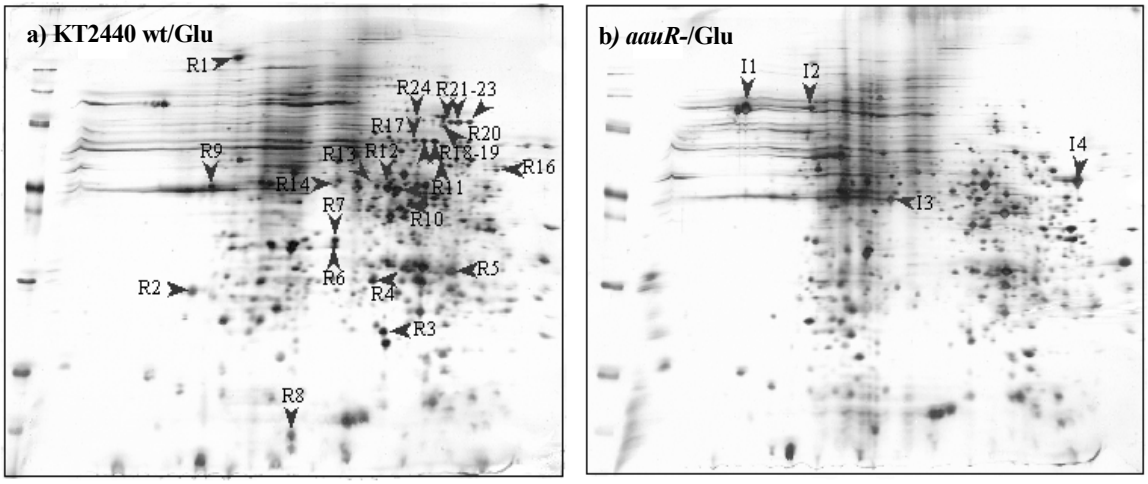


Figure 4.24: Two-dimensional electrophoresis of differentially expressed proteins in *P. putida aauR*- mutant during growth on 10 mM Glu. Protein spots a) repressed and b) induced in mutant are represented as RI-R24 and I1-I4 , respectively.

Proteins less strongly expressed in the *aauR*- mutant during growth on Glu

Protein spot	Protein name	Locus
R1	General amino acid ABC transporter, Periplasmic binding protein	PP1297
R3	Superoxide dismutase (Fe)	PP0915
R4	2-dehydro-3-deoxy-phosphogluconate aldolase	PP1024
R5	6-phosphogluconolactonase	PP1023
R8	50S ribosomal protein L14	PP0464
R11	Transcriptional regulator, GntR family	PP0620
R12	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein	?
R14	Translation elongation factor Tu	PP0440
R16	Inosine-5-monophosphate dehydrogenase	PP1031
R17	2-oxoglutarate dehydrogenase, lipoamide dehydrogenase component	PP4187
R19	Dihydrolipoamide dehydrogenase	PP5366
R21-23	Quinoprotein ethanol dehydrogenase	PP2674

Proteins more strongly expressed in the *aauR*- mutant during growth on Glu

Protein spot	Protein	Locus
I1	Flagellin C	PP4378
I2	Dna K protein	PP4727
I4	Acetyl-CoA carboxylase, biotin carboxylase	PP5347

Table 4.9: List of the identified differentially expressed proteins in the aauR- mutant during growth in glutamate containing medium.

For the identification of the differentially expressed protein spots, a total of 28 spots (R1–R24 and I1-I4, see Fig. 4.24) were selected, cut from the Coomassie-stained gels and pooled. The cut gel pieces were then digested with trypsin and the tryptic peptides were analyzed by MALDI-TOF mass spectroscopy as described in section 3.28.1. For identification, several modification parameters such as modification of cysteine by carbamidomethylation and oxidation of methionine were considered. The searching parameters used were a peptide mass tolerance of 0.1 Da and missed cleavages not allowed. The MS data obtained was directly used as input for the Mascot™ program. From the proteins R1-R24, 14 could be identified and matched to entries of the *P. putida* KT2440 genome with high scores. Proteins R21-R23 were identified as isoforms of a single protein, quinoprotein ethanol dehydrogenase. The results of protein identification are summarized in Tables 4.9. Surprisingly, a comparison of these tables with table 4.2 does not show any overlap, none of the proteins appears in both tables.

4.20 Overexpression of the AauR protein

For expression of the AauR protein, the pGEX expression system was used. In this system expression is under the control of the *tac* promoter, which is induced by the lactose analogue isopropyl β-D thiogalactoside (IPTG).

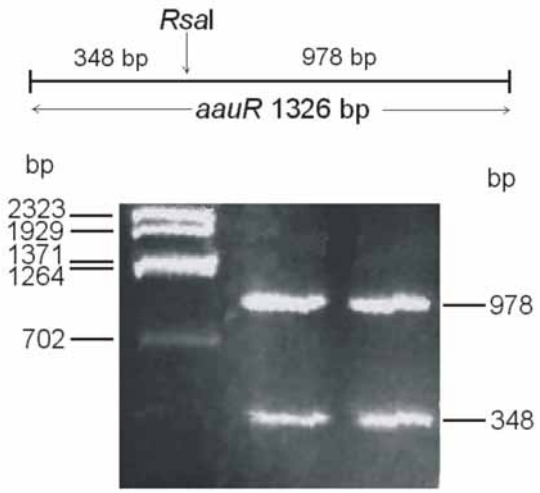


Figure 4.25: Confirmation of the aauR cloning into the pGEX vector by restriction digestion analysis with a *RsaI* enzyme.

pGEX also contain an internal *lacIq* gene. The *lacIq* gene product is a repressor protein that binds to the operator region of the *tac* promoter, preventing expression until induction by IPTG, thus maintaining tight control over expression of the insert. Cloning of *aauR* gene into the compatible sites of pGEX-6P-3 (see section 3.22.2) was confirmed by cutting the vector with *EcoRI* and *BamHI* and digesting the released insert (1326 bp) with *RsaI*. As shown by Fig. 4.25, the expected fragments (978 and 348 bp) were found. Using this construct, AauR was overexpressed in a protease-deficient *E. coli* BL21(DE3) IL strain as a fusion protein with GST.

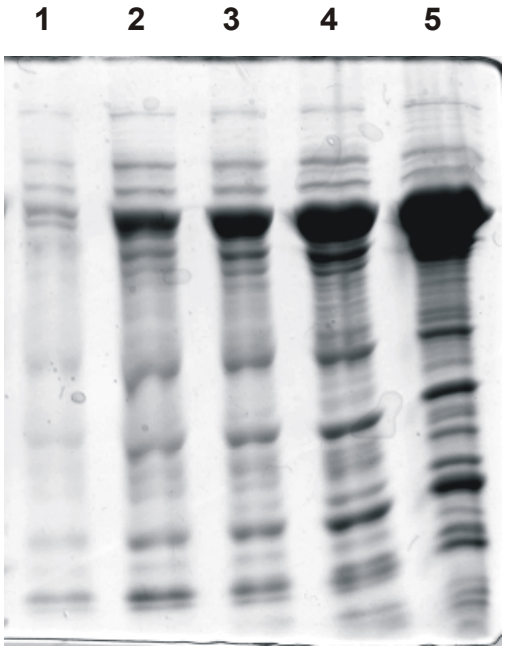


Figure 4.26: Induction kinetics of the AauR protein. The figure shows SDS-PAGE of crude cell extracts before induction with IPTG (lane 1) and 0.5, 1, 2 and 3 h after induction (lanes 2-5).

In order to obtain optimum yields of the protein, we examined the dependence of AauR expression on incubation time after IPTG induction. For these studies, a 50 ml pre-culture was grown overnight and added to 500 ml of LB medium supplemented with chloram- phenicol and ampicillin. When OD_{600} had reached 0.7, IPTG was added to a final concentration of 1 mM. Samples were removed at suitable time intervals up to 24 h after induction with IPTG. As no significant increase in the yield of protein was observed at times longer than 3 h, cells were harvested after this time (see Fig. 4.26).

4.21 Purification of the AauR protein

Purification of the *P. putida* KT2440 AauR protein was accomplished in a three-step procedure starting with a sonication of cells to generate a crude extract, followed by affinity chromatography on glutathione sepharose 4B and finally cleavage of the GST fusion with PreScission protease. Fig.4.27 shows material from one such purification separated on 12% SDS-polyacrylamide gel before and after cutting with PreScission protease. As shown by the figure, about 80 % purity was achieved by affinity chromatography. Additional steps like gel filtration will be necessary to obtain the AauR protein in a homogeneous form. Extracts from 500 ml cultures afforded 50-60 mg of protein.

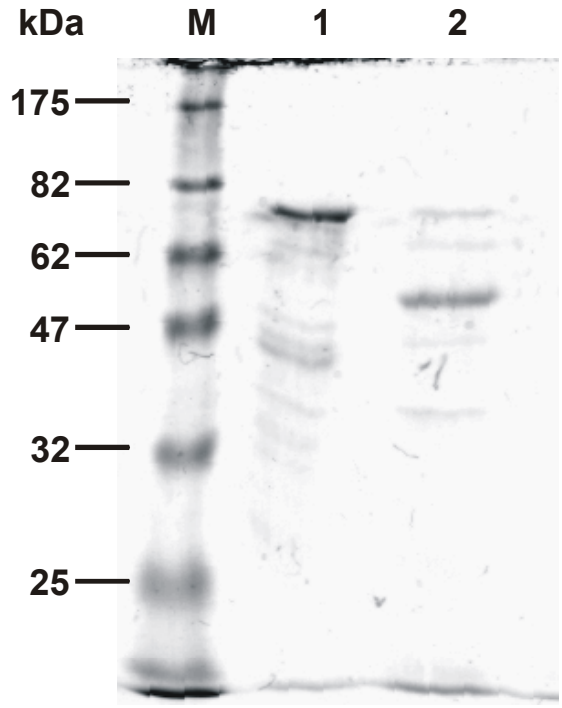


Figure 4.27: Purification of the overexpressed AauR-GST fusion protein. M, Marker; Lane1, AauR-GST fusion protein (uncut); Lane 2, AauR protein (after cutting with PreScission protease).

By analytical SDS-PAGE, the molecular mass of the GST-AauR fusion protein was estimated to be 78.0 kDa and that of AauR protein as 48.7 kDa (see Fig. 4.27). These values are in good agreement with those predicted from the respective amino acid sequences.

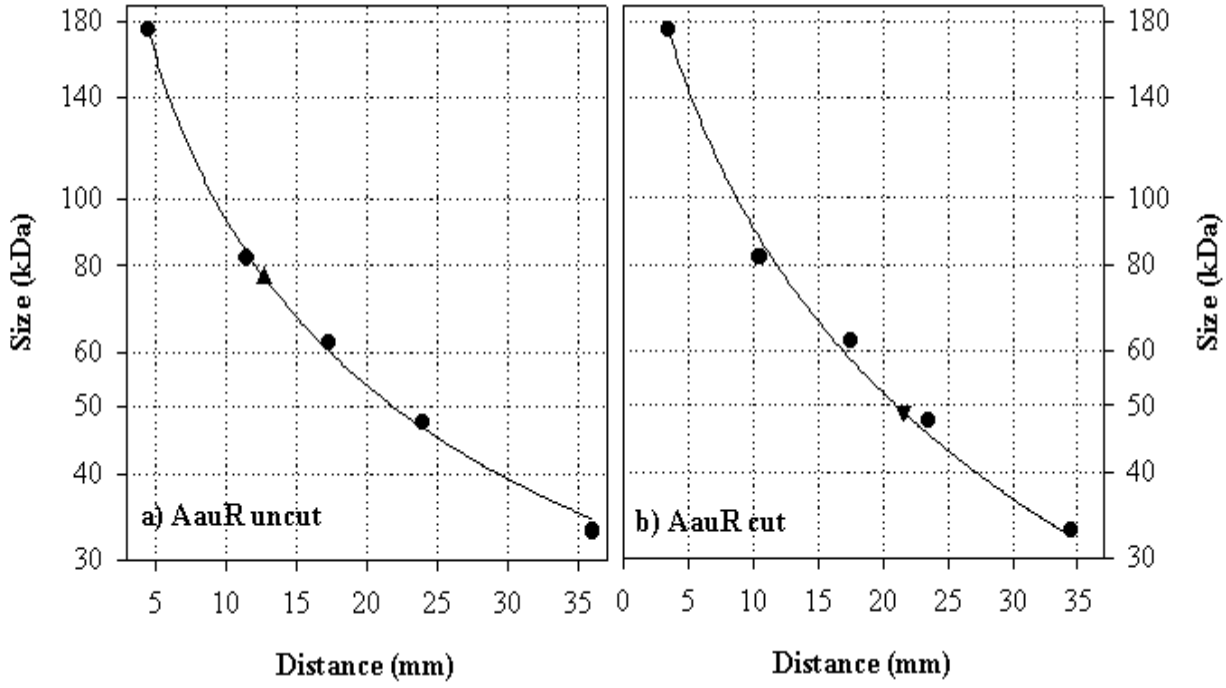


Figure 4.28: AauR purified protein a) before Protease PreScission treatment (uncut) , b) after protease treatment (cut). -●-, standard protein markers; -▼-, AauR protein.

So far, no functional characterization of the purified AauR protein has been carried out. However, sufficient amounts of purified protein are now available for further functional characterization such as DNA binding studies and an analysis of the mode of phosphorylation.

5. Discussion

Bacteria have an unique ability to occupy very hostile environments and to adapt to rapid changes of the environmental conditions. In this respect, they are much more adaptable than mammalian cells which enjoy rather constant and well-regulated conditions of their environment. Therefore, the mechanisms that regulate bacterial gene expression in response to environmental signals are of major interest to basic research. Such studies also may find practical applications in novel methods for fighting bacterial infections or in the use of bacteria to improve the quality of soils by removing toxic compounds or enhance plant growth. Today, the study of bacterial gene regulation is greatly facilitated by the availability of a large number of complete genomes. Together with increasingly powerful methods for the analysis of differential protein expression (the so-called proteome research) and the sensitivity of analytical methods in the field of gene expression, it is now possible to obtain a wealth of detailed information on the responses of bacterial cells to environmental factors.

5.1 Amino acid utilization by *P. putida*

In the present work, we studied the utilization of acidic amino acids and their amides by *P. putida* strain KT2440. As already discussed in the introduction, this organism, like other *Pseudomonas* strains, has the ability to grow on a very broad range of carbon sources and is also a plant growth-promoting organism (PGPR) with possible applications in agriculture. Another pseudomonad that has attracted much attention due to medical reasons is the human pathogenic species *P. aeruginosa*. Comparative sequence analysis revealed that the genomes of *P. aeruginosa* PAO1 and *P. putida* KT2440 have an about 85% sequence similarity. The pseudomonad genomes also are among the largest of all bacterial genomes so far, and also contain the largest proportion of regulatory genes (Nelson *et al*., 2002, Shingler, 2003).

The main goal of this study was to identify factors in *P. putida* KT2440 that regulate the uptake and metabolism of acidic amino acids and their amides. This line of investigation was based on previous data from our laboratory which showed that expression of periplasmic glutaminase/asparaginase (PGA) of several pseudomonads is stringently regulated by induction and repression (Hüser, 1999; Klöppner, 1999). The activation of systems for the utilization of amino acids is one of the most important mechanisms for the adaptation of bacteria to nitrogen starvation, i.e. a lack of a preferred nitrogen source like NH_4^+. As already discussed in the introduction, this reaction which is known as 'nitrogen response' was studied

in much detail in *E. coli* and other enterobacteria while little information on the Ntr system is available for the pseudomonads.

In the present work, we first showed that the general patterns of amino acid utilization and its regulation in *P. putida* KT2440 are similar to those of the strains previously studied by Hüser and Klöppner (see Table 4.1; Figs. 4.2 and 4.3). Asp and Glu as well as their amides (Asn, Gln) supported rapid growth of strain KT2440. As already noted, this differs from the situation in *E. coli* where NH_4^+ is a much better nitrogen source than amino acids (Reitzer, 1996).

5. 2 Regulation of PGA activity

The acidic amino acids and their amides not only supported rapid growth of *P. putida* KT2440, they were also the only amino acids capable of inducing PGA. A somewhat different situation was observed in *P. fluorescens* ATCC 13525 (Klöppner, 1999), in which the kinetics of induction indicated that aspartate and glutamate rather than asparagine and glutamine are the actual inducers. This paradoxical situation is difficult to explain. However, one may expect that in most situations in which pseudomonads are naturally exposed to asparagine and glutamine, the dicarboxylates aspartate and glutamate should be available as well. It could also be due to different activation mechanisms in different strains. It was further shown by Klöppner (1999) that root-colonizing biocontrol strains express PGA at much higher activities than non-colonizers. When supported by additional data, it would suggest an important role of acidic amino acids and their amides for interactions in the rhizosphere.

In the present study, we found that preferred sources of carbon like glucose and intermediates of the tricarboxylic acid (TCA) cycle strongly repress PGA synthesis in *P. putida* KT2440. This phenomenon is known as 'carbon catabolite repression' (CCR). Bacterial genes encoding carbon catabolite enzymes are often regulated in response to the available carbon source. It is well established that intermediates of the TCA cycle often repress the catabolic pathways of other carbon sources (Collier *et al*., 1996). If a rapidly metabolizable carbon source such as glucose is present in excess amounts the synthesis of peripheral catabolic enzymes is reduced. In contrast to *E. coli* or *B. subtilis*, carbon catabolite repression in pseudomonads is not well understood. In enteric bacteria, uptake of glucose indirectly leads to a low level of cyclic AMP (cAMP) which results in repression of activation of cAMP mediated genes responsible for degradation of secondary carbon sources (Postma *et al*., 1993). For *Pseudomonas* species, organic acids like succinate, citrate are

among the preferred carbon sources. In the presence of any of these substrates, the activity of the enzymes involved in the transport and/or catabolism of amides and amino acids like histidine is reduced (Ng and Dawes, 1967; Philipps and Mulfinger, 1981; Smyth and Clark, 1975). As Gln and Asn are eventually degraded to intermediates of the TCA cycle (2-oxoglutarate and fumarate, respectively), the CCR of their catabolism makes sense biochemically. When intermediates of the cycle or metabolites that feed it are already available, there is no need to utilize these amino acids.

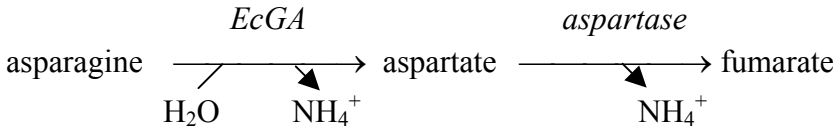
The only example of CCR in *P. putida* that was investigated in some detail is the degradation of phenols (Müller *et al*., 1996). However, the available data do not yet provide a consistent picture of the mechanisms involved (Petruschka *et al*., 2001). It is known, however, that cAMP and the CAP protein do not play any role in pseudomonads. Instead, a carbon catabolite regulatory protein (crc) was identified that is involved in CCR in ways which have still to be characterized (Hester *et al*., 2000). Although, in the present work the mechanism of CCR was not characterized in detail, the regulation of PGA activity may become an interesting model system to study carbon catabolite repression in pseudomonads. Another organism interesting to examine would be *P. fluorescens* Pf-5, which is markedly less subject to CCR than the other *Pseudomonas* strains examined so far (Klöppner, 1999).

As already noted by Klöppner (1999) for wild type strains of *P. fluorescens* and *P. putida*, the findings summarized above that in *P. putida* KT2440 the acidic amino acids mainly serve as sources of carbon which are dispensable when other, more readily metabolizable C sources are available.

5.3 Role of PGA in *P. putida* KT2440

In *E. coli*, the PGA homolog asparaginase II (EcGA) is under the control of the *fnr* gene product and thus only expressed by anaerobically growing cells (Jerlström *et al*., 1987). This finding indicates that *E. coli* asparaginase II mainly serves to supply precursors for the so-called fumarate respiration. In this pathway, which is used by *E. coli* to produce ATP in anaerobic conditions, fumarate is used as an final electron acceptor in an electron transfer

chain that generates a proton gradient over the plasma membrane which eventually is used for ATP production. The fumarate required for this process can be synthesized from Asn by the following two-step sequence



P. putida as a predominantly aerobic organism does not use this pathway. To elucidate the role of PGA in amino acid utilization in *P. putida* KT2440, a disruption mutant defective in *ansB* expression was constructed. As shown in section 4.4, the disruption mutant was unable to grow on Gln as sole source of carbon and nitrogen. As PGA is a purely periplasmic activity, this result suggests that *P. putida* KT2440 does not possess an efficient uptake system for glutamine and thus has to hydrolyze Gln to Glu in the periplasm to be able to use exogenous Gln as a source of carbon and nitrogen. Direct evidence for this assumption was provided by the results of amino acid transport studies (see section 4.5) which indicate that the disruption was also unable to take up glutamine from the medium to support growth.

Fig. 5.1 shows an alignment of several PGA sequences deduced from *Pseudomonas* genomes with two PGA homologues from enterobacteria (i.e. type II asparaginases from *Escherichia coli* and *Shigella flexneri*). Clearly, all these enzymes share a high degree of sequence similarity. Differences between the *Pseudomonas* enzymes from those of enteric bacteria are mainly seen in the region of the leader peptides and the N-termini while all the catalytically important residues (marked by ●) are strictly conserved.

Polar effects of the mutation on downstream genes are unlikely to contribute to the observed consequences of *ansB* disruption. An analysis of the *ansB* region in the *P. putida* KT2440 genome (Fig. 5.2) shows that *ansB* is flanked by an *end*X homolog on one side and a putative operon (*rbsABC*) on the other which encodes a ribose uptake system. Thus, the genes flanking *ansB* in *P. putida* KT2440 are entirely unrelated to amino acid metabolism. A similar case was reported by Hüser *et al*. (1999) for *P.* flu*orescence* ATCC 13525, where the *ansB* gene is also followed by an ORF (*end*X) encoding an endonuclease of unknown function.

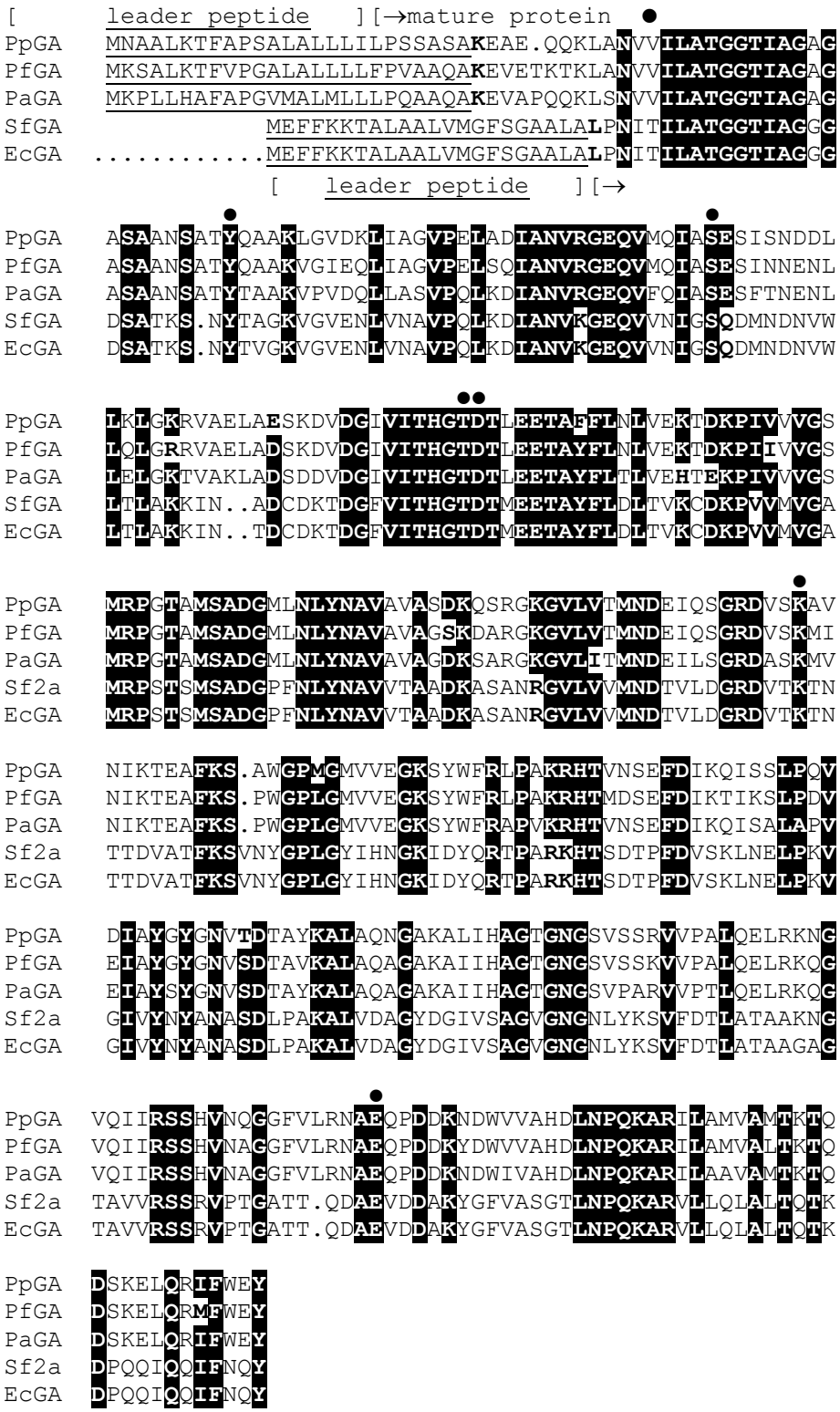


Figure 5.1: Alignment of the derived amino acid sequence of periplasmic glutaminase/ asparaginase enzymes from *P. putida* KT2440 (PpGA), *P. fluorescens* ATCC13525 (PfGA), *Pseudomonas aeruginosa* PA01 (PaGA), *Shigella flexneri* 2a strain 301 (Sf2a), and *Escherichia coli* K12(EcGA). Sequence motifs conserved in all of these enzymes are shown in black boxes. Catalytically important active site residues are marked by ●.

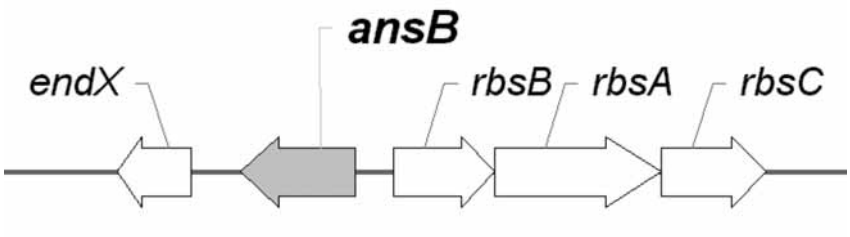


Figure 5.2: Genetic organization of the *ans*B region in *P. putida* KT2440

In addition, the size of the *ansB* transcript indicated that *ans*B expression is monocistronic. Taken together, these findings and our present results suggest that Gln utilization by *P. putida* KT2440 strictly depends on the activity of PGA whereas Asn can be taken up and metabolized by independent systems. In *P. fluorescens* a cytosolic amidohydrolase with marked specificity for Asn (*Pseudomonas* asparaginase, PA) was found (Klöppner, 1999). If this enzyme is also present in *P. putida* KT2440, it could mediate the utilization of asparagine in cooperation with an Asn uptake system that has still to be identified.

5.4 Further Glu-responsive genes in *P. putida* KT2440

In order to identify further genes that are up-regulated during growth on acidic amino acids and their amides, we selected a proteomics approach. 2D-PAGE was used to follow the time course of protein synthesis in cells transferred from NH_4^+/glucose minimal medium i) to the same medium, ii) to minimal media containing Glu as the sole source of carbon and nitrogen, iii) or to media containing the CCR-active compound fumarate in addition to Glu. In each case, several samples were taken from the same culture at different time intervals and analyzed under exactly the same conditions to minimize the risk of artifacts (Figs. 4.6-4.8). To keep the experimental effort within an acceptable limit, only Glu was used as an inducer. This appeared justified as studies by Klöppner (1999) showed that in *P. fluorescens* the same set of proteins were induced by all four amino acids (Asp, Asn, Glu, Gln). Differentially expressed proteins were identified by mass spectrometry (MS). At the time when these experiments were done, the annotated genome of *P. putida* KT2440 was not yet available in the databases. Therefore, the rather simple method of spot identification from the mass distribution of tryptic peptides could not be

used for all proteins (about 5500 in strain KT2440). Instead we selected a series of about 40 possible candidate genes from the unfinished genome and calculated the respective peptide masses ourselves. In this way only three spots could be identified in an unequivocal fashion. For the other spots the more complicated (and expensive) MALDI-PSD technique had to be used which yields sequences of the respective peptides and thus is more reliable.

The key advantages of protein identification by mass spectrometry are the sensitivity of the method, the amount of information generated and the speed of the analysis and identification. With a sensitivity limit of 10^{-15} to 10^{-18} mol of protein, any protein spot that can be detected with Coomassie blue staining also contains adequate amounts of protein for identification. Further, the analysis is sufficiently automated that peptides are detected and product-ion-spectra recorded in a single analysis. The high percentage of successfully identified spots (11 out of 13 in this work) demonstrates the efficiency of the approach followed. The use of MALDI-TOF and PSD was fundamental to obtain a high number of high-quality mass spectra, even from peptides present in very low abundance.

The set of Glu-induced proteins of *P. putida* KT2440 included at least 9 major members. Many of these proteins were found to perform functions that relate them to amino acid uptake and metabolism. In parallel, during growth on NH_4^+/ glucose at least four proteins were up-regulated which appear to be involved in sugar uptake and metabolism. Most of the genes induced during growth on glutamate were repressed by growth on glucose-containing medium. This result strongly indicates that expression not only of PGA but of the whole set of Glu-responsive genes is subject to CCR. Most of these genes were also repressed by fumarate. Possible functions of the proteins specifically upregulated in response to glutamate were derived from sequence comparisons with the respective genes in the genomes of *P. aeruginosa* and other related species. In the following the assignments made are discussed in more detail.

Transcription termination factor Rho (Pp1)

Spot Pp1 was identified as the Rho protein which has functions in the termination of transcription. *E. coli* has two known modes for termination of RNA transcription. One is intrinsic to the function of RNA polymerase, which can spontaneously terminate transcription. The other mode is dependent upon the action of Rho. The Rho protein is, for instance, known to participate in the regulation of tryptophanase expression in *E. coli*.

Here, tryptophan enhances the transcription of tryptophanase via Rho-mediated antitermination (Konan and Yanofsky, 2000). It is still unknown whether comparable mechanisms also operate in the regulation of glutamine and glutamate metabolism in *P. putida*.

Periplasmic glutaminase/asparaginase (Pp3/4)

Protein spots Pp3/4 were identified as PGA. Although, Pp4 has about the same mass as Pp3, it has a slightly lower isoelectric point. Still the origin of minor spot Pp4 (see Fig 4.7) was not investigated in detail, but it may correspond to an isoform of PGA in which one or more asparagine or glutamine residues have been converted to the respective dicarboxylates. However, we cannot exclude the existance of a phosphorylated or otherwise covalently modified form of the enzyme. At present it is a matter of speculation whether this second form of PGA is an artifact or has a functional role in cellular processes.

ABC transporter ATP-binding protein (Pp5)

Pp5 was assigned to the ATP-binding subunit of an ABC transporter. Pp5 showed a strong similarity to sequences of ATP binding proteins of many different bacterial ABC transporters specific for amino acid uptake, among them the *glnQ* gene product from *Helicobacter pylori* and the ATP binding protein of a histidine transporter from *B. subtilis*. In *P. putida KT2440* the closest match is PP1068 which is annotated as 'amino acid ABC transporter, ATP-binding protein'.

PGA has equal specificity towards Asn and Gln and thus can generate both dicarboxylates (Glu and Asp) for uptake by transport systems in the inner membrane. The ATP-binding protein Pp5 could belong to such a transport system of the ABC type which mediates the uptake of the acidic amino acids and/or their amides. As already discussed above, this assumption is supported by the genetic organization of certain Glu-related genes in *P. putida* KT2440, *P. aeruginosa* PAO1, and *P. fluorescens* (Fig. 4. 22).

Aspartate ammonia lyase (Pp6)

Pp6 was identified as aspartate ammonia lyase (aspartase). In many microorganisms L-asparagine is utilized through the consecutive action of two enzymes (Sun and Setlow, 1991). The product of the PGA catalysed reaction, L-aspartate is converted to fumarate and ammonium by aspartate ammonia lyase. Under aerobic conditions this reaction feeds the tricarboxylic acid cycle with the carbon skeletons of Asn and Asp. In *E. coli* and other

enterobacteria, the enzyme also assists in anaeorobic fumarate respiration by providing fumarate from aspartate (see above).

Outer membrane porin D (Pp8)

Pp8 showed strong similarity to the product of the *oprD* gene of *P. aeruginosa* which encodes a porin involved in the uptake of amino acids and/or peptide (Trias and Nikaido, 1990). The product of the corresponding *P. putida* gene (PP1206) was also annotated as a type D porin. Ochs *et al*. (1999) further showed that expression of OprD in *P. aeruginosa* is strongly enhanced by amino acids (including Glu, Arginine and Alanine) and repressed by succinate. The arginine-mediated induction of OprD was mediated by the regulatory protein ArgR, whereas the glutamate-induced expression of OprD was independent of ArgR, indicating the presence of more than a single activation mechanism. These findings suggest that the *oprD* gene product facilitates the passage of amino acids through the outer membrane.

Carboxyphosphonoenol pyruvatephosphonomutase (Pp9)

Pp9 yielded peptides with similarities to the sequence of a putative carboxyphosphono-enolpyruvate phosphonomutase. Thus, unlike the proteins discussed above, Pp9 has no apparent relation to amino acid metabolism. The only known function of this enzyme is to catalyze a step in the biosynthesis of the antibiotic bialaphos in *Streptomyces hygroscopicus* (Lee *et al*., 1995). However, the deduced amino acid sequence of Pp9 is also similar to those of more common phosphopyruvate hydratases from intermediary metabolism (e. g. enolase, EC 4.2.1.11**,** Lee *et al.*, 1995**)**. Thus the annotation of PP1389 as a PEP phosphonomutase may be erroneous. Clearly, further experiments are required to characterize the role of Pp9 in *P. putida* KT2440.

Four protein spots were identified that were preferentially expressed during growth on NH_4^+/Glc. Thus it appears that the corresponding proteins are not required during growth on glutamate. The proteins upregulated during growth on glucose/NH_4^+ (Pp10-Pp13) can all be related to the uptake and degradation of glucose (Pp11 and Pp12) or diamines (Pp10 and Pp13), respectively.

2,4-Diaminobutyrate 2-oxoglutarate transaminase (Pp10)

Pp10 was identified as 2,4-diaminobutyrate-2-oxoglutarate transaminase. A transaminase of this type (type III) was shown to catalyze a step in the biosynthesis of 1,3-diaminopropane by *Acinetobacter baumannii* (Ikai and Yamamoto, 1997). This enzyme

is known to catalyse the transfer of the side-chain amino group of 2,4-diaminobutyrate to 2-oxoglutarate to yield aspartic-4-semialdehyde and glutamate. However, other functions of a type III transaminase appear also to be possible.

Fumarase (Pp11)

Pp11 was identified as fumarase C . This is a 'housekeeping' enzyme that converts fumarate to malate (or malate to fumarate) in the tricarboxylic acid cycle and in this way contributes to an effient degradation of glucose.

Sugar ABC transporter, sugar binding protein (Pp12)

The increased synthesis of a sugar uptake system during growth on glucose is not surprising. Pp12 corresponds to a periplasmic sugar-binding protein associated with an ABC transporter. The closest homolog of Pp12 in *P. aeruginosa* (PA3190) was shown to be involved in glucose uptake (Sage *et al*., 1996).

Putrescine ABC transporter, putrescine binding protein (Pp13)

Pp13 was identified as the ATP-binding subunit of an ABC transporter responsible for the uptake of putrescine (PP5181). Like Pp10, this protein seems to be involved in the utilization of diamines. At present it is difficult to explain, why putrescine uptake is up-regulated during growth on NH_4^+/glucose. However, there are indications that diamines play a role in the interactions between root bacteria and their host plants. Putrescine, a normal component of root exudates, was shown to inhibit growth of *P. fluorescens* WCS365 and its ability to colonize tomato roots (Kuiper *et al*., 2001). Sauer and Camper (2001), studying changes in the gene expression during attachment of *P. putida* to surfaces, found that 15 proteins that were up-regulated following bacterial adhesion and 30 proteins were down-regulated. The proteins down-regulated after attachment include the *potF* gene product (Pp13) as well as PGA (Pp3/4) and other proteins involved in amino acid uptake and metabolism. Although these findings are difficult to interpret at present,

they support the notion that profound changes in the metabolism of amino acids and polyamines accompany the change from free-living to sessile growth in pseudomonads.

A model summarizing the possible involvement of the various identified Glu-responsive genes in uptake and utilization of acidic amino acids and their amides is shown in Fig 5.3. Most of the assigned functions are speculative at this time. Entry of Gln, Asn, Glu and Asp into the cell (top) is facilitated by a porin of the outer membrane (oprD, Pp8). In the periplasmic space glutaminase/asparaginase (PGA, Pp3/4) hydrolyzes Gln and Asn to yield additional Glu and Asp. These amino acids are bound by one or more periplasmic binding proteins associated with ABC transporters. The ATP-binding subunit of one ABC transporters was identified in the present work as Pp5. Klöppner (1999) showed that in *P. fluorescens* a corresponding amino acid binding protein is induced by acidic amino acids and their amides. After uptake of Glu and Asp, the latter can be converted to fumarate by aspartase (Pp6) and in this way channeled into the TCA cycle. One of the TCA cycle enzymes (fumarase, Pp11) is also differentially expressed in the absence and presence of amino acids. Finally, DABA transaminase (Pp10) might have a role in linking diaminobutyrate (DABA) with Glu and aspartic-β-semialdehyde which can be synthesized from Asp.

No specific role in the context of amino acid metabolism can yet be assigned to the transcription termination factor Rho (Pp1) and to the putative carboxyphosphonoenol pyruvatephosphonomutase (Pp9). Of course, for most of the Glu-responsive proteins identified here, detailed physiological studies with disruption mutants have to be performed to confirm their putative roles in the metabolism of acidic amino acids. To date, this has only be done for PGA.

5.5 Dependency of Glu-responsive genes on σ^{54}

Our finding that most of the Glu-responsive gene products identified by 2D-PAGE are not expressed in a *rpoN*- mutant of *P. putida* KT2440 (see Fig. 4.9) gives additional support to our assumption that the respective genes are regulated in a coordinate fashion. By contrast, expression of the proteins up-regulated during growth on glucose/NH_4^+ (Pp10-Pp13) are not dependent on a functional σ^{54}.

Bacterial sigma (σ) factors are RNA polymerase subunits required for the initiation of transcription, i. e. for the formation of an active RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$). A different choice of sigma factors allow the cells to respond to a variety of environmental stimuli (see introduction). The core polymerase (E) associated with the σ^{70} ($E\sigma^{70}$) translates housekeeping genes like those encoding enzymes of intermediary metabolism. The alternate factors σ^S, σ^{32} and σ^E are involved in various responses to stress like depletion of nutrients, extreme temperaturs or pH values or oxidarive stress.

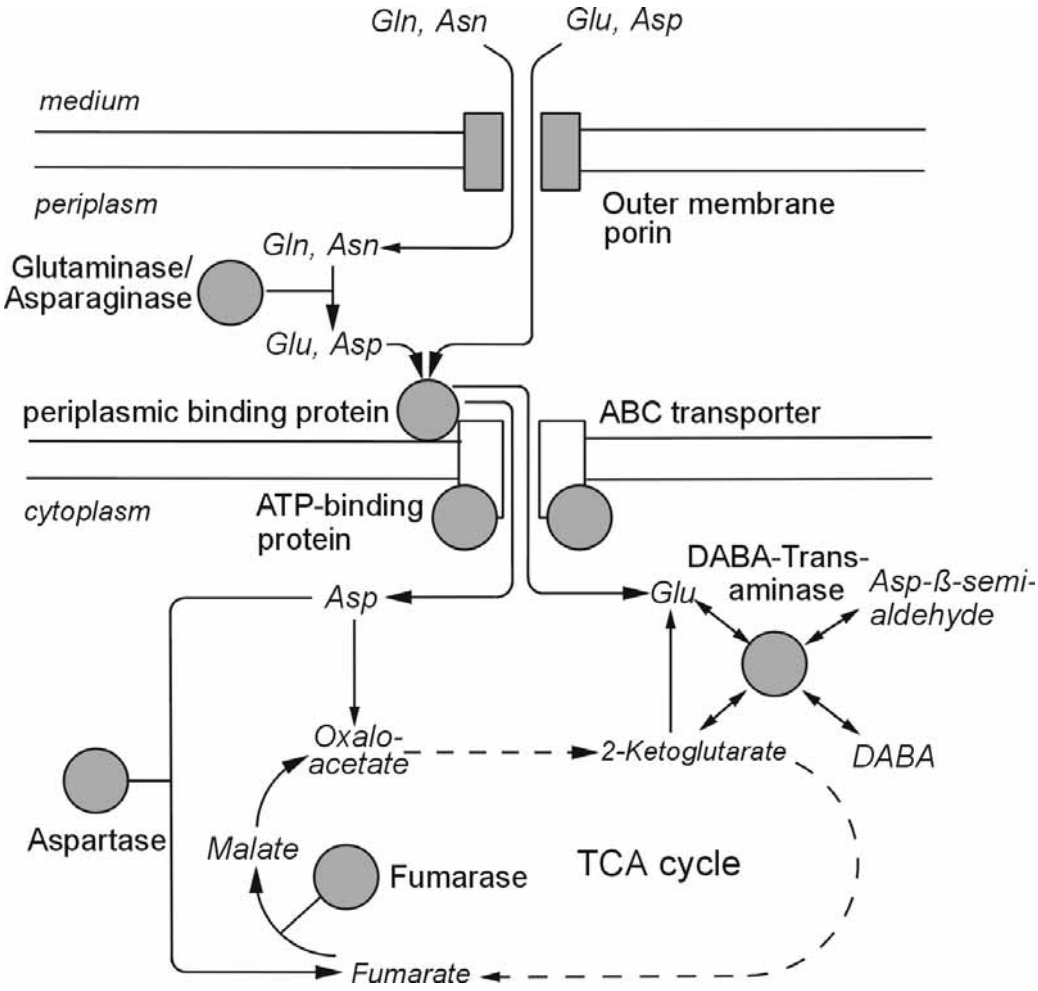


Figure 5.3: Pathways for uptake and utilization of acidic amino acids and their amides. See text. DABA - diaminobutyric acid transaminase.

The alternate factor σ^{54} is the only sigma factor that is not homolgous to σ^{70}. Orginally it was thought to control only nitrogen assimilation and therefore the gene encoding σ^{54} was called *rpoN* (Kustu *et al.*, 1989). In most bacteria, the downstream open reading frames in the *rpoN* region are also highly conserved. Their products probably act as co-inducers. From the analysis of bacterial gernomes, especially that of *E. coli*, it became clear that not only the Ntr response but many other genes also depend on σ^{54} for expression. These additional genes encode the uptake and metabolism of NH_3, Gln and Arg, the degradation of propionate and acetoacetate, several hydrogenases, flagellar movement and zinc tolerance (Reitzer and Schneider, 2001). Transcription from all known σ^{54}-dependent promoters is absolutely dependent on the presence of additional proteins with ATPase activity that stimulate transcription (Collado-Vides *et al*., 1991, Buck *et al*., 2000). The function of these proteins is to facilitate conversion of the closed promoter complex to an open one with consumption of ATP. Usually the enhancer proteins are so-called response regulators i. e. proteins that transmit environmental signals from a membrane-bound sensor kinase to the transcription complex (Chang and Stewart, 1998) are involved in these processes. A well-known example is the pair NtrB-NtrC (NR_{II}/NR_I) which mediates the Ntr response (see Introduction). In *E. coli* another sensor/ regulator system, AtoC/AtoS, activates degradation of acetoacetate, while ZrA/ZrS control the expression of proteins involved in zinc tolerance. In *P. putida*, σ^{54} is also required for the expression of genes responsible for the degradation of toluene and xylenes (Shingler, 2003). In *P. aeruginosa*, the products of *rpoN* and the downstream ORF2 act as a coinducers of genes involved in the assimilation of Gln (Jin *et al*., 1994). In *P. fluorescens*, the expression of *ansB* was also shown to dependent on σ^{54}. In addition the putative σ^{54} binding site was identified, which is located at positions -95/-105 from the translation start site (Hüser *et al.* 1999). The upstream region of the *P. putida* KT2440 *ansB* gene also contains a σ^{54} recognition site at a suitable distance from the translation start site. The dependence of KT2440 PGA expression on σ^{54} is further supported by the observation that PGA was downregulated in the *P. putida* KT2440 *rpoN* negative mutant (see Fig. 4.9).

A set of individual genes and/or operons the expression of which is controlled by one and the same response regulator is called a regulon. In summary, our present data indicate that the proteins upregulated by Glu (and other proteins not yet identified) may all be the products of a regulon responsible for their uptake and metabolism. In order to substantiate this assumption it has to be demonstrated that a single response regulator binds to and

enhances transcription of these genes. This question will be adressed in future experiments.

5.6 Glutamate synthase as a mediator of the Ntr response

Transposon mutagenesis is a very useful tool for the identification of bacterial genes that regulate cellular processes. Gene knockout or targeted gene disruption techniques provide important informations about the biological functions of unknown genes. As targeted gene replacements by homologous recombination are rare events, they require highly selective screening procedures for detection. Once transconjugants of interest have been isolated, the identification of sequences flanking the transposon is required for characterization of the gene product affected by the insertion. The most popular transposon for use with non-*E. coli* species is Tn5 for several reasons:

1) Tn5 transposes into many different Gram-negative bacteria with high frequency

2) it generally inserts with little target sequence specificity, and

3) it exhibits low probability of genome rearrangements upon transposition and a high stability once integrated in a genome.

In this work, a self cloning Tn5 derivative was used to screen for genes involved in the regulation of PGA induction. Surprisingly, we found that in most transconjugants the transposon had integrated in to the *gltB* gene coding for large subunit of glutamate synthase (GOGAT). Only three of these transconjugants (Tn-SM2, Tn-SM15, and Tn-SM30) were characterized in detail. Growth studies on different carbon and nitrogen sources showed that growth was severely impaired in media containing amino acids as sole source of carbon and nitrogen, and also on amino acids in combination with NH_4^+.

A search of the literature showed that the importance of GOGAT in the regulation of nitrogen metabolism in Gram-negative bacteria has been known for a long time. The first observations were made 30 years ago with *gltB*- mutants of *Klebsiella* (Nagati *et al*., 1971; Brenchley *et al*., 1973). Later on it was found that GOGAT deficiency in *E. coli* (Pahel *et al*., 1978; Magasanik, 1996) *Bradyrhozobium japonicum* (O'Gara *et al*., 1984) and *Rhizobium meliloti* (Lewis *et al*., 1990) also caused pleiotropic nitrogen assimilation defects, leading to the so called Asm^- (nonassimilatory) phenotype which is characterized by inability to grow on poor nitrogen sources such as amino acids. These and other

findings showed that function of the nitrogen regulatory system (Ntr) of Gram-negative bacteria crucially depends on the activity of GOGAT. The reasons for this are still not known with certainty. One possible explanation is that GOGAT-deficient mutants are unable to deplete the internal glutamine pool and thus cannot derepress the Ntr system which is mainly controlled by the ratio [Gln]/[2-oxoglutarate]. This hypothesis, however, creates a paradox (Goss *et al*., 2001), i.e. that in *gltB* mutants Gln levels appear to be too high for derepression of the Ntr system but too low to support growth. An alternative model, which was also proposed by Bender and coworkers, suggests that GOGAT-deficient mutants of *E. coli* or *Klebsiella aerogenes* do not suffer from excessive intracellular Gln levels but are rather starved for Glu (Goss *et al*., 2001). Such a situation, on the other hand, could occur only if intracellular glutaminase actitivites are too low to generate sufficient amounts of Glu from Gln. Another hypothesis proposed for *E. coli*, suggest that inactivation of *gltB* creates polar effects on the neighbouring nitrogen metabolizing *gltF* gene which is involved in regulation of the Ntr response. In case of *P. putida* KT2440, the polar effect of *gltB* disruption on *gltF*, can be ruled out as KT2440 genome search did not revealed any homolog of the *gltF* gene in the *gltB* region.

In *E. coli* the Ntr^- phenotype resulting from *gltB* disruption was suppressed by mutations in *NtrB,* a component of Ntr system. These mutants were observed to synthesize glutamine synthetase constitutively even under nitrogen excess conditions. In some bacteria, GOGAT activity is influenced by the *gltC* gene product which aberrantly regulates the levels of GOGAT activity. In *B. subtilis,* the GltC protein stimulates expression of *gltA* as well as the *gltB* operon (Bohannon and Sonenshein, 1989).

The phenotype resulting from *gltB* disruption in *P. putida* KT2440 is similar to that observed in *E .coli*. Of the amino acids checked, growth of the *gltB* mutant was impaired on Glu, Gln and Asp, while Asn supported growth at significant rates after 12 h (see Fig. 4.17 and Table 4.6). Growth of the mutants on Asn is probably due to the combined action of an Asn-uptake system and the cytosolic Asn-specific asparaginase which has been shown to exist at least in *P. fluorescens* (Klöppner, 1999). GOGAT mutants of other bacteria were also reported to show poor or no growth at all on amino acids as sole nitrogen source (Kondorosi *et al.*, 1977; Eberl *et al*., 2000).

While the GOGAT-negative transconjugants and the targeted disruption mutant were unable to utilize amino acids as the sole source of carbon and nitrogen, they grew at almost normal rates in media containing amino acids in combination with glucose. On the other hand amino acids supplemented with NH_4^+ did not support growth (Table 4.6). This clearly indicates that these mutants had lost the ability to utilize amino acids as carbon source but can use amino acids as sources of nitrogen. Thus, it appears that in *P. putida* KT2440 GOGAT activity is not required for ammonia assimilation but rather for a prerequisite for reactions that channel the carbon skeleton of Glu and Asp into the tricarboxylic acid cycle or other catabolic pathways. In agreement with results of Eberl *et al*. (2000) we also found that GOGAT-negative mutants were severely impaired in their motility as well as their chemotactic response towards acidic amino acids (Table 4.7). Both motility and chemotaxis play an important role in the interactions of rhizobacteria with host plants (O'Toole and Kolter, 1998; Pratt and Kolter, 1998). For many bacterial species amino acids act as strong chemoattractants. Gaworzewka and Carlile (1982) showed that *Rhizobium* and *Bradyrhizobium* spp. are attracted by amino acids and dicarboxylic acids present in the root exudates. In *E. coli*, chemotaxis involves a membrane-bound sensor that either binds to the amino acid directly or interacts with the binding protein loaded with the amino acid, while in *Rhodobacter sphaeroides*, chemotaxis is thought to require for both uptake and the metabolism of the amino acid (Jacobs *et al*., 1995). Similarly, *P. aeruginosa* like most other motile bacteria, exhibits a chemotactic response towards a wide range of chemical stimuli, including amino acids (Craven and Montie, 1985). The taxis toward amino acids is subject to control by nitrogen availability in a manner similar to the control of other enzymes of nitrogen metabolism. Biochemical evidence indicates that the chemotaxis of *P. aeruginosa* towards amino acids is mediated by methyl-accepting chemotaxis proteins.

As mentioned above, it has been proposed that the phenotype resulting from *gltB* inactivation is the result of starvation for Glu (Goss *et al*., 2001). In contrast to this proposal we show here that *gltB*-mutants of *P. putida* KT2440 are not at all starved for Glu, but rather progressively accumulate Glu as well as Gln, apparently without being able to metabolize it (Fig. 4.21). As GOGAT is inactive in the mutant, the accumulation of Glu is most probably due to the action of glutamate dehydrogenase which increasingly deplete the levels of intracellular 2-oxoglutarate (see scheme 1.1). As Gln is formed at the same

time, the ratio [Gln]/[2-oxoglutarate] will rise until the Ntr response is shut off. This, in turn, could trigger the loss of motility and chemotaxis and permanently suppress the synthesis of proteins necessary for Glu and Asp utilization. For *E. coli,* it was observed that the cells synthesize more Glu and accumulate K^+ as a counter ion to restore the cell's turgor pressure under osmotic upshift conditions (Csonka and Hanson, 1991). Later, Csonka and coworkers (1994) showed that GOGAT-deficient mutants of *Salmonella enterica* serovar Typhimurium are sensitive to osmotic stress when grown under ammonia-limiting conditions. These authors proposed that the synthesis of glutamate is necessary for growth in hyperosmotic conditions. Still it is not clear whether increased synthesis of glutamate is required for the survival during prolonged periods of nitrogen starvation.

At present, it is difficult to give a satisfactory explanation for these rather diverse findings. The intracellular levels of the acidic amino acids and their amides are affected by a large number of enzymes and transport systems (see Fig. 1.1). All of these activities must be thoroughly studied in wild type and the mutant cells to obtain a consistent picture of the metabolic changes resulting from *gltB* disruption. One factor that, in our opinion, was not sufficiently considered in previous discussions of the *gltB*- phenotype is the competition for substrates and coenzymes. The carbon skeletons of Glu and Asp can be channeled into the tricarboxylic acid cycle by oxidative deamination of Glu (cf. Fig. 1. 1, GDH) or by eliminating deamination of Asp (enzyme 6, aspartase). Aspartase is one of the enzymes which is induced by Glu in wild type *P. putida* and thus should be repressed in the *gltB*- mutant. As GOGAT and GDH have substrates (2-oxoglutarate, Glu) and the coenzyme (i. e. NADP/ NADPH) in common, both enzymes will influence each other. When GOGAT is inactive, the concentrations of NADPH and 2-oxoglutarate will increase, and the GDH equilibrium is shifted towards formation of glutamate. This may be the reason why the *gltB*- mutant accumulates Glu even though GOGAT is missing. If aspartase is also inactive, more Glu will be formed via transamination of Asp (enzyme 7, aspartate transaminase). The fact that glutamine was formed in our *gltB*- disruption strain indicates that glutamine synthetase (GS, enzyme 3) is active in the mutant. This is supported by our finding that GOGAT-negative strains grow well on glucose and Glu, i. e. they must be able to synthesize Gln for *de novo* protein synthesis and other biochemical functions and thus should contain sufficient GS activity. Glucose is probably required because its

degradation provides the ATP necessary for the synthesis of Gln and Asn. In fact, Gln and Asn which can be converted to Glu and Asp without expense of ATP allowed much faster growth of the mutants. If GS is active in the presence of high concentrations of glutamine, the Ntr system of *P. putida* KT2440 must significantly differ from the that of *E. coli* where GS would be repressed under these conditions. Clearly, more detailed comparative studies of the amino acids metabolism in *gltB* mutants and the wild type cells are necessary to elucidate the events that lead to the observed phenotype and to discriminate between causes and effects.

5.7 aauR/aauS as a novel two-component system in *P. putida* KT2440

In this work we have identified a novel two-component system (aauS-aauR) in *P. putida* KT2440 which is involved in the regulation of Gln and Glu utilization. This is the first such system known to regulate the metabolism of acidic amino acids and their amides. As described in section 4.17, our decision to investigate this particular system (PP1067/PP1066) was based on the fact that in *P. aeruginosa* PAO1 the closest homologs of PP1067/PP1066 are clustered with several genes that clearly have roles in the metabolism of glutamate. We therefore speculated that PP1067/PP1066 might have a similar role. Our present results suggest that this is indeed the case. Mutants with an inactive sensor kinase or a response regulator failed to grow efficiently on Glu and showed pleiotropic defects in Glu-induced protein induction. For this reason we designated the system aau (for **a**cidic **a**mino acids **u**tilization). The letter S stands for 'sensor kinase' while R refers to 'response regulator'.

The sequence analysis of *aauS* predicts an open reading frame of 1914 base pairs encoding a product with 636 amino acids and a molecular mass of 70,513 kDa, while *aauR* (1329 base pairs) encodes a protein of 443 amino acids and a calculated mass of 48,710 kDa. Genes that encode a two-component system are usually linked in an operon. In *P. putida* KT2440 the *aauR* open reading frame (ORF) has a translation initiation codon that overlaps with the *aauS* termination codon, suggesting that expression of *aauS* and *aauR* is probably also translationally coupled. Annotated sequence alignments of *aauS* and *aauR* with several related genes are shown in Figs. 5.5 – 5.6. They indicate that *aau* belongs to the same family of the well-characterized *dct* system which is involved in the regulation of dicarboxylate uptake by *Rhizobium leguminosarum* (recently reviewed by

Janausch *et al*., 2002). A schematic overview of the domain structure of the DctB and the DctD is shown in Fig. 5.4. The sensor histidine kinase DctB (top) is composed of a sensor domain which is inserted in the inner membrane by two transmembrane helices (TM1 and TM2). In between, there is a periplasmic ligand binding domain. The cytoplasmic part of the molecule consists of a dimerization domain where autophosphorylation of the activated sensor kinase at a histidine residue (H) takes place and a C-terminal kinase domain which contains several conserved ATP binding motifs (G1, F and G2) and a so-called N-Box the function of which is unknown.

Fig. 5.5 shows that these highly conserved segments all can be found in the predicted sequence of AauS as well. Not unexpectedly, AauR, the response regulator component of the aau system shows a high degree of sequence homology with DctD, the respective regulator of the *R. leguminosarum dct* system (Fig. 5.6). Both proteins contain a N-terminal receiver domain where the aspartate residue (D) is located which can become phosphorylated by phosphate transfer from the histidyl phosphate residue of DctB. This part of the molecule is followed by an extended σ^{54} interaction domain which binds to the σ^{54} component of the initial transcription complex (see introduction).

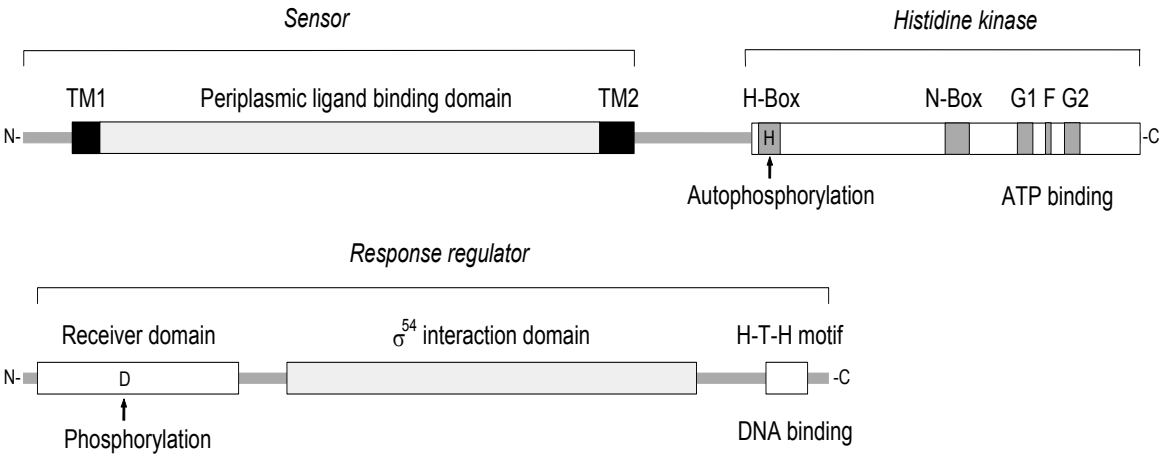


Figure 5. 4: Predicted domain structure of AauS and AauR, based on sequence similarity with DctB and DctD from *R. leguminosarum*

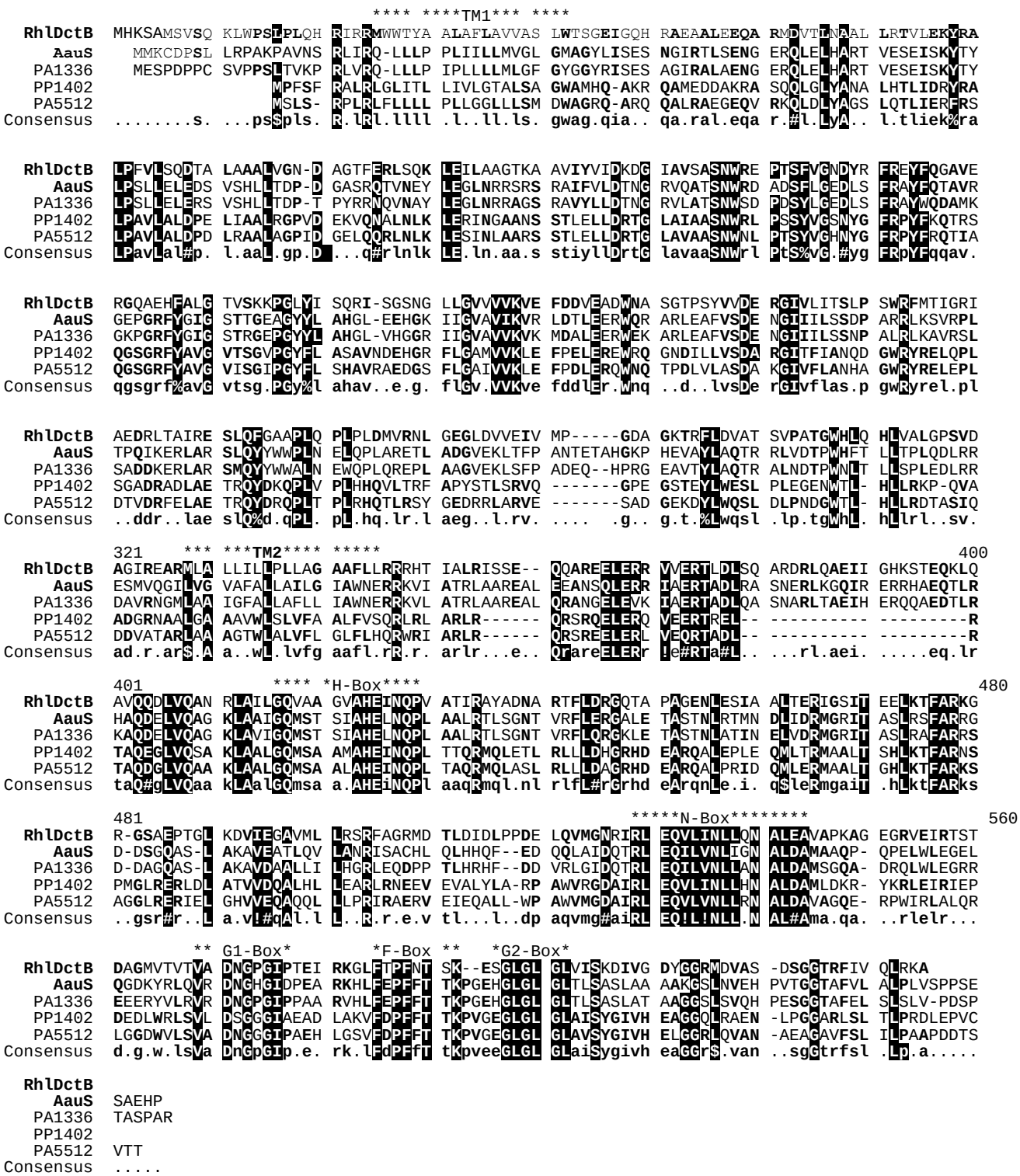
At the C terminus there is a DNA-binding domain with a helix-turn-helix motif. In addition to the sequences of the *aau* and *dct* proteins the alignments shown in Figs. 5.5 and 5.6 contain further closely related protein sequences derived from the genomes of *P. aeruginosa* PAO1 (PA 1335, PA1336, PA5512) or *P. putida* KT2440 (PP0263, PP1401,

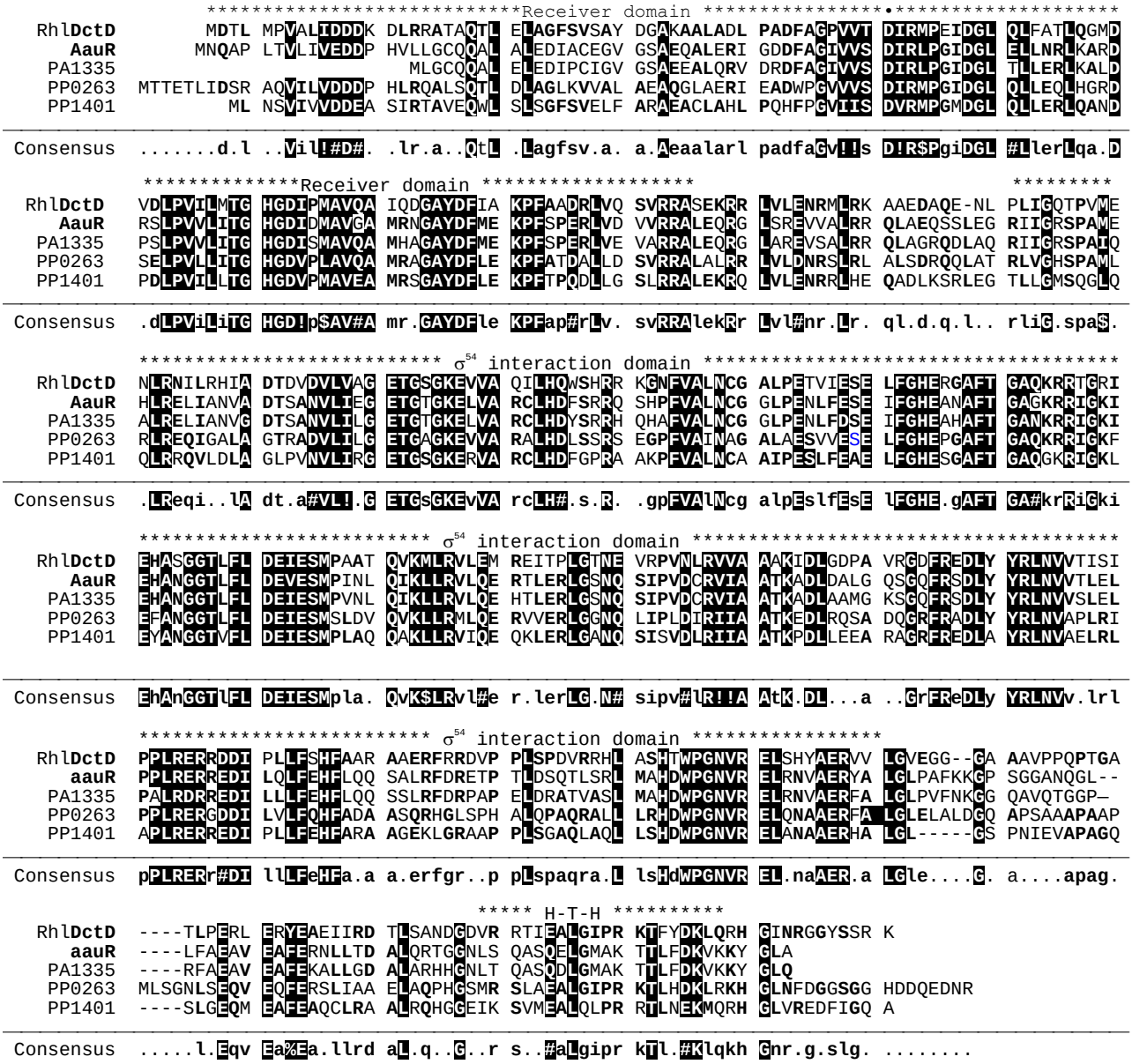
PP1402). None of these two-component systems has been functionally characterized so far.

The experimental data described in this thesis strongly suggest that the *aau* system of *P. putida* KT2440 has an important regulatory functions in the utilization of glutamate and probably also of glutamine. Growth defects of both the *aauS*- and *aauR*- mutants on different C and N sources were observed. The growth kinetics of both mutants on amino acids (Asp, Glu, Asn, Gln) as sole carbon and nitrogen source showed that the range of amino acids that could be used by these two strains was identical, except that the *aauS*-mutant grew poorly on Asp (see Fig. 4.23 and table 4.8). Both mutants grew well on Asn. However, the expression of PGA was greatly impaired in both the *aauS*- and the *aauR*-mutants which indicates that a functional *aau* is required for the expression of the *ansB* gene. To confirm this in a more direct way it has to be shown that AauR binds to the *ansB* promoter. Experiments with this aim were initiated but could not yet be completed.

By 2D-PAGE analysis we further showed that in an aauR-negative strain more than 20 proteins were not expressed during growth on Glu as sole source of carbon and nitrogen (see Fig. 4. 24). This indicates that the *aau* system not only affects *ansB* transcription but has a more general role in the regulation of amino acid metabolism.

In rhizobia, transport and catabolism of dicarboxylic acids play a very important role in symbiotic nitrogen fixation. In *Rhizobium leguminosarum* and *Sinorhizobium meliloti*, transport of dicarboxylic acid such as succinate which is provided by the plant host is taken up by the C_4-dicarboxylic transport (Dct) system (Engelke *et al*., 1987; Finan *et al*., 1983; Glenn *et al*., 1980, Reid and Poole, 1998). This system consist of three genes: *dctA*, which encodes the transporter, and two genes, *dctB* and *dctD*, which code for the two-component system already mentioned above. DctB/DctD activate transcription of *dctA* in response to the presence of dicarboxylates (Engelke *et al*., 1989; Jording *et al*., 1992; Ronson *et al*., 1987). In *R. meliloti, Bradyrhizobium janpnicum,* and *Azorhizobium cauinodans,* σ^{54} has been shown to control C_4-dicarboxylate utilization (Kullik *et al*., 1991; Ronson *et al*., 1987; Stigler *et al*., 1993). DctD has been shown to interact directly with both σ^{54} and the β-sununit of RNA polymerase (Huala *et a*l., 1992; Lee and Hoover, 1995; Lee *et al*., 1994). Mutations in any of the three *dct* genes result in the loss of the ability to transport and growth on C_4-dicarboxylates. Our observation that aauS and aauR-negative mutants of *P. putida* KT2440 grow well when succinate is supplied as sole source of carbon indicates that the aau system in *P. putida* is different from the Dct system.


**** ****TM1*** ****
RhlDctB MHKSAMSVSQ KLWPSLPLQH RIRRMWWTYA ALAFLAVVAS LWTSGEIGQH RAEAALEEQA RMDVTINAAL LRTVLEKYRA
AauS MMKCDPSL LRPAKPAVNS RLIRQ-LLLP PLIILLMVGL GMAGYLISES NGIRTLSENG ERQLELHART VESEISKYTY
PA1336 MESPDPPC SVPPSLTVKP RLVRQ-LLLP IPLLLLMLGF GYGGYRISES AGIRALAENG ERQLELHART VESEISKYTY
PP1402 MPFSF RALRLGLITL LIVLGTALSA GWAMHQ-AKR QAMEDDAKRA SQQLGLYANA LHTLIDRYRA
PA5512 MSLS- RPLRLFLLLL PLLGGLLLSM DWAGRQ-ARQ QALRAEGEQV RKQLDLYAGS LQTLIERFRS
Consensus ....s. ...psSpls. R.lRl.llll .l..ll.ls. gwag.qia.. qa.ral.eqa r.#l.LyA.. l.tliek%ra
RhlDctB LPFVLSQDTA LAAALVGN-D AGTFERLSQK LEILAAGTKA AVIYVIDKDG IAVSASNWRE PTSFVGNDYR FREYFQGAVE
AauS LPSLLELEDS VSHLLTDP-D GASRQTVNEY LEGLNRRSRS RAIFVLDTNG RVQATSNWRD ADSFLGEDLS FRAYFQTAVR
PA1336 LPSLLELERS VSHLLTDP-T PYRRNQVNAY LEGLNRRAGS RAVYLLDTNG RVLATSNWSD PDSYLGEDLS FRAYWQDAMK
PP1402 LPAVLALDPE LIAALRGPVD EKVQNALNLK LERINGAANS STLELLDRTG LAIAASNWRL PSSYVGSNYG FRPYFKQTRS
PA5512 LPAVLALDPD LRAALAGPID GELQQRLNLK LESINLAARS STLELLDRTG LAVAASNWNL PTSYVGHNYG FRPYFRQTIA
Consensus LPavLal#p. l.aaL.gp.D ...q#rlnlk LE.ln.aa.s stiyllDrtG lavaaSNWrl PtS%vG.#yg FRpYFqqav.
RhlDctB RGQAEHFALG TVSKKPGLYI SQRI-SGSNG LLGVVVKVE FDDVEADWNA SGTPSYVVDE RGIVLITSLP SWRFMTIGRI
AauS GEPGRFYGIG STTGEAGYYL AHGL-EEHGK IIGVAVIKVR LDTLEERWQR ARLEAFVSDE NGIIILSSDP ARRLKSVRPL
PA1336 GKPGRFYGIG STRGEPGYYL AHGL-VHGGR IIGVAVVKVK MDALEERWEK ARLEAFVSDE NGIIILSSNP ALRLKAVRSL
PP1402 QGSGRFYAVG VTSGVPGYFL ASAVNDEHGR FLGAMVVKLE FPELEREWRQ GNDILLVSDA RGITFIANQD GWRYRELQPL
PA5512 QGSGRFYAVG VISGIPGYFL SHAVRAEDGS FLGAIVVKLE FPDLERQWNQ TPDLVLASDA KGIVFLANHA GWRYRELEPL
Consensus qgsgrf%avG vtsg.PGy%l ahav..e.g. flGv.VVKve fddlEr.Wnq ..d..lvsDe rGIvflas.p gwRyrel.pl
RhlDctB AEDRLTAIRE SLQFGAAPLQ PLPLDMVRNL GEGLDVVEIV MP-----GDA GKTRFLDVAT SVPATGWHLQ HLVALGPSVD
AauS TPQIKERLAR SLQYYWWPLN ELQPLARETL ADGVEKLTFP ANTETAHGKP HEVAYLAQTR RLVDTPWHFT LLTPLQDLRR
PA1336 SADDKERLAR SMQYYWWALN EWQPLQREPL AAGVEKLSFP ADEQ--HPRG EAVTYLAQTR ALNDTPWNLT LLSPLEDLRR
PP1402 SGADRADLAE TRQYDKQPLV PLHHQVLTRF APYSTLSRVQ -------GPE GSTEYLWESL PLEGENWTL- HLLRKP-QVA
PA5512 DTVDRFELAE TRQYDRQPLT PLRHQTLRSY GEDRRLARVE -------SAD GEKDYLWQSL DLPNDGWTL- HLLRDTASIQ
Consensus ..ddr..lae slQ%d.qPL. pL.hq.lr.l aeg..l.rv. .... .g.. g.t.%Lwqsl .lp.tgWhL. hLlrl..sv.
321 *** ***TM2**** ***** 400
RhlDctB AGIREARMLA LLILLPLLAG AAFLLRRRHT IALRISSE-- QQAREELERR VVERTLDLSQ ARDRLQAEII GHKSTEQKLQ
AauS ESMVQGILVG VAFALLAILG IAWNERRKVI ATRLAAREAL EEANSQLERR IAERTADLRA SNERLKGQIR ERRHAEQTLR
PA1336 DAVRNGMLAA IGFALLAFLL IAWNERRKVL ATRLAAREAL QRANGELEVK IAERTADLQA SNARLTAEIH ERQQAEDTLR
PP1402 ADGRNAALGA AAVWLSLVFA ALFVSQRLRL ARLR------ QRSRQELERQ VEERTREL-- ---------- ---------R
PA5512 DDVATARLAA AGTWLALVFL GLFLHQRWRI ARLR------ QRSREELERL VEQRTADL-- ---------- ---------R
Consensus ad.r.arS.A a..wL.lvfg aafl.rR.r. arlr...e.. QrareELERr !e#RTa#L.. ...rl.aei. .....eq.lr
401 **** *H-Box**** 480
RhlDctB AVQQDLVQAN RLAILGQVAA GVAHEINQPV ATIRAYADNA RTFLDRGQTA PAGENLESIA ALTERIGSIT EELKTFARKG
AauS HAQDELVQAG KLAAIGQMST SIAHELNQPL AALRTLSGNT VRFLERGALE TASTNLRTMN DLIDRMGRIT ASLRSFARRG
PA1336 KAQDELVQAG KLAVIGQMST SIAHELNQPL AALRTLSGNT VRFLQRGKLE TASTNLATIN ELVDRMGRIT ASLRAFARRS
PP1402 TAQEGLVQSA KLAALGQMSA AMAHEINQPL TTQRMQLETL RLLLDHGRHD EARQALEPLE QMLTRMAALT SHLKTFARNS
PA5512 TAQDGLVQAA KLAALGQMSA ALAHEINQPL TAQRMQLASL RLLLDAGRHD EARQALPRID QMLERMAALT GHLKTFARKS
Consensus taQ#gLVQaa kLAalGQmsa a.AHEiNQPl aaqRmql.nl rlfL#rGrhd eArqnLe.i. qSleRmgaiT .hLktFARks
481 *****N-Box******** 560
RhlDctB R-GSAEPTGL KDVIEGAVML LRSRFAGRMD TLDIDLPPDE LQVMGNRIRL EQVLINLLQN ALEAVAPKAG EGRVEIRTST
AauS D-DSGQAS-L AKAVEATLQV LANRISACHL QLHHQF--ED QQLAIDQTRL EQILVNLIGN ALDAMAAQP- QPELWLEGEL
PA1336 D-DAGQAS-L AKAVDAALLI LHGRLEQDPP TLHRHF--DD VRLGIDQTRL EQILVNLLAN ALDAMSGQA- DRQLWLEGRR
PP1402 PMGLRERLDL ATVVDQALHL LEARLRNEEV EVALYLA-RP AWVRGDAIRL EQVLINLLHN ALDAMLDKR- YKRLEIRIEP
PA5512 AGGLRERIEL GHVVEQAQQL LLPRIRAERV EIEQALL-WP AWVMGDAIRL EQVLVNLLRN ALDAVAGQE- RPWIRLALQR
Consensus ..gsr#r..L a.v!#qAl.l L..R.r.e.v tl...l..dp aqvmg#aiRL EQ!L!NLL.N AL#Ama.qa. ..rlelr...
** G1-Box* *F-Box ** *G2-Box*
RhlDctB DAGMVTVTVA DNGPGIPTEI RKGLFTPFNT SK--ESGLGL GLVISKDIVG DYGGRMDVAS -DSGGTRFIV QLRKA
AauS QGDKYRLQVR DNGHGIDPEA RKHLFEPFFT TKPGEHGLGL GLTLSASLAA AAKGSLNVEH PVTGGTAFVL ALPLVSPPSE
PA1336 EEERYVLRVR DNGPGIPPAA RVHLFEPFFT TKPGEHGLGL GLTLSASLAT AAGGSLSVQH PESGGTAFEL SLSLV-PDSP
PP1402 DEDLWRLSVL DSGGGIAEAD LAKVFDPFFT TKPVGEGLGL GLAISYGIVH EAGGQLRAEN -LPGGARLSL TLPRDLEPVC
PA5512 LGGDWVLSVA DNGGGIPAEH LGSVFDPFFT TKPVGEGLGL GLAVSYGIVH ELGGRLQVAN -AEAGAVFSL ILPAAPDDTS
Consensus d.g.w.lsVa DnGpGIp.e. rk.lFdPFfT tKpveeGLGL GLaiSygivh eaGGr$.van ..sgGtrfsl .Lp.a.....
RhlDctB
AauS SAEHP
PA1336 TASPAR
PP1402
PA5512 VTT
Consensus .....



Figures 5.5 and 5.6: Sequence alignment of *P. putida* KT2440 AauS and AauR with related proteins (see text). RhlDctB/D – DctB/DctD from *R. leguminosarum*; PP – protein sequences predicted from the *P. putida* KT2440 genome; PA – protein sequences predicted from the *P. aeruginosa* PAO1 genome

Nishijyo *et al*. (2001) have recently described a two-component system (CbrA-CbrB) in *P. aeruginosa* that is required for the utilization of several amino acids such as arginine, histidine and proline. Although there are several other two-component systems which, by homology, are thought to be involved in amino acid metabolism, none of these has been characterized in detail so far.

5.8 Expression and purification of AauR

In the present work, the response regulator AauR from *P. putida* KT2440 was overexpressed in *E. coli* as a fusion protein with GST and purified by a standard protocol involving affinity chromatography. The GST part of the fusion protein binds to immobilized glutathione and the impurities are removed by washing with binding buffer. The fusion protein is then eluted under mild, non-denaturing conditions which are conducive to preserve the protein in a native form. As the GST tag may interfere with protein folding, it can be removed in simple way by proteolytic cleavage. The preparation obtained in this way is already sufficiently pure for functional studies such as DNA binding experiments while additional purification steps will be necessary before a detailed structural characterization of the protein can be started.

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7. Summary

Pseudomonas putida KT2440 has the ability to utilize a wide range of amino acids as carbon and nitrogen sources. Rapid growth of this organism is supported by the acidic amino acids (Asp and Glu) and their amides (Asn and Gln) when supplied as sole source of carbon and nitrogen, or in combination with other carbon and nitrogen sources such as glucose and NH_4^+. The acidic amino acids and their amides specifically induce the expression of certain nitrogen- metabolizing genes such as the *ansB* gene which encodes periplasmic glutaminase/asparaginase (PGA). Glucose, a preferred carbon source and intermediates of the citric acid cycle excerted a carbon catabolite repressing effect on the expression of PGA. In order to check the role of PGA in the assimilation of amino acids, a *ansB* knock-out mutant was constructed by homologous recombination. This *ansB*- strain failed to grow in glutamine (Gln) and also did not take up Gln from the medium. Thus PGA seems to have an important role in the utilization of Gln.

A proteomics study based on two-dimensional gel electrophoresis revealed that during growth of *P. putida* KT2440 in Glu containing medium a set of at least 9 major proteins were up-regulated in a coordinate fashion, whereas 4 other proteins were specifically induced during growth in NH_4^+/Glucose. Most of the identified proteins have some role in the uptake and utilization of amino acids. Based up on their assigned functions and genetic organization of the respective genes, we propose that they form a regulon involved in the metabolism of amino acids. Most of the Glu-induced proteins were subject to carbon catabolite repression by fumarate, whereas the proteins up-regulated by NH_4^+/Glucose were unaffected. In addition, most of the Glu-responsive proteins seem to depend on the alternate sigma factor σ^{54} for expression, as indicated by their greatly reduced expression profile in an otherwise isogenic *rpoN*- mutant. A semi-quantitative RT-PCR study showed that the effect of Glu observed on the proteomics level is taking place at the level of transcription.

By transposon mutagenesis it was found that the expression of *ansB* depends on a functional *gltB* gene which encodes the major subunit of glutamate synthase (GOGAT). Transconjugants with inactivated *gltB* and a targeted *gltB* disruption mutants were unable to utilize amino acids as sole source of carbon and nitrogen, whereas amino acids in

combination with glucose supported rapid growth of all mutants. In contrast to wild-type *P. putida* KT2440, transconjugants with insertions in the *gltB* gene were nonmotile and failed to show any chemotactic response towards Asn, Asp, Gln or Glu. Thus, GOGAT activity also affects the expression of motility as well as the chemotactic genes. In contrast to the wild-type strain, the GOGAT-negative strains also failed to survive for longer times in nitrogen-depleted medium. The *gltB*- strain accumulated large amounts of both glutamine and glutamate within the cells while the wild type did not. The central roles of GOGAT in nitrogen metabolism and the metabolic basis of the *gltB*- phenotype are discussed.

Finally, a novel two-component system (*aau*) was identified which seems to be involved in the utilization of acidic amino acids. Disruption mutants defective in the response regulator (AauR) and the sensor kinase component (AauS), respectively, were constructed and the resulting phenotype analyzed. Growth of both mutants was severely impaired in glutamate and glutamine-containing media. By contrast, both strains grew at normal rates when succinate was supplied in addition to amino acids. This finding indicate that the *aau* system is related to, but not identical with the *dct* two-component system which is involved in the utilization of succinate by rhizobia. 2D electrophoresis experiments showed that more than 20 proteins were no longer induced by Glu in the AauR-negative mutant. This suggests that the aau system has a central role in the regulation of glutamate metabolism. The AauR protein was overexpressed in *E. coli* as a fusion protein with GST. After purification of this protein, AauR was released by proteolytic cleavage and is now available for functional studies.

7. Zusammenfassung

Pseudomonas putida KT2440 ist in der Lage, Aminosäuren als einzige Kohlenstoff-und Stickstoffquelle zu nutzen. Die sauren Aminosäuren (Asp, Glu) und ihre Amide (Asn, Gln) allein oder im Kombination mit weiteren C- und N-Quellen führen zu raschem Wachstum von *P. putida* KT2440. Diese Aminosäuren induzieren auch die Expression verschiedener Gene aus dem Stickstoff-Stoffwechsel u. a. das *ansB*-Gen, das für eine periplasmatisch lokalisierte Glutaminase/Asparaginase (PGA) kodiert. Glucose, eine gute C-Quelle, und Intermediate des Citrat-Zyklus reprimieren die *ansB*-Expression im Sinne einer Katabolit-Repression. Um die Rolle der PGA bei der Aminosäure-Assimilation zu untersuchen, wurde eine knock-out Mutante konstruiert und durch homologe Rekombination in das KT2440-Genom eingeführt. Diese *ansB*- Mutante war nicht mehr in der Lage, auf Glutamin (Gln) als einziger C- und N-Quelle zu wachsen oder Gln aus dem Medium aufzunehmen. Dies zeigt, dass die PGA in der Verwertung von Gln eine zentrale Rolle spielt.

Ein Proteomics-Ansatz auf der Basis der 2D-Gelelektrophorese zeigte, dass beim Wachstum von *P. putida* KT2440 auf Glutamat (Glu) mindestens 9 Proteine in koordinierter Weise verstärkt exprimiert wurden, während einige andere Proteine beim Wachstum auf NH_4^+-Glucose induziert wurden. Die meisten der durch Glu induzierten Gene scheinen an der Aufnahme und am Stoffwechsel von Aminosäuren beteiligt zu sein und bilden möglicherweise ein Regulon, d. h. einen durch ein und dasselbe Regulatorprotein induzierten Satz funktionell verwandter Gene. Die Mehrzahl der durch Glu induzierten Gene zeigte auch Katabolit-Repression durch Fumarat, während die auf NH_4^+-Glucose verstärkt exprimierten Gene nicht auf Fumarat ansprachen. Außerdem scheinen alle Glu-aktivierten Gene zur Expression den alternativen Sigma-Faktor σ^{54} zu benötigen, da Glu in einer sonst isogenen σ^{54}-negativen Mutante keine induzierenden Eigenschaften zeigte. Durch semiquantitative RT-PCR wurde nachgewiesen, dass die im Proteom beobachteten Effekte von Glu auf Transkriptionsebene stattfinden.

Durch Transposon-Mutagenese wurde weiterhin gezeigt, dass die Expression von *ansB* vom intakten Zustand des *gltB*-Gens abhängt, das für die große Untereinheit der Glutamat-Synthase (GOGAT) kodiert. Einige Transposon-Mutanten mit Insertionen in *gltB* sowie eine gezielt hergestellte *gltB*-knockout-Mutante waren nicht mehr in der Lage,

Aminosäuren als einzige C- und N-Quelle zu nutzen, während ihr Wachstum normal war, wenn diese Aminosäuren zusammen mit Glucose angeboten wurden. Transkonjuganten mit Insertionen im *gltB*-Gen waren – im Gegensatz zum Wildtyp - außerdem unbeweglich und nicht mehr zur Chemotaxis gegenüber Asn, Asp, Glu und Gln befähigt. Dies zeigt, dass die Aktvität der Glutamat-Synthase indirekt auch Chemotaxis und Motilität von *P. putida* KT2440 kontrolliert. Die erwähnten Mutanten waren schließlich auch nicht mehr in der Lage, in Abwesenheit einer Stickstoffquelle längere Zeit zu überleben. Die intrazellulären Spiegel von Gln und Glu waren jedoch im Vergleich zum Wildtyp stark erhöht. Die Bedeutung dieser Befunde im Hinblick auf die Rolle der GOGAT im Stickstoffstoffwechsel wird diskutiert.

In der vorliegenden Arbeit wurde schließlich ein zuvor nicht beschriebenes Zweikomponentensstem (*aau*) identifiziert, das bei der Regulation des Stoffwechsels der sauren Aminosäuren eine zentrale Rolle zu spielen scheint. Die für die beiden Komponenenten des Systems kodierenden Gene (*aauR* und *aauS*) wurden durch homologe Rekombination inaktiviert. Die Transkonjuganten zeigten stark vermindertes Wachstum auf Glu und Gln, während die Verwertung von Asn und Asp weniger beeinträchtigt war. Auch das Wachstum auf Succinat als C-Quelle war normal. Dies zeigt, dass *aau* mit dem gut untersuchten *dct*-System, das die Aufnahme von Dicarbonsäuren in Rhizobien reguliert, zwar verwandt aber nicht identisch ist. Die Bedeutung des *aau*-Systems geht auch aus der Tatsache hervor, dass in einer *aauR*-negativen Mutante im Vergleich zum Wildtyp mehr als 20 Proteine nicht mehr durch Glu induzierbar waren. Das AauR-Protein, der Reponse-Regulator des Systems, wurde als Fusionsprotein mit GST in *E. coli* überexprimiert. Nach dessen Reinigung durch Affinitätschromatographie wurde AauR proteolytisch in weitgehend homogener Form freigesetzt und steht damit für funktionelle Untersuchungen zur Verfügung.

8. Appendix

8.1 Abbreviations

8.1.1 Amino acids

Ala (A)	**A**lanine
Arg (R)	**A**rginine
Asn (N)	**A**sparagine
Asp (D)	**A**spartate
Cys (C)	**C**ysteine
Gln (Q)	**G**lutamine
Glu (E)	**G**lutamic acid
Gly (G)	**G**lycine
His (H)	**H**istidine
Ile (I)	**I**soleucine
Leu (L)	**L**eucine
Lys (K)	**L**ysine
Met (M)	**M**ethionine
Phe (F)	**P**henylalanine
Pro (P)	**P**roline
Ser (S)	**S**erine
Thr (T)	**T**hreonine
Tyr (Y)	**T**yrosine
Val (V)	**V**aline

8.1.2 Antibiotics

Amp	**A**mpicillin
Cb	**C**arbenicillin
Cmp	**C**hloramphenicol
Gm	**G**entamycin
Kan	**K**anamycin
Tet	**T**etracycline

8.1.3 Enzymes

AR	**A**denylyl **r**emoving
ATase	**A**denylyl**t**ransferase
CIP	**C**alf **i**ntestinal alkaline **p**hosphatase
GDH	**G**lutamate **d**ehydrogenase
GOGAT	**G**lutamate **s**ynthase
GS	**G**lutamine **s**ynthetase
HPK	**H**istidine **p**rotein **k**inase
PGA	**P**eriplasmic **g**lutaminase/**a**sparaginase
UR	**U**ridylyl **r**emoving
UT	**U**ridylyl**t**ransferase

8.1.4 Microorganisms

A. brasilense	*Azospirillum brasilense*
B. subtilis	*Bacillus subtilis*
E. chrysanthemi	*Erwinia chrysanthemi*
E. coli	*Escherichia coli*
K. pneumoniae	*Klebsiella pneumoniae*
P. aeruginosa (PA)	*Pseudomonas aeruginosa*
P. putida (Pp)	*Pseudomonas putida*
R. meliloti	*Rhizobium meliloti*
S. aureus	*Staphylococcus aureus*
S. meliloti	*Sinorhizobium meliloti*
S. typhimurium	*Salmonella typhimurium*

8.1.5 General

α-KG	**A**lpha-**k**eto**g**lutarate
2-D	**T**wo-**d**imensional
A	**A**bsorption
Å	Ångstrom [10^{-8} cm]
aau	**A**cidic **a**mino acid **u**tilization
aauR	Gene encoding response regulator AauR

aauS	Gene encoding sensor kinase AauS
ABC	**A**TP **b**inding **c**assette
ADP	**A**denine **di**p**h**osphate
AMP	**A**denine **m**ono**p**hosphate
Amt	**A**mmonium **t**ransport system
ansB	Gene encoding periplasmic glutaminase/asparaginase
APS	**A**mmonium **p**ersulfate
ATP	**A**denine **t**riphosphate
bp	**B**ase **p**air
cAMP	**C**yclic **a**denine **m**ono**p**hosphate
CCR	**C**arbon **c**atabolite **r**epression
cDNA	**C**omplementary **D**NA
CIP	**C**alf **i**ntestinal **p**hosphatase
crc	**C**arbon **c**atabolite regulatory protein
Da	**D**alton
DABA	**Di**a**m**ino**b**utyric **a**cid transaminase
dct	**Di**c**a**rboxylic **t**ransport system
DNA	**D**eoxy**n**ucleic **A**cid
DTT	**Di**t**h**io**t**hreitol
EDTA	**E**thylene**d**iamine**t**etracetic **a**cid
fnr	**F**umarate **n**itrate **r**eductase
Fum	**F**umarate
g	**R**elative centrifugal force
gltB	Gene encoding major subunit of glutamate synthase
GSP	**G**ene **s**pecific **p**rimer
GST	**G**lutathione **S**-**t**ransferase
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
IEF	**I**so**e**lectric **f**ocussing
IEP_{calc}	**C**alculated **i**so**e**lectric **p**oint
IHF	**I**ntegration **h**ost **f**actor
IPGs	**I**mmobilized **p**H **g**radient **s**trips
IPTG	**I**so**p**ropyl β-D **t**hio**g**alactoside

IR	**I**nverted repeats
IS	**I**nsertion **s**equences
kb	**K**ilo**b**ase
kDa	**K**ilo**d**alton
kV	**K**ilo**v**olt
L-AHA	**L**-**a**spartic acid β-**h**ydroxamate
LB medium	**L**uria-**B**ertani medium
LPS	**L**ipo**p**oly**s**accharide
M	**M**olar
Maldi-PSD	**M**atrix **a**ssisted **l**aser **d**esorption **i**onization-**p**ost **s**ource **d**ecay
Maldi-TOF	**M**atrix **a**ssisted **l**aser **d**esorption **i**onization **T**ime-**o**f-**f**light
MCP	**M**ethyl accepting **c**hemotactic **p**rotein
mg	**m**ili**g**ram
min	**M**inute
MOPS	3-[N-**M**orpholino]-**p**ropane **s**ulphonic acid
MS	**M**ass **s**pectrometry
N	**N**itrogen source
Na_2CO_3	**S**odium **c**arbonate
$NaIO_4$	**S**odium per**i**odide
nt	**N**ucleo**t**ide
Ntr	**N**itrogen **r**egulatory system
OAA	**O**xalo**a**cetic **a**cid
^oC	**d**egree **c**entigrade
OD	**O**ptical **d**ensity
ORF	**O**pen **r**eading **f**rame
OX	**O**xoglutarate
PCR	**P**olymerase **c**hain **r**eaction
PGPR	**P**lant **g**rowth **p**romoting **r**hizobacteria
Pi	**I**norganic **p**hosphate
pI	**I**soelectric **p**oint
PITC	**P**henyl**i**so**t**hio**c**ynate
PMSF	**P**henyl**m**ethane**s**ulfonyl **f**luoride
RNA	**R**ibo**n**ucleic **a**cid

RP	**R**everse **p**hase
rpm	**R**evolutions **p**er **m**inute
rpoN	gene encoding σ^{54} factor
RR	**R**esponse **r**egulator
RT-PCR	**R**everse **t**ranscriptase **p**olymerase **c**hain **r**eaction
σ	**S**igma factor
SDS	**S**odium **d**odecyl **s**ulfate
SDS-PAGE	**S**odium **d**odecyl **s**ulfate-**p**oly**a**crylamide **g**el **e**lectrophoresis
Sucr	**S**ucrose
TBE	**T**ris **b**orate-**E**DTA buffer
TCA	**T**ri**c**arboxylic **a**cid
TE	**T**ris-**E**DTA buffer
TEMED	N' N' N' N'-**T**etra**m**ethylen**d**iamine
TFA	**T**ri**f**luoro**a**cetic acid
Tn	Transposon
Tris	Tris(hydroxymethyl)-aminomethane
U	Activity unit ($\mu mol.min^{-1}$)
UTP	**U**ridine **t**ri**p**hosphate
UV	**U**ltra **v**oilet
V	**V**olt
v/v	**V**olume by **v**olume
W	**W**att
w/v	**W**eight by **v**olume
WT	**W**ild **t**ype

8.2 Peptide sequences

Amino acid sequences of proteins identified by MALDI-PSD-MS as compared to the respective gene sequences derived from the *P. putida* KT2440 genome. Internal peptide sequences identified by MS data are highlighted and underlined

Transcription termination factor (Pp1)

MNLTELKQKPITDLLEMAEQMGIENMARSRKQDVIFALLKKHAK**SGEEISGDGVLEILQD**
GFGFLRSADASYLAGPDDIYVSPSQIRRFNLRTGDTIVGK**IRPPKEGERYFALLKVDTIN**
FDRPENAKNKILFENLTPLFPNKRLK**MEAGNGSTEDLTGRVIDLCAPIGK**GQR**GLIVAPP**
KAGKTIMLQNIAANITRNNPECHLIVLLIDERPEEVTEMQR**TVRGEVVASTFDEPPTRHV**
QVAEMVIEKAKRLVEHK**KDVVILLDSITR**LARAYNTVIPSSGK**VLTGGVDAHALEKPKRF**
FGAARNIEEGGSLTIIATALVETGSKMDEVIYEEFKGTGNMELPLDRRIAEK**RVFPAINI**
NRSGTR**REELLTADDELQRMWILRKLLHPMDEIAAIEFLVDK**LKQTK**TNDEFFLSMKRK**

ABC transporter ATP-binding protein (Pp5)

MAGQAPAKKDLRMISIKNVNKWYGDFQVLTDCSTEVKKGEVVVVCGPSGSGKSTLIKCVN
ALEPFQKGDIVVDGTSIADPKTNLPKLRSRVGMVFQHFELFPHLTITENLTIAQRKVLGR
SEAEATKKGLALLDRVGLSAH**AKKHPGQLSGGQQQR**VAIARALAMDPIVMLFDEPTSALD
PEMVSEVLDVMVQLAQEGMTMMCVTHEMGFARKVANRVIFMDKGSIIEDCTKEEFFGDQS
ARDQRTQHLLSKILQH

Aspartase ammonia lyase (Pp6)

MIYIMSSAASFRVEKDLLGTLEVPADAYYGIQTLRAANNFH**LSGVPLSHYPK**LVVALAMV
KQAAADANRELGHLSDAKHAAISAACARLIKGDFHDQFVVDMIQGGAGTSTNMNANEVIA
NVALEAMGHQK**GEYQYLHPNNDVNMAQSTNDAYPTAIR**LGLLLGHDALLASLDSLIQAFA
AKGKEFDHVLKMGR**TQLQDAVPMTLGQEFR**AFATTMTEDLQRLRSLAPELLTEINLGGTA
IGTGINADPGYQALAVQRLATISGHPLVPAADLIEATSDMGAFVLFSGMLKRTAVKLSK**I**
CNDLRLLSSGPRTGINEINLPARQPGSSIMPGKVNPVIPEAVNQVAFAIMGNDLALTVAA
EGGQLQLNVMEPLIAYK**IFDSIR**LLQR**AMDMLREHCIVGITANEQRCRELVEHSIGLVTA**
LNPYIGYENATRIARVALESGR**GVLELVR**EEKLLDDAMLDDILRPENMIAPRLVPLKA

Outer membrane protein (Pp8)

MRVMKWSMIALAVSAGTSQLAMASAQDESKGFIEDSKLSVKTRMLYFSRDFRNNEGGQSR
REETGLGFVGTFESGFTQGTVGVGVDAIGMLGLKLDSGKGRAGTGLFPTGSDGRSQDDYS
KGGGAVKFRISDTVLK**VGDQFTALPVFATDDSR**LLPEIAQGTLITSNEIEGLTLHAGHFT
SLTAQEQTNRDSFGLKEANVVGGTYAFTDNLSTSLYYSKVEDYWRKYYANVNWALPISDN
QGLVFDFNIYDTKSEGSAEYRAFDGDKLDNRAFSLSGAYNIGAHTFTLAYQKVTGDGDYG
YGIDGGGTIFLANSVARSDFNAEDEKSWQARYDLNFAEYGIPGLTFMTRYVRGSDANVAG
TSNGKEWERDVDIKYVLQEGPAKDLSFRVRQATYRSSDGVYYDSPSIDELRLIVEYPLSI
L

2,4-Diaminobutyrate 2-oxoglutarate transaminase (Pp10)

MPQPLYEFTDSPLLQRQQQQESNARSYPRRIPLALRRARGIHVEDVEGRQFIDCLAGAGT
LALGHNHPVVVEAIQRVLADELPLHTLDLTTPVKDRFVQDLFGILPEALRREAKVQFCGP
TGTDAVEAALKLVRTATGRSTVLAFQGAYHGMSQGALNLMGSHGPKQPLGALLGNGVQFM
PYPYDYR**CPFGLGGEAGVK**ANLHYLENLLLDPESGVPLPAAVILEVVQGEGGVVPADIEW
LKGVR**RITEQAGVALIVDEIQSGFARTGRMFAFEHAGIVPDVVTLSKAIGGSLPLAVVVY
R**DWLDTWKPGAHAGTFRGNQMAMAAGSAVINYLVEHRLAEHAEAMGQRLRGHLQRLQR**DY
PQLGDIRGR**GLMLGVELVDPQGQPDALGHPPANRDLAPKVQRECLK**RGLILELGGR**HGAV
VRFLPPLIISAEQIDEVAQRFSAAVAAAVGSV

Sugar ABC transporter, sugar-binding protein (Pp12)

MNSTLRLAAAISFASLIPLGAQAADAKGSVEVVHWWTSGGEKAAVDVLKAQVEKDGFIWK
DGAVAGGGGATAMTVL**KSRAVAGNPPGVAQIK**GPDIQDWAATGLLDADVLKDVAKEGKWD
SLLDKKVADTVKYDGDYVAVPVNIHRINWLWINPEVFKKAGIDKAPTTLDEFYAAADKLK
AAGFIPLAHGGQPWQDSTVFESVVLSVMGVDGY**KKALVDLDSATLTGPQMVK**ALTEL**KKV
ATYMDPDGKGQDWNLEAAK**VING**KAGMQIMGDWAKSEWTLAKK**TAGKDYQCVPFPGTDKS
FLYNIDSLVVF**KQNNAGTSAGQQDIARKVLGEDFQK**VFSINKGSIPVRNDMLADMGKYGF
DACAQTSAKDFLADAKTGGLQPSMAHNMATTLAVQGAFFDVVTNYINDPKADPADAAKKL
AAAIKAAQ

8. 3 Curriculum vitae

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Marburg (Molecular Enzymology Group, Prof. Röhm)
"Regulation of Pseudomonas putida genes involved in the utilization of amino acids".

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8.5

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin zur Promotionsprüfung eingereichte Arbeit mit dem Titel „Regulation of *Pseudomonas putida* genes involved in the metabolism of acidic amino acids“ im Institut für Physiologische Chemie, Arbeitsgruppe Molekulare Enzymologie, unter der Leitung von Herrn Prof. Dr. Röhm ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angegebenen Hilfmittel benutz habe. Ich habe bisher an keinem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Teile der vorliegenden Arbeit wurden bereits wie folgt veröffentlicht:

Originalarbeiten

Sonawane A., Klöppner, U., Derst, C. and Röhm, K. H. (2003). Utilization of acidic amino acids and their amides by Pseudomonads: role of periplasmic glutaminase-asparaginase. Arch. Microbiol. 179: 151-159.

Sonawane A., Klöppner, U., Sven, H., Völker, U.and Röhm, K. H. (2003). Identification of *Pseudomonas* proteins coordinately induced by acidic amino acids and their amides: a two-dimensional electrophoresis study. Microbiology 149, 2909-2918.

Sonawane, A. and Röhm, K.H. (2003). A functional *gltB* gene is essential for utilization of acidic amino acids and expression of periplasmic glutaminase/asparaginase by *Pseudomonas putida* KT2440. Mol. Gen.Genet., in press.

Posterpräsentationen

American Society of Microbiology (ASM) Conference
18-24th May, 2003, Washington D.C.
Sonawane, A. and Röhm, K. H.: Identification in *Pseudomonas putida* KT2440 of a two-component system involved in the utilization of acidic amino acids

Pseudomonas 2003 Conference
5-10th September, 2003, Laval University, Quebec, Canada
Sonawane A. and Röhm, K. H.: Proteomics based identification of differentially expressed proteins in *Pseudomonas putida* KT2440 using two-dimensional electrophoresis

Marburg, den 6. November 2003

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