Identification of *Pseudomonas* proteins coordinately induced by acidic amino acids and their amides: a two-dimensional electrophoresis study

Avinash Sonawane, Ute Klöppner, Sven Hövel, Uwe Völker and Klaus-Heinrich Röhm

1Philips-University Marburg, Institute of Physiological Chemistry, D-35032 Marburg, Germany
2Philips-University Marburg, Department of Biology, Laboratory for Microbiology, D-35032, Marburg, Germany
3Max-Planck-Institute for Terrestrial Microbiology, D-35043 Marburg, Germany
4Ernst-Moritz-Arndt-University, Medical Faculty, Laboratory for Functional Genomics, D-17487 Greifswald, Germany

The acidic amino acids (Asp, Glu) and their amides (Asn, Gln) are excellent growth substrates for many pseudomonads. This paper presents proteomics data indicating that growth of *Pseudomonas fluorescens* ATCC 13525 and *Pseudomonas putida* KT2440 on these amino acids as sole source of carbon and nitrogen leads to the induction of a defined set of proteins. Using mass spectrometry and N-terminal sequencing, a number of these proteins were identified as enzymes and transporters involved in amino acid uptake and metabolism. Most of them depended on the alternative sigma factor $\sigma^{34}$ for expression and were subject to strong carbon catabolite repression by glucose and citrate cycle intermediates. For a subset of the identified proteins, the observed regulatory effects were independently confirmed by RT-PCR. The authors propose that the respective genes (together with others still to be identified) make up a regulon that mediates uptake and utilization of the abovementioned amino acids.

INTRODUCTION

Plant-growth-promoting rhizobacteria (PGPRs) are microorganisms with a potential to enhance crop yields. They can contribute to plant growth by biofertilization, by secretion of growth hormones or by the production of antibiotics that control pathogenic fungi and competing bacteria (Bloemberg & Lugtenberg, 2001). In recent years, several groups have initiated projects with the aim of elucidating the interactions between plant roots and rhizosphere microorganisms. In most of these studies, fluorescent pseudomonads have been used; these inhabit the rhizospheres of most crop plants. Although progress is still slow, it is now well established that root exudates play a central role in plant–PGPR interactions. Exudate components are thought to attract beneficial micro-organisms to the rhizosphere and also to promote their survival in this special environment. One approach to characterize the effects of exudate components on rhizobacteria is to search for bacterial genes that are differentially expressed in the rhizosphere, or in the presence of root exudates (van Overbeek & van Elsas, 1995; Bayliss *et al*., 1997). Although a number of such genes have been identified, the precise roles of most of them have remained elusive.

The main organic components of root exudates are sugars, various organic acids and a number of amino acids (Fan *et al*., 1997). 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. On the other hand, it was shown that root exudates can induce bacterial enzymes that are involved in the metabolism of amino acids such as proline (Vilchez *et al*., 2000a, b) or lysine (Espinosa-Urgel & Ramos, 2001).

The predominant amino acids in root exudates are the acidic amino acids aspartate (Asp) and glutamate (Glu) and their amides asparagine (Asn) and glutamine (Gln) (Barber & Gunn, 1974; Jones & Darrah, 1993; Jones & Darrah, 1993). Glu and Gln are key intermediates in nitrogen metabolism. In *E. coli* and other enterobacteria all nitrogen-containing compounds derive their nitrogen from Glu or Gln (Reitzer, 1996a, b).
Nevertheless, Glu and Gln are inferior to NH₄⁺ in supporting the growth of enteric bacteria. In pseudomonads the situation is different. We have recently shown that several strains of *Pseudomonas fluorescens* and *Pseudomonas putida* rapidly grow on acidic amino acids and their amides, even when supplied as the sole source of carbon and nitrogen (Sonawane et al., 2003). All of these amino acids strongly and specifically induce periplasmic glutaminase/asparaginase (PGA) (Hüser et al., 1999). On the other hand, PGA is subject to carbon catabolite repression by glucose and dicarboxylic acids such as succinate, fumarate and 2-oxoglutarate. A PGA knockout mutant was unable to utilize Gln whereas growth on Glu, Asn and Asp was unimpaired.

In order to examine whether the acidic amino acids and their amides, in addition to regulating PGA expression, induce a more general response in pseudomonads, we used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to compare gene expression in pseudomonads in the presence and absence of these amino acids.

### METHODS

**Bacterial strains and growth conditions.** *P. fluorescens* ATCC 15325 or *P. putida* KT2440 (Bagdasarian & Timmis, 1982) and an isogenic rpoN-null mutant of the latter strain (Köhler et al., 1989) were used for the experiments. The cells were cultivated with shaking at 30 °C in LB broth or in M9 minimal medium (Sambrook et al., 1989) supplemented with various carbon and nitrogen sources. To investigate the effect of nutrients on growth and enzyme activity, cultures were first pregrown overnight in M9 medium supplemented with NH₄Cl (19 mM) and glucose (22 mM). Then the cells were washed and aliquots transferred to fresh M9 medium supplemented with (a) NH₄⁺/glucose as above, (b) amino acids (10 mM) as the sole source of carbon and nitrogen, or (c) glutamate (10 mM) + alternative carbon sources (glucose, sucrose, fumarate, 2-oxoglutarate, 10 mM each).

**PGA assay.** Glutaminase/asparaginase (PGA) activities were measured with L-aspartic acid β-hydroxamate as the substrate (Derst et al., 1992). Briefly, 20 µl enzyme solution was added to 30 µl of 1 mM substrate in 50 mM MOPS, pH 7-0. After an incubation for 5–60 min at room temperature, the reaction was terminated and colour developed by adding 240–5–60 min at room temperature, the reaction was terminated and colour developed by adding 240 µl stop solution (1 M Na₂CO₃ containing 2%, w/v, 8-hydroxyquinoline in dimethyl sulfoxide and 1%, w/v, NaIO₄). After 5 min, the A₂₅₄ was measured in a Bio-Rad 3550-UV microplate reader. Protein concentrations were determined by the BCA method using bovine serum albumin as the standard. One unit of PGA activity is the amount of enzyme hydrolysing 1 mmol L-aspartic acid β-hydroxamate min⁻¹ under these conditions.

**Sample preparation for 2D gel electrophoresis.** Bacteria were usually harvested 4–6 h after transfer to fresh medium and collected by centrifugation. After washing with M9 salt solution, the pellet was resuspended in TE/PMSF buffer (10 mM Tris, 1 mM EDTA, 0-1 mM PMSF, pH 7-5) cooled on ice and disrupted by sonication (Sonopuls, Bandelin; 15 x 4 s). The homogenate was centrifuged for 10 min at 10000 r.p.m. and then twice for 30 min at 14000 r.p.m. Proteins were precipitated by the addition of 5 vols ice-cold acetone (analytical grade), incubated overnight at −20 °C, and then collected by centrifugation at 0 °C.

**2D gel electrophoresis.** For isoelectric focussing (IEF), proteins were solubilized in a rehydration solution containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 28 mM DTT, 1:3% (v/v) Pharmalytes, pH 3–10 and bromophenol blue. After rehydration for 24 h under low-viscosity paraffin oil, Immobiline DryStrips (IPG strips 18 cm NL; Amersham Biosciences) covering a pH range of 3–10 were subjected to isoelectric focussing with the following voltage/time profile: linear increase from 0 to 500 V for 1000 V h, 500 V for 2000 V h, linear increase from 500 to 3500 V for 10 000 V h and a final phase of 3500 V for 35 000 V h up to a total of 48 000 V h. After IEF, the individual strips were consecutively incubated in equilibration solutions A and B, each for 15 min (50 mM Tris/HCl, pH 6-8, 6 M urea, 30% (v/v) glycerol, 4% (w/v) SDS, with 5-7 mg DTT ml⁻¹ (solution A); or 45 mg iodoacetamide ml⁻¹ instead of DTT (solution B)). In the second dimension, proteins were separated on 12-5% SDS-polyacrylamide gels with the Investigator System (Perkin Elmer Life Sciences) at 2 W per gel. For routine use proteins were visualized by silver staining. Gels intended for MALDI-TOF analysis were stained with PhastGel Coomassie R350 according to the manufacturer’s instructions (Amersham BioSciences). Scanned images were analysed with the Melanie3 software package (Bio-Rad) to facilitate identification of differentially expressed spots. For quantitative densitometry the BandLeader program (Magnitec) was used. Separate gels of each condition were analysed and only spots were labelled that displayed the same pattern (i.e. up- or down-regulation) in all replicates.

**Protein identification by N-terminal sequencing.** Spots of interest were transferred to a PVDF membrane by electroblotting using the semi-dry method (Kyhse-Anderson, 1984). Sequencing was performed by Dr D. Linder, Giessen.

**Protein identification by peptide mass fingerprinting.** Protein spots were excised from stained 2D gels. Pooled extracts from three to nine gels were destained and digested with trypsin (Promega). Peptides were extracted according to Otto et al. (1996). They were purified with C18 tips according to the manufacturer’s instructions (Millipore) and eluted with 75% acetonitrile/2% trifluoroacetic acid (v/v). Peptide solutions were mixed with an equal volume of saturated z-cyano-3-hydroxycinnamic acid solution in 50% acetonitrile/0-1% trifluoroacetic acid (v/v) and applied to a sample template for a MALDI-TOF mass spectrometer. Peptide masses were determined in the positive ion reflector mode in a Voyager DE RP mass spectrometer (PerSeptive Biosystems) with internal calibration. Mass accuracy was usually in the range between 10 and 50 p.p.m. Peptide mass fingerprints were compared to databases using the program MS-Fit (http://prospector.ucsf.edu). Spots that could not be identified by the above method were further analysed by MALDI-Post Source Decay (PSD) sequencing (Protagen AG, Bochum, Germany).

**Semi-quantitative RT-PCR.** RNA was isolated from mid-exponential-phase cells using the RNeasy minikit (Qiagen). Residual DNA was removed by digestion for 30 min at 37 °C with 1 U µl⁻¹ RNase-free DNase (Promega). The reaction was stopped by adding 1 µl RQ1 DNase stop solution and incubated at 65 °C for 10 min. The reverse transcription was carried out using 1 µg RNA in a 20 µl reaction mixture containing 1 X RT buffer, 0.1 mM dNTP, 50 pmol oligo(dT) primer, 5 mM MgCl₂, 20 mM DTT, 2 U RNaseOUT Recombinant RNase inhibitor, 0.25 U SUPERSCRIPT II RT (Invitrogen). PCR was carried out in 50 µl reaction mixtures, containing 2 µl of the RT reaction as template for Pfu Turbo DNA polymerase (0-5 U, Strategene), 100 pmol of each primer (Table 1), 0-2 mM dNTP, and amplified for 26 cycles. The PCR sequence used was 94 °C, 60 s; 54 °C, 30 s; 72 °C, 4 min and 72 °C, 5 min. cDNA-specific primers (listed in Table 1) were derived from the *P. putida* KT2440 genome (Nelson et al., 2002). Ten micro litres of RT-PCR products was then subjected to electrophoresis in a 1-5% agarose gel and visualized by staining with ethidium bromide.
RESULTS

Selection of strains

In a previous study we addressed the question whether amino acids in root exudates are involved in the communication between root-colonizing pseudomonads and their host plants. We compared type strains of *P. fluorescens* and *P. putida* and a number of root-colonizing pseudomonads as to their ability to utilize amino acids as sources of carbon and/or nitrogen (Sonawane *et al*., 2003). Extracts from *P. fluorescens* ATCC 13525 were then used to study proteins differentially expressed in the presence of amino acids. While these experiments were in progress, the full genome sequence of the efficient root colonizer *P. putida* KT2440 became available (Nelson *et al*., 2002). For this reason *P. putida* KT2440 was selected for further 2D-PAGE experiments.

Regulation of PGA expression by amino acids and alternative carbon sources

As reported previously, the expression of periplasmic glutaminase/asparaginase (PGA) in pseudomonads is strongly and specifically enhanced by acidic amino acids and their amides, while good carbon sources such as glucose or tricarboxylic acid cycle intermediates repress PGA production (Hüser *et al*., 1999). Surprisingly, in *P. fluorescens* ATCC 13525, Asp and Glu rather than Asn and Gln were found to be the actual inducers, while in *P. putida* KT2440 the time-courses of PGA induction by Asn and Asp on the one hand, or Gln and Glu on the other, were almost the same (Sonawane *et al*., 2003).

As in *P. fluorescens* ATCC 13525, the expression of PGA in *P. putida* KT2440 is subject to carbon catabolite repression by good carbon sources. Fig. 1 shows the effects of sugars (glucose, sucrose) and intermediates of the citric acid cycle (2-oxoglutarate, fumarate) on PGA expression. Glucose, fumarate and 2-oxoglutarate almost completely prevented PGA induction by Glu, while sucrose, which is not metabolized by *P. putida* KT2440, was much less effective as a repressor. As described elsewhere (Sonawane *et al*., 2003), PGA induction by Asn and Asp is delayed as compared to that by Glu and Gln. Therefore, full PGA induction by Asp or Asn was only seen in samples taken after 24 h.

Protein production during growth of *P. fluorescens* ATCC 13525 on amino acids

In order to identify further proteins differentially expressed in the presence of acidic amino acids and/or their amides, we

| Table 1. Oligonucleotides used for RT-PCR experiments in this study (for, forward; rev, reverse) |
|---|---|---|
| **Protein** | **Gene** | **Primers** |
| ABC transporter ATP-binding protein | ? | for: | rev: |
| Aspartase | aspA | for: | rev: |
| Porin D | oprD | for: | rev: |
| Fumarase C | fumC | for: | rev: |
performed 2D-PAGE experiments with cell extracts prepared at different times (2–6 h) after transfer from NH$_4^+$/glucose to media containing amino acids alone or in combination with fumarate. Fig. 2 shows such gels obtained with *P. fluorescens* ATCC 13525. The extracts were prepared 6 h after transfer from NH$_4^+$/glucose to the same medium (Fig. 2a) or to M9 containing L-Asn (Fig. 2b), L-Asp (Fig. 2c) or L-Asn in combination with 10 mM fumarate (Fig. 2d). Major spots upregulated under these conditions (Pf1–Pf6, Pf for *P. fluorescens*) are marked by squares and circles.

Two of the spots marked in Fig. 2 could be assigned to known proteins. By MALDI-TOF mass spectrometry, protein Pf1 was identified as PGA. N-terminal sequencing of Pf2 yielded the sequence AELTGTLKKINDXGT, which closely matches the N-termini of several periplasmic binding proteins associated with ABC transporters. They include

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**Fig. 2.** Two-dimensional electrophoresis maps (pH 4–9) of soluble proteins expressed by *P. fluorescens* ATCC 13525 during growth on M9 medium containing 5 mM NH$_4$Cl and 22 mM glucose (NH$_4^+$/Glc, a), M9 medium supplemented with 10 mM l-asparagine (Asn, b), with 10 mM l-aspartate (Asp, c), or with 10 mM Asn and 10 mM fumarate (Asp+Fum, d). Major protein spots upregulated in each case are marked by squares (a) or circles (b–d). Spots used as references in densitometric measurements (see Fig. 3) are marked by arrows in (a).
the P. aeruginosa PA1342 gene product, the product of the P. putida KT2440 gene PP1071 as well as putative Gln-binding proteins from Bacillus subtilis (Wu & Welker, 1991) and Rhodospirillum rubrum (glnA, [gi:2599566]).

Spots Pf1–Pf6 responded in a similar fashion to different carbon and nitrogen sources. Fig. 3 compares their relative densities estimated by quantitative densitometry. Although the extent of induction and carbon catabolite repressions varied to some extent, the same general pattern was seen with all spots examined. As compared to growth on NH₄⁺/glucose, their expression was strongly upregulated by Asn and Asp, while the effect of Asn was markedly reduced by fumarate.

**Fig. 3.** Differential protein expression during growth of P. fluorescens ATCC 13525 on different media: M9 minimal medium supplemented with 5 mM NH₄Cl and 22 mM glucose (NH₄⁺/Glc), with 10 mM asparagine (Asn), with 10 mM aspartate (Asp), or with 10 mM asparagine and 10 mM fumarate (Asn+Fum). Cell extracts containing equal amounts of protein were analysed by 2D gel electrophoresis. Spots Pf1–Pf6 (see Fig. 2) of each gel were quantified by densitometry. The figure shows integrated densities of these spots as estimated by the program BandLeader. They were calibrated by reference to spots not significantly affected by medium composition (marked by arrows in Fig. 2a).

**Identification of Glu-induced proteins in P. putida KT2440**

2D gels loaded with extracts from P. putida KT2440 yielded comparable results to those seen with P. aeruginosa. A group of 8–10 major spots were coordinately upregulated by Glu and repressed by good carbon sources. Other proteins were more strongly expressed in cells grown on NH₄⁺/glucose. In the experiment illustrated in Fig. 4, a total of 13 spots (Pp1–Pp13, Pp for P. putida) were selected, extracted from several gels, pooled, digested with trypsin and analysed by MALDI-TOF or MALDI-PSD mass spectroscopy. Except for Pp2 and Pp7, all spots could be identified and assigned to proteins deduced from the P. putida KT2440 genome. Table 2 summarizes the results of the mass spectrometric analysis. The table lists the names of the encoding genes, their known or putative functions, as well as isoelectric points, and molecular masses calculated from the genome data. In addition, the dependency of induction/repression on the presence of the alternative sigma factor RpoN and the

**Fig. 4.** Two-dimensional electrophoresis maps (pH 4–9) of soluble proteins expressed by P. putida KT2440 during growth on M9 medium containing 5 mM NH₄Cl and 22 mM glucose (NH₄⁺/Glc) and M9 medium supplemented with 10 mM glutamate (Glu) as the sole source of C and N. Selected proteins upregulated during growth on Glu (Pp1–Pp9) and NH₄⁺/Glc (Pp10–Pp13) are marked by circles.
influence of carbon catabolite repression elicited by the presence of fumarate in the growth medium are indicated.

**RT-PCR analysis of Glu-induced gene expression in P. putida KT2440**

To ascertain whether the effects of Glu detected at the protein level are indeed taking place at the level of transcription, we used RT-PCR to examine the levels of mRNA for several of the proteins differentially expressed in the absence or presence of Glu. Total RNA was isolated from cells grown on NH$_4^+$/Glc or Glu and transcribed into cDNA, which was then amplified by PCR. Care was taken to use equal amounts of cells (based on protein content) and identical reaction conditions in PCR for all samples. The primers used were derived from the KT2440 genome data. The observed effects of Glu on the amounts of RT-PCR amplificates (Fig. 5) showed the same general pattern as the changes of the respective spot densities in 2D-PAGE. Glu-responsive proteins previously identified in 2D gels (cf. Table 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 were readily detected during growth on Glu. The mRNAs for PGA (Pp3), the outer-membrane porin D (Pp8) and the Rho

<table>
<thead>
<tr>
<th>Spot</th>
<th>Identified as</th>
<th>Locus</th>
<th>pI,calc</th>
<th>Mass,calc (kDa)</th>
<th>Expressed in RpoN$^-$ strain</th>
<th>Repressed by fumarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf1</td>
<td>Glutaminase/asparaginase (PGA)</td>
<td>ansB</td>
<td>6-6</td>
<td>36.2±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pf2</td>
<td>ABC transporter amino-acid-binding protein</td>
<td>Seq</td>
<td>7-0</td>
<td>30.7±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pp1</td>
<td>Transcription termination factor Rho</td>
<td>rho (PP5214)</td>
<td>7-7</td>
<td>47.0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pp2</td>
<td>Not identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp3, Pp4</td>
<td>Glutaminase/asparaginase (PGA)</td>
<td>ansB (PP2453)</td>
<td>6-6</td>
<td>36.1±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pp5</td>
<td>ABC transporter ATP-binding protein</td>
<td>? (PP1068)</td>
<td>8-2</td>
<td>28.1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pp6</td>
<td>Aspartate ammonia-lyase (aspartase)</td>
<td>aspA (PP5338)</td>
<td>5-7</td>
<td>51.5</td>
<td>+</td>
<td>(-)</td>
</tr>
<tr>
<td>Pp7</td>
<td>Not identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp8</td>
<td>Outer-membrane porin D</td>
<td>oprD (PP 1206)</td>
<td>4-8</td>
<td>46.1±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pp9</td>
<td>Carboxyphosphonooenolpyruvate phosphomutase (putative)</td>
<td></td>
<td></td>
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*PM (n), identified from peptide masses (n=number of peptides). PSD (n), identified by MALDI-PDS (n=number of matching peptides). Seq, identified by N-terminal sequencing.

†Mass calculated without leader peptide.

**Fig. 5.** RT-PCR analysis of gene expression in P. putida KT2440. Total RNA was extracted from cells grown on M9 medium containing 5 mM NH$_4$Cl and 22 mM glucose (NH$_4^$/Glc) and M9 medium supplemented with 10 mM glutamate (Glu) as the sole source of C and N. RT-PCR of the genes indicated was carried out as described in Methods. See Table 1 for identities of proteins.
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 suppressed by Glu. However, as we used only one pair of primers per gene and did not include a Glu-independent control gene in the RT-PCR experiments, the results shown in Fig. 5 are still qualitative rather than quantitative.

Many of the Glu-inducible genes require $\sigma^{54}$ for expression

$\text{rpoN}$ encodes the alternative sigma factor $\sigma^{54}$ ($\sigma^N$) which is required for the expression of genes involved in the utilization of alternative nitrogen and carbon sources as well as diverse other functions (Merrick, 1993). Therefore, a comparative proteome analysis of the wild-type strain KT2440 (Fig. 6a) and its isogenic $\text{rpoN}$ mutant (Fig. 6b) was performed to investigate the $\text{rpoN}$-dependency of the changes in the protein profile discussed above. As reported by Köhler et al. (1989), an $\text{rpoN}$ mutant of strain KT2440 displayed a severe growth defect when amino acids were given as sole source of carbon and nitrogen. The data presented in Fig. 6 clearly show that the accumulation of Glu-responsive proteins was almost completely prevented in the strain lacking $\text{RpoN}$, supporting the notion that induction of their genes following transfer from $\text{NH}_4^+$/Glc medium to Glu medium requires $\sigma^{54}$. The only exception was Pp6 (aspartase), the spot density of which was similar in the mutant (see Fig. 6).

To corroborate our conclusion that the Glu-responsive proteins identified in the present study all depend on $\sigma^{54}$ for expression, we screened the $\text{P. putida}$ KT2440 genome to localize potential $\sigma^{54}$ recognition sequences in the upstream regions of the respective genes using the program PROMSCAN (Studholme et al., 2000). $\sigma^{54}$ recognition sequences are typically located at $-12/-24$ relative to the start of transcription. In the consensus sequence (see Table 3) a GG and a TG pair, both separated by 9 nucleotides, are strictly conserved (Buck et al., 2000). For all Glu-responsive genes identified here, sequences with the expected properties were

### Table 3. Putative $\sigma^{54}$ recognition sites of Glu-responsive genes in $\text{P. putida}$ KT2440

<table>
<thead>
<tr>
<th>Protein</th>
<th>Potential $\sigma^{54}$-binding site</th>
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<tbody>
<tr>
<td>Pf2 Gln-binding protein</td>
<td>TGGCACgactcATGCC*</td>
</tr>
<tr>
<td></td>
<td>TGGTACgcgtTGGCT†</td>
</tr>
<tr>
<td>Pp1 Rho termination factor</td>
<td>GCCCACggtttTGGCT</td>
</tr>
<tr>
<td>Pp3/4 Glutaminase/asparaginase (PGA)</td>
<td>TGGTACgaaaaATGCC†</td>
</tr>
<tr>
<td></td>
<td>TGGTACaatccTGGCT*</td>
</tr>
<tr>
<td>Pp6 Aspartate ammonia lyase</td>
<td>TGGCACggtgcTGGCT†</td>
</tr>
<tr>
<td>Pp8 Outer-membrane porin D</td>
<td>cggcacagatgtca†</td>
</tr>
<tr>
<td>Pp9 Phosphoenolpyruvate mutase</td>
<td>CGGTGCgacagtTGGCT†</td>
</tr>
<tr>
<td>Consensus sequence:</td>
<td>TGGCACGnnnTGGCT</td>
</tr>
</tbody>
</table>

*In $\text{P. fluorescens}$.
†In $\text{P. putida}$ KT2440.
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 will be necessary to locate the transcription start in each case. So far, this has only been done for the *ansB* gene of *P. fluorescens* ATCC 13525 (Hüser et al., 1999).

**DISCUSSION**

In the present communication we show that the growth of *P. fluorescens* ATCC 13525 and *P. putida* KT2440 on acidic amino acids and their amides as sole source of carbon and nitrogen leads to the coordinated expression of a well-defined set of genes. Our data suggest (but do not prove) that in both organisms all four amino acids (Asn, Asp, Gln and Glu) induce the same proteins. Most of the genes induced during growth on amino acids of this group are also subject to carbon catabolite repression by intermediates of the citric acid cycle and other good carbon sources. Many of the proteins differentially expressed from glucose/NH$_4^+$ to amino acids as sole carbon and nitrogen source were shown either to be involved in sugar metabolism or to perform functions that relate them to amino acid uptake and metabolism.

The Rho protein (spot Pp1) is known to participate in the regulation of tryptophanase expression in *E. coli*; tryptophanase enhances the transcription of tryptophanase by Rho-mediated antitermination (Konan & Yanofsky, 2000). It is unknown whether comparable mechanisms also operate in the regulation of Gln and Glu metabolism of other bacteria. The essential role of PGA (Pf2; Pp3/4) in the utilization of Glu by *P. putida* KT2440 has been established previously (Sonawane et al., 2003). The origin of the minor spot Pp4 (cf. Fig. 4) has not yet been investigated in detail. It may correspond to a PGA variant in which one or more Asn or Gln residues have been degraded to the respective dicarboxylates. However, the existence of a phosphorylated or otherwise covalently modified form of the enzyme cannot be excluded.

PGA hydrolyses Asn and Gln at similar rates and thus can generate both dicarboxylates (Glu and Asp) for uptake by transport systems in the inner membrane. The amino-acid-binding protein Pf2 (encoded by PP10171 in *P. putida* KT2440) and the ATP-binding protein Pp5 (encoded by PP1068) could both belong to such a transport system of the ABC type which possibly mediates the uptake of the acidic amino acids and/or their amides. This assumption is based on the genetic organization of certain Glu-related genes in *P. aeruginosa*, *P. putida* and *P. fluorescens* (see Fig. 7). In *P. aeruginosa* PAO1, a series of eight consecutive genes (PA1342–PA1335) encode proteins that all appear to be involved in the uptake and utilization of acidic amino acids. PA1342–1339 code for an ABC transporter that, by sequence similarity, mediates amino acid uptake. Two subsequent genes, PA1338 and PA1337, encode a γ-glutamyltransferase and PGA (*ansB*), respectively, while PA1336 and PA1335 encode a two-component system with strong similarity to dctBD, a system controlling dicarboxylate utilization in rhizobia (Wang et al., 1989). In *P. putida* KT2440, the genes for a closely related two-component system (PP1067–1066) are immediately adjacent to those encoding the ABC transporter mentioned above (PP1071–1068) while the *ansB* gene (PP2453) and the γ-glutamyltransferase gene (PP4659) are located elsewhere. The sequence identity between the ABC transporters of the two strains (PP1071–1068 and PA1342–1339) is 85 % at the protein level while the two-component systems (PP1067–1066 and PA1336–1335) have 76 % of the amino acid residues in common. A similar arrangement of genes for an ABC transporter and a two-component system was detected in the unfinished genome data of *P. fluorescens* SBW25 (available at the Sanger Institute, http://www.sanger.ac.uk/Projects/P_fluorescens/). The respective open reading frames which are contained in fragment Pflu346g05.q1kb show a very high degree of sequence similarity to PP1065–1071. The predicted amino acid sequence of gene 2 (see Fig. 7), which encodes a periplasmic solute-binding protein, contains the complete N-terminal sequence determined for spot Pp2 (i.e. AELTGLKINGXT, see Table 2), and the predicted sequence of gene 5 (the ATP-binding component of the ABC transporter) shows 93 % identity with the predicted sequence of PP1068.

Aspartate ammonia lyase (aspartase, Pp6) converts aspartate to fumarate and ammonia. Under aerobic conditions this reaction feeds the tricarboxylic acid cycle with the carbon

![Fig. 7. Genetic organization of Glu-related genes in the genomes of *P. putida* KT2440, *P. aeruginosa* PAO1 and *P. fluorescens* SBW25 (see text).](image-url)
skeletons of Asn and Asp. In *E. coli* and other enterobacteria, the enzyme also assists in anerobic fumarate respiration by providing fumarate from aspartate. Unlike the other spots induced by Glu, Pp6 was also expressed in the *rpoN* mutant and only weakly repressed by fumarate.

The outer-membrane porin D (Pp8) facilitates the uptake of amino acids and/or peptides by *P. aeruginosa* (Trias & Nikaido, 1990). Ochs et al. (1999) further showed that *oprD* expression in this organism is strongly enhanced by amino acids (including Glu, Arg and Ala) and repressed by succinate. The arginine-mediated induction of *oprD* was mediated by the regulatory protein ArgR, whereas the Glu-induced expression of OprD was independent of ArgR, indicating the presence of more than a single activation mechanism.

Unlike the proteins discussed above, Pp9, a putative carboxyphosphonoenolpyruvate phosphonomutase, has no apparent relation to amino acid metabolism. The only carboxyphosphonoenolpyruvate phosphonomutase, has no apparent relation to amino acid metabolism. The amino acid sequence of Pp9 also shows similarity to more common phosphopyruvate hydratases from intermediary metabolism (e.g. enolase; Lee et al., 1995). Thus, the annotation of PP1389 as a PEP phosphonomutase may be erroneous. Further experiments are required to characterize the role of Pp9 in *P. putida* KT2440.

The proteins upregulated during growth on NH₄Cl/glucose (Pp10–Pp13) can be related to the uptake and degradation of glucose (Pp11, Pp12) or diamines, respectively (Pp10 and Pp13). The transaminase Pp10 was shown to catalyse a step in the biosynthesis of the antibiotic bialaphos in *Streptomyces hygroscopicus* (Lee et al., 1995). However, the deduced amino acid sequence of Pp9 shows similarity to more common phosphopyruvate hydratases from intermediary metabolism (e.g. enolase; Lee et al., 1995). Thus, the annotation of PP1389 as a PEP phosphonomutase may be erroneous. Further experiments are required to characterize the role of Pp9 in *P. putida* KT2440.

The increased synthesis of a sugar uptake system during growth on glucose (spot Pp12) is not surprising, while the upregulation of putrescine uptake (Pp13, gene: *potF*) is more difficult to explain. Putrescine, a 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 & Camper (2001), studying changes in gene expression during attachment of *P. putida* to surfaces, found that 15 proteins were upregulated following bacterial adhesion and 30 proteins were downregulated. The downregulated proteins 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.

Our present data further indicate that most of the proteins upregulated by Glu depend on the alternative sigma factor $\sigma^{54}$ (RpoN) for expression. With the possible exception of aspartase (Pp6), none of the Glu-responsive proteins was synthesized in an RpoN $^-$ mutant of strain KT2440. As a result of this diminished presence of amino-acid-metabolizing enzymes, this strain exhibits a severe growth defect in media lacking glucose and NH₄Cl (Köhler et al., 1989). It is now well established that activation of the $\sigma^{54}$-RNA polymerase holoenzyme requires additional enhancer-binding proteins with ATPase activity to stimulate transcription (Buck et al., 2000). The function of these proteins is to facilitate conversion of the closed promoter complex to an open one. 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 & Stewart, 1998). A set of individual genes and/or operons controlled by one and the same response regulator is called a regulon. In our opinion, the present data indicate that the *P. putida* proteins upregulated by acidic amino acids and their amides (and other proteins not yet identified) may all be products of a regulon responsible for their uptake and metabolism. In order to substantiate this hypothesis, it has to be demonstrated that a single response regulator binds to and enhances transcription of these genes. Experiments aiming at the identification of such a response regulator are now under way in our laboratory.

**REFERENCES**


