Mol Gen Genet (1991) 229:41–51
002689259100276C

MGG

Relationship between tryptophan biosynthesis and indole-3-acetic acid production in *Azospirillum*: identification and sequencing of a *trpGDC* cluster

Wolfgang Zimmer, Christine Aparicio, and Claudine Elmerich

Unité de Physiologie Cellulaire, CNRS URA 1300, Institut Pasteur. Dept. des Biotechnologies, 25, rue du Dr. Roux, 75724 Paris Cedex 15, France

Received February 14, 1991

Summary. Screening the tryptophan (Trp)-dependent indole-3-acetic acid (IAA) production of different *Azospirillum* species revealed that *A. irakense* KA3 released 10 times less IAA into the medium than *A. brasilense* Sp7. A cosmid library of strain Sp7 was transferred into *A. irakense* KA3 with the aim of characterizing genes involved in IAA biosynthesis. Trp-dependent IAA production was increased in two transconjugants which both contained an identical 18.5 kb *Hin*dIII fragment from Sp7. After Tn*5* mutagenesis, cosmids carrying Tn*5* insertions at 36 different positions of the 18.5 kb fragment were isolated and transferred into strain KA3. IAA production by the recipient strains was screened by HPLC. The Tn*5* insertions of 4 clones with decreased IAA production were mapped on a 2 kb *Sal*I–*Sph*I fragment. Recombination of Tn*5* insertions at this locus into the genome of strain Sp7 led to Trp auxotrophic mutants. A 5.2 kb *Eco*RI–*Sal*I fragment including the 2 kb *Sal*I–*Sph*I fragment was sequenced and six open reading frames were identified. Three of them were clustered and their deduced amino acid sequences showed significant similarity to TrpG, TrpD and TrpC, which are enzymes involved in tryptophan biosynthesis. One of the remaining open reading frames probably encodes an acetyltransferase. The region responsible for the enhanced Trp-dependent IAA production in strain KA3 corresponded to *trpD*, coding for the phosphoribosyl anthranilate transferase.

Key words: *Azospirillum* – *trpGDC* – Acetyltransferase gene – Indole-3-acetic acid – Anthranilate

Introduction

Bacteria of the nitrogen-fixing genus *Azospirillum* live in association with the roots of species of Gramineae. In addition to the formation of nitrite by dissimilatory nitrate reduction (Zimmer et al. 1988), the release of phytohormones has been proposed as being responsible for the proliferation of the host-plant root system. It has been reported that *Azospirillum* releases the phytohormone indole-3-acetic acid (IAA) into the culture medium, when grown in the presence of tryptophan (Trp) (Tien et al. 1979; Reynders and Vlassak 1979). Whereas the genetic analysis of *Azospirillum* genes involved in nitrogen fixation is in progress (for review see Elmerich et al. 1991), little information about the genes and enzymes involved in IAA biosynthesis is available. Up to now is has not been possible to isolate *Azospirillum* mutants after chemical or Tn*5* mutagenesis which are completely unable to synthesize IAA (Marocco et al. 1983; Abdel-Salam and Klingmüller 1987) and only strains producing reduced amounts of IAA have been isolated. It was therefore proposed that *Azospirillum* possesses either more than one copy of the genes involved in IAA biosynthesis or more than one IAA synthetic pathway. A regulatory mechanism presumably controls IAA synthesis, since the release of this compound was only observed when the bacteria were supplied with Trp (Zimmer and Bothe 1988).

The only genetically analysed microbial pathway for IAA biosynthesis includes two enzymes, a Trp monoxygenase and an indoleacetamide hydrolase. The corresponding genes have been isolated and sequenced from the phytopathogen *Pseudomonas syringae*. from the pTi of *Agrobacterium tumefaciens* and from a *Bradyrhizobium* spp. (Yamada et al. 1985; Klee et al. 1984; Sekine et al. 1989). Physiological studies performed with the intermediate indoleacetamide, and hybridization experiments, failed to detect an analogous pathway in *Azospirillum* (Hartmann et al. 1983; Zimmer and Elmerich 1991). Consequently a different pathway for IAA synthesis was proposed for *Azospirillum*, involving the intermediates indole-3-pyruvate and indole-3-acetaldehyde. The initial reaction, the conversion of Trp into indole-3-pyruvate, can be catalysed by aromatic aminotransferases. Four of these enzymes have been identified in *A. lipoferum* (Ruckdäschel et al. 1988). As these enzymes

Offprint requests to: W. Zimmer

were found to be specific for different aromatic amino acids and not only for Trp, the detection of these proteins is not proof that IAA is synthesized via indole-3-pyruvate in *Azospirillum*. Enzymes or genes involved in the remaining part of this putative pathway have not yet been detected in *Azospirillum*.

The present paper reports the identification of a locus in *A. brasilense*, which enhanced Trp-dependent IAA production in the IAA low-producing strain *A. irakense* KA3. Analysis of the DNA sequence revealed that a *trpGDC* cluster was responsible for the observed phenotype. Part of this work has been presented at the Fifth International Symposium on the Molecular Genetics of Plant-Microbe Interaction, in Interlaken, Switzerland and a summary has been published in the proceedings of the meeting (Zimmer and Elmerich 1991).

Materials and methods

Strains, bacteriophages, cosmids and plasmids. The strains, bacteriophages, cosmids and plasmids which were used or isolated in this study are listed in Table 1. The maps of the plasmids are given in Fig. 1.

Media and growth conditions. Azospirillum cultures were grown in 50 ml Erlenmeyer flasks in 5 ml liquid minimal K medium (Franche and Elmerich 1981) at 30° C on a shaker or on agar plates containing the same medium or nutrient broth (Gauthier and Elmerich 1977). *Escherichia coli* was grown on Luria-Bertani medium (Sambrook et al. 1989). Derivatives of the bacteriophage M13 were propagated in *E. coli* TG1 (Messing 1983). Antibiotics were used at the following concentrations (μg/ml): ampicillin (Ap), 100; chloramphenicol (Cm), 50; kanamycin (Km); tetracycline (Tc), 5.

Trp, IAA and anthranilate assays. HPLC analyses were performed with 25 μl of supernatant from 5-ml cultures grown for 48 h at 30° C in minimal medium supplemented with 100 mg/l tryptophan and suitable antibiotics. Components of the samples were separated on a Nucleosil 5 μm C18 reversed phase column on a Perkin Elmer Series 3B Liquid Chromatograph. The solvent was 40:60 (v/v) methanol/1% H_3PO_4 in water and the flow rate was 1 ml/min. Trp and IAA were detected at 280 nm, and anthranilate at 310 nm. In addition IAA concentrations were estimated colorimetrically with the Salkowsky reagent (Tang and Bonner 1947). Two

Table 1. Bacterial strains, phages, cosmids and plasmids

Designation	Relevant characteristics	Source of reference
Escherichia coli strains:		
S17.1	*pro, thi, hsd*^{R}, *recA*, chromosomal integration of RP4-2-Tc::Mu−Km::Tn*7*, Sm^R, Tp^R, Tra$^+$, strain for conjugal transfer, cloning host	Simon et al. 1983
S17.1−Tn*5*	S17.1 derivative containing Tn*5* in the chromosome	Bozouklian et al. 1986
TG1	*Δ(lac−pro), supE, thi, hsdD5 F', traD36, lacI*^{q}, *ΔlacZ*M15, host for M13 phages, cloning host	Wain-Hobson et al. 1985
Azospirillum strains:		
A. brasilense Sp7	wild type (ATCC29145)	Tarrand et al. 1978
A. brasilense Sp7030	Sp7 mutant lacking the 115 MDa plasmid	Franche and Elmerich 1981
A. brasilense Sp7853	Km^R, Trp$^-$, Tn*5* insertion in *trpD*	this work
A. brasilense Sp7854	Km^R, Trp$^-$, Tn*5* insertion in *trpC*	this work
A. irakense KA3	wild type	Khammas et al. 1989
A. irakense KBC1	wild type	Khammas et al. 1989
A. lipoferum Sp59	wild type (ATCC19707)	Tarrand et al. 1978
A. lipoferum Br17	wild type	Tarrand et al. 1978
A. lipoferum USA5a	wild type	Tarrand et al. 1978
A. amazonense Y1	wild type	Magalhaes et al. 1983
A. halopraeferens Au4	wild type	Reinhold et al. 1987
Phages, cosmids, plasmids:		
M13mp18	derivative of M13, source for single-stranded DNA	Norrander et al. 1983
M13mp19	derivative of M13, source for single-stranded DNA	Norrander et al. 1983
pVK100	Tc^R, Km^R, IncP, Tra$^-$, cos	Knauf and Nester 1982
pAB1005	cosmid of a *Hin*dIII gene bank in pVK100 Tc^R, IncP, Tra$^-$, cos	Bozouklian et al. 1986
pAB1289	cosmid of an *Hin*dIII gene bank in pVK100	Bozouklian et al. 1986
pBluescript II KS+	Ap^R, pUC derivative, F1 replication origin	Stratagene
pSUP202	Ap^R, Cm^R, Tc^R, Mob, ColE1 replicon	Simon et al. 1983
pAB10053A	Ap^R, pBluescript II KS+derivative, carries a 3 kb *Sal*I fragment from pAB1005	this work
pAB10053	Ap^R, Cm^R, pSUP202 derivative, carries a 3 kb *Sal*I fragment from pAB1005	this work
pAB10054A	Ap^R, pBluescript II KS+derivative, carries an 8.2 kb *Eco*RI−*Hin*dIII fragment from pAB1005	this work
pAB10054	Ap^R, Cm^R, pSUP202 derivative, carries an 8.2 kb *Eco*RI−*Hin*dIII fragment from pAB1005	this work

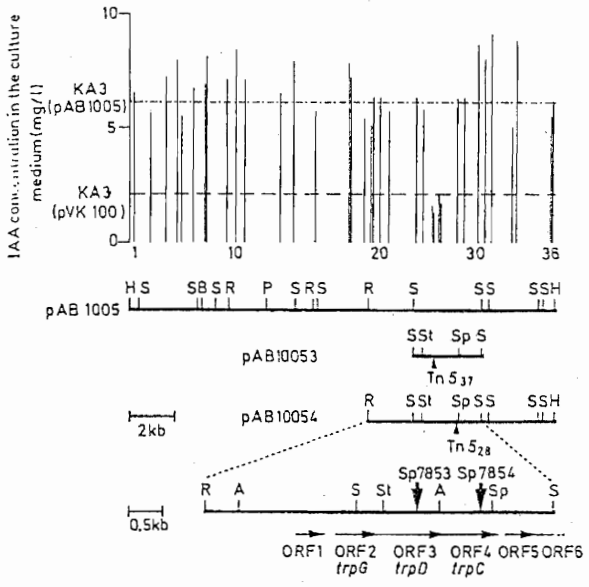


Fig. 1. Physical map of pAB1005, pAB10053 and pAB10054, and indole-3-acetic acid (IAA) production by *Azospirillum irakense* KA3 clones carrying Tn*5* insertions in pAB1005. Restriction sites: A, *Apa*I; B, *Bgl*II; H, *Hin*dIII; P, *Pvu*II; R, *Eco*RI; S, *Sal*I; Sp. *Sph*I; St, *Stu*I. The *vertical lines* in the diagram represent the amount of IAA released into the culture medium by *A. irakense* KA3 clones, carrying a Tn*5* insertion in pAB1005 at the corresponding position. Tn*5* insertions in pAB1005 are numbered from left to right as indicated by the numbers below the diagram. IAA production by strain KA3 carrying pVK100 or pAB1005 are shown by *interrupted horizontal lines*. IAA concentrations were determined by reversed phase HPLC (see the Materials and methods) of supernatants from cultures incubated for 2 days in minimal medium supplemented with 100 mg/l tryptophan and 5 mg/l kanamycin. The physical map below pAB10054 corresponds to the 5.2 kb fragment which was sequenced. The *arrows* indicate the open reading frames with a G+C content of >85% in the third codon position. The positions of the Tn*5* insertions of mutant Sp7853 and Sp7854 are marked by arrows on the map

hundred microlitres of culture supernatant were mixed with 0.8 ml Salkowsky reagent. After 30 min the extinction at 520 nm was determined against a blank containing 0.2 ml H_2O/0.8 ml Salkowsky reagent incubated for the same amount of time as the sample.

Plasmid and phage construction. Transformation, DNA isolation, restriction analysis, and DNA hybridization were performed by conventional techniques (Sambrook et al. 1989). Plasmid pAB10053 was constructed as follows: a 3 kb *Sal*I fragment from pAB1005 was subcloned in the *Sal*I site of the pBluescript II KS+ polylinker to yield pAB10053A. From this plasmid the 3 kb *Sal*I fragment was recovered as a *Hin*dIII−*Xho*I fragment and ligated into *Hind*sIII+*Sal*I-digested pSUP202 to yield pAB10053. Plasmid pAB10054 was constructed by cloning an 8.2 kb *Eco*RI−*Hin*dIII fragment of pAB1005 between the *Eco*RI/*Hin*dIII sites of the pBluescript II KS+ polylinker (pAB10054A). The inserted fragment was recovered as a *Hin*dIII−*Bam*HI fragment and cloned in *Hin*dIII+*Bam*HI-digested pSUP202 to yield pAB10054.

Tn5 mutagenesis and construction of Trp⁻ mutants. Tn*5* insertions in the inserts of pAB1005 and pAB10053 were isolated as previously described (Bozouklian et al. 1986) using strain S17.1-Tn*5*. The insertions in pAB1005 were named pAB1005-*Tn5*$_1$ to pAB1005-Tn*5*$_{36}$, numbered from left to right according to the map in Fig. 1. The Tn*5* insertion derivative of pAB10053 was named pAB10053−Tn*5*$_{37}$. Tn*5* insertion 28 was transferred from pAB1005 to pAB10054 by homogenotization. Insertions 28 and 37 were subsequently recombined into the Sp7 genome to yield mutants 7854 and 7853, respectively (Fig. 1).

DNA sequencing. A 2.2 kb *Eco*RI−*Sal*I frament of pAB10054A, a 2 kb *Sal*−*Sph*I fragment of pAB10053A and a 1 kb *Sph*I−*Sal*I frament of pAB10053A were independently subcloned in both M13mp19 and M13mp18 to allow sequencing of both DNA strands. Progressive deletions of these phages were performed using a deletion kit (Cyclone I Kit, International Biotechnologies, New Haven, Connecticut). To enhance efficiency of the deletion in the M13mp18 derivatives an annealing reaction (5 min incubation at 65° C, following by slow cooling to 30° C) was performed with the *Hin*dIII oligomer of the kit before digestion with *Hin*dIII at 30° C. When necessary oligomers were synthesized (Cyclone ™Plus DNA Synthesizer, MilliGen/Biosearch, Division of Millipore, Middlesex Turnpike, Burlington) to be used as primers for the sequence reaction. In particular this was done to establish that the three sequenced fragments were adjacent using pAB10053A and pAB10054A as a matrix. The sequence of the 5.2 kb fragment was determined on both strands by the dideoxy chain-termination technique (Sanger et al. 1977) using the TAQuence ™Sequencing kit (United States Biochemical Corporation, Cleveland, Ohio). Each clone was sequenced in parallel with both dGTP and 7-deaza dGTP. The EMBL accession number for the nucleotide sequence is X57853.

Results and discussion

Screening of wild-type Azospirillum *strains for IAA production*

Trp-dependent IAA production by *A. lipoferum*, *A. brasilense*, *A. amazonense* and *A. irakense* strains was measured by HPLC, and the amount of IAA present in the supernatants of cultures grown for 48 h in minimal medium supplemented with Trp is shown in Table 2. *A. irakense* KA3 and *A. irakense* KBC1 showed the lowest IAA production while their growth behaviour was similar to that of strain Sp7. *A. irakense* KA3 produced only one-tenth as much IAA as did strain Sp7, but released ten times more anthranilate into the medium. Consequently this strain was chosen to screen a genomic library of *A. brasilense* for fragments carrying genes involved in IAA biosynthesis. The clones harbouring such

Table 2. Consumption of tryptophan and production of anthranilate and indole-3-acetate by strains of *Azospirillum*

Strains/conditions		Tryptophan consumption	Anthranilate content	Indole-3-acetate content
A. brasilense Sp7		55.2	0.3	16.2
A. lipoferum Sp59		54.5	nd	5.8
A. amazonense Y1		49.7	nd	2.5
A. irakense KA3		61.2	16.0	1.6
A. irakense KBC1		64.4	16.5	1.7
Sp7(pVK100)	+Tc	56.3	0.3	17.4
Sp7(pAB1005)	+Tc	55.0	0.3	17.0
KA3(pVK100)	+Tc	64.6	17.2	2.0
KA3(pAB1005)	+Tc	65.8	1.5	6.5
KA3(pAB1289)	+Tc	65.2	1.5	6.8
Mutant 7853	+Km	72.5	0.3	20.7
+ 20 mg/l anthranilate	+Km	74.6	19.5	18.3
+100 mg/l anthranilate	+Km	74.5	90.0	19.5
Mutant 7854	+Km	70.5	0.4	22.1
+ 20 mg/l anthranilate	+Km	75.4	18.8	23.2
+100 mg/l anthranilate	+Km	71.7	80.6	21.0

Strains were grown aerobically in minimal medium supplemented with 100 mg/l tryptophan. After 2 days (OD 600 nm = 1.7) the concentrations of tryptophan, indole-3-acetate and anthranilate were determined by reversed phase HPLC (see the Materials and methods). Data are given in mg/l of culture medium. When added, the concentration of the antibiotics was 5 mg/l kanamycin (Km) or 20 mg/l tetracycline (Tc); nd, not determined

fragments could be detected by an enhanced IAA production.

Cloning and mapping of an A. brasilense *Sp7 locus enhancing IAA production in* A. irakense *KA3*

Four hundred cosmids from a library of *A. brasilense* Sp7, constructed in the *Hin*dIII site of the broad host range vector pVK100 (Bozouklian et al. 1986) were transferred one by one by conjugation from *E. coli* S17.1 into *A. irakense* KA3. The Trp-dependent IAA production of the transconjugants was determined independently for each clone with the Salkowsky reagent (see the Materials and methods). Two KA3 transconjugants containing pAB1005 and pAB1289 showed a significantly enhanced IAA production. Quantification by HPLC revealed that the IAA production of the KA3 transconjugants carrying pAB1005 or pAB1289 was more than three times higher than in the control strain containing pVK100 (Table 2). The anthranilate release by KA3 (pAB1005) or KA3 (pAB1289) was ten times lower than KA3 (pVK100) (Table 2). The amount of IAA released by strain KA3 (pAB1005) was similar to that of wild-type *A. lipoferum* Sp59. Transfer of pAB1005 into wild-type Sp7 did not enhance Trp-dependent IAA production. Restriction analysis of the two cosmids revealed that both contained an identical 18.5 kb *Hin*dIII insert (Fig. 1). A 2 kb *Sal*I–*Sph*I fragment (from a 3 kb *Sal*I fragment derived from pAB1005), was used as a probe for hybridization experiments with genomic DNA of strain Sp7 (Fig. 2). The fragment hybridized to a 3 kb *Sal*I and an 18.5 kb *Hin*dIII band of Sp7 DNA, indicating that the 18.5 kb insert of pAB1005 corresponded to a genuine fragment of the Sp7 genome. pAB1289 contained an additional 1.1 kb *Hin*dIII insert not present in pAB1005 (not shown).

Identification of the locus on pAB1005 responsible for the enhanced IAA production by strain KA3

In order to localize the region on pAB1005 responsible for the enhanced IAA production by strain KA3, Tn*5* mutagenesis was performed. Thirty-six different insertions in the 18.5 kb *Hin*dIII fragment were mapped (Fig. 1). The mutated cosmids were transferred by conjugation into *A. irakense* KA3, and the Trp-dependent IAA production of the recipient clones was assayed by HPLC (Fig. 1). Four transconjugants showed a level of IAA production similar to that of the strain containing pVK100. The Tn*5* insertions responsible were clustered

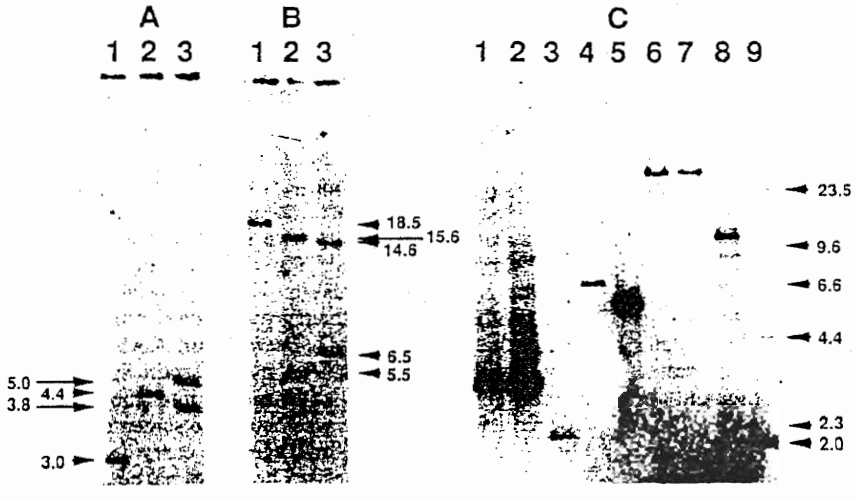


Fig. 2 A–C. Hybridization pattern of a 2 kb *Sal*I-*Sph*I fragment of pAB10053 with A *Sal*I- and B *Hin*dIII- digested total DNA of strain Sp7 (lane 1), 7854 (lane 2) and 7853 (lane 3). Hybridization pattern of C the 3 kb *Sal*I fragment with *Sal*I-digested total DNA of *A. brasilense* Sp7 (lane 1), Sp7030 (lane 2), *A. lipoferum* Sp59 (lane 3), Br17 (lane 4), USA5a (lane 5), *A. irakense* KA3 (lane 6), KBC1 (lane 7), *A. amazonense* Y1 (lane 8), *A. halopraeferens* Au4 (lane 9). Sizes on the left or right of the autoradiographs are given in kb. The time of exposure to Kodak X-OMAT films was 18 h at −80° C

in a region of 1 kb on a 3 kb *Sal*I fragment of pAB1005. Transconjugants, carrying pAB1005 with Tn*5* insertions upstream or downstream of this region, produced about three times more IAA than KA3 (pVK100) which was comparable to the amount released by strain KA3 containing the parental cosmid pAB1005 (Fig. 1).

Hybridization experiments were performed using the 3 kb *Sal*I fragment as a probe against restriction enzyme-digested total DNA of various *Azospirillum* species (Fig. 2). All the *Azospirillum* species tested contained fragments that hybridized to the probe. This indicated that the locus was also present in the IAA low-producing strains such as *A. irakense* KA3 and *A. irakense* KBC1. As IAA production of strain KA3 was enhanced in the presence of pAB1005, the transcription rate of the homologous gene in the *A. irakense* wild-type genome is presumably submaximal. It was assumed that the locus enhancing IAA production in *A. irakense* corresponded to a gene involved in IAA biosynthesis in strain Sp7.

Construction of A. brasilense *Sp7 mutants carrying Tn5 at the locus responsible for enhanced IAA production of strain KA3*

By recombination of Tn*5* insertions into the locus of strain Sp7, which was responsible for enhanced IAA synthesis in strain KA3, we expected to isolate mutants defective in IAA production. Therefore pAB10053 – $Tn5_{37}$ and pAB10054 – $Tn5_{28}$ were constructed (see the Materials and methods). After a conjugational transfer of these plasmids into the strain Sp7, attempts to isolate recombinants on minimal medium containing Km were unsuccessful. As IAA biosynthesis starts from Trp, it was suspected that the synthesis of IAA and Trp might share a common regulatory mechanism. Were this the case, disruption of the regulatory gene would lead to mutants impaired in Trp metabolism. Therefore, the conjugation experiments were repeated in the presence of Trp. Selection on minimal medium containing Trp led to the isolation of two mutants of *A. brasilense* Sp7 with a Tn*5* insertion in the expected locus. Mutant 7853 carried the Tn*5* insertion in the region which has been identified to be responsible for the enhanced IAA production in the *A. irakense* transconjugants. Mutant 7854 carried the Tn*5* insertion at a position adjacent to the locus responsible for the enhanced IAA production of strain KA3 (Fig. 1). Recombination of Tn*5* in the correct position was verified by Southern hybridization (Fig. 2). the two mutants were found to be Trp auxotrophs and were also unable to grow in the presence of anthranilate, an intermediate of Trp biosynthesis. This indicated that the enzymatic conversion of anthranilate to Trp was interrupted by the Tn*5* insertions. Surprisingly the Trp-dependent IAA production of the mutants 7853 and 7854 was not reduced (Table 2). As the level of anthranilate release was changed in the KA3 strain carrying pAB1005, the effect of anthranilate on IAA production by the mutants was studied. However, adding anthranilate to the Trp-containing growth medium did not significantly influence IAA production by mutants 7853 and 7854 (Table 2). Obviously there was a discrepancy between the phenotypes conferred by mutations at the locus in *A. irakense* KA3 and its homologue in *A. brasilense* Sp7.

Sequencing of the locus and identification of six open reading frames

The DNA sequence of the locus was determined, to allow the identificaiton of possible functions by a computer-aided gene bank search. The nucleotide sequence of a 5.2 kb *Eco*RI – *Sal*I fragment of pAB10054 (Fig. 1) was established (Fig. 3) which included the locus responsible for enhanced IAA production in strain KA3 and the loci identified to be essential for Trp biosynthesis in strain Sp7. Six open reading frames ORF1 to ORF6 (Fig. 1) were identified with high G+C contents (99.2%, 94.4%, 94.4%, 93.5%, 88.2% and 94.5% respectively) in the third codon position. These data were in the same range as the values previously observed for other *Azospirillum* genes: *glnA*, 95%; *glnB*, 87%; *hisB*, 93%; *nifH*, 92%; *nodP*, 87%; and *nodQ*, 89% (for references see Fani et al. 1989; Vieille and Elmerich 1990; de Zamaroczy et al. 1990). Start and stop codons of the ORFs were at positions: 1341–1791 (ORF1), 1963–2551 (ORF2), 2550–3615 (ORF3), 3614–4400 (ORF4), 4438–4897 (ORF5), 4896–? (ORF6) (the numbers correspond to the nucleic acid positions in Fig. 3). The complete sequence of ORF6 has not been established. Four of the ORFs were preceded by putative ribosome-binding sites (Shine and Dalgarno 1974): GAAGGA (ORF2), GGAGA (ORF3), GAGGAG (ORF5), GGAGA (ORF6). The length (in amino acids) and M_r of the deduced amino acid sequences of ORF1 to ORF5 were 150 (16.3 kDa), 196 (21.4 kDa), 355 (36.7 kDa), 262 (28.1 kDa) and 153 (17.1 kDa) respectively.

Homology search with ORF1, ORF5 and ORF6

No significant homology was found for the deduced amino acid sequences of ORF1 and ORF6 by computer-aided gene bank search. In the case of ORF5, a weak similarity to three different acetyltransferases was detected (Fig. 4). ORF5 was most similar to the C-terminal part of *E. coli* ArgA (Brown et al. 1987), the N-acetylglutamate synthase, and was also similar to *E. coli* RimI (Yoshikawa et al. 1987) which was described as an enzyme acetylating the N-terminus of the ribosomal S18 protein. The third highest score of the sequences identified as similar was that of *Streptomyces lavendulae* StaT (Horinouchi et al. 1987) which is an enzyme transferring an acetyl group from acetyl coenzyme A to the beta-amino-group of the beta-lysine moiety of streptothricin. Therefore it was assumed that ORF5 also encoded an acetyltransferase.

Homology of the deduced amino acid sequence of ORF2 and glutamine amido transferases

The deduced amino acid sequence of ORF2 shared high similarities with a gene family encoding the glutamine

EcoRI
*GAATTC*TCCTCAAGCGGGACCGGCGGTCACCCGCCGGTGTGATGGGCGCGCACGACGGCGCTGACCGGAACCAGGACGAAGCCGCGTTTC
91
TGCACCTCCGGCATCCAGGAGGCCAGTGCGTCGATTGTCGCATCGTGCGGGTGGCCGATGGCGATGGCGTAGCCCTGCTTGCGCGCCACC
181
TGCTCCGTCTTGGCAAGCTGGCCGCGCACCGCCGGCACGGTCATCTCGTTGTCCAGGAAGATGTCGCGCCCGGCGAAGGGCATCTGAAGC
271
TCCCGCGCGAGGGTCAGGCCGGCGCTCTTCGCCGTGGTCCGGCTGTCCAGCCACAACAGGCCGCGCCGCGCGATCTCCCCGAGCACGGGC
361
GCCATGGCCGCGGAGTCGGCGGTGAAGCGGCTGCCCATGTGGTTGTTCACCCCGACATAGCCGTCGAAGCTGGTCCCAGCGCCGCCTTGG
451 *ApaI*
TCCGGCGCAGGATCTCCCCCTTGTCGAGCGACACGCGCACGCCTG*GGGCCCC*CGGATCGGCAGCGCCGCTGGGTTCCATGGGCAACGATGC
541
AGCATCAGCTCGTGCCCGGCGGCGCGGGCGGCGCGGGCCTGGGCCGGCAGCTCGTGGGCGTAGGGCAGCCACGCCAGCGCTCACGCGCCG
631
GCAGCGACACCGCGCGGTTCGAACGTTGCCGGTCCACGCCCATGTCGTCGATGACGATGGCGATGGCCGGCTTGCCCGGCGGGGCGCGGA
721
AGGGCAGCGCGTTCTTCTTCCACAGCGGCGATCCCGGCGCCACCTTCGGCGGTTCCAGCGGGGCGACGGGAGGCGGCAGCATCGCCACCA
811
CCGGCTTCGGCGTCATGGCCGGCGGCGGGGGCGGTGCGGGAACCGGAACGGGGGCCGGAGCCTCGGTCACTTGGTGAGGCGGTGCCGGAA
901
CGGGCGCCGGAGCCGGTTCGGCCTCGCGAACCGGCGGAGGCCGGAGGGCGGGGGCGGCGCATCTGCGACCTTGGGCGGCGGCGTCTTGAT
991
CGGCGCGGCCGGGGCGATGACCGGCGCCTCCCGCTCCACGACGGTCTCCGTCTCCGGCGCGTCGCGCCCGATGATGAATCGCGCGCCGAG
1081
CCGCACGCCGAAGACCGCGATCACACCGGCCAGCGCCAGCAGGACCGGCGAGACGGCGCGCCCCGATGCGGCGGCGGGCGTTTTCGGCGA
1171
TGGCTTCCGTGCCGCGGCTTTCTGGGCGTTGCGCGGCGTTTTCTTGGCCATGGGAAGGAATCTCGAACCCTGGTTTCCGGTTTGCGCGCA
1261
AGGTGGCACCCCCGCACCGTCCCTGCAACAGCGGAGCGGGGTGGAGGCGCCTGCCGGCTGGGACTATGCTGTGACCCGCCATGCTCCGCA
M L R T
1351
CCCTGATCCTCCTGACGCTCCTCATCACCGCCACCGCCGGCCCCGCCCTCGCGAACGAGGTCCGCTGCCCGGCCAGCCTGACCGTGCAGG
L I L L T L L I T A T A G P A L A N E V R C P A S L T V Q A
1441
CCCAGCCGGAGGCGCCCGGCGGCTGGTCGCCCTACCCGGCGAAGGACCAGCACGCCTTCGCCGGCGTCACCCTGGTGGAGGGCGACCGGG
Q P E A P G G W S P Y P A K D Q H A F A G V T L V E G D R A
1531
CGGCGCAGATGGCCGCCCCGGCCCCGGCGGCGCTGGAGCCGGACCGCAGCCTGCGGCGGGGCCGCTCGGAGATCCGGCAATGGGACTTCC
A Q M A A P A P A A L E P D R S L R R G R S E I R Q W D F P
1621
CCGCCGCGCGGCGCGACAACGTCTTCCTGATCTGCCGCTACGCCGGAACGCAGGCCACGCTGGCCATCGACCTGCCGCGCACGGTGCGGC
A A R R D N V F L I C R Y A G T Q A T L A I D L P R T V R R
1711
GCTGCCAGATCACCGAGGAGACCGACGCGCGCGGCATGGTGCTGGACAAGCCGGCGACGGCGCCGCAGTTCCTCTGCCGCTGATTCTCCG
C Q I T E E T D A R G M V L D K P A T A P Q F L C R *
1801
CCGAGTCGAGGTTCGGACGGGCTGGAAATCCACCGCTCCAGCCGCTAGGGTACCCGCCTTGGAACAAGGCCGGAACGCTATTCCGGGACG
1891
ATCGGGCCGGGAACAATGGACCCGAGACCGACGGACCGGAAACGACAGGCCG*GAAGGA*TGCCGGGGCGCCCC*ATG*CTGCTGCTCATCGAT
M L L L I D
1981
AATTACGACAGCTTCACCTACAACCTCGTCCATTACCTGGGCGAACTGGGCGCCGAACTGGACGTCCGCCGCAACGACAGCCTGACGGTG
N Y D S F T Y N L V H Y L G E L G A E L D V R R N D S L T V
2071
GAGGAGGCCATGGCGCTCCGCCCCGAAGGGATCGTGCTGTCGCCCGGCCCCTGCGACCCGGACAAGGCGGGCATCTGCCTGCCGCTGATC
E E A M A L R P E G I V L S P G P C D P D K A G I C L P L I
2161
GACGCCGCCGCCAAGGCCGCGGTTCCGCTGATGGGCGTGTGCCTGGGCCATCAGGCCATCGGGCAGCCGTTCGGCGGCACGGTCGTGCGC
D A A A K A A V P L M G V C L G H Q A I G Q P F G G T V V R
2251 *SalI*
GCGCCGGTGCCGATGCACGGCAAG*GTCGAC*CGCATGTTCCACCAGGGGCGCGGCGTCCTGAAGGACCTGCCCTCGCCCTTCCGGGCCACC
A P V P M H G K V D R M F H Q G R G V L K D L P S P F R A T
2341
CGCTACCACTCGCTGATCGTCGAGCGCGCCACCCTGCCCGCCTGCCTGGAGGTGACCGGCGAGACGGAGGACGGGCTGATCATGGCGCTG
R Y H S L I V E R A T L P A C L E V T G E T E D G L I M A L
2431
TCCCACCGCGAGCTGCCGATCCACGGCGTGCAGTTCCACCCGGAAAGCATCGAGAGCGAGCACGGGCACAAGATCCTGGAAAACTTCCTG
S H R E L P I H G V Q F H P E S I E S E H G H K I L E N F L
2521
AACACGACCCGCCGGCT*GGAGA*CCGCCGC*ATG*AGCACGCCGTCCGCGCCGCACGGCGACCTGACCGACATGAAGGCGATCCTCGCCAAGG
N T T R R L E T A A *
M S T P S A P H G D L T D M K A I L A K V
2611 *ScuI*
TCGCCGCCGGCAACGCCCTGAACGAAGCGG*AGGCCT*CGCTGGCCTTCGACATCATCATGTCGGGCAACGCCACCCCGTCGCAGATGGGCG
A A G N A L N E A E A S L A F D I I M S G N A T P S Q M G G
2701
GCTTCCTGATGGCGCTGCGCGTGCGCGGCGAGACGGTGGACGAGATCACCGGGGCCGCCCGCGTCATGCGCGCCAAGGCGATCCCGGTGG
F L M A L R V R G E T V D E I T G A A R V M R A K A I P V E
2791
AGGCGCCGGACGGCACCATCGACACCTGCGGCACCGGCGGCGACGGCTCGGGCACCTACAACATCTCCACCGCCGCGGCCGTGGTCATCG
A P D G T I D T C G T G G D G S G T Y N I S T A A A V V I A

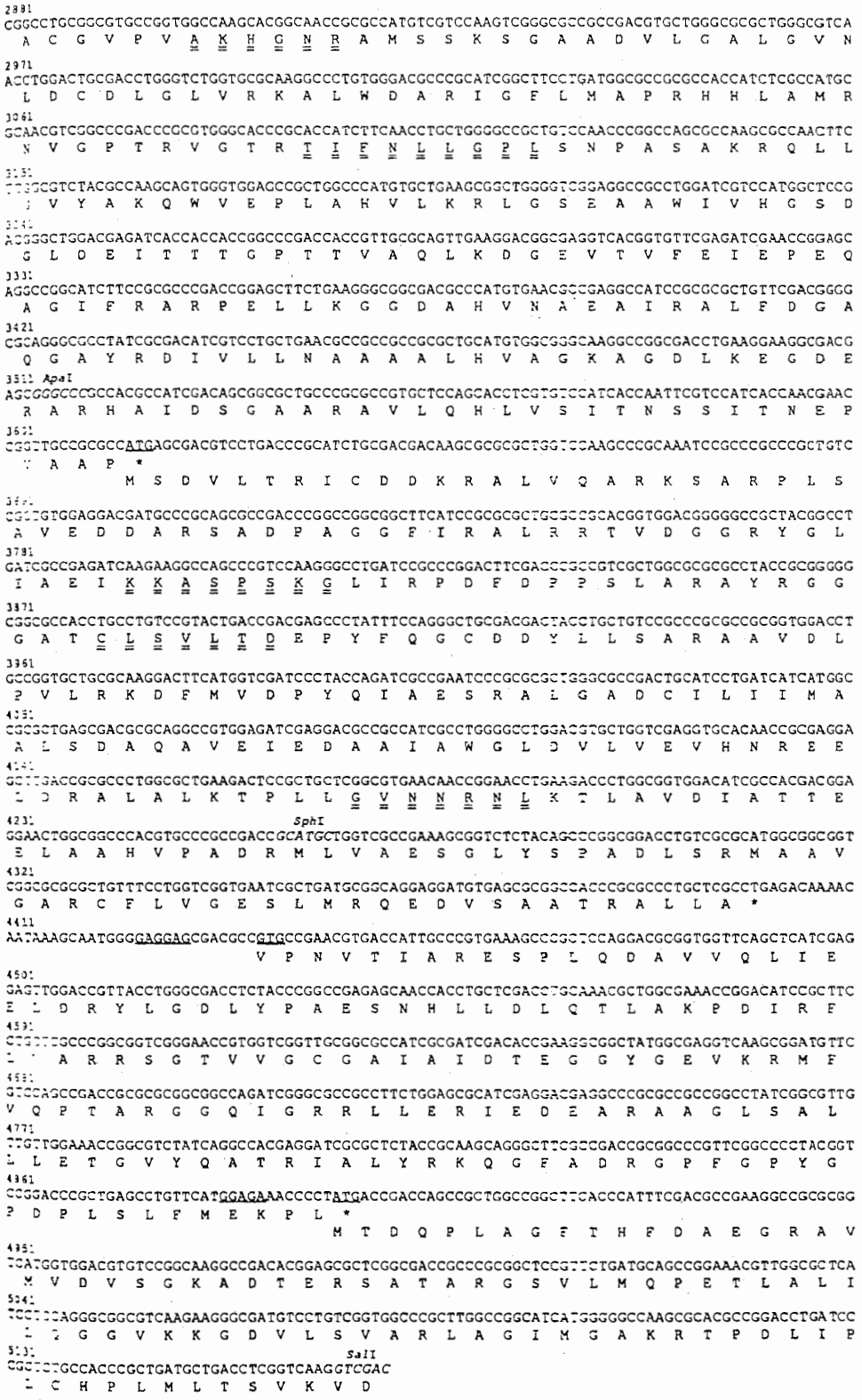
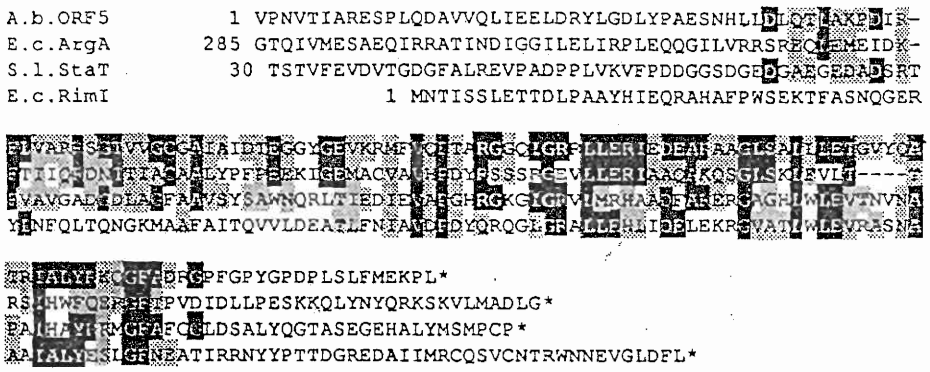
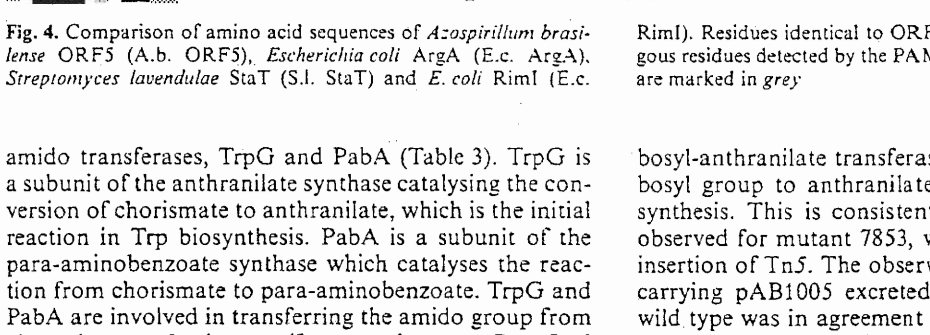


Fig. 3. Nucleotide sequence of the 5.2 kb *Eco*RI – *Sal*I fragment of pAB10054 containing *trpGDC* of *A. brasilense* and the flanking regions. The deduced amino acids are printed below the corresponding codons of the open reading frames. Probable Shine-Dalgarno sequences and start codons are *underlined*. *Asterisks* are used to indicate the stop codons. Relevant restriction sites are indicated above the sequence. Conserved regions according to Crawford (1989) in the deduced amino acid sequence of *trpG*, *trpD* and *trpC* are *double underlined*





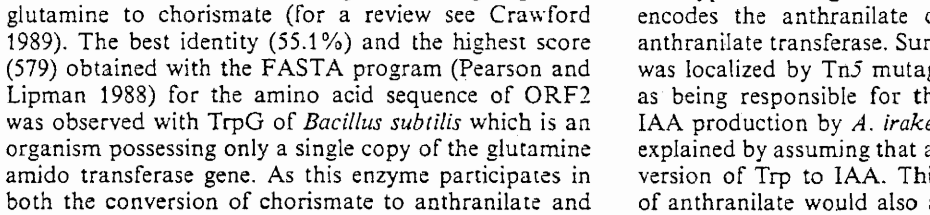


Fig. 4. Comparison of amino acid sequences of *Azospirillum brasilense* ORF5 (A.b. ORF5), *Escherichia coli* ArgA (E.c. ArgA), *Streptomyces lavendulae* StaT (S.l. StaT) and *E. coli* RimI (E.c. RimI). Residues identical to ORF5 are marked in *black*. Homologous residues detected by the PAM250 matrix (Dayhoff et al. 1978) are marked in *grey*

amido transferases, TrpG and PabA (Table 3). TrpG is a subunit of the anthranilate synthase catalysing the conversion of chorismate to anthranilate, which is the initial reaction in Trp biosynthesis. PabA is a subunit of the para-aminobenzoate synthase which catalyses the reaction from chorismate to para-aminobenzoate. TrpG and PabA are involved in transferring the amido group from glutamine to chorismate (for a review see Crawford 1989). The best identity (55.1%) and the highest score (579) obtained with the FASTA program (Pearson and Lipman 1988) for the amino acid sequence of ORF2 was observed with TrpG of *Bacillus subtilis* which is an organism possessing only a single copy of the glutamine amido transferase gene. As this enzyme participates in both the conversion of chorismate to anthranilate and to para-aminobenzoate, it is called amphibolic. Another high score (575) was obtained with the TrpG of *Acinetobacter calcoaceticus*, which is also amphibolic (Kaplan et al. 1984). It is therefore probable that *Azospirillum* also carries only one glutamine amido transferase gene encoding an amphibolic protein. In agreement with this conclusion, all hybridization experiments performed with fragments of pAB1005 revealed only one copy of the *trpG* region in the genome (as an example see Fig. 2). A score of only 321 was obtained for the best alignment between the deduced amino acid sequence of *A. brasilense trpG* and Trp(G) of *Rhizobium meliloti*, which is phylogenetically the most closely related organism listed in Table 3. Even similarities to fungal polypeptides were greater (Table 3). This surprising observation might be associated with the different organization of the polypeptide in the two organisms. Whereas *trp(G)* of *R. meliloti* is contranslated with *trpE* resulting in the fusion protein TrpE(G), TrpG of *Azospirillum* is a single and independent protein.

The amino acid sequence of ORF3 shows similarity to TrpD

The amino acid sequence encoded by *A. brasilense* ORF3 was deduced and used for sequence comparisons. The highest degree of identity was with the *R. meliloti* TrpD (56.9%, Table 3). The *trpD* gene encodes the phosphoribosyl-anthranilate transferase, which adds a phosphoribosyl group to anthranilate in the second step of Trp synthesis. This is consistent with the Trp$^-$ phenotype observed for mutant 7853, where ORF3 is disrupted by insertion of Tn*5*. The observation that *A. irakense* KA3 carrying pAB1005 excreted less anthranilate than the wild type was in agreement with the finding that ORF3 encodes the anthranilate consuming phosphoribosyl-anthranilate transferase. Surprisingly, only ORF3 (*trpD*) was localized by Tn*5* mutagenesis of pAB1005 (Fig. 1) as being responsible for the enhanced Trp-dependent IAA production by *A. irakense*. This phenotype can be explained by assuming that anthranilate inhibits the conversion of Trp to IAA. This putative regulatory effect of anthranilate would also account for the observation that *A. brasilense* releases IAA only in the presence of Trp (Zimmer and Bothe 1988). In the absence of Trp, the genes for Trp synthesis are transcribed, translated and the anthranilate synthase of *Azospirillum* is active (Hartmann et al. 1983). Consequently the level of anthranilate, the first intermediate of the pathway, increases. According to the proposed regulatory mechanism this inhibits IAA synthesis and prevents loss of Trp from the cells. In the presence of external Trp, anthranilate synthesis in *Azospirillum* is blocked (Hartmann et al. 1983) and the intracellular level of anthranilate is low, enabling IAA synthesis. A mutant of *A. brasilense* Sp245 carrying a Tn*5*—*mob* insertion in a 85 MDa plasmid has been described which differs from the wild type in both enhanced anthranilate release and decreased IAA production (Katzy et al. 1990). The surprising combination of the two phenotypes can also be explained by the putative inhibitory activity of anthranilate on IAA biosynthesis. However, external anthranilate did not inhibit Trp-dependent IAA synthesis in the TrpD$^-$ strain 7853 (Table 3), perhaps due to the fact that anthranilate did not accumulate in the cells.

The amino acid sequene of ORF4 is similar to TrpC

The highest degree of identity of the ORF4-encoded amino acid residues was with the *R. meliloti* TrpC

Table 3. Comparison of ORF2, ORF3 and ORF4 amino acid sequences with TrpG/PabA, TrpD and TrpC of other organisms

Organism	Reference	Identity in %	Number of gaps	Optimal score
Azospirillum brasilense ORF2:				
Bacillus subtilis TrpG	Slock et al. 1990	55.1	1	579
Salmonella typhimurium PabA	Kaplan et al. 1985	55.6	1	575
Acinetobacter calcoaceticus TrpG	Kaplan et al. 1984	53.6	3	575
Escherichia coli PabA	Kaplan and Nichols 1983	53.5	1	558
Klebsiella aerogenes PabA	Kaplan et al. 1985	53.0	1	556
Pseudomonas putida TrpG	Essar et al. 1990	55.6	3	549
Serratia marcescens PabA	Kaplan et al. 1985	53.1	3	533
Thermus thermophilus TrpG	Sato et al. 1988	52.5	2	515
Penicillium chrysogenum Trp(G)	Penalva and Sanchez 1987	44.4	2	410
Aspergillus nidulans Trp(G)	Mullaney et al. 1985	42.9	2	409
Saccharomyces cerevisiae Trp(G)	Zalkin et al. 1984	39.8	0	406
Aspergillus niger Trp(G)	Kos et al. 1988	42.9	2	399
Escherichia coli Trp(G)	Yanofsky et al. 1981	37.2	3	341
Rhizobium meliloti Trp(G)	Bae et al. 1989	35.2	1	321
A. brasilense ORF3:				
R. meliloti TrpD	Crawford*	56.9	2	n.d.
P. putida TrpD	Essar et al. 1990	39.8	4	608
Bacillus pumilis trpD	Rivas et al. 1990	37.7	2	590
A. calcoaceticus TrpD	Kaplan et al. 1984	38.3	4	589
B. subtilis TrpD	Henner et al. 1985	38.0	2	571
S. typhimurium Trp(D)	Horowitz et al. 1983	35.0	2	547
E. coli Trp(D)	Yanofsky et al. 1981	35.5	3	544
Brevibacterium lactofermentum TrpD	Matsui et al. 1986	35.0	6	459
A. brasilense ORF4:				
R. meliloti TrpC	Crawford*	56.7	0	n.d.
P. putida TrpC	Essar et al. 1990	51.3	1	640
A. calcoaceticus TrpC	Kaplan et al. 1984	47.9	2	604
B. subtilis TrpC	Henner et al. 1985	38.4	2	468
S. typhimurium Trp(C)	Horowitz et al. 1983	37.3	3	451
E. coli Trp(C)	Yanofsky et al. 1981	37.6	3	450
B. lactofermentum Trp(C)	Matsui et al. 1986	33.8	3	398
Haloferax volcani TrpC	Lam et al. 1990	36.9	4	384
P. chrysogenum TrpC	Penalva and Sanchez 1987	36.4	3	384

The table shows results of the best alignments performed by the FASTA program (Pearson and lipman 1988). Trp(G), Trp(D), Trp(C) indicate that the polypeptide is part of a fusion protein, whereas TrpG, TrpD, TrpC and PabA are independent proteins

* As the amino acid sequences of TrpC and TrpD of *R. meliloti* are not present in the GenPept bank of Genebank, no FASTA scores are calculated for these sequences. The *R. meliloti* sequences were supplied by the laboratory of I. Crawford

(56.7%, Table 3). TrpC is the indoleglycerolphosphate synthase which catalyses the fourth step in the Trp biosynthetic pathway. This explained the Trp$^-$ phenotype of mutant strain 7854 which carries a Tn*5* insertion in ORF4. All the highly conserved regions known for TrpG, TrpD and TrpC polypeptides (Crawford 1989) were found in the corresponding polypeptides of *A. brasilense* (double underlined in the amino acid sequence in Fig. 3).

Organization of the Trp genes in A. brasilense

No sequence reminescent of a σ^{70} promotor was identified in the region located 172 bp downstream of ORF1 and upstream of *trpG*. The three genes *trpG*, *trpD* and *trpC* are likely to be cotranscribed in *A. brasilense*, as they are clustered with overlapping start and stop codons. Since none of pAB1005-Tn*5* cosmids having Tn*5* either in *trpD* or *trpC* could complement the *trpD*$^-$ strain 7853 or the *trpC*$^-$ strain 7854 (data not shown), cotranscription of *trpD* and *trpC* was indicated. However, complementation of strains 7853 and 7854 was observed with cosmids pAB1005–Tn5_{22} and pAB1005–Tn5_{23} carrying Tn*5* in *trpG*, which indicates either that a new transcription initiation site was created inside *trpG* by the Tn*5* insertion or that a transcription initiation site is located in *trpG*. A similar conclusion was indicated by analysis of *A. irakense* transconjugants carrying pAB1005$-$Tn5_{22} or pAB1005$-$Tn5_{23}, as the Trp-dependent IAA production of these two transconjugants was not reduced (Fig. 1), suggesting that the *trpG* mutations were non-polar and not affecting *trpDC*. In the regions located 2062 bases upstream of *trpG* and 768 bases downstream of *trpC* no homology to other genes involved in Trp metabolism was detected. Similarly in both *Pseudomonas putida* and *Acinetobacter calcoaceticus trpG*, *trpD* and *trpC* are organized as an operon that is independent of the other *trp* genes (Essar et al. 1990; Kaplan et al. 1984). Besides the *his* and *gln* genes (Fani et al. 1989; Bozouklian and Elmerich 1986; de Zamaroczy et al. 1990) the *trpGDC* cluster is the third locus described for *Azospirillum* whch is involved in amino acid biosynthesis.

Differences in Trp metabolism between A. brasilense *Sp7 and* A. irakense *KA3*

In appear from results reported above that IAA production in *Azospirillum* is probably repressed by anthranilate, which is an intermediate in Trp biosynthesis and catabolism. Aerobic growth experiments performed in minimal medium containing Trp revealed that *A. irakense* can grow when Trp is the sole carbon and nitrogen source whereas *A. brasilense* needs an additional carbon source for growth (data not shown). Together with the observed difference in the anthranilate release by the two strains (Table 2), this indicates that the Trp catabolism in *A. irakense* is more effective than that in *A. brasilense*. Moreover, the release of anthranilate during Trp catabolism suggests that TrpD, the phosphoribosyltransferase, seems to be inactive or that *trpD* is not transcribed in wild-type *A. irakense*. In contrast TrpD, encoded by the *trpGDC* cluster of *A. brasilense*, is functional in *A. irakense* when grown in Trp-containing medium. since the introduction of pAB1005 into strain KA3 led to a reduced release of anthranilate into the medium due to the conversion of anthranilate by TrpD (Table 2). In contrast, as anthranilate is apparently not produced via Trp catabolism in strain Sp7, the Trp-dependent IAA synthesis cannot be regulated through anthranilate accumulation or consumption in either the wild type or the *trpD* mutant. Further investigations are necessary to detail the differences in Trp metabolism and in IAA biosynthesis between the two *Azospirillum* stins, to characterize the other genes involved in the process and to study the transconjugants and mutants for altered plant growth promoting activities in association with their host plants.

Acknowledgements. The authors wish to thank Professor J.-P. Aubert and Professor H. Bothe for helpful discussion and A. Edelman for reading the manuscript. W.Z. was recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. This work was supported by research funds from the University of Paris 7.

References

Abdel-Salam MS, Klingmüller W (1987) Transposon Tn*5* mutagenesis in *Azospirillum lipoferum*: isolation of indole acetic acid mutants. Mol Gen Genet 210:165–170

Bae YM, Homgren E, Crawford IP (1989) *Rhizobium meliloti* anthranilate synthase: cloning, sequence and expression in *Escherichia coli*. J Bacteriol 171:3471–3478

Bozouklian H, Elmerich C (1986) Nucleotide sequence of the *Azospirillum brasilense* Sp7 glutamine synthetase structural gene. Biochimie 68:1181–1187

Bozouklian H, Fogher C, Elmerich C (1986) Cloning and characterization of the *glnA* gene of *Azospirillum brasilense* Sp7. Ann Inst Pasteur Microbiol 137:3–18

Brown K, Finch PW, Hickson ID, Emmerson PT (1987) Complete nucleotide sequence of the *Escherichia coli argA* gene. Nucleic Acids Res 15:10586

Crawford IP (1989) Evolution of a biosynthetic pathway: The tryptophan paradigm. Annu Rev Microbiol 43:567–600

Dayhoff M, Schwarz RM, Orcutt BC (1978) In: Dayhoff M (ed) Atlas of protein sequence and structure, Vol 5, Suppl 3. Natl Biomed Res Found, Silver Spring, Md, pp 345–352

de Zamaroczy M, Delorme F, Elmerich C (1990) Characterization of three different nitrogen-regulated promoter regions for the expression of *glnB* and *glnA* in *Azospirillum brasilense* Sp7. Mol Gen Genet 224:421–430

Elmerich C, Zimmer W, Vieille C (1991) Associative nitrogen fixing bacteria. In: Evans H, Burris R, Stacey G (eds) Biological nitrogen fixation. Chapman and Hall, New York, pp 211–257

Essar DW, Eberly L, Crawford IP (1990) Evolutionary differences in chromosomal locations of four early genes of the tryptophan pathway in fluorescent *Pseudomonas*: DNA sequences and characterization of *Pseudomonas putida trpE* and *trpGDC*. J Bacteriol 172:867–883

Fani R, Bazzicalupo M, Damiani G, Bianchi A, Schipani C, Sgaramella V, Polsinelli M (1989) Cloning of the histidine genes of *Azospirillum brasilense*: Organisation of the *ABFH* gene cluster and nucleotide sequence of the *hisB* gene. Mol Gen Genet 216:224–229

Franche C, Elmerich C (1981) Physiological properties and plasmid content of several strains of *Azospirillum brasilense* and *A. lipoferum*. Ann Inst Pasteur Microbiol 132a:3–17

Gauthier D, Elmerich C (1977) Relationship between glutamine synthetase and nitrogenase in *Spirillum lipoferum*. FEMS Microbiol Lett 2:101–104

Hartmann A, Singh M, Kingmüller W (1983) Isolation and characterization of *Azospirillum* mutants excreting high amounts of indoleacetic acid. Can J Microbiol 29:916–923

Henner DJ, Band L, Shimotsu H (1985) Nucleotide sequence of the *Bacillus subtilis* tryptophan operon. Gene 34:169–177

Horinouchi S, Furuya K, Nishiyama M, Suzuki H, Beppo T (1987) Nucleotide sequence of the streptothricin acetyltransferase gene from *Streptomyces lavendulae* and its expression in heterologous hosts. J Bacteriol 169:1929–1937

Horowitz H, van Arsdell J, Platt T (1983) Nucleotide sequence of the *trpD* and *trpC* genes of *Salmonella typhimurium*. J Mol Biol 169:775–797

Kaplan JB, Nichols BP (1983) Nucleotide sequence of *Escherichia coli pabA* and its evolutionary relationship to *trp(G)D*. J Mol Biol 168:451–468

Kaplan JB, Goncharoff P, Seibold AM, Nichols BP (1984) Nucleotide sequence of the *Acinetobacter calcoaceticus trpGDC* gene cluster. Mol Biol Evol 1:456–472

Kaplan JB, Merkel WK, Nichols BP (1985) Evolution of glutamine aminotransferase genes: nucleotide sequences of the *pabA* genes from *Salmonella typhimurium*, *Klebsiella aerogenes* and *Serratia marcescens*. J Mol Biol 183:327–340

Katzy EI, Iosipenko AD, Egorenkov DA, Zhuravleva EA, Panasenko VI, Ignatov VV (1990) Involvement of *Azospirillum brasilense* plasmid DNA in the production of indole acetic acid. FEMS Microbiol Lett 72:1–4

Khammas KM, Ageron E, Grimont PAD, Kaiser P (1989) *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soils. Res Microbiol 140:679–693

Klee H, Monotoya A, Horodyski F, Lichtenstein C, Garfinkel D, Fuller S, Flores C, Peschon J, Nester E, Gordon M (1984) Nucleotide sequence of the *tms* genes of the pTiA6NC octopine Ti plasmid: Two gene products involved in plant tumorigenesis. Proc Natl Acad Sci USA 81:1728–1732

Knauf VC, Nester EU (1982) Wide host range vectors: a cosmid clone bank of an *Azospirillum* Ti plasmid. Plasmid 8:45–54

Kos T, Kuijvenhoven A, Hessing HGM, Pouwels PH, van den Hondel CAMJJ (1988) nucleotide sequence of the *Aspergillus niger trpC* gene: structural relationship with analogous genes of other organisms. Curr Genet 13:137–144

Lam WL, Cohen A, Tsouluhas D, Doolittle WF (1990) Genes for tryptophan biosynthesis in the archaebacterium *Haloferax volcanii*. Proc Natl Acad Sci USA 87:6614–6618

Magalhaes FM, Baldani JI, Souto SM, Kuykendall JR, Döbereiner J (1983) A new acid-tolerant *Azospirillum species*. An Acad Bras Cien 55:417–430

Marocco A, Bazzicalupo M, Perenzin M (1983) Forage grasses inoculation with gentamycine and sulfaguanidine resistant mu-

tants of *Azospirillum brasilense*. In: Klingmüller W (ed) Azospirillum II: Genetics, physiology, ecology. Springer-Verlag, Berlin, pp 149–158

Matsui K, Sano K, Ohtsubo E (1986) Complete nucleotide and deduced amino acid sequences of the *Brevibacterium lactofermentum* tryptophan operon. Nucleic Acids Res 14:10113–10114

Messing J (1983) New M13 vectors for cloning. Methods Enzymol 101:20–79

Mullaney EJ, Hamer JE, Roberti KA, Yelton MM, Timberlake WE (1985) Primary structure of the *trpC* gene from *Azospirillum nidulans*. Mol Gen Genet 199:37–45

Norrander J, Kempe T, Messing J (1983) Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26:101–106

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448

Penalva MA, Sanchez F (1987) The complete nucleotide sequence of the *trpC* gene from *Penicillium chrysogenum*. Nucleic Acids Res 15:1874

Reinhold B, Hurek T, Fendrik I, Pot B, Gillis M, Kersters K, Thielemans S, De Ley J (1987) *Azospirillum halopraeferens* sp. nov., a nitrogen-fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth). Int J Syst Bacteriol 37:43–51

Reynders L, Vlassak K (1979) Conversion of tryptophan to indoleacetic acid by *Azospirillum* sp. Soil Biol Biochem 11:547–548

Rivas MV, Jarvis ED, Rudner R (1990) The structure of the *trpE*, *trpD* and 5' *trpC* genes of *Bacillus pumilus*. Gene 87:71–78

Ruckdäschel E, Kittell BL, Helinski DR, Klingmüller W (1988) Aromatic amino acid aminotransferases of *Azospirillum lipoferum* and their possible involvement in IAA biosynthesis. In: Klingmüller W (ed) *Azospirillum* IV: Genetics, physiology, ecology. Springer-Verlag, Berlin, pp 49–53

Sambrook J, Fritsch E, Maniatis T (1989) molecular cloning, 2nd edn, vol 1–3. Cold Spring Habor Laboratory, Cold Spring Harbor, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5466

Sato S, Nakada Y, Kanaya S, Tanaka T (1988) Molecular cloning and nucleotide sequence of *Thermus thermophilus* HB8 *trpE* and *trpG*. Biochim Biophys Acta 950:303–312

Sekine M, Watanabe K, Syono K (1989) Nucleotide sequence of a gene for indole-3-acetamide hydrolase from *Bradyrhizobium japonicum*. Nucleic Acids Res 17:6400

Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram-negative bacteria. Bio/Technology 1:784–791

Shine J, Dalgarno L (1974) The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. Proc Natl Acad Sci USA 71:1341–1346

Slock J, Stahly DP, Han C-Y, Crawford IP (1990) An apparent *Bacillus subtilis* folic acid biosynthetic operon containing *pab*, an amphibolic *trpG*, a third gene required for synthesis of para-aminobenzoic acid, and the dihydropteroate synthase gene. J Bacteriol 172:7211–7226

Tang YW, Bonner J (1947) The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch Biochem 13:11–25

Tarrand JJ, Krieg NR, Döbereiner J (1978) A taxonomic study of the *Spirillum lipoferum* group, with the description of a new genus *Azospirillum* gen. nov. and two species, *Azospirillum brasilense* sp. nov. Can J Microbiol 24:967–998

Tien TM, Gaskins MH, Hubbel DH (1979) Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum*). Appl Environm Microbiol 37:1016–1024

Vieille C, Elmerich C (1990) Characterization of two *Azospirillum brasilense* Sp7 plasmid genes homologous to *Rhizobium meliloti nod PQ*. Mol Plant-Microbe Interact 3:389–400

Wain-Hobson S, Sonigo P, Danos O, Cole S, Alison M (1985) Nucleotide sequence of the AIDS virus, LAV. Cell 40:9–17

Yamada T, Palm CJ, Brooks B, Kosuge T (1985) Nucleotide sequences of *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. Proc Natl Acad Sci USA 82:6522–6526

Yanofsky C, Platt T, Crawford IP, Nichols BP, Christie GE, Horowitz H, van Cleemput M, Wu AM (1981) The complete nucleotide sequence of the tryptophan operon of *Escherichia coli*. Nculeic Acids Res 9:6647–6668

Yoshikawa A, Isono S, Sheback A, Isono K (1987) Cloning and nucleotide sequencing of the genes *rimI* and *rimJ* which encode enzymes acetylating ribosomal proteins S18 and S5 of *Escherichia coli* K12. Mol Gen Genet 209:481–488

Zalkin H, Paluh JL, van Cleemput M, Moye WS, Yanofsky C (1984) Nucleotide sequence of *Saccharomyces cerevisiae* genes TRP2 and TRP3 encoding bifunctional anthranilate synthase: indole-3-glycerol phosphate synthase. J Biol Chem 259:3985–3992

Zimmer W, Bothe H (1988) The phytohormonal interactions between *Azospirillum* and wheat. Plant Soil 110:239–247

Zimmer W, Elmerich C (1991) Regulation of the synthesis of indole-3-acetic acid in *Azospirillum*. In: Hennecke H, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions, vol 1. Kluver Academic Publishers, London, pp 465–468

Zimmer W, Roeben K, Bothe H (1988) An alternative explanation for the plant growth promotion by bacteria of the genus *Azospirillum*. Planta 176:333–342

Communicated by A. Kondorosi