REGULATION OF THE SYNTHESIS OF INDOLE-3-ACETIC ACID IN *AZOSPIRILLUM*

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

ABSTRACT. Bacteria of the genus *Azospirillum* are associated with the roots of *gramineae* and produce the phytohormone indole-3-acetic acid (IAA). In order to characterize genes involved in IAA biosynthesis, a cosmid library of *A. brasilense* Sp7 was transferred by conjugation into *A. irakense* KA3, a low producer of IAA. IAA production was increased in two transconjugants, both containing an identical 18.5 kb *Hin*dIII fragment of the *A. brasilense* genome. Tn*5* mutagenesis was performed with one of these cosmids. Tn*5* insertions at 36 different positions in the 18.5 kb fragment were transferred into strain KA3 and the IAA production of the recipient clones 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 two of these Tn*5* insertions into the genome of strain Sp7 led to Trp auxotrophic mutants. Sequencing of the region revealed that it contains part of *trpG*, complete *trpD* and part of *trpC*, organized in a single operon. To explain that introducing *trpD* into strain KA3 enhances IAA production, a model is proposed in which anthranilate inhibits IAA synthesis. Several experimental results corroborate this model.

1. Introduction

Production of phytohormones by *Azospirillum* ,which lives in association with the roots of grasses, has been proposed to be responsible for the increase of the root surface of the host-plant (reviewed by Elmerich et al. 1990). It has been established that indole-3-acetic acid (IAA) production by *Azospirillum* is tryptophan (Trp)-dependent (Tien et al. 1979, Zimmer and Bothe 1988). Mutants of *A. lipoferum* obtained by random Tn*5* mutagenesis (Abdel-Salam and Klingmüller 1987) showed a reduced IAA production rate. No mutant, completely unable to synthesize IAA was isolated, suggesting that *Azospirillum* possesses more than a single pathway for IAA synthesis. The biosynthetic pathway(s) has(have) not yet been elucidated. Moreover, a regulation mechanism probably controls IAA synthesis, since the release of this compound was only observed when the bacteria were supplied with Trp. Results reported here show that IAA synthesis in *Azospirillum* is inhibited by anthranilate.

2. Results and Discussion

2.1. SEARCH FOR THE PATHWAY OF INDOLE-3-ACETIC ACID SYNTHESIS IN *AZOSPIRILLUM BRASILENSE*

The best investigated pathway for IAA synthesis was first described in the phytopathogen *Pseudomonas syringae* (Magie et al. 1963). A Trp monoxygenase catalyses the conversion of Trp into indole-3-acetamide (IAM). The second step, the formation of IAA is catalysed

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by an IAM hydrolase.The genes coding for Trp monoxygenase (*iaaM*) and for IAM hydrolase (*iaaH*) of *P. syringae*, and for the IAM hydrolase (*bam*) of *Bradyrhizobium* have been isolated and sequenced (Yamada et al. 1985; Sekine et al. 1989). No hybridization signal was detected between *A. brasilense* total DNA and intragenic probes from *iaaM* and *iaaH* of *P. syringae*, or *bam* of *Bradyrhizobium* (data not shown). In addition HPLC-analyses showed that *Azospirillum* was unable to convert IAM to IAA. It was therefore concluded that this pathway is not present in *Azospirillum*.

2.2. CLONING AND MAPPING OF AN *A. BRASILENSE* SP7 LOCUS INVOLVED IN THE SYNTHESIS OF INDOLE-3-ACETIC ACID

A cosmid library of strain Sp7 constructed by cloning *Hin*dIII fragments in the broad host range cosmid vector pVK100 (Fogher et al. 1985) was used to screen for genes involved in IAA synthesis. 400 cosmids were transferred by conjugation into *A. irakense* KA3, a recently described *Azospirillum* species (Khammas et al. 1989), which was found to produce only 1/10 of the IAA amount of *A. brasilense* after 3 days of incubation in a Trp (100 mg/l) containing medium. Two transconjugants, containing pAB1005 and pAB1289 showed a significantly enhanced Trp-dependent IAA production when compared to the control strain, which contained pVK100 without any insert (Fig. 1). Cosmids pAB1005 and pAB1289 carried an identical 18.5 kb *Hin*dIII fragment, which was verified to be a genuine fragment of strain Sp7 genome by hybridization with *Hin*dIII digested total DNA of strain Sp7 (data not shown). The region was mapped by restriction analysis (Fig. 1). To localize the locus responsible for the enhancement of the IAA production, Tn*5* mutagenesis was performed with pAB1005. 36 different insertions were obtained in the 18.5 kb *Hin*dIII fragment. After transfer into *A. irakense* KA3, the IAA production of the recipient clones was assayed by reversed phase HPLC. Four transconjugants showed an IAA production level comparable to that of the strain containing the control cosmid pVK100. The others produced as much IAA as strain KA3 containing the wildtype cosmid pAB1005 (Fig. 1). The Tn*5* insertions of the 4 clones with reduced IAA production were mapped on a 3 kb *Sal*I fragment. Two of the Tn*5* containing fragments were subcloned in the suicide vector pSUP202 and recombined into the genome of wildtype strain Sp7. Surprisingly, the two recombinant strains were Trp auxotroph and their Trp-dependent IAA production was almost as high as that of the wildtype strain Sp7.

2.3. SEQUENCING OF THE LOCUS AND IDENTIFICATION OF A *trp*-OPERON

The nucleotide sequence of the 2 kb *Sal*I/*Sph*I fragment of pAB10053 (Fig. 1) was established. The 4 Tn*5* insertions which abolished the enhanced IAA production in *A. irakense*, were mapped in this 2 kb fragment. One complete open reading frame (ORF) encoding a polypeptide of 355 amino acids was localized in the middle of the 2 kb fragment (Fig. 1). The amino acid sequence showed homology with TrpD of *E.coli* (Yanofsky et al. 1981). The highly conserved regions found in TrpD polypeptides (Crawford 1989) were present in the amino acid sequence coded by this ORF. Another incomplete ORF was localized upstream from the 5'end of *trpD*. The deduced amino acid sequence of this ORF showed homology with the C-terminal part of *E. coli* TrpG. A third incomplete ORF starts directly at the 3'end of *trpD* (Fig. 1). Homology between the amino acid sequence coded by this ORF and the N-terminal part of TrpC of *E. coli* was detected. As the three genes *trpG*, *trpD* and *trpC* were adjacent, with the stop codon of the previous gene overlapping the start codon of the next, it is likely that the 3 *trp* genes of *A. brasilense* Sp7 are organized in a single operon.

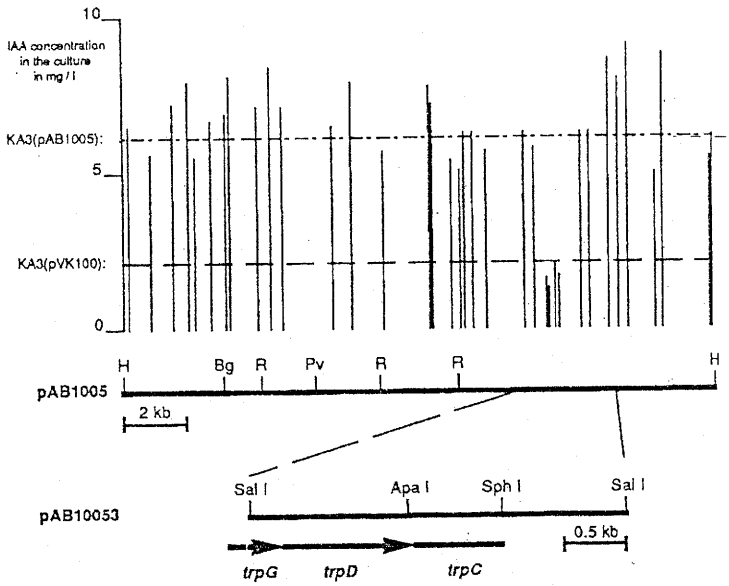

Figure 1. Physical map of pAB1005 and pAB10053 and IAA-production by *A. irakense* KA3 clones, carrying Tn*5* insertions in pAB1005. Restriction sites: H: *Hin*dIII, Bg: *Bg*lII, R: *Eco*RI, Pv: *Pvu*II. The vertical lines in the diagram represent the IAA amount, released in the culture medium by *A. irakense* KA3 clones, carrying a Tn*5* insertion in pAB1005 at the corresponding position. IAA amounts produced by strain KA3 carrying pVK100 and pAB1005 are shown by interrupted horizontal lines. Determination of IAA was performed by reversed phase HPLC (C_{18} column, detection at 278 nm, flow rate 1ml/min, mobile phase methanol : 1% H_3PO_4, 40/60 V/V) after 2 days of incubation in minimal medium supplemented with 100 mg/l Trp and 5 mg/l kanamycin. The arrows under the physical map of pAB10053 indicate the open reading frames established by sequencing.

2.4. MODEL FOR THE REGULATION OF INDOLE-3-ACETIC ACID SYNTHESIS IN *AZOSPIRILLUM*

The *trpD* gene encodes the phosphoribosylanthranilate transferase, which phosphoribosylates anthranilate in the second step of Trp synthesis. HPLC analysis of culture supernatants of *A. irakense* KA3 grown in the presence of Trp (100 mg/l) revealed that this strain consumed Trp faster than *A. brasilense* Sp7. Moreover it was found that strain KA3 released more anthranilate (16 mg/l in 2 days) into the medium than strain Sp 7 (less than 0.5 mg/l in 2 days), suggesting that strain KA3 was able to convert Trp into anthranilic acid. In contrast, strain KA3 released about 1.6 mg/l IAA whereas strain Sp7 released 16 mg/l under these conditions. The production of anthranilate by transconjugants of strain KA3 carrying the *trpD* gene of strain Sp7 on pAB1005 was reduced to 1/10 and the synthesis of IAA was enhanced more than 3 fold compared to the control strain containing pVK100.

These observations led us to propose a model for the regulation of IAA synthesis in *Azospirillum*. According to this model anthranilate inhibits the conversion of Trp to IAA.

In addition to the phenotypes of the KA3 mutants, this model accounts also for the observation that *Azospirillum 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 and translated. Consequently, the level of anthranilate, the first intermediate of the pathway, increases. According to the model, this inhibits IAA synthesis and prevents a loss of Trp from the cells. In the presence of external Trp, internal Trp synthesis is blocked and the intracellular level of anthranilate is low, enabling IAA synthesis.

In strain KA3, where the anthranilate concentration is high even in the presence of Trp, IAA synthesis is blocked. The anthranilate concentration in cultures of clones carrying *trpD* of strain Sp7 on pAB1005 was found to be 90% decreased suggesting that TrpD is functional in strain KA3. According to the model, the lower level of anthranilate enabled the observed enhanced IAA production.

The model is also in agreement with the behavior of the Tn*5* insertion TrpD$^-$-mutants of strain Sp7 (mentioned above), whose Trp-dependent IAA production was decreased by adding anthranilate to the medium, without affecting growth. Other experiments are in progress to corroborate this model.

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