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Analysis of IAA Biosynthesis in *Azospirillum lipoferum* and Tn5 Induced IAA Mutants

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Abstract

The presence of different IAA biosynthetic pathways in *A. lipoferum* was studied. In culture supernatants only intermediates of the transamination pathway could be detected. To a small extent (5% as compared to tryptophan) tryptamine could be converted to IAA when added to the culture medium. However, the *in vitro* IAA synthesis was dependent on the presence of α-ketoglutaric acid and a conversion of tryptamine or indole-3-acetamide to IAA could not be observed. Previously isolated Tn5 induced IAA mutants (Abdel Salam et al., 1987) showed no loss of an aromatic aminotransferase and the *in vitro* IAA synthesis was similar to that of the wildtype. It therefore is suggested that genes for the excretion of IAA into the medium are affected in these mutants. The Tn5 integration sites of two different mutants were cloned and the corresponding wildtype DNA sequences were isolated from a gene bank of *A. lipoferum*. The two mutations mapped in the same genetic region of the DNA within a 2.7 kb HpaI/BglII fragment. An EcoRI fragment carrying the Tn5 integration site of mutant Al37 was cloned into the suicide vector pSUP202. Then the Tn5 was substituted by a Cm resistance cartridge. The resulting plasmid was used to introduce a second mutation in other Tn5 mutants. However, strains carrying two different IAA mutations still produced IAA.

Keywords: indole-3-acetic acid, Azospirillum, iaa – genes

Abbreviations: IAA: indole-3-acetic acid, ILA: indole-3-lactic acid, IPyA: indole-3-pyruvic acid, ICA: indole-3-carboxylic acid, IEtOH: indole-3-ethanol, IMeOH: indole-3-methanol, IAM: indole-3-acetamide, IAN: indole-3-acetonitril

1. Introduction

The production of the plant hormone indole-3-acedic acid (IAA) by *Azospirillum lipoferum* and *A. brasilense* is already well documented (Hartmann et al., 1983; Crozier et al., 1988). However, the role of IAA in the plant bacteria interaction has not yet been elucidated. Therefore mutants are desirable that do not produce IAA. By using Tn5 mutagenesis Abdel Salam et al. (1987) isolated mutants of *A. lipoferum* that produced less IAA than the wildtype. But no completely IAA negative mutant could be obtained, indicating that the mutagenesis of one gene is not sufficient for the isolation of completely IAA minus mutants. The aims of this study therefore were to analyse the pathways involved in IAA biosynthesis, to characterize the isolated Tn5 mutants and to construct new mutants harbouring two different IAA mutations.

2. Materials and Methods

Bacterial strains and growth conditions

Azospirillum lipoferum (ATCC 29708) and iaa minus mutants Al37, Al320, Al106, Al13 and Al99 (Abdel Salam et al., 1987) were grown at 30°C in MAZ medium (Albrecht and Okon, 1980) supplemented with 100 μg/ml tryptophan. For the mutants 50 μg/ml neomycin was added.

Extraction of indole compounds

The indole compounds were extracted from culture supernatants with ethylacetate in the presence of 5 mM diethyldithiocarbamate (Crozier et al., 1988) and IAA was detected with a Shimadzu RF 535 (Shimadzu Corp., Kyoto, Japan) fluorescence detector (exitation 280 nm, emission 350 nm) connected to a Beckman System Gold HPLC station (Beckman Corp., FRG) equipped with an ODS C_{18} column. Isocratic elution with 35% methanol in 1% acetic acid was used to separate IAA from other indole compounds. The IAA peak was quantified by using indole-3-propionic acid as an internal standard (Akiyama et al., 1983). As a control 100 μg/ml tryptophan or IAA were added to MAZ medium without bacteria. No spontaneous breakdown of tryptophan and no decomposition of IAA to ICA was observed.

In vitro *IAA synthesis*

Crude cell extracts were generated by sonication and protein concentrations were determined by using the Coomasie Protein Assay reagent (Pierce

Corp., The Netherlands). The assay mix for the *in vitro* experiments contained 100 mM Tris/HCl, pH 8.3, 0.1 mM pyridoxalphosphate, 20 mM α-ketoglutarate, 3 mM NAD, 1 mM DTT, 5 mM tryptophan and 100 μg of protein in a final volume of 500 μl. After incubation for 6 hr the reaction products were analysed by HPLC. The IAA production was found to be linear for at least 8 hr. The detection limit was at 2 ng IAA synthesized per min and mg of protein.

DNA manipulation

Recombinant DNA procedures were done by the method of Sambrook et al. (1989). Enzymes were used according to the manufacturer's instructions.

Bacterial matings and marker exchange

For bacterial matings the procedure of Eppendorf conjugation (Singh et al., 1986) was used. For marker exchange the site-directed mutagenesis technique of Ruvkun and Ausubel was applied using the suicide plasmid pSUP202 (Tc^r, Amp^r) as described (Singh et al., 1986).

3. Results

Analysis of culture supernatants

Bacteria were grown to stationary growth phase in MAZ medium without tryptophan supplementation. Then the cells were harvested and resuspended in fresh MAZ medium to which now 100 μg/ml tryptophan was added. After a further incubation for 16 hr the culture supernatants were extracted with ethylacetate and analysed by reversed phase HPLC. In chromatograms of the acidic extract IAA, ILA and a breakdown product of IPyA were detected (Fig. 1). This breakdown product also was described by McQueen-Mason et al. (1989). Forty-eight hours after the addition of tryptophan, IAA and ILA were the dominant substances in the chromatogram. In cultures grown for 96 hr in the presence of tryptophan also ICA – a break down product of IAA was detected. In neutral extracts IEtOH and especially in cultures older than 72 hr, IMeOH were detected. Neither tryptamine nor IAM or IAN could be found.

Conversion of precursor substances to IAA

In a second type of experiments we tested whether different IAA precursor substances could be converted to IAA, both, when added to culture media

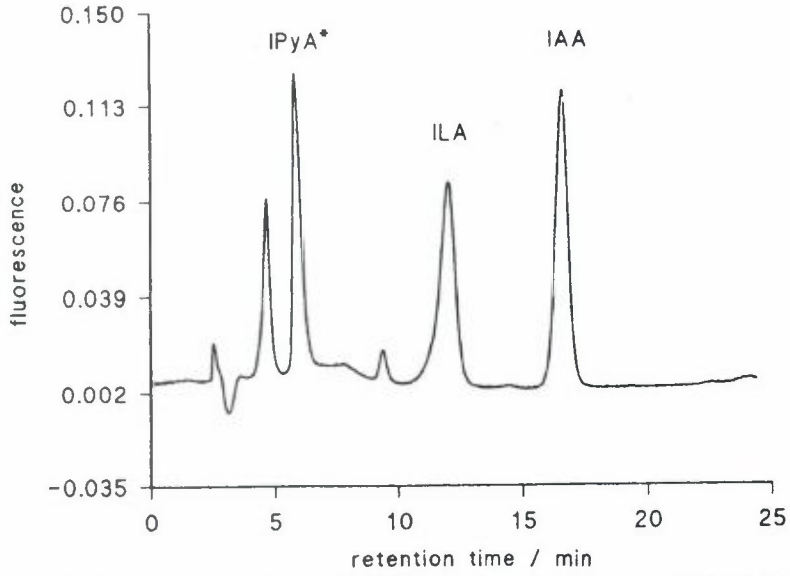

Figure 1. Chromatogram of an acidic extract of a culture supernatant of *A. lipoferum* after incubation in tryptophan supplemented MAZ medium for 16 hr. IPyA* refers to a breakdown product of IPyA.

(*in vivo*) and when added to cell extracts (*in vitro*). After the addition of tryptophan to the media of stationary phase cultures, about 3.2 μg/ml IAA accumulated to the supernatant. However, *in vitro* only tryptophan was converted to IAA (Table 1). Furthermore, in the absence of α ketoglutaric acid, tryptophan was not converted to IAA *in vitro*. The detection limit of IAA in these *in vitro* experiments was 2 ng IAA per mg protein and min. We therefore conclude that *A. lipoferum* produces IAA mainly via the transamination pathway, but might possess a minor pathway via tryptamine.

Analysis of Tn5 mutants

Based on the results obtained with the wildtype, we examined different Tn5-induced IAA minus mutants. No accumulation of an intermediate substance was found in culture supernatants and no loss of one of the four aromatic aminotransferases (Ruckdäschel et al., 1987) was detected on polyacrylamide gels (data not shown). Furthermore, the *in vitro* IAA synthesis was comparable to that of the wildtype, although the *in vivo* IAA production is reduced to about 10–20% (Table 2). We therefore suggest, that genes for the excretion of IAA are affected in these mutants.

Table 1. Conversion of different precursor substances to IAA. The substances were added in a concentration of 100 μg/ml either to stationary phase cultures (*in vivo*) or to a cell extract (*in vitro*). IAA production *in vivo* was determined after an incubation time of 16 hr. The *in vitro* experiments were done as described in Materials and Methods. Each value represents the mean of at least three different experiments

Precursor	IAA production		
	in vivo μg/ml		*in vitro* ng $mg^{-1}min^{-1}$
tryptophan	3.20	(100%)	83
tryptamine	0.18	(5.6%)	< 2
indoleacetamide	0.04	(1.2%)	< 2
indoleacetonitrile	0.05	(1.7%)	< 2
none	0.04	(1.2%)	< 2
controls without bacteria:			
tryptophan	< 0.02		< 2
tryptamine	< 0.02		< 2
indoleacetamide	< 0.02		< 2
indoleacetonitrile	< 0.02		< 2

Table 2. Comparison of IAA production of wildtype strain and different Tn5 mutants. Each value represents the mean of three different experiments. The experimental conditions were the same as for the experiments described in Table 1.

	IAA production from tryptophan	
	in vivo μg/ml	*in vitro* ng $mg^{-1}min^{-1}$
wildtype	3.2	72.4
Al37	0.3	66.6
Al320	0.4	71.4
Al106	0.7	68.3

Cloning of putative IAA excretion genes

We then decided to clone the corresponding wildtype DNA region from a cosmid library of *A. lipoferum* (Ruckdäschel et al., 1987). At first a 20 kb EcoRI fragment containing the integrated Tn5 of mutant Al37 was cloned in the vector pACYC 184 (plasmid p37-1). Then a fragment next to the Tn5 was used as a probe to screen the cosmid library by colony hybridization. Different overlapping clones were obtained that comprise about 40 kb of the genomic DNA (cosmids p37K1, p37K2 and p37K3; see Fig. 2). Further analysis revealed that the integration site of another IAA minus mutant (Al 106) maps only 200 bp distant from that of mutant Al 37 on a 2.7 kb HpaI/BglII fragment (Fig. 2).

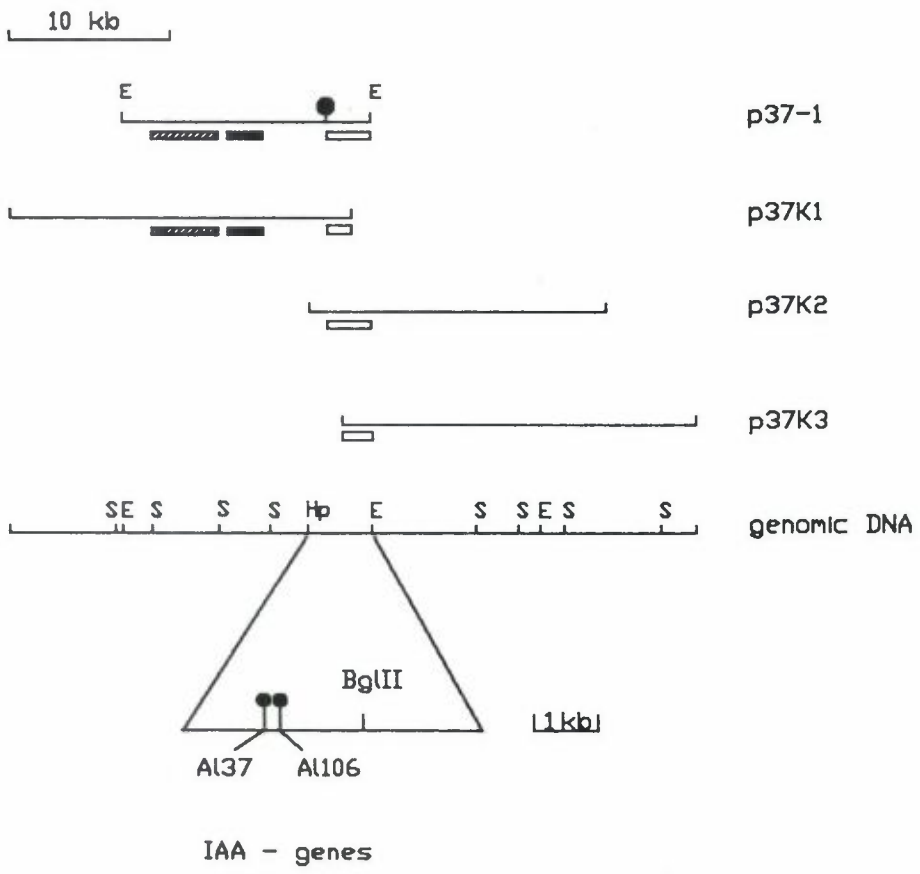

Figure 2. Physical map of the chromosomal region of *A. lipoferum* containing IAA genes. The plasmid names refer to different overlapping gene bank clones containing DNA of this region. p37-1 consists of an EcoRI fragment of mutant Al37 containing the Tn5 and the vector pACYC 184. Al37 and Al106 mark the locus of the Tn5 integration in the mutants Al37 and Al106, respectively. The bars indicate different probes and the corresponding homologous fragments used to verify the physical map.

Construction of IAA double mutants

The analysis of previously isolated Tn5 mutants (Abdel Salam et al., 1987) indicated that the mutagenesis of one gene is not sufficient to obtain completely IAA negative mutants. We therefore wanted to know whether a combination of two different mutations in one cell would result in completely IAA negative mutants. To address this question, we at first cloned the EcoRI fragment containing the integrated Tn5 of mutant Al 37 into the suicide vector pSUP202. Then the Tn5 was cut out of this plasmid by restriction with HpaI. In the

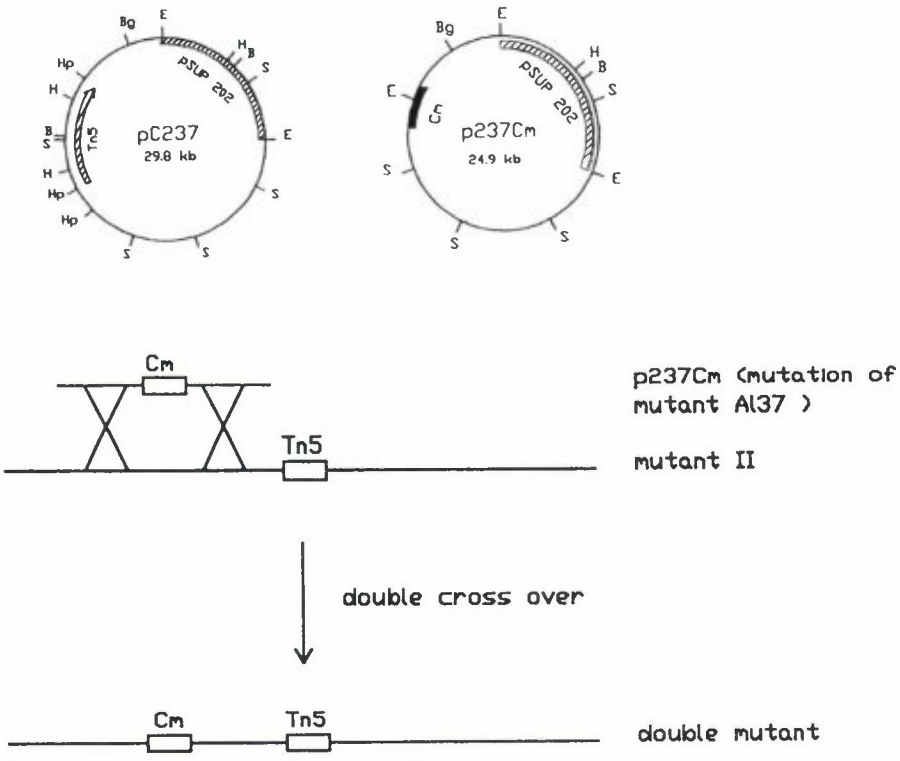

Figure 3. Strategy for the construction of IAA double mutants. The plasmids pC237 and p237Cm are described in the text.

Table 3. Effect of the integration of a Cm cassette from plasmid p237Cm in *A. lipoferum* and different mutants on the IAA production *in vivo*. IAA production was measured after an incubation time of 72 hr at which all cultures had an optical density (A_{578}) of about 1.5. The strains 708Cm, Al13Cm and Al99Cm are double mutants carrying the Cm resistance cassette in addition to the Tn5 and were grown in the presence of 20 μg/ml Cm. The values represent the mean of three, in the case of 708Cm, Al13Cm and Al99Cm of 20 different experiments.

Strain	IAA (μg/ml)
708 (wildtype)	4.4
Al13	2.5
Al99	3.3
708Cm	0.5
Al13Cm	0.6
Al99Cm	0.7

remaining HpaI site then a Cm resistance cartridge (Schneider et al., 1990) was cloned (plasmid p237Cm; see Fig. 3). After the conjugation of this plasmid into other IAA minus mutants, site-directed mutants carrying both the Tn5 and the Cm cassette were isoalted by their Neo^r Cm^r Te^s phenotype. As derivatives of pSUP202 cannot replicate in *Azospirillum* the loss of Tc resistance indicates an integration of the Cm cassette into the chromosome by homologous recombination, as outlined in Fig. 3. In each case 20 colonies were purified and tested for IAA production. The results showed that the introduction of a second mutation reduced the IAA production but did not cause a complete loss of IAA production (Table 3).

4. Discussion

Our results show that *A. lipoferum* produces IAA mainly via the transamination pathway. But similar to *A. brasilense* (Hartmann et al., 1983), *A. lipoferum* might possess a second, minor pathway via tryptamine. The occurrence of several IAA pathways in addition to the existence of multiple aromatic aminotransferases that can catalyse the initial reaction of the transamination pathway (Ruckdäschel et al., 1987), renders the task of isolating completely IAA negative mutants unpromising. However, the analysis of Tn5 mutants revealed the existence of genes that affect the production of IAA without affecting its biosynthesis. Abdel Salam et al. (1987) already demonstrated a normal growth rate and tryptophan uptake of the Tn5 mutants. We therefore suggest that the mutants investigated harbour a defect in genes involved in the excretion of IAA into the medium. The elimination of IAA excretion could result in completely IAA negative mutants. This adds an interesting new aspect to the isolation of IAA minus mutants that has to be exploited in future research work.

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