Symbiosis, 13 (1992) 151–158
Balaban, Philadelphia/Rehovot

IAA Synthesis in *Azospirillum brasilense* Sp6: Analysis of a Mutant Impaired in IAM-Hydrolase

A. COSTACURTA[1,*], E. PRINSEN[2], H. VAN ONCKELEN[2],
K. MICHIELS[1], J. VANDERLEYDEN[1] and M.P. NUTI[3]
[1]*F.A. Janssens Laboratory of Genetics, Catholic University of Leuven*
W. de Croylaan 42, B-3001 Heverlee, Belgium
Tel. 32 (16) 220921, Fax 32 (16) 220761
[2]*Department of Biology, University of Antwerp, Universiteitsplein 1*
B-2610 Wilrijk, Belgium
[3]*Department of Biotechnology, University of Padova, Via Gradenigo 6, Italy*

Received October 20, 1992; Accepted April 13, 1992

Abstract

A. brasilense SpM7918, a previously isolated *A. brasilense* Sp6 Tn*5* mutant, is shown to produce high amounts of indoleacetamide, which is a precursor of indole-3-acetic acid in the indoleacetamide pathway, and to excrete very low amounts of indole-3-acetic acid. From genomic DNA of the mutant, the Tn*5* containing restriction fragment was cloned in pUC19 and used as a hybridization probe to screen a genomic library of *A. brasilense* Sp245. Seven positive clones were isolated and conjugated into the original mutant restoring IAA production. One transconjugant was further analyzed for indoleacetamide and indole-3-acetic acid production by HPLC.

Keywords: *A. brasilense*, indole-3-acetic acid mutant, IAM pathway

1. Introduction

Bacteria of the genus *Azospirillum* are diazotrophs associated with the roots of grasses and cereals (reviewed by Bashan et al., 1990). The capacity of

0334-5114/92 /$03.50

azospirilla to promote plant growth, although not fully understood, is presumed to depend on several bacterial properties. One of these factors could well be the production of phytohormones, e.g. indole-3-acetic acid (IAA), (Barbieri et al., 1986). In order to elaborate further on this property, more knowledge on IAA biosynthesis by *Azospirillum* is needed, especially on the biochemical pathway(s) and the determining genes.

The best investigated pathway for IAA biosynthesis is described for the phytopathogen *Pseudomonas syringae*. Tryptophan-2-monooxygenase converts tryptophan to indoleacetamide (IAM) and then indolacetamide-hydrolase catalyzes the conversion of IAM to IAA. *iaa*M and *iaa*H, and *tms*-1 and *tms*-2, the genetic determinants for these enzymatic conversions, respectively in *Pseudomonas syringae* and *Agrobacterium tumefaciens*, have been cloned and sequenced (Yamada et al., 1985; Klee et al., 1984; Gielen et al., 1984). Also in *Bradyrhizobium japonicum* the *bam* gene, which encodes for the indolacetamide-hydrolase has been cloned (Sekine, 1989), but there is no evidence that in this bacterium the IAM pathway operates in symbiotic conditions.

In *A. lipoferum* only the presence of the transamination pathway has been reported, while the excretion of IAM in the supernatants of bacterial cultures was not detected (Ruckdaschel, et al., 1990). Recently genes involved in the IAA synthesis have been localized on the 85 MDa plasmid of *A. brasilense* Sp245, by isolating a Tn*5-mob* insertion mutant with less IAA production and accumulation of anthranilic acid (Katzy et al., 1990). A possible regulation role of anthranilic acid on IAA biosynthesis in *Azospirillum brasilense* has been proposed (Zimmer and Elmerich, 1991). Since no mutant completely unable to synthesize IAA was isolated after Tn*5* mutagenesis in *A. lipoferum* (Abdel-Salam and Klingmuller, 1987) and *A. brasilense* (Barbieri et al., 1990), it was suggested that *Azospirillum* possesses more than one pathway for IAA synthesis.

The biosynthetic pathway to indole-3-acetic acid in *Azospirillum* is still unclarified. Here we present data consistent with the existence of the IAM pathway in *A. brasilense*.

2. Materials and Methods

Bacterial strains

Strains used in this study are listed in Table 1.

Table 1. Bacterial strains

Strains	Relevant properties	Source/Reference
E. coli HB101	F$^-$ *hsd*20 (r_{B-} m_{B-}), *recA*13 *ara*$^-$14, *proA2*, *lacY1*, *galK2* *rpsl20*, (*Sm*^{r}) *xyl*$^-$ *5*, *mtl*$^-$ *l* *supE44*$^-$	Boyer et al. (1969)
E. coli S17-1	*pro*, *thi*, *hsdR*, *hsdM*$^+$, *recA*, Tp^r, Sm^r, (RP4-2-Tc::Mu::Km::Tn7)	Simon et al. (1983)
A. brasilense Sp7 (ATCC29145)		Tarrand et al. (1978)
A. brasilense Sp245		Baldani et al. (1987)
A. brasilense Sp6		Bani et al. (1980)
A. brasilense SpM7918	Tn*5*-induced IAA mutant of Sp6	Barbieri et al. (1990)
A. lipoferum Sp59		Tarrand et al. (1978)
A. lipoferum Br17		Tarrand et al. (1978)
A. irakense KBC1		Khammas et al. (1989)
A. halopraeferens Au4		Rheinhold et al. (1987)

Growth media

Azospirillum strains were grown at 30°C in L*, which is LB supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ or in MMAB medium (Vanstockem et al., 1987). For screening the IAA synthesis, growth medium was supplemented with 0.1 mg/ml of tryptophan. *Escherichia coli* strains were maintained on M9 glucose agar plates or LB agar plates and grown in LB broth at 37°C (Sambrook et al., 1989). When required, antibiotics were added in the following concentrations (μg/ml): tetracycline (Tc), 10; kanamycin (Km), 30; ampicillin (Ap), 100.

DNA manipulation

Isolation of total DNA and plasmid DNA, DNA ligations, Southern hybridizations, and transformations were performed using standard techniques (Sambrook et al., 1989). Plasmid profiles of *A. brasilense* were visualized by gel electrophoresis according to Kado and Liu (1981).

DNA restriction fragments for cloning and for ^{32}P-labelling were isolated from agarose gel pieces, after electrophoresis, by a freeze and thaw method (Sambrook et al., 1989).

Bacterial conjugations

One ml portions of log phase cultures of donor, recipient, and when necessary, helper strain, were centrifuged and cells were resuspended in 100 μl LB broth. Thirty μl of the appropriate suspensions were then mixed and spread as a patch on D agar plates, which contained per liter: 8 g Bacto Nutrient Broth (Difco Laboratories, MI, USA), 0.25 g $MgSO_4 \cdot 7H_2O$, 1 g KCl, 0.01 g $MnCl_2$. After overnight incubation at 30°C in a humid atmosphere, bacteria were scraped from the agar surface, suspended in 1 ml sterile 0.85% NaCl, and plated on selective media.

Detection of indole-3-acetic acid

IAA was colorimetrically determined with the Salkowsky reagent in supernatants of *Azospirillum* cultures grown for 72 hr in MMAB with 0.1 mg/ml tryptophan (Kaper and Veldstra, 1958).

3. Results and Discussion

From a collection of Tn*5*-induced mutants of *A. brasilense* Sp6, a particular mutant SpM7918 with reduced IAA production was previously isolated (Barbieri et al., 1990) using a screening with Salkowsky reagent (Kaper and Veldstra, 1958). Lower IAA production by this mutant was confirmed and IAM accumulation was found by H.P.L.C. analysis (Table 2), suggesting that the IAM pathway is present and the IAM-hydrolase is impaired.

Table 2. H.P.L.C.-analysis[a] of *A. brasilense* strains for tryptophan (TRP), indolacetamide (IAM), and indole-3-acetic acid (IAA)

Strains	Minimal medium without tryptophan			Minimal medium with tryptophan	
	TRP	**IAM**	**IAA**	**IAM**	**IAA**
Sp6	12549	28.6	86882	15696	1392379
SpM7918	46943	408.6	1946	59388	80812
SpM7918(p0.2)	ND	ND	ND	787	465906

Data are given in picomol per 10 ml culture supernatant. Values are averages obtained from samples taken at the 2nd, 3rd, 4th day of growth. Sp6 is an *A. brasilense* wild type, SpM7918, a Tn*5* insertional mutant, and SpM7918(p0.2) an exconjugant with a positive hybridizing clone.

[a]Analyses were carried out as described by Prinsen et al. (1991)

From *Sal* I digested genomic DNA of the mutant SpM7918, a 13.5 Kb *Sal* I fragment containing a Tn*5* flanking region of 11 Kb and the kanamycine resistance half of Tn*5* was cloned in pUC19.

Other *Azospirillum* spp. were analysed for the presence of the sequence which had been mutated in SpM7918 (Fig. 1). Total DNA was isolated from *A. brasilense* Sp7, *A. brasilense* Sp245, *A. lipoferum* Sp59, *A. lipoferum* Br17, *A. irakense* KBC1 and *A. halopraeferens* Au4, and digested with *Sal* I. The fragments were separated by gel electrophoresis and blotted onto nitrocellulose filters. These were hybridized against the 13.5 Kb *Sal* I fragment, cut from agarose gel and ^{32}P-labelled. Two positive signals, ca. 13.5 and 7 Kb were detected, with the Tn*5* insertional mutant SpM7918, representing, as expected, the right and left flanking sequences of the inserted Tn*5* (since this transposon has one unique central *Sal* I site). Hybridization signals were found for all the strains tested except *A. irakense*, indicating that this sequence is generally present among *Azospirillum* spp.

The same 13.5 Kb *Sal* I fragment hybridized also with the chromosomal DNA band on plasmid profiles obtained by the protocol of Kado and Liu (Kado and Liu, 981) showing its localization in the chromosome (data not shown).

In order to clone the corresponding sequences, a pLAFR1 cosmid genomic library of *A. brasilense* Sp245 was screened by colony hybridization with the 13.5 Kb fragment cloned from the mutant SpM7918. Seven pLAFR1 clones, all containing a 6.5 Kb EcoRI fragment, were identified and subsequently transferred into *E. coli* S17-1 strain. Plasmids were subsequently mobilized into *A. brasilense* SpM7918 in a biparental mating with the *E. coli* S17-1 transformants. Exconjugants were selected on minimal medium plates that contained tetracycline and kanamycine. To determine if the IAA production was restored, the seven exconjugants were grown on minimal medium with the appropriate antibiotics and 0.1 mg/ml tryptophan. After 3 days, IAA production was colorimetrically demonstrated with Salkowsky reagent in the supernatants of all the exconjugants. As a control, an exconjugant, where only the cosmid pLAFR1 was mobilized, did not show any positive reaction (data not shown).

One of the exconjugants, named SpM7918(po.2), was further investigated by H.P.L.C. analysis. Samples of culture supernatant were analyzed at the 2nd, 4rd, and 4th day of growth. Data indicate that the IAA production of the complemented clone increased five times compared with the mutant, abolishing the accumulation of IAM. The fact that IAA production was not fully restored to the wild type level might be due to the presence of other genes, structural as well as regulatory, involved in the IAA biosynthesis, on the multicopy plasmid used for complementation.

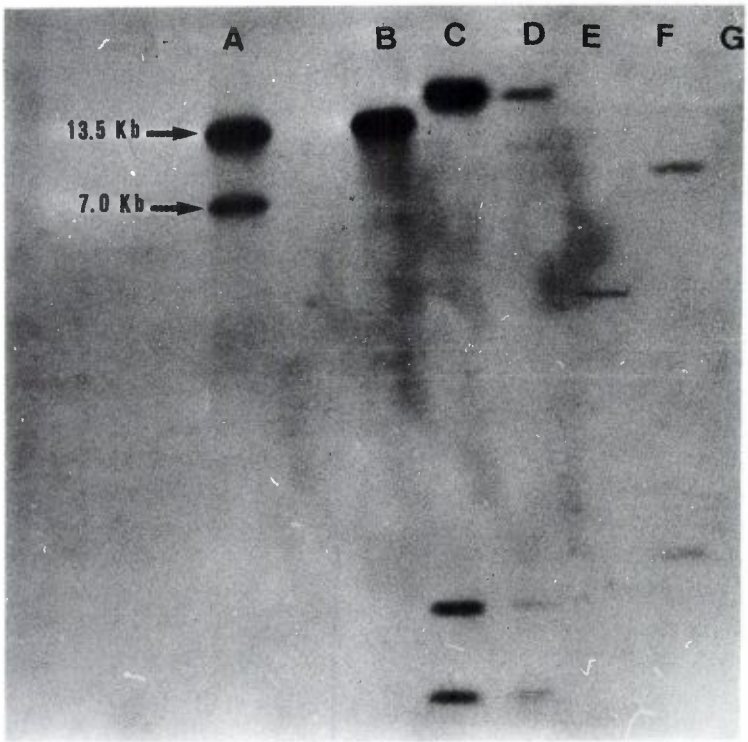

Figure 1. Hybridization of blotted total DNA from several *Azospirillum* strains digested with *Sal* I. The probe was the Tn*5*-containing 13.5 Kb *Sal* I ^{32}P-labelled fragment cloned in pUC19 from the mutant SpM7918.
(a) *A. brasilense* Sp6 Tn*5* insertional mutant SpM7918
(b) *A. brasilense* Sp7
(c) *A. brasilense* Sp245
(d) *A. lipoferum* Sp59
(e) *A. lipoferum* Brl7
(f) *A. halopraeferens* Au4
(g) *A. irakense* KBC1

From previous papers, it is clear that IAA biosynthesis in *A. brasilense* appears to be quite complex. Nevertheless our results clearly demonstrate that *A. brasilense* contains a functional gene that encodes conversion of IAM to IAA. Further experiments to elucidate the structure of this gene and its regulation in *A. brasilense* will help to understand the IAA biosynthetic pathways in *A. brasilense.*

Acknowledgements

This work was funded by the Nationaal Fonds voor Wetenschappelijk Onderzoek (N.F.W.O., contract 3.0093.89), and by the Onderzoeksraad K.U. Leuven (contract OT/88/20). A.C. wishes to thank the National Food Biotechnology Centre of the University College Cork, Ireland, for helpful discussions during a predoctoral stay and A. Squartini (University of Padova) for helpful suggestions during the course of this work. A.C. acknowledges the receipt of a predoctoral fellowship of the "Fondazione Aldo Gini" (University of Padova) and a training grant of BIOMERIT (EEC COMETT II).

REFERENCES

Abdel-Salam, M.S. and Klingmuller, W. 1987. Transposon Tn*5* mutagenesis in *Azospirillum lipoferum*: isolation of indole acetic acid mutants. *Mol. Gen. Genet.* **210:** 165–170.

Bani, D., Barberio, C., Bazzicalupo, M., Favilli, F., Gallori, E., and Polsinelli, M. 1980. Isolation and characterization of glutamate synthase mutants of *Azospirillum brasilense. J. Gen. Microbiol.* **119:** 239–244.

Barbieri, P., Baggio, C., Bazzicalupo, M., Galli, E., Zanetti, G., and Nuti, M.P. 1990. *Azospirillum-Gramimae* interaction: effect of indole-3-acetic acid. *Nitrogen Fixation*, Vol. 48. M. Polsinelli, R. Materassi and M. Vincenzini, eds. Kluwer Acad. Publ., Dordrecht, pp. 161–168.

Barbieri, P., Zanelli, T., Galli, E., and Zanetti, G. 1986. Wheat inoculation with *Azospirillum brasilense* Sp6 and some mutants altered in nitrogen fixation and indole-3-acetic acid production. *FEMS Microbiol. Lett.* **36:** 87–90.

Bashan, Y. and Levanony, H. 1990. Current status of *Azospirillum* as a challenge for agriculture. *Can. J. Microbiol.* **36:** 591–608.

Boyer, H.W. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli. J. Mol. Biol.* **41:** 459–465.

Gielen, J., De Beuckeleer, M., Seurinck, J., Deboeck, F., de Greve, H., Lemmers, M., Van Montagu, M., and Schell, J. 1984. The complete nucleotide sequence of the TL-DNA of the *Agrobacterium tumefaciens* plasmid pTiAch5. *EMBO J.* **3:** 835–846.

Kado, C.I. and Liu, S.T. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **145:** 1365–1373.

Kaper, J.M. and Veldstra, H. 1958. On the metabolism of tryptophan by *A. tumefaciens*. *Biochim. Biophys. Acta* **30:** 401–406.

Katzy, E.I., Isopienco, A.D., Egorenkov, D.A., Zhuravleva, E.A., Panasenko, V.I., and Ignatov, V.V. 1990. Involvement of *Azospirillum brasilense* plasmid DNA in the production of indole acetic acid. *FEBS Lett.* **72:** 1–4.

Khammas, K.M., Ageron, E., Grimont, P.A.D., and 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., Montoya, A., Horodyski, F., Lichtenstein, C., Garfinkel, D., Fuller, S., Flores, C., Pechon, J., Nester, E., and Milton, G. 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.

Prinsen, E., Chauvaux, N., Schmidt, I., John, M., De Greef, J., and Van Onckelen, H. 1991. Stimulation of indole-3-acetic acid production in *Rhizobium* by flavonoids. *FEBS Lett.* **282:** 53–55.

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

Ruckdaschel, E., Ramschultz, S., Kraft, K., and Klingmuller, W. 1990. Genetic and biochemical studies on the production of indole-3-acetic acid (IAA) in *Azospirillum lipoferum*; *Nitrogen Fixation.* Vol. 48. M. Polsinelli, R. Materassi, M. Vincenzini, eds. Kluwer Acad. Publ., Dordrecht, pp. 301–302.

Sambrook, J., Fritsch, E., and Maniathis, T. 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York.

Simon, R., Priefer, U., and Puhler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio. Technology* **1:** 784–791.

Tarrand, J.J., Krieg, N.R., and Döbereiner, J. 1978. A taxonomic study of the *Spirillum lipoferum* group with description of a new genus, *Azospirillum* gen. nov., and two species, *Azospirillum lipoferum* (Beyerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can. J. Microbiol.* **24:** 967–980.

Vanstockem, M., Michiels, K., Vanderleyden, J., and Van Gool, A. 1987. Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*: Physiological analysis of Tn*5* and Tn*5mob* insertional mutants. *Appl. Environ. Microbiol.* **53:** 1387–1405.

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

Zimmer, W. and Elmerich, C. 1991. Regulation of the synthesis of indole-3-acetic acid in *Azospirillum*. In: *Advances in Molecular Genetics of Plant-Microbe Interactions.* Vol. 1. H. Hennecke and D.P.S. Verma, eds. Kluwer Acad. Publ., Dordrecht, pp. 465–468.