# Azospirillum brasilense Indole-3-Acetic Acid Biosynthesis: Evidence for a Non-Tryptophan Dependent Pathway

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Bacteria of the nitrogen-fixing genus Azospirillum live in association with roots of many plants. Bacterial phytohormone synthesis is proposed to influence the host plant root proliferation. Analysis of tryptophan (Trp), indole-3-acetamide (IAM), and indole-3-acetic acid (IAA) synthesis of the mutant Azospirillum brasilense strain SpM7918 showed an indoleacetamide accumulation concomitant with reduced indoleacetic acid synthesis. The IAA deficiency, and IAM accumulation could be reversed with a specific cosmid from an A. brasilense Sp245 library. The identity of the indoleacetic acid and indoleacetamide produced was confirmed by HPLC with on-line mass spectrometry. Specific radioactivities of tryptophan, indoleacetamide, and indoleacetic acid formed during $^3$H-IAM and $^3$H-Trp feeding experiments revealed multiple IAA biosynthetic pathways in Azospirillum: the indoleacetamide pathway, a second tryptophan-dependent, and a tryptophan-independent pathway, the latter being predominant in case no tryptophan was supplied to the medium. This report is the first to demonstrate tryptophan-independent indoleacetic acid synthesis in bacteria.

Additional keywords: auxin, auxin mutant.

Bacteria of the nitrogen-fixing genus Azospirillum live in association with roots of many plants (for a review, see Döbereiner and Pedrosa 1987). Like most rhizosphere bacteria, Azospirillum species produce phytohormones such as cytokinins, indoleacetic acid (IAA), and gibberellins (Tien et al. 1979; Reynders and Vlassak 1979; Horemans et al. 1986). This excretion of phytohormones by associated bacteria may promote plant growth. Indeed root colonization by Azospirillum affects host plant root hair branching (Jain and Patriquin 1985) and root elongation (Harari et al. 1988). In several

studies, the increased plant growth observed after inoculation with Azospirillum was proposed to be attributable to IAA and not to enhanced nitrogen supplied by the bacteria (Barbieri et al. 1986; Bashan et al. 1989). These effects could be mimicked by exogenous auxins (Tien et al. 1975; Reynders and Vlassak 1979). More direct evidence for the role of the bacterial IAA production was presented recently by Barbieri and Galli (1993) showing that, compared with the wild-type strain, a mutant producing very low IAA had reduced ability to promote root system development in the host plant. Although IAA production by Azospirillum has been intensively investigated, little is known so far about the pathway(s) involved. Based on the observation that IAA could only be detected in Trp supplemented media, Zimmer and Bothe (1988) proposed a Trp-dependent regulation of IAA biosynthesis in Azospirillum. However, the failure to detect IAA in nonsupplemented media could also be attributed to the poor detection limit of the methods used. The best-investigated pathway for IAA synthesis is the IAM pathway, which is shown to operate in Pseudomonas savastanoi (Kosuge et al. 1966; Comai and Kosuge 1982) and Agrobacterium-transformed plant cells (Schröder et al. 1984; Thomashow et al. 1984; Van Onckelen et al. 1985; 1986). Tryptophan-2-monooxygenase converts Trp to IAM, which is converted to IAA by an indoleacetamide-hydrolase. iaaM and iaaH, and tms1 and tms2, the genetic determinants for these enzymatic conversions, respectively, in P. savastanoi and Agrobacterium tumefaciens, have been cloned and sequenced (Yamada et al. 1985; Klee et al. 1984; Gielen et al. 1984). In Bradyrhizobium japonicum, the Bam gene, which encodes for an IAM-hydrolase, has also been cloned (Sekine et al. 1989a, 1989b; Kawaguchi et al. 1990). In Azospirillum, Bar and Okon (1992) recently showed a partial homology with cloned genes of the IAM pathway as well as evidence for the conversion of IAM into IAA, which contradicts the results of Zimmer and Elmerich (1991) and Hartmann et al. (1983). Indole-3-pyruvic acid and indole-3-acetaldehyde were proposed as IAA intermediates in A. lipoferum (Ruckdäschel et al. 1988; 1990; Ruckdäschel and Klingmüller 1992). Indole-3-ethanol, indole-3-methanol, and indole-lactic acid are also present in the supernatant of A. brasilense cultures (Crozier et al. 1988). Moreover, Azospirillum IAA

synthesis is inhibited by anthranilate (Zimmer *et al.* 1991).

Mutants completely unable to synthesize IAA could not be isolated in *A. lipoferum* (Abdel-Salam and Klingmüller 1987) and *A. brasilense* (Barbieri *et al.* 1990). Therefore, multiple IAA pathways were suggested. Using the IAA mutant SpM7918 (Barbieri *et al.* 1990) and its transconjugant restored for IAA production (Costacurta *et al.* 1992), we attempted to unravel the *A. brasilense* IAA biosynthetic pathway(s). Data presented in this report provide evidence for multiple IAA biosynthetic pathways, including an important tryptophan-independent pathway.

## RESULTS AND DISCUSSION

### Identification of IAM and IAA synthesized by the bacteria.

Retention times and UV signal of the IAM and IAA samples obtained from culture supernatant extracts after HPLC with on-line mass spectrometry (LC-MS) (cfr. M&M) were the same as those obtained with pure compounds (3.8 min [IAM] and 4.4 min [IAA]). Mass spectra are shown in Figures 1 (IAM) and 2 (IAA). No important ion masses other than the $[MH]^+ = 175$ and $[MNa]^+ = 197$ (at retention time 3.8, specific diagnostic ions for IAM) (Fig. 1) resp. $[MH]^+ = 176$ and $[MNa]^+ = 198$ (at retention time 4.4, specific diagnostic ions for IAA) (Fig. 2) were observed, identifying IAM and IAA. No additional peak signals could be found in the LC-

chromatogram (data not shown), indicating that the samples are pure after the purification used.

### Kinetics of endogenous Trp, IAM, and IAA production.

Figure 3 summarizes growth (A) and endogenous IAA (B), IAM (C), and Trp (D) levels of the *A. brasilense* Sp6 derived strains SpF94, SpM7918, and SpM7918(p0.2) analyzed during 100-hr growth kinetic in the absence of exogenous Trp. Growth characteristics (Fig. 3A) are comparable for all strains analyzed and were identical with the growth kinetics we observed for the wild-type strain Sp6 (data not shown). Stationary stage was reached after 40 hr of culture. By expressing hormone concentrations per $OD_{600}$, small growth differences among strains are taken into account.

Even in the absence of Trp, all strains clearly showed IAA biosynthesis (Fig. 3B) from the stationary stage on. The data obtained with the parent strain SpF94 and the wild-type strain Sp6 were comparable (data not shown). Lower levels of endogenous IAA were found in the culture medium of the IAA mutant strain SpM7918 (Fig. 3B) representing a 90% reduction of IAA synthesis compared to the parent strain. This reduced IAA synthesis confirms the results described for this mutant by Barbieri *et al.* (1990). The transconjugant SpM7918(p0.2) showed a complete recovery of the IAA synthesis compared with the parent strain.
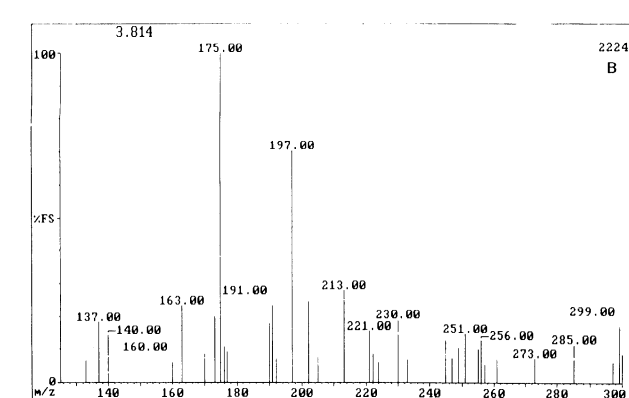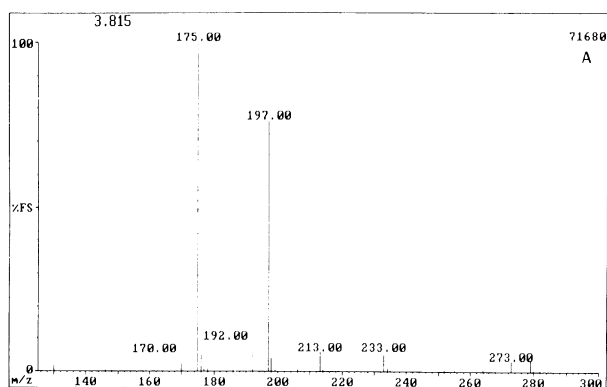


**Fig. 1.** Mass spectra of an IAM reference (**A**) and sample (**B**) extracted from an *Azospirillum brasilense* strains SpM7918 culture medium. $[MH]^+ = 175$ and $[MNa]^+ = 197$; retention time 3.8.
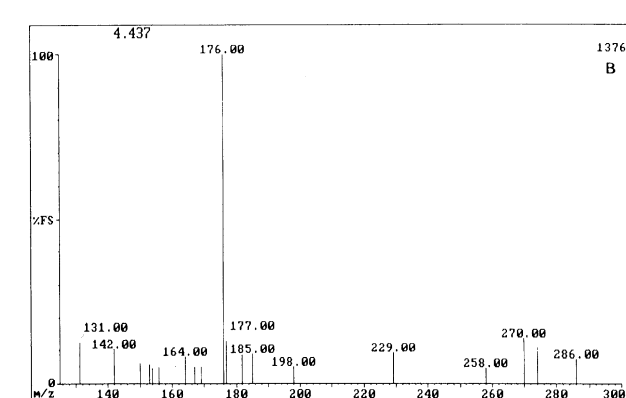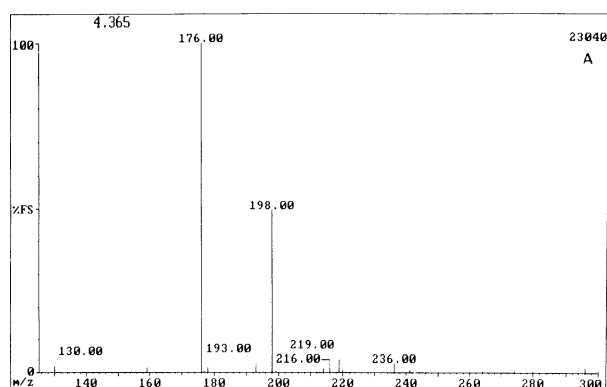


**Fig. 2.** Mass spectra of an IAA reference (**A**) and sample (**B**) extracted from an *Azospirillum brasilense* strain SpM7918(p0.2) culture medium. $[MH]^+ = 176$ and $[MNa]^+ = 198$; retention time 4.4.

Analysis of IAM levels (Fig. 3C) revealed that the reduced IAA synthesis of the mutant SpM7918 was accompanied by a clear IAM accumulation. Like the reduced IAA level, this IAM accumulation was reversed in strain SpM7918(p0.2), giving rise to IAA and IAM levels comparable to the control strain.

In comparison with the IAA levels obtained, the amount of Trp (Fig. 3D) excreted to the medium remains 10–100 times lower, indicating that tryptophan may be limiting for IAA biosynthesis in all *Azospirillum* strains analyzed. Only the IAA mutant strain SpM7918 shows somehow elevated Trp levels during the stationary stage, which could be correlated to the reduced IAA biosynthesis described. Our data concerning the endogenous IAA and IAM concentrations of *A. brasilense* culture media give evidence for the presence of the IAM pathway, although the IAM accumulation did not match the reduced IAA synthesis on a molarity base. Indeed, the existence of an IAM pathway in *A. brasilense* was recently proposed by Bar and Okon (1992), who demonstrated tryptophan-2-monooxygenase enzymatic activity in *A. brasilense*, and found DNA homology to the *Pseudomonas savastanoi iaa*M gene encoding this enzyme. The IAM accumulation and reduced IAA synthesis shown for the mutant SpM7918, and which could be reversed in the

transconjugant, lead to the suggestion of impaired IAM-hydrolase activity (Costacurta *et al.* 1992).

Figure 4 shows growth and endogenous IAA and indoleacetamide (IAM) levels in the culture medium of the same bacterial strains grown on a medium supplemented with tryptophan. Growth curves (Fig. 4A) are comparable to growth curves obtained in absence of L-Trp. Trp supplementation resulted in a 20-fold increase of the IAA levels. Even the auxin mutant SpM7918 showed a significantly enhanced IAA biosynthesis (Fig. 4B). Complementation of this strain with plasmid p0.2 resulted in elevated IAA biosynthesis, although the level of the parent strain SpF94 (Fig. 4B) was not reached. IAM levels excreted to the media were 40- to 100-fold higher than the values reached without additional Trp for all strains (Fig. 4C). As in the nonsupplemented culture, a clear IAM accumulation was observed in the late exponential and early stationary stages of the auxin mutant. However, at a later growth stage, this accumulated IAM disappeared again. IAM accumulation was suppressed in the complemented strain (Fig. 4C).

These data, obtained by feeding the bacterial cultures with excess L-tryptophan, might indicate the presence of an additional Trp-dependent IAA biosynthetic pathway. This was also suggested by Abdel-Salam and Klingmüller
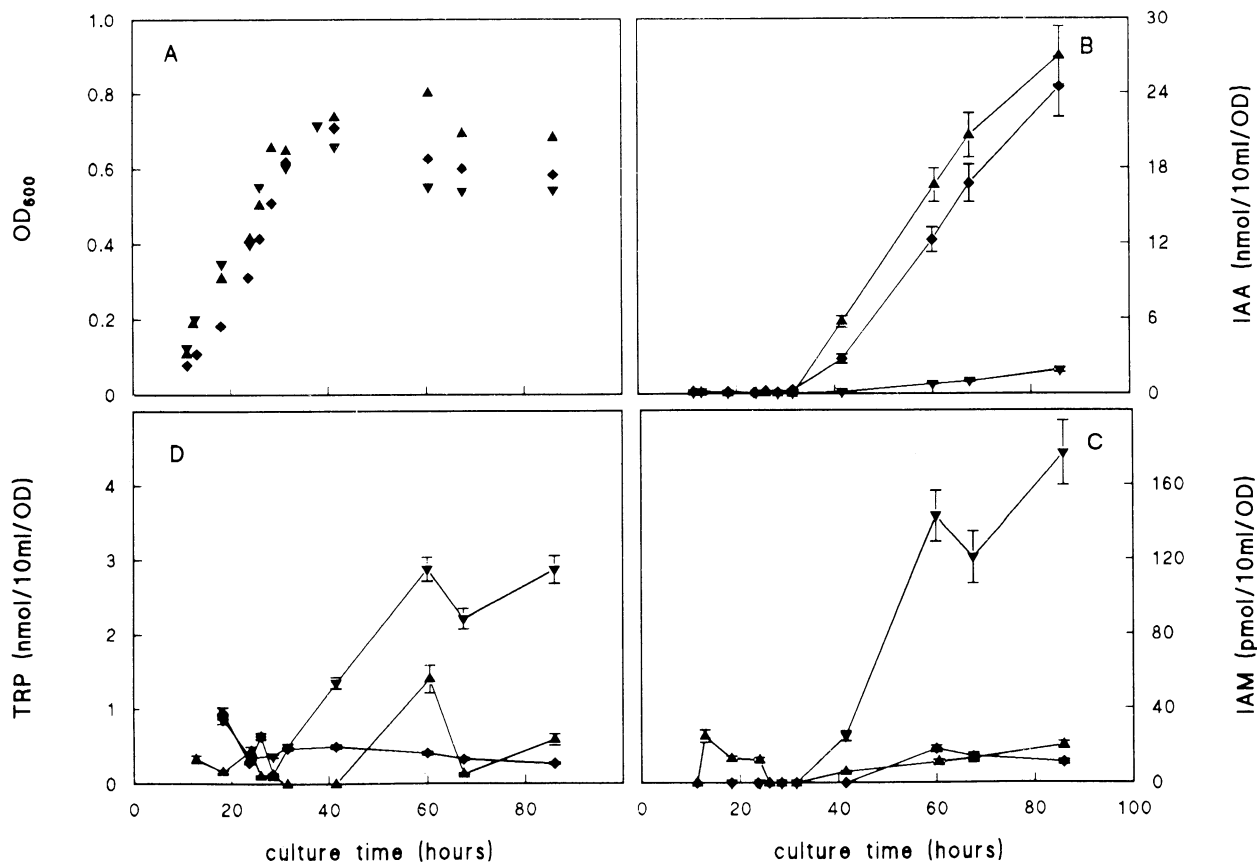


Fig. 3. Growth curve ($OD_{600}$) (A) and endogenous IAA (B), IAM (C), and Trp (D) concentrations (p- or $nmol/OD_{600}/10$ ml) ± error in the culture media of different *Azospirillum brasilense* derived strains (▲SpF94, ▼SpM7918 and ◆SpM7918(p0.2)) during a 100-hr growth kinetics in the absence of exogenous Trp.

(1987) for *A. lipoferum* and by Barbieri *et al.* (1991) for *A. brasilense*, to explain their failure to isolate a complete IAA deficient mutant.

### *In vivo* IAM and IAA synthesis.

Specific radioactivities of IAM and IAA after feeding the bacterial cultures with $^3$H-IAM are summarized in Table 1. The reduced specific radioactivity of IAM after labeling of the endogenous IAM pool of strain SpM7918 compared to SpF94 and SpM7918(p0.2) confirmed the IAM accumulation shown in Figures 3C and 4C. The surprisingly low specific radioactivity of IAA compared to IAM shows that less than 0.1% of IAA in the parent strain is synthesized via the IAM pathway in case no Trp is supplied. Furthermore, the mutant synthesizes more IAA from IAM than does the parent strain (almost 0.5% compared to less than 0.1% from the parent strain). These findings are in contrast to the previously presented hypothesis of impaired IAM-hydrolase activity in mutant SpM7918 (Costacurta *et al.* 1992).

Specific radioactivities of IAM and IAA after labeling the endogenous Trp pool by feeding the bacterial cultures with $^3$H-L-Trp are summarized in Table 2. For all strains analyzed, the specific radioactivity of IAM equals the specific radioactivity of the $^3$H-L-Trp pool. This proves that the L-Trp-pool is labeled and indicates that L-Trp is the IAM precursor in all strains. These results confirm the

presence of Trp-monooxygenase activity shown by Bar and Okon (1993). A substantial amount of radiolabeled IAA is present; however, $^3$H-IAA specific radioactivities were 10-fold diluted compared to $^3$H-Trp specific radioactivity for both the parent and mutant strain. Therefore, we conclude that 10% of the IAA is produced by a non-IAM but Trp-dependent pathway (the IAM pathway accounts only for less than 0.1%, see above). An additional *de novo* IAA synthesis without tryptophan as an intermediate accounts for about 90% of the IAA synthesized in case no excess Trp is supplied. It is noteworthy that Baldi *et al.* (1991) and Wright *et al.* (1991) have already shown that a non-Trp pathway is the primary route of IAA synthesis in *Lemna* and maize. The nature of the mutation in SpM7918 and of the genes present on the recombinant plasmid p0.2 are, however, not yet clear at this stage.

**Table 1.** Specific radioactivities expressed as dpm/pmol ± error of indole acetamide (IAM) and indoleacetic acid (IAA) in a $^3$H-IAM feeding experiment

| | Specific radioactivity(dpm/pmol)[a] | |
|---|---|---|
| | **IAM** | **IAA** |
| SpM7918 | 5,100 ± 200 | 24 ± 2 |
| SpM7918(p0.2) | 18,000 ± 600 | 1.0 ± 0.1 |
| SpF94 | 17,000 ± 600 | 2.5 ± 0.2 |

[a] dpm = Disintegrations per minute.
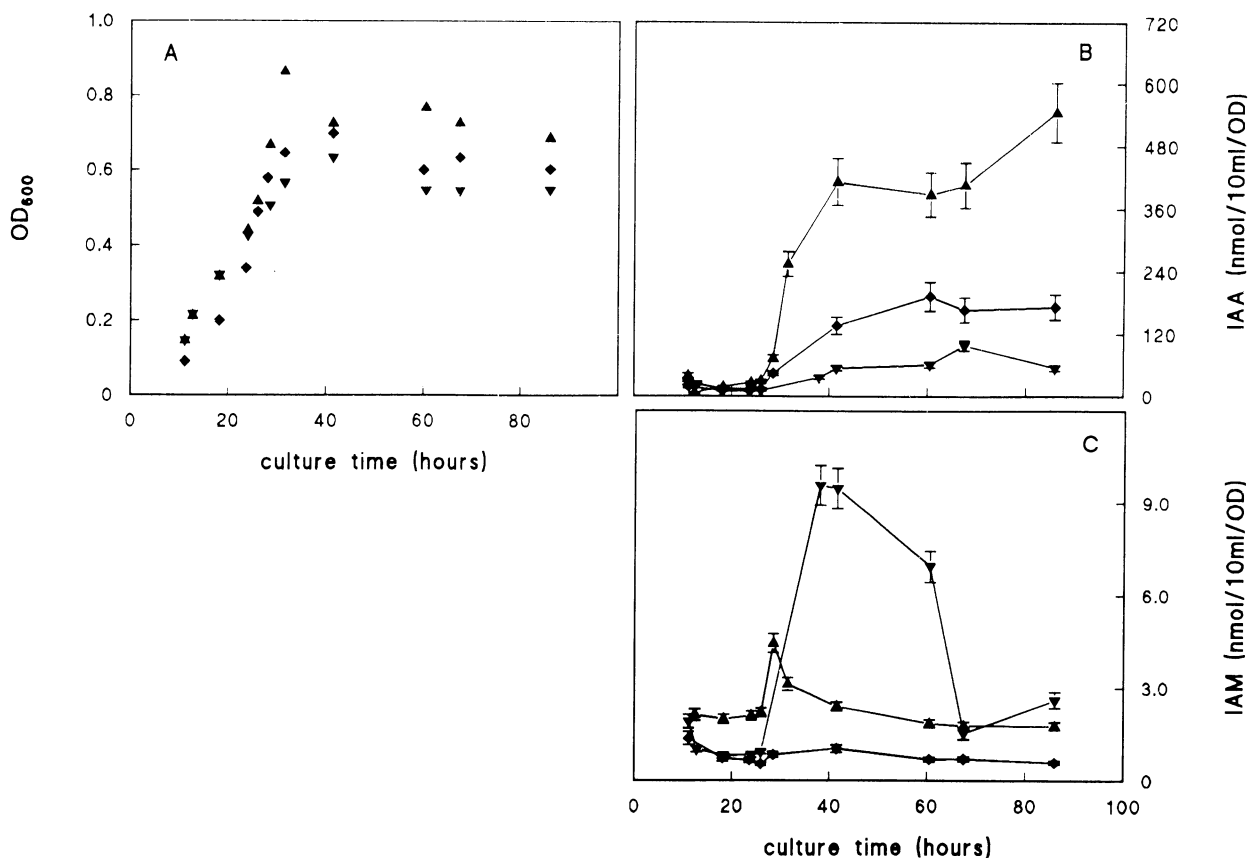


**Fig. 4.** Growth curve (OD$_{600}$) **(A)** and endogenous IAA **(B)** and IAM **(C)** concentrations (nmol/OD$_{600}$/10 ml) ± error in the culture media of different *Azospirillum brasilense* strains (▲SpF94, ▼SpM7918, ◆SpM7918[p0.2]) during a 100-hr growing period after exogenous Trp supply.

Based on the feeding experiments, the following model for IAA synthesis of *A. brasilense* might be postulated (Fig. 5). The observation that in both the parent and mutant strain, 90% of the IAA is synthesized through the non-Trp pathway (Table 2) rules out the possibility that this pathway is blocked in the mutant. Inhibition of the IAM pathway in SpM7918, as we suggested previously (Costacurta *et al.* 1992), now seems rather unlikely since this pathway accounts only for less than 0.1% in the total IAA biosynthesis, whereas IAA production in SPM7918 is reduced with 90% (Fig. 3B). Moreover an accumulation of IAM, as is observed in the mutant strain, certainly does not prevent the IAA production by other routes (Tables 1 and 3). This points to the possibility that the mutation present in strain SpM7918 is located in the non-IAM L-Trp encoded pathway, which accounts for 10% of the *A. brasilense* IAA biosynthesis (Fig. 5). The difference in Trp levels between the parent and mutant strain (Fig. 3D) is indeed about 10% of the IAA accumulated in the culture media of the parent strain (Fig. 3B). IAA reduction in the mutant strain is, however, more drastic, which made us postulate that accumulation of an unknown intermediate, "X", in the mutant, exerts a negative control on the non-Trp pathway. A postulated positive, regulation of "X" on the IAM pathway explains the IAM accumulation shown for the mutant strain SpM7918 in both presence and absence of Trp as well as the enhanced importance of this pathway shown for the mutant strain SpM7918 (Table 1). Similar $^3$H-IAM specific radioactivities in both the parent and the mutant strain after $^3$H-L-Trp feeding (Table 2), revealed the enhanced relative importance of the IAM-pathway due to

the accumulation of IAM, caused by the positive control of "X" on this pathway in the mutant strain. Clone p0.2 may restore the conversion of "X" into IAA, alleviating the activation of the IAM pathway as well as the suppression of the non-Trp pathway. The observation that clone p0.2 does not completely restore IAA synthesis in the presence of excess Trp (Fig. 4B), may be explained by the heterologous origin of this clone. p0.2 is a cosmid clone containing *A. brasilense* Sp245 DNA, and possibly the complementing gene is not optimally expressed in the Sp6 background. Alternatively, it can not be excluded that clone p0.2 may encode a gene product that catabolizes "X" without converting "X" into IAA. The only Trp-encoded pathway present being the IAM pathway would in this case explain why complementation could not restore completely IAA synthesis in presence of excess Trp (Fig. 4).

## GENERAL CONCLUSION

From the data presented here we may conclude that at least three IAA biosynthetic pathways are present in *A. brasilense*: The IAM pathway, a second Trp-dependent pathway, and a Trp-independent pathway accounting, respectively, for 0.1, 10, and 90% of the IAA produced in case no exogenous Trp is supplied. There is a strong regulatory interaction between the pathways described here.

This work is the first to provide data supporting the existence of Trp-independent IAA synthesis in bacteria.

## MATERIALS AND METHODS

**Bacterial strains and media.**

*A. brasilense* SpF94 is a Rif$^r$ strain derived from strain Sp6 (Fani *et al.* 1988). *A. brasilense* SpM7918 is an SpF94 Tn5 mutant with reduced IAA synthesis (Barbieri *et al.* 1990). Part of the Tn5 and flanking DNA from SpM7918 was cloned and used as hybridization probe to screen a genomic library of *A. brasilense* Sp245. p0.2 is one of seven positive clones from this screening which, upon introduction into the mutant, restored IAA synthesis (Costacurta *et al.* 1992).

*Azospirillum* strains were grown at 30° C in liquid MMAB minimal medium (Vanstockem *et al.* 1987). In case of exogenous Trp supply, 100 μg/ml L-Trp (Janssen Chimica, Geel, Belgium) was added. At different time intervals, the OD$_{600}$ of the bacterial cultures were measured and after centrifugation the bacterial cells and

**Table 2.** Specific radioactivities expressed as dpm/pmol ± error of tryptophan (Trp), idoleacetamide (IAM) and indoleacetic acid (IAA) in a $^3$H-Trp feeding experiment

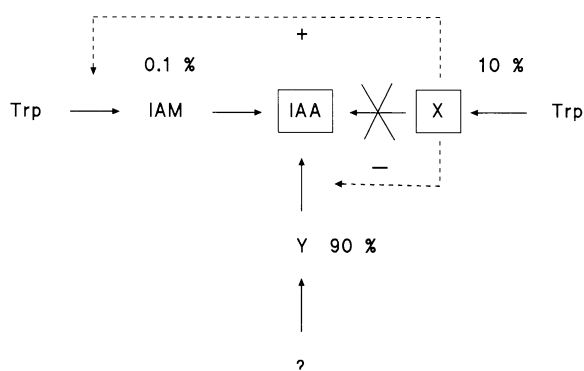|  | Specific radioactivity (dpm/pmol) | | |
|  | L-Trp | IAM | IAA |
| --- | --- | --- | --- |
| SpM7918 | 3,100 ± 150 | 2,400 ± 160 | 200 ± 12 |
| SpM7918(p0.2) | 4,100 ± 200 | 4,200 ± 280 | 2.1 ± 0.1 |
| SpF94 | 2,300 ± 110 | 2,900 ± 210 | 230 ± 15 |



**Fig. 5.:** Hypothetical model for IAA biosynthesis of *Azospirillum brasilense* in absence of exogenously supplied Trp. The crossed arrow in the biosynthetic pathway indicates the mutation. The dashed arrows indicate a hypothetical positive (+) and negative (−) control of an accumulated compound X.

**Table 3.** Specific radioactivities expressed as dpm/pmol ± error of indoleacetamide (IAM) and indoleacetic acid (IAA) in a $^3$H-IAM feeding experiment; 1 mM unlabeled IAM was also added to the culture media

|  | Specific radioactivity (dpm/pmol) | |
|  | IAM | IAA |
| --- | --- | --- |
| SpM7918 | 1.01 ± 0.04 | 0.67 ± 0.06 |
| SpM7918(p0.2) | 1.01 ± 0.04 | 0.25 ± 0.02 |
| SpF94 | 1.01 ± 0.04 | 0.18 ± 0.02 |

the supernatants were frozen in liquid nitrogen and stored at $-80°$ C until analysis.

## IAA, IAM, and Trp in bacterial growth media.

The method described for the analysis of IAA and IAM in bacterial cultures (Prinsen *et al.* 1991) was used and modified for Trp analysis. To 5 ml of culture supernatant 3-[5(n)-$^3$H]-IAA (250 Bq, 777 GBq/mmol, Amersham), 3-[5(n)-$^3$H]-IAM (250 Bq, 777 GBq/mmol, for preparation see Van Onckelen *et al.* 1985) and L-[G(n)-$^3$H]Trp (250 Bq, 2.46 TBq/mmol, Amersham) were added. When 5 ml of 0.1 N HCl was added, the acidified sample was purified using a bond-elut C-18 column (Analitichem Int.) equilibrated with 0.05 N HCl. The retained IAA and IAM were eluted from the C-18 column with 5 ml of diethyl ether; Trp was subsequently eluted with 10 ml of acetonitrile. The ether and acetonitrile phase were separately evaporated *in vacuo*, dissolved in 2 × 0.5 ml of 100% MeOH, respectively, 2 × 0.5 ml of acetonitrile, both samples were dried in a speed-vac and resolved each in 100 μl of the liquid phase prior to HPLC.

IAA and IAM were separated during a preparative ion suppression (IS)-reversed phase (RP)-HPLC run (50/49.5/0.5; $H_2O$/MeOH/HAc; 0.5 ml/min; Rosil C18, 10 cm, 3-μm column, Alltech-RSL). For IAM an additional preparative RP-HPLC run (60/40; phosphate 0.01 M, pH 6.6/MeOH; same column and flow) was performed. IAA and IAM were analyzed by an analytical ion pairing (IP)-RP-HPLC run (60/40; 0.001 M phosphate, 0.01 M Tetra butyl ammonium hydroxide [TBAH] pH 6.6/MeOH; same column and flow) and measured on line with a Shimadzu RF530 fluorescence detector (excitation at 285 nm, emission at 360 nm). Trp was analyzed using IS-RP-HPLC (80/20; Phosphate 0.01 M pH 3.5/acetonitrile) with on-line fluorescence detection (same specifications as above). The endogenous IAA, IAM, or Trp contents were calculated following the principles of isotope dilution and concentrations were expressed as p- or nmol in 10 ml/$OD_{600}$.

## *In vivo* IAM and IAA synthesis
### $^3$H-IAM-feeding experiment.

Bacterial MMAB cultures (5 ml) were fed with 3-[5(n)-$^3$H]-IAM (33.3 kBq, 777 GBq/mmol) and grown for 96 hr in presence or absence of 1 mM IAM. Cultures were sampled and purified as described before.

### $^3$H-Trp-feeding experiment.

Bacterial MMAB cultures (5 ml) were fed with L-[G(n)-$^3$H]Trp (26.7 kBq, 2.46 TBq/mmol, Amersham) and grown for 48 hr. Cultures were sampled and purified for Trp, IAM, and IAA as described before without adding $^3$H-labeled tracers. After the analytical IP-RP-HPLC run specific radioactivities of Trp, IAA, and IAM were calculated, taking into account the radioactivity present at the compound's specific retention time and its specific fluorescence. Specific radioactivies were expressed in dpm/pmol.

## Identification of IAA and IAM
## LC-MS.

The IAA and IAM samples obtained from culture supernatant extracts after analytical IP-HPLC were acidified to pH 3 with HCl and desalted on a C18 cartridge before identification with LC-MS. IAA and IAM were analyzed by LC-MS (Waters 600MS HPLC, VG thermospray-plasmaspray probe coupled to a VG TRIO2000 quadrupole mass spectrophotometer) under optimized thermospray conditions (repeller 200 V; Capillary T 160° C; source T 225° C; 40% MeOH/ 0.5% HCOOH isocratic conditions, Rosil C18 HL, 10 cm, 3 μm column, 0.8 ml/min) UV signal at 260 nm (Waters 486 MS) was measured.

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