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 ¹H-IAM and ³H- 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; Reyners 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; Reyners 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-monoxygenase converts Trp to IAM, which is converted to IAA by an indoleacetamide-hydrolase, iaAM and iaAM, 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 Bum 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

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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 (Costacura 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&K) 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 OD600, 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](image1.png)  
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](image2.png)  
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-monoxygenase enzymatic activity in *A. brasilense*, and found DNA homology to the *Pseudomonas savastanoi iaaM* 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 indoleacetic acid (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.

![Figure 3](image-url)

**Fig. 3.** Growth curve (OD₆₀₀ (A) and endogenous IAA (B), IAM (C), and Trp (D) concentrations (p- or nmol/OD₆₀₀/10 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 3H-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 IAM 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 3H-l-Trp are summarized in Table 2. For all strains
analyzed, the specific radioactivity of IAM equals the
specific radioactivity of the 3H-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

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

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<th>Specific radioactivity (dpm/pmol)*</th>
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<tr>
<td></td>
<td>IAM</td>
</tr>
<tr>
<td>SpM7918</td>
<td>5,100 ± 200</td>
</tr>
<tr>
<td>SpM7918(p0.2)</td>
<td>18,000 ± 600</td>
</tr>
<tr>
<td>SpF94</td>
<td>17,000 ± 600</td>
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IAA               |
| 600              |
| 480              |
| 360              |
| 240              |
| 120              |
| 60               |
| 0                |

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<th>IAA</th>
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<td></td>
<td>600</td>
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<td>480</td>
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<td>360</td>
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<td>120</td>
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<td>60</td>
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4dpm = 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.

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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 3H-IAM specific radioactivities in both the parent and the mutant strain after 3H-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 cannot 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° 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 OD600 of the bacterial cultures were measured and after centrifugation the bacterial cells and

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

<table>
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<th>Specific radioactivity (dpm/μmol)</th>
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<tr>
<td></td>
<td>IAM</td>
</tr>
<tr>
<td>SpM7918</td>
<td>3,100 ± 150</td>
</tr>
<tr>
<td>SpM7918(p0.2)</td>
<td>4,100 ± 200</td>
</tr>
<tr>
<td>SpF94</td>
<td>2,300 ± 110</td>
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**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/μmol ± error of indoleacetic acid (IAM) and indoleacetic acid (IAA) in a 3H-IAM feeding experiment; 1 mM unlabeled IAM was also added to the culture media**

<table>
<thead>
<tr>
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<th>Specific radioactivity (dpm/μmol)</th>
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<tbody>
<tr>
<td></td>
<td>IAM</td>
</tr>
<tr>
<td>SpM7918</td>
<td>1.01 ± 0.04</td>
</tr>
<tr>
<td>SpM7918(p0.2)</td>
<td>1.01 ± 0.04</td>
</tr>
<tr>
<td>SpF94</td>
<td>1.01 ± 0.04</td>
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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)-3H]-IAA (250 Bq, 777 GBq/mmol, Amersham), 3-[5(n)-3H]-IAM (250 Bq, 777 GBq/mmol, for preparation see Van Onckelen et al. 1985) and L-[G(n)-3H]Trp (250 Bq, 2.46 TBq/mm, 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 (Analytichem 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, H2O/MeOH/HAc; 0.5 ml/min; Rosil C18, 10 cm, 3-µm column, Alltech-RSL). For 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-

**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.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


