Growth and indole-3-acetic acid biosynthesis of *Azospirillum brasilense* Sp245 is environmentally controlled

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**Abstract**

Batch and fed batch cultures of *Azospirillum brasilense* Sp245 were conducted in a bioreactor. Growth response, IAA biosynthesis and the expression of the *ipdC* gene were monitored in relation to the environmental conditions (temperature, availability of a carbon source and aeration). *A. brasilense* can grow and produce IAA in batch cultures between 20 and 38°C in a standard minimal medium (MMAB) containing 2.5 g l⁻¹ l-malate and 50 µg ml⁻¹ tryptophan. IAA synthesis requires depletion of the carbon source from the growth medium in batch culture, causing growth arrest. No significant amount of IAA can be detected in a fed batch culture. Varying the concentration of tryptophan in batch experiments has an effect on both growth and IAA synthesis. Finally we confirmed that aerobic growth inhibits IAA synthesis. The obtained profile for IAA synthesis coincides with the expression of the indole-3-pyruvate decarboxylase gene (*ipdC*), encoding a key enzyme in the IAA biosynthesis of *A. brasilense*.

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**Keywords:** *Azospirillum brasilense*; Fermentor; Indole-3-acetic acid

1. Introduction

The genus *Azospirillum* consists of diazotrophic gram-negative bacteria occurring in the rhizosphere and intercellular spaces of the roots of several plant species. There are numerous reports on the improvement of plant growth and crop yield upon *Azospirillum* inoculation on plant roots. The observed plant response to *Azospirillum* inoculation is attributed to the production of indole-3-acetic acid (IAA) by these bacteria [1–3]. IAA is the most important naturally occurring auxin and is implicated in many aspects of plant growth and development. In addition to its production in plants, IAA biosynthesis has been recorded in a few fungi, some protozoa and is widespread among plant-associated bacteria [4–8]. Tryptophan (Trp) is a precursor for bacterial IAA biosynthesis for which different pathways have been described [9,10]. The indole-3-pyruvate decarboxylase (IPDC) is a key enzyme for IAA biosynthesis in *Azospirillum brasilense*, as an *ipdC* knock out mutant was found to produce only 10% of the wild type IAA production level [11]. This gene is up-regulated by IAA as well as by synthetic auxins such as 1-naphthalene acetic acid and chlorophenoxy acids [12]. Genes encoding IPDC have also been cloned from some other IAA producing bacterial species [13–17].

Results obtained from *Azospirillum* field inoculations have been somewhat inconsistent [18] suggesting that
bacterial IAA biosynthesis may be drastically affected by environmental factors. A number of contradicting reports regarding factors that may influence the IAA biosynthesis of A. brasilense have been published [12,15,19]. A precise and careful monitoring and control of culture conditions by the use of a fermentor could provide a better understanding of environmental factors controlling IAA biosynthesis. Results from some of such studies have been extrapolated to predict survival and activity of A. brasilense in the fields [20]. The work presented here is an attempt to define environmental conditions which may come into play in the regulation of growth, ipdC expression and the IAA biosynthesis of A. brasilense.

2. Materials and methods

2.1. Culture conditions

A. brasilense Sp245 (pFAJ64) [12] was routinely maintained on L-malate minimal medium (MMAB) [21] supplemented with tetracycline (25 μg ml⁻¹). Fermentations were conducted in a 5 l BioFlow 3000 bench-top fermentor (New Brunswick Scientific; USA). Sterile medium components were separately added to the fermentor (containing cool, sterile water) to make up a 3 l (2.5 l when in fed-batch mode) working volume. Unless stated otherwise, the set-points for the fermentations were temperature 30 °C, pH 6.8, dissolved oxygen (DO) 3%, and agitation 50–500 rpm. The DO-agitation cascade was selected in the controller to maintain the DO at set-point by automatically adjusting agitation speed in response to oxygen demand. 4 N H₃PO₄ was used as the pH control solution. A pre-culture used to inoculate the fermentor was initiated by inoculating 100 ml of MMAB medium in a 250-ml Erlenmeyer flask with a loop of cells taken from an overnight plate culture and cultivating in an incubator shaker for 14 h at 30 °C and 180 rpm. To enable significant IAA accumulation and measurable ipdC gene expression levels, cultures were supplemented with 50 μg ml⁻¹ Trp. In addition to the fermentor’s internal controller, the bioprocess software (AFS-BioCommand, New Brunswick Scientific, USA) was routinely used to supervise the process. All data from the fermentor were transmitted to a computer loaded with the ‘AFS-BioCommand’ software. Samples of 10-ml used for measuring absorbance, residual L-malate, IAA and Trp concentrations, were withdrawn aseptically and divided into sub-samples for analysis. 5 ml of culture used for the analysis of IAA and Trp were centrifuged and the supernatant frozen until analysed. Samples used for quantitative β-glucuronidase were stored at 4 °C and analysed immediately at the end of the fermentation. In order to exclude any complications that may arise if the external signals such as DO and pH are not kept constant, cultivation was stopped when it became difficult to maintain the culture at set points. This time point is regarded as the elapsed fermentation time (EFT).

2.2. Analysis of cells and growth medium

Cell growth was estimated by measuring absorbance at 600 nm with a Genesys 6 spectrophotometer (Electronic Instruments, Rochester, New York, USA). For the purposes of calculating growth rates, cell number was estimated from a calibration curve of OD versus CFU. The specific growth rate was determined with regression analysis from plots of In CFU versus time at points during the exponential growth phase. l-malate concentrations were determined using a test kit from Roche (R-Biopharm, Germany). IAA and Trp were purified from 5 ml fermentation medium supernatant by solid phase extraction and analysed by gas chromatography/mass spectrometry (GC/MS) [22] as pentafluorobenzyl (PFB) ester [23]. For recovery purposes, [phenyl-13C₆]-indole-3-acetic acid (Cambridge Isotope Laboratories, Andover, MA, USA) and [2',4',5',6',7'-d₅]-l-Trp (CDN isotopes, Quebec, Canada) (100 ng each) were added as internal tracers. Quantitative analysis of β-glucuronidase activity was assayed in microtiter plates using the GUS extraction buffer and p-nitophenyl-β-D-glucuronide as substrate [24]. The measured β-glucuronidase activity expressed as Miller units [25] was used to monitor ipdC gene expression and represents the mean of three replicates.

3. Results

Three sets of fermentations were performed for all cases reported and all data shown represent the average of at least three replicates.

3.1. Initial experiments

A. brasilense has been isolated from different climatic regions and because their rhizosphere competence depends on their ability to grow on available nutrients in the rhizosphere [26], the influence of temperature and nutritional status on cell growth was investigated. Growth tests in the fermentor were conducted at different temperatures in N-free and N-containing MMAB. Cell growth was measured after 20 h of growth. A. brasilense developed rapidly with NH₄⁺ as the N source but no significant growth was recorded in N-free medium. The bacterium could grow between 20 and 38 °C with a maximum at 30 °C. At 40 °C, the culture grew slowly and started to flocculate after 10 h of fermentation. No significant growth was recorded at temperatures below 20 °C and above 40 °C. Additional tests were done in minimal medium in which l-malate was substituted with
other single carbon sources namely d-glucuronic, d-fructose and dl-lactate, each at a concentration of 0.02 M. The mean OD values ± SD (n = 3) were 1.596 ± 0.06, 1.338 ± 0.06*, 0.824 ± 0.07* and 0.682 ± 0.06*, respectively, for cultures grown in minimal medium with l-malate, dl-lactate, d-gluconate, and d-fructose as carbon source. *P value is <0.05 relative to l-malate OD values as reference. Because IAA biosynthesis of A. brasilense has been shown to be cell density dependent [12], and the highest OD was recorded with l-malate as carbon source, further tests were therefore conducted at 30°C in l-malate minimal medium (MMAB) with NH4+ as N source. Unless specified otherwise, 50 µg ml−1 Trp was added to this medium.

3.2. Influence of carbon status

Azospirillum spp. are most often isolated from poor soils with low carbon content and carbon availability may be a limiting factor for microbial growth in the rhizosphere [27]. To investigate the influence of carbon status on IAA biosynthesis, two batch fermentations, ‘A’ and ‘B’ with 2.5 and 5.0 g l−1 initial l-malate concentrations, respectively, and fed batch fermentation with 2.5 g l−1 initial l-malate concentration were performed. For the fed-batch process, A. brasilense was first grown in batch mode and then switched to fed-batch mode after 5 h by feeding a 10 g l−1 l-malate solution at the rate of 0.004 l h−1. Table 1 summarises the data for OD, l-malate and IAA concentrations and the ipdC expression for the respective cultures. l-malate was rapidly depleted in the batch cultures but was available throughout the course of fermentation in the fed batch process. Culture ‘A’ yielded the least cell concentration as estimated by absorbance at 600 nm but allowed the highest levels of IAA and ipdC expression. Neither IAA production nor ipdC expression was detected in the fed batch culture, indicating that carbon stress is required for IAA biosynthesis.

3.3. Tryptophan modulates growth and IAA biosynthesis in a variety of ways

The production of IAA by Azospirillum cultures in MMAB is very low unless Trp is added. However, the effect of Trp on growth is not known. Growth experiments were therefore conducted in the absence and in the presence of Trp (50 and 200 µg ml−1). The growth patterns for these fermentations are illustrated by the OD plots in Fig. 1. In the logarithmic phase of growth, two distinct growth patterns were observed; a first fast growing phase (µ1) followed by a second slow one (µ2). The transition was seen at 8 h of EFT coinciding with the point where l-malate concentration dropped below 0.05 g l−1 (data not shown). The mean specific growth rates for cultures grown without Trp and with 50 and 200 µg l−1 Trp, respectively, were µ1: 0.23 ± 0.006, 0.20 ± 0.005 and 0.19 ± 0.007; µ2: 0.07 ± 0.011, 0.09 ± 0.05 and 0.1 ± 0.07. The stationary phase was markedly short in culture supplemented with 50 µg ml−1 Trp. In medium supplemented with 200 µg ml−1 Trp, the culture exhibited the capacity to resume growth again after an intervening stationary phase. To further clarify the effect of Trp on growth, the fermentation profiles are presented in the DO and agitation trend graph in Fig. 2. At the beginning of the log phase of growth, the DO remained approximately at 100% of air saturation until inoculation, and then declined rapidly to the 3% setpoint. In the absence of Trp, this decline was fast and agitation speed was high reflecting increasing oxygen demand. Fig. 2 reveals that the log phase (reflected by an increasing agitation profile) was extended for a further 2 and 4 h of EFT in the presence of 50 and 200 µg l−1 Trp supplement, respectively. The stationary phase (reflected by a constant agitation and DO profile) was remarkably short in the presence of 50 µg ml−1 Trp. The agitation pattern at 200 µg ml−1 Trp supplement was strikingly different; characterized by a second rise in agitation speed after an intervening constant profile of about 10 h. These results are consistent with observations in Fig. 1.

The influence of Trp on IAA production and the expression of the ipdC gene are shown in Fig. 3. Without Trp, IAA production and ipdC expression were very low and were only observed at the late stationary phase of growth. When medium was supplemented with Trp, ipdC expression and IAA synthesis were enhanced several fold and were detected at the early stationary phase of growth. This early boost in IAA biosynthetic activity was higher in culture supplemented with 200 µg ml−1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Batch-cultures</th>
<th>Fed-batch culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial l-malate concentration (g l−1)</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Maximum OD at 600 (g l−1)</td>
<td>1.609 ± 0.06</td>
<td>2.045 ± 0.05</td>
</tr>
<tr>
<td>Maximum IAA concentration (µg ml−1)</td>
<td>3.731 ± 0.06</td>
<td>1.849 ± 0.05</td>
</tr>
<tr>
<td>Maximum ipdC expression (Miller units)</td>
<td>443 ± 0.40</td>
<td>250 ± 0.30</td>
</tr>
<tr>
<td>Residual l-malate (g l−1)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD, n = 3 of samples taken after 18 h of EFT.
ND, not detected.
* Values for fermentation A, serving as control.
P < 0.05 relative to control.
Trp but in contrast to culture containing 50 μg ml⁻¹ Trp the boost decreased as the culture progressed.

3.4. Aerobic conditions inhibit IAA biosynthesis

A. brasilense is attracted to zones of low oxygen concentrations and in aerobic or microaerobic conditions uses O₂ as terminal electron acceptor [28,29]. Okon et al. [30] reported that the IpdC pathway for IAA biosynthesis is elevated under anaerobic conditions. In contrast, however, Brandl and Lindow [31] did not see any effect of O₂ availability on the IAA biosynthesis and the expression of the ipdC promoter in Erwinia herbicola. To investigate the effect of

Fig. 1. Cell concentration (OD) for batch fermentations of A. brasilense cultures without tryptophan (black squares) and with 50 μg ml⁻¹ (black triangles) and 200 μg ml⁻¹ (black circles) tryptophan.

Fig. 2. Agitation (black symbols) and DO% (white symbols) trend graphs for the batch cultures of A. brasilense without tryptophan (black squares, white squares), 50 μg ml⁻¹ (black triangles, white triangles) and 200 μg ml⁻¹ (black circles, white circles) tryptophan supplement.
oxygen availability, *A. brasilense* was also cultured under aerobic condition (30% DO). Aerobic conditions allowed a longer EFT but biomass concentration was not significantly affected. IAA production and the expression of the *ipdC* were drastically reduced (Fig. 4).

4. Discussion

The absence of IAA biosynthesis and *ipdC* expression during exponential growth in micro-aerobic batch cultures of *A. brasilense* and during growth in fed-batch cultures clearly indicate that carbon limitation and
reduction in growth rate are necessary for IAA biosynthesis in *A. brasilense*. However, under aerobic conditions, neither the exhaustion of the carbon source nor a reduction in growth rate can trigger IAA biosynthesis. This implies that (i) IAA biosynthesis in *A. brasilense* is triggered by stress factors (including carbon and oxygen stress, and an entry into the stationary phase of growth) and (ii) these factors must act in concert to enable IAA biosynthesis. These results agree with a previous observation that the overproduction of the stationary phase sigma factor RpoS in *Enterobacter cloacae* and *Pseudomonas putida* GR12-2 enhanced IAA synthesis several folds [32,33]. The requirement for carbon starvation and a reduction in growth rate are similar features of other secondary metabolites produced by microorganisms. The inability of *Azospirillum* to produce IAA when a carbon source is available may provide an explanation for the failure of plant growth promotion following *Azospirillum* inoculations in rich or highly fertilized soils [2,34]. The effect of Trp on the growth of *A. brasilense* has received little attention in the studies of IAA biosynthesis of *A. brasilense*. Our current findings contrast the long held view that adding Trp to the culture medium of *A. brasilense* affects neither the growth rate nor the length of the logarithmic phase of growth [35]. Trp decreased the growth rate, increased the length of the logarithmic phase and allowed an increase in cell concentration. The degradation of Trp to indole, pyruvate and ammonia has been reported in *Escherichia coli*. Trp has also been implicated as the sole source of carbon and nitrogen in some species of Pseudomonadaceae and Bacillaceae [36–40]. The resumption of growth after an intervening stationary phase when Trp was present in Fig. 1 suggests that a similar system may exist in *A. brasilense*. Similar amounts of Trp were detected in the supernatants of the different cultures at the end of the fermentation (data not shown), so it would be plausible to argue that Trp was used for other purposes (most probably growth) rather than for IAA biosynthesis. Trp has been known as the precursor for IAA biosynthesis in bacteria, but the question on whether the effect of Trp is merely due to an increase in precursor supply and/or the induction of one or more synthetases of the biosynthetic pathway has received conflicting answers. There have been reports on an increase in *ipdC* gene expression in *A. brasilense* Sp7 and *Pseudomonas putida* GR12-2, respectively, in response to exogenous tryptophan [33,41]. In contrast, the *ipdC* promoter in *Erwinia herbicola* 299R is not influenced by exogenous Trp [31]. In this study, we observed that all conditions that deter IAA biosynthesis affect *ipdC* expression negatively. For instance, irrespective of the presence of 50 µg ml⁻¹ of Trp in the medium, aerobic conditions suppress both IAA production and *ipdC* expression. Results from this work suggest that the up-regulation of *ipdC* expression is concomitant with the appearance of IAA in the growth medium and is independent of the presence of Trp (Figs. 3 and 4). These observations lend credence to our earlier report on the autoinduction of the *ipdC* promoter in *A. brasilense* by IAA [12]. No physiological function is yet ascribed to IAA production in *A. brasilense*. In all culture conditions that favour IAA biosynthesis, the stationary phase is remarkably shorter suggesting that the presence of IAA in the medium could have some antibacterial effects upon accumulation in the bioreactor. Indeed, IAA had been shown to inhibit the growth of *Saccharomyces cerevisiae* [42]. *A. brasilense* is also reputed to be capable of restricting the proliferation of a number of rhizosphere bacteria [43]. Having previously demonstrated the up-regulation of *ipdC* expression by IAA, this observation apparently implies that IAA production is suicidal for *Azospirillum*. This will probably not be the case in the rhizosphere since bacterial IAA is taken up by plant roots and any residual IAA will be expected to dissipate in the rhizosphere and possibly be a factor in providing the proliferation of a number of rhizosphere bacteria [43]. Moreover, Fig. 3 indicates another phenomenon in the IAA biosynthesis of *A. brasilense* that may cushion the effect of overproduction of IAA. A 200 µg ml⁻¹ Trp supplement results in the accumulation of very high levels of IAA at the early stationary phase of growth but in contrast to medium supplemented with 50 µg ml⁻¹ Trp, IAA levels did not increase and did not result in higher *ipdC* expression as growth progressed. A possible explanation is that when IAA is present at high concentrations at the early phase of growth, it may act as a signal for the cell indicating potentially lethal levels of IAA in the growth medium. This signal then initiates a downregulation of *ipdC* gene expression. The excess Trp is then metabolised thereby alleviating the carbon stress and thus arrest further IAA biosynthesis. This effectively implies that the IAA biosynthesis of *Azospirillum* is favoured by low tryptophan concentrations. Oxygen is an important environmental factor because in aerobic or microaerobic conditions, *A. brasilense* uses O₂ as terminal electron acceptor. The IpdC pathway for IAA biosynthesis is elevated under anaerobic conditions [30]. In contrast however, Brandl and Lindow [31] did not see any effect of O₂ availability on the IAA biosynthesis and the expression of the *ipdC* promoter in *Erwinia herbicola*. We previously demonstrated the prevalence of microaerobic conditions in the lower part of wheat roots by the use of nifH-gusA reporter construct in *Azospirillum* [44]. Furthermore, oxygen consumption by microbes and/or by roots can lead to low oxygen levels in the rhizosphere [45]. The elevation of IAA production in microaerobic condition may therefore be an adaptation by *A. brasilense* for optimal IAA production. Since the oxygen status in the rhizosphere depends on the texture, structure and the water content of the soil [46], it is of utmost importance to
consider these factors during field applications of *Azospirillum* cultures.

In conclusion, our findings suggest that IAA is only produced when the nutritional limits (low carbon and nitrogen conditions) are in place and when there is an associative relationship with plant roots resulting in low oxygen and the presence of Trp. The evolutionary associative relationship with plant roots resulting in nitrogen conditions) are in place and when there is an produced when the nutritional limits (low carbon and

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