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FEMS Microbiology Letters 246 (2005) 125–132

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

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

Ositadinma Ona [a], Jan Van Impe [b], Els Prinsen [c], Jos Vanderleyden [a,*]

[a] *CMPG, KULeuven, Kasteelpark Arenberg 20, B-3001 Heverlee, Belgium*
[b] *BioTeC, KULeuven, W. de Croylaan 46, B-3001 Heverlee, Belgium*
[c] *Department of Biology, University of Antwerp, Universiteitsplein 1, B-2610 Antwerpen, Belgium*

Received 10 February 2005; received in revised form 30 March 2005; accepted 30 March 2005

First published online 9 April 2005

Edited by Y. Okon

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

* Corresponding author. Tel.: +32 16 321631; fax: +32 16 321966.
E-mail address: jozef.vanderleyden@agr.kuleuven.ac.be (J. Vanderleyden).

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doi:10.1016/j.femsle.2005.03.048

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 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 fermentation 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 $H_3PO_4$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^{R}' 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 (Spectronic 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 ln 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-$^{13}C_6$]-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 NH_4^+ 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

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