Symbiosis, 13 (1992) 191–198
Balaban, Philadelphia/Rehovot

Induction of Indole-3-Acetic Acid Synthesis and Possible Toxicity of Tryptophan in *Azospirillum brasilense* Sp7

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Received October 20, 1991; Accepted April 15, 1992

Abstract

Addition of high concentrations of tryptophan to *Azospirillum brasilense* Sp7 cultures caused a toxic response, expressed by growth inhibition, color change of cultures, specific changes in DNA transcription and changes in the pattern of protein synthesis.

A time-induced indole-3-acetic acid production system was developed using a pulse of tryptophan as the induction factor. It was found that indole-3-acetic acid production depended on culture age and amount of tryptophan supplement. Indole-3-acetic acid production after tryptophan addition by pulse injection was linear versus time, and higher in young cultures.

We suggest that indole-3-acetic acid biosynthesis from tryptophan and its immediate excretion to the growth medium of *A. brasilense* Sp7 is a way by which *A. brasilense* Sp7 reduces toxic levels of tryptophan.

Keywords: *Azospirillum brasilense* Sp7, tryptophan, indole-3-acetic acid, pulse induction, toxicity

1. Introduction

Free-living diazotrophic soil bacteria of the genus *Azospirillum* have a potential for application as a biofertilizer, probably due to their ability to excrete phytohormones (Fallik et al., 1989; Bottini et al., 1989; Tien et al., 1979).

0334-5114/92 /$03.50

Phytohormones excretion might be involved in the close association of the bacterium with the roots of many economically important plants (for review see Okon, 1985; Döbereiner and Pedrosa, 1987).

Investigation of the indole-3-acetic acid acid (IAA) biosynthetic pathway(s) in *A. brasilense* has been difficult, due to the absence of a fast and well-characterized system for IAA production from its precursor tryptophan (Trp), and accurate determination of IAA.

In previous studies, Trp was added to the medium at the time of inoculation, and it was reported that auxins are not formed during the logarithmic growth phase of batch cultures, and it was suggested that auxin formation in the stationary growth phase is connected to Trp release from dead cells (Zimmer et al., 1988), or that Trp, as well as other amino acids (Hartmann et al., 1988), may be utilized by the bacteria as an N source (Tien et al., 1979), followed by IAA production and excretion (De Francesco et al., 1985).

Addition of Trp at high concentrations inhibits bacterial growth, and IAA accumulating in the medium increases with Trp concentration (Tien et al., 1979). The possibility of Trp toxicity in *A. brasilense* Sp7 which may be expressed in IAA synthesis and excretion was examined in this study.

A time-induced IAA production system was developed in order to examine, at different culture ages, the pattern and amount of IAA production, enabling us to analyse simultaneously, proteins and RNA synthesis.

2. Materials and Methods

Bacteria and growth conditions

A. brasilense Sp7 (ATCC 29145) Sm^R cells were grown in flasks in a shaking water bath under batch culture conditions at 30°C, in the medium described by Zimmer and Bothe (1988) containing 5 mM NH_4Cl and 0.25 $g \cdot l^{-1}$ streptomycin sulfate (Sigma). Trp was sterilized by filtration before addition to the growth medium.

Auxin determination by high-performance liquid chromatography (HPLC)

Cell-free cultures were filtered and injected into an HP 1090 HPLC, using a Merck 50943 LiChrospher 100RP-18 column equilibrated with metanol:1% glacial acetic acid in water (40:60, v/v) at room temperature at a flow rate of 0.5 $ml \cdot min^{-1}$. Substances were quantitated by integrating the areas under the peaks (U.V. detector, 278 nm), using an HP 9121 integrator and IAA as the standard.

IAA induction by L-Trp pulse

During growth, 30 ml samples of the cell suspension (300 ml in 1 l flasks) were transferred and L-Trp was added to a final concentration of 0.5 mM, 0.5% (v:v). Samples were taken for HPLC analysis.

^{3}H-uridine incorporation to A. brasilense *Sp7 RNA*

^{3}H-uridine (Nuclear Research Center – Negev, Israel, 1 mCi·ml^{-1}) was added to culture samples (1 ml) taken 2, 4, 8 and 24 hr after L-Trp pulse (2 μCi·ml^{-1}), and shaking was continued for an additional 30 min at 30°C. Nucleic acids were obtained by Na-acetate mini-prep. Total cell nucleic acids were resuspended in Insta-gel II (Packard), and radioactivity was determined with a scintillation counter.

Statistics

All experiments were done on 2 cultures of independent origin. In each replicate, 4 samples were taken from each culture for determination of 0.D., IAA content or ^{3}H-uridine incorporation. Data were analyzed by one-way model I analysis of variance (ANOVA) (Sokal and Rohlfe, 1981) in order to detect significant differences between replicates. In all experiments, comparison of the variance ratio (Fs) to the critical values showed that $P \leq 0.05$.

Influence of Trp on culture growth was analyzed by two-way model I ANOVA. No significant differences were found among treatments when concentrations were lower than 10 mM or higher than 20 mM.

Data from L-Trp pulse experiments were analyzed by linear regression (after analysis by one-way model I ANOVA), and correlation coefficient (r) was determined according to Spiegel (1961).

3. Results

Effects of L-Trp concentration on A. brasilense *Sp7*

IAA production by *A. brasilense* Sp7 was dependent upon addition of L-Trp to the medium and the amount of IAA accumulating in the medium increased with L-Trp concentration (data not shown).

Cell aggregation was observed and the medium turned from transparent to orange, in the presence of high L-Trp concentration (5 mM). In all L-Trp concentrations tested, as well as in the control without L-Trp, a final pH of 8.8 was reached in 48 hr cultures. No IAA was found in cells.

Effect of D,L-Trp and L-Trp concentration in medium on growth rate

Possible Trp toxicity (Tien et al., 1979; Smidt and Kosuge, 1978) was examined by addition of several concentrations of D,L-Trp and L-Trp to the medium (10^{-3}, $2{\cdot}10^{-3}$, 1, 2, 10, 20, 30 and 50 mM).

No significant influence on culture growth was evident when less than 10 mM Trp was added, and the stationary phase was reached within 24 hr (data not shown).

An inhibition 16% in growth was caused by 10 mM L-Trp and 24% by 20 mM L-Trp 10 hr after inoculation, while 10 mM D,L-Trp inhibited growth by 29% and 20 mM D,L-Trp inhibited growth by 63% (Fig. 1). Higher concentrations of Trp (30 or 50 mM) caused almost total growth inhibition.

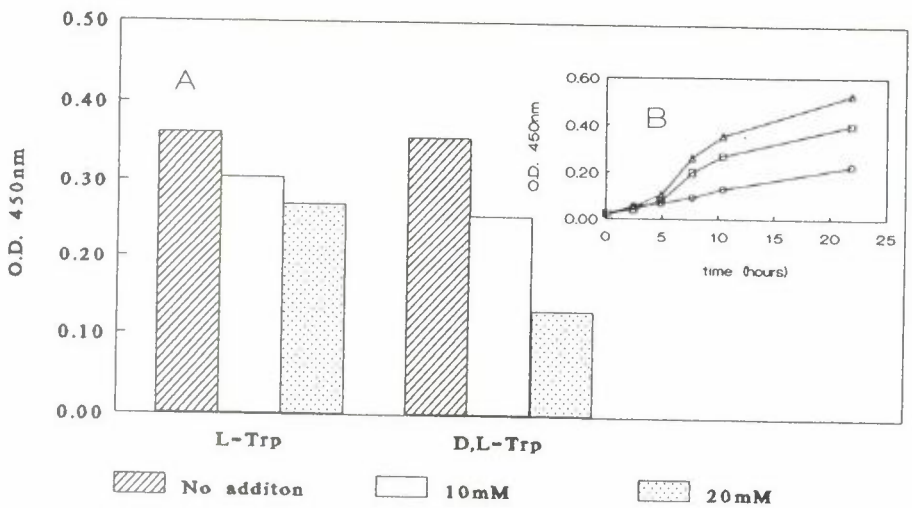

Figure 1. (a) Growth inhibition by 10 mM (blank) and 20 mM (dotted) L-Trp and D,L-Trp, as compared to the control (no Trp addition, lines), 10 hr after inoculation, as measured by absorbance at 450 nm.
(b) Growth of *A. brasilense* Sp7. Without Trp supplement (△), with 20 mM L-Trp (□) and with 20 mM D,L-Trp (○).

Induction of IAA production by L-Trp pulses

In order to determine the pattern and amount of bacterial conversion of L-Trp to IAA, L-Trp was added to *A. brasilense* Sp7 cultures of various ages.

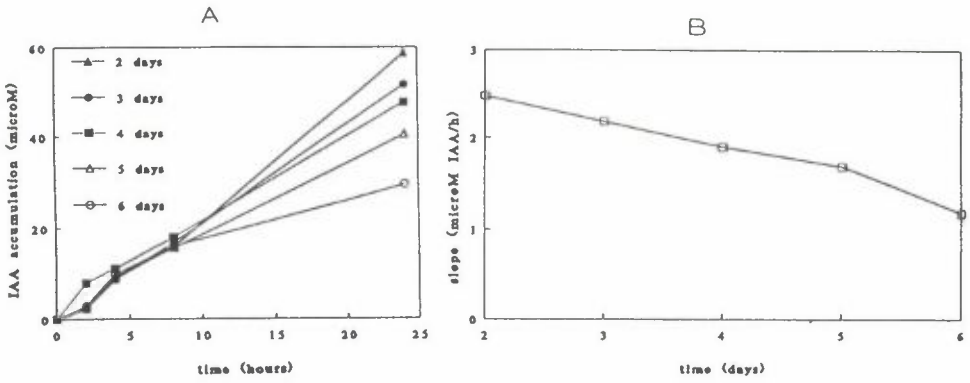

Figure 2. (a)IAA accumulation in *A. brasilense* Sp7 growth medium after Trp pulse. Pulse was given at different culture ages, 2–6 days after inoculation, r=0.96–0.99.
(b) Slopes of IAA accumulation versus time at different culture ages, r=0.99.

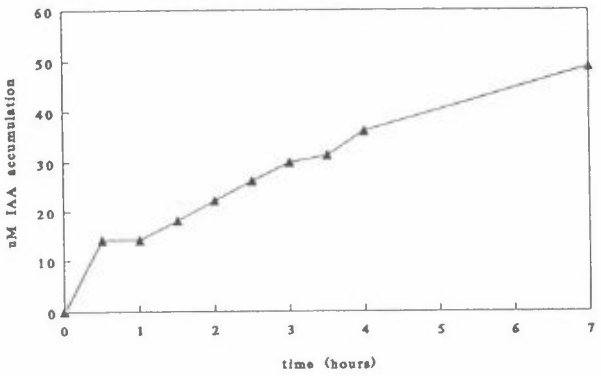

Figure 3. IAA accumulation in 4 day *A. brasilense* Sp7 culture after Trp pulse to final concentration of 0.5 mM, r=0.97.

IAA biosynthesis proved linear versus time (Fig. 2a). The rate of IAA production (e.g. slope of IAA accumulated in the medium versus time) shows dependence on culture age. The greatest amount of IAA was found to be produced at the youngest culture detected (2-day-old). Old cultures partially lost their ability for IAA synthesis; in 6-day-old cultures, 50% of the IAA production ability was lost 24 hr after L-Trp pulse, as compared to 2-day-old cultures. Decreasing of IAA amount in the growth medium was not correlated to the amount of dead cells, which does not extend over 5% of the total cell

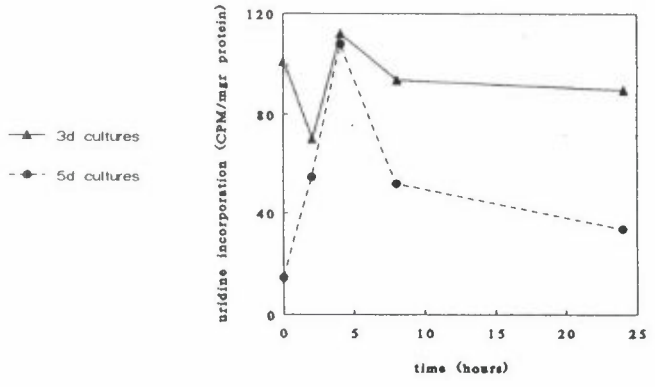

Figure 4. ^{3}H-uridine incorporation, after Trp pulse, to 3 (▲) and 5 (●) days cultures of *A. brasilense* Sp7, 30 min after incubation. Data obtained were not significantly different 24 hr after Trp pulse, as compared to the basic level of the culture.

content. Loss of IAA production ability was found to be linear versus time (Fig. 2b).

Sort-time production of IAA, as a response to L-Trp pulse, was examined, and found to be linear versus time. IAA was found in the growth medium 30 min after L-Trp addition (Fig. 3).

Effect of culture age on total RNA synthesis after L-Trp pulse

Changes in DNA transcription are expressed as changes in RNA synthesis, and can be determined by measuring incorporation of labelled uridine into the newly formed RNA (McKellar and Cholette, 1987). RNA synthesis was determined at different culture ages after L-Trp pulse. Prior to L-Trp pulse, ^{3}H-uridine incorporation was higher in young cultures than in old ones, as would be expected (Fig. 4). Cultures reached equal amounts of ^{3}H-uridine incorporation 4 hr after L-Trp pulse. In young cultures, bacteria showed a decrease in ^{3}H-uridine incorporation 2 hr after L-Trp pulse (Fig. 4).

4. Discussion

The amounts of IAA accumulating in the medium were higher when a high concentration of L-Trp was supplied with bacterial inoculation, and high concentrations of either L-Trp or D,L-Trp, inhibited bacterial growth, as was previously reported for D,L-Trp (Tien et al., 1979). It was also observed that cell

aggregation and color change to orange corresponded to L-Trp concentration. These responses are typical stress reactions in *A. brasilense* (this laboratory, unpublished data).

The phenomenon of Trp-toxicity, followed by the bacterial toxic response, lead us to develop a system for the induction of IAA production using L-Trp as the induction factor.

IAA production after L-Trp pulse, was linear versus time, indicating that the rate of IAA production after L-Trp pulse was equivalent in every time period. IAA production was higher in young cultures and began less than 30 min after L-Trp pulse. This may indicate that at least a few IAA regulatory and synthetic enzymes are regular constituents of the cells, and following addition of L-Trp, their activity and/or synthesis is increased. The development of an induction system allowed to follow changes in DNA transcription, which is expressed by an increase in RNA synthesis and can give an additional indication of Trp toxicity, as well as changes in specific protein's synthesis (Bar and Okon, 1991).

Metabolic steady-state may be disturbed by an alteration in transcription or translation processes caused by toxins. Changes in RNA synthesis may be due to inhibition of protein synthesis or increased synthesis of unique transcriptional products. Both of these phenomena may be caused by toxic substances (Gilchrist, 1983). Total RNA synthesis is the net result of these increasing and decreasing processes. In young cultures, where the initial level of RNA synthesis was high, inhibition of RNA synthesis was observed 2 hr after L-Trp pulse, probably due to disturbance of the processes associated with metabolic steady-state and was expressed as a decrease in total protein synthesis. In older cultures, total RNA synthesis increased 2 hr after L-Trp pulse, probably because of the initial low level of RNA synthesis present, combined with an increase in specific RNA synthesis which is the dominant process. Four hr after L-Trp pulse, the level of RNA synthesis was the same in young and old cultures. This level may represent the maximum level of specific RNA synthesis. Both cultures returned gradually to the initial amount of RNA synthesis, indicating continuation of the regular steady-state metabolic processes of the cell. Induction of specific protein's synthesis was examined by ^{35}S-methionine labeling. New protein bands appeared after L-Trp pulse (Okon et al., 1990).

We suggest that Trp conversion to IAA and the immediate excretion of IAA to the medium is a way by which *A. brasilense* Sp7 reduces toxic levels of Trp in the medium.

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