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FEMS Microbiology Letters 233 (2004) 15–21

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Aromatic amino acid aminotransferase activity and indole-3-acetic acid production by associative nitrogen-fixing bacteria

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Received 22 July 2003; received in revised form 28 October 2003; accepted 14 January 2004

First published online 14 February 2004

Abstract

In this work, we report the detection of aromatic amino acid aminotransferase (AAT) activity from cell-free crude extracts of nine strains of N_2-fixing bacteria from three genera. Using tyrosine as substrate, AAT activity ranged in specific activity from 0.084 to 0.404 μmol min^{-1} mg^{-1}. When analyzed under non-denaturating PAGE conditions; and using tryptophan, phenylalanine, tyrosine, and histidine as substrates *Pseudomonas stutzeri* A15 showed three isoforms with molecular mass of 46, 68 and 86 kDa, respectively; *Azospirillum* strains displayed two isoforms which molecular mass ranged from 44 to 66 kDa and *Gluconacetobacter* strains revealed one enzyme, which molecular mass was estimated to be much more higher than those of *Azospirillum* and *P. stutzeri* strains. After SDS–PAGE, some AAT activity was lost, indicating a differential stability of proteins. All the strains tested produced IAA, especially with tryptophan as precursor. *Azospirillum* strains produced the highest concentrations of IAA (16.5–38 μg IAA/mg protein), whereas *Gluconacetobacter* and *P. stutzeri* strains produced lower concentrations of IAA ranging from 1 to 2.9 μg/mg protein in culture medium supplemented with tryptophan. The IAA production may enable bacteria promote a growth-promoting effect in plants, in addition to their nitrogen fixing ability.

Keywords: AAT activity; IAA production; Nitrogen-fixing bacteria

1. Introduction

Diverse groups of microorganisms, including soil, epiphytic and endophytic bacteria, and some cyanobacteria [1] have been found to synthesize indole-3-acetic acid (IAA). Several of these groups are implicated in plant pathogenesis, while others stimulate plant growth [2]. Examples of the latter group include nitrogen-fixing bacteria such as *Azospirillum*, *Pseudomonas*, *Rhizobium* and *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*) [2–4].

Some members of the genuses *Azospirillum*, *Gluconacetobacter* and *Pseudomonas* are associative nitrogen-fixing bacteria, often referred to as plant growth-promoting rhizobacteria [5]. *G. diazotrophicus* which contributes to plant yield through N_2-fixation and auxins biosynthesis, was first isolated from sugarcane in Brazil [3,6,7]. Recently, two new *Gluconacetobacter* N_2-fixing species were isolated from the rhizosphere of coffee plants in México: *Gluconacetobacter johannae* and *Gulconacetobacter azotocaptans* [8]. Although IAA biosynthesis was reported for *G. diazotrophicus* [3], nothing is known of the AAT activity. Similarly, the AAT activity and IAA production for *G. johannae* and *G. azotocaptans* are also unknown.

Alcaligenes faecalis A15 was reclassified as *Pseudomonas stutzeri* A15 [9]. This bacterium is widely used as a rice inoculant in China due to its N_2-fixing ability.

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0378-1097/$22.00
doi:10.1016/j.femsle.2004.01.047

However, the production of growth regulating substances has not been reported.

Some *Azospirillum* strains can produce significant amounts of IAA and can increase IAA production when medium is supplemented with tryptophan (Trp) [10,11]. The biosynthesis of IAA, among other growth regulators, by *Azospirillum* could explain the enhancement of plant root development and the concomitant improvement of mineral and water uptake by roots [12,13]. Such effects make these microorganisms beneficial as commercial inoculum in agronomical important crops [14].

IAA is a metabolite derive from Trp by several pathways and can also be synthesized by Trp-independent route in plants and bacteria. More that one pathway could be present in bacteria [2]. Physiological evidence for different Trp-dependent pathways for IAA synthesis in *Azospirillum brasilense* has been reported [15,16]. The indole pyruvate (IPyA) route is one of the main pathways for IAA synthesis from Trp [Trp → IPyA → indole-3-acetaldehyde (IAald) → IAA], catalyzed by the key enzyme indole pyruvate decarboxylase (IPDC) [17]; and for the aromatic amino acid aminotransferases (AAT) [2]. These enzymes are ubiquitous and catalyze reversibly the conversion of amino acids to the corresponding α-ketoacids; in the presence of an α-keto-glutaric acid acceptor of an amino group, which is converted into glutamic acid. The AATs are homodimeric proteins and bacteria can possess several isoforms which are involved in multiple pathways [18].

Currently, four aromatic amino acid aminotransferases have been identified in *Azospirillum lipoferum* ATCC 29708 [19] and two in *A. brasilense* UAP14 [11]. There are no reports of AAT activity detected in N_2-fixing *Gluconacetobacter* or in *P. stutzeri* A15. In *Azospirillum*, the enzymatic activity was detected in zymograms after non-denaturing polyacrylamide gel electrophoresis (PAGE); however, aminotransferase activity can also be detected after denaturing PAGE, using sodium dodecyl sulfate (SDS) [20,21].

In this work, we report the detection of AAT activity in non-denaturing and denaturing PAGE in five Azospirilla strains, three *Gluconacetobacter* strains and in *P. stutzeri* A15. This is the first report on AAT activity and IAA production for some of these strains.

2. Materials and methods

2.1. Cell-free crude extract preparation

The bacterial strains and growth culture media used in this work are listed in Table 1. Cell-free extracts were prepared as indicated by Baca et al. [11] but with some modifications. The different strains were cultured in 150 ml of the corresponding medium and harvested at the stationary phase of growth (10,000 rpm, 10 min at 4 °C).

Table 1
Bacterial strains and culture media used

Strain	Culture media	Reference
Azospirillum brasilense Sp7	NFb	[34]
A. brasilense Sp245	NFb	[35]
A. brasilense UAP14	NFb	[11]
A. brasilense R07	NFb	[36]
Azospirillum lipoferum USA5a	NFb	[34]
Pseudomonas stutzeri A15	LB	[9]
Gluconacetobacter diazotrophicus PAL5	LGI-TA	[6]
Gluconacetobacter azotocaptans CFN-Ca 54^T	LGI-TA	[8]
Gluconacetobacter johannae CFN-Cf55^T	LGI-TA	[8]

Cells (2 g) were washed in 50 ml of 3 M NaCl. After being stirred for 30 min at 4 °C, the cells were centrifuged (10,000 rpm, 10 min at 4 °C); the supernatant was discarded, and the cells suspended in 100 ml of 50 mM Tris–HCl, pH 7.5. The mixture was stirred 30 min at 4 °C and then centrifuged to collect the cells (10,000 rpm, 10 min at 4 °C). The pellet was suspended in 50 ml of TES buffer with lysozyme (500 $\mu g\,ml^{-1}$); 1 mM phenyl methylsulfonylfluoride (PMSF), and 10% sucrose. The mixture was stirred overnight at 4 °C, stored at −70 °C for 30 min, thawed in a water bath at 45 °C, and finally centrifuged (10,000 rpm, 10 min at 4 °C). The final supernatant (cell-free crude extract) was stored in sterile vials at 4 °C and used for the following steps 24 h after obtained.

2.2. Enzymatic activity assay

Tyrosine aminotransferase activity was assessed by measuring the concentration of *p*-hydroxyphenyl pyruvate (*p*HPP) produced, according to the method of Diamondstone [22], incubating at 45 °C for 45 min. The amount of *p*HPP was estimated spectrophotometrically at 331 nm.

2.3. Protein quantification

Protein concentration was determined by the method of Bradford [23] using bovine-serum albumin as standard.

2.4. Non-denaturing PAGE, SDS–PAGE conditions, and aromatic amino acid aminotransferase activity staining

Electrophoretic analysis under non-denaturing conditions was performed according to Laemmli [24] in a discontinuous system, using Tris–glycine running buffer (25 and 192 mM, respectively; pH 8.3). The separating gel consisted of 10% acrylamide and the stacking gel of 4% acrylamide. Samples of cell-free crude extracts (100

μg of protein) were diluted with an equivalent volume of Laemmli loading buffer and loaded into the gel wells. Gels were run at room temperature (25 °C) at 3.4 $V\,cm^{-1}$ during 60 min and then at 5.1 $V\,cm^{-1}$ until the blue color (blue bromophenol colorant) of loading buffer begin to migrate out of the gel. After electrophoresis, the gels were assayed for AAT activity as was previously described (11), briefly: the reaction mixture consisted of 20 ml of Tris–HCl buffer (0.1 M, pH 8.6) containing 5.5 mM Trp, or another L-amino acid (phenylalanine, histidine or tyrosine); 0.2 mM pyridoxal phosphate; 12.5 mM α-ketoglutaric acid; 98 μM phenazine methosulfate, and 0.6 mM nitroblue tetrazolium. Gels were incubated at 37 °C in the dark until the appearance of bands (30 min).

The electrophoresis PAGE–SDS conditions were the same as mentioned previously, with the exception that SDS (0.1% w/v) was included. Samples of cell-free crude extracts were prepared as above, but without PMSF, 100 μg of protein were diluted with an equivalent volume of Laemmli loading buffer without β-mercaptoethanol and loaded into the gel wells without heating. The samples were loaded in duplicates to compare the protein profiles observed with Coomassie brilliant blue R-250 and with the AATs reaction as above. After electrophoresis, the gels were split into two parts and rinsed with distilled water. One half of the gel, containing the molecular weight marker, was stained with Coomassie brilliant blue. The other half of the gel was assayed for AAT activity.

2.5. Indolic compounds detection

A colorimetric method was used to detect indolic compounds from the nine bacterial strains assessed, according to Glickmann and Dessaux [25]. The reaction consisted of 500 μl of supernatant of cell culture (See HPLC analysis) and 500 μl of Salkowski reagent, followed by incubation in the dark for 30 min at room temperature. Indolic compounds were determined spectrophotometrically at 540 nm, using IAA (Sigma) as standard.

2.6. Indole-3-acetic acid determination by HPLC

For IAA determination, all Azospirilla strains and *P. stutzeri* A15 were grown in K-lactate medium supplemented with Trp (100 $\mu g\,ml^{-1}$), while the *Gluconacetobacter* strains were grown in LGI-TA medium plus Trp (100 $\mu g\,ml^{-1}$). Bacteria were grown at 32 °C, in a shaker (120 rpm), for 72 h. IAA production was determined by HPLC following the conditions as previously described [16]. IAA was separated on a 4.6 mm × 14.5 cm, 5 μm C-18 reverse phase column on a Beckman Gold Liquid Chromatograph. Samples were analyzed under isocratic conditions with acetonitrile: 1% acetic acid in water (40:60 v/v) as separation solvent, at a flow rate of 1 $ml\,min^{-1}$. Eluates were detected by spectrophotometry at 280 nm. IAA was quantified by reference to the peak area obtained for the IAA standard (Sigma).

3. Results and discussion

3.1. Experimental evidence for AAT activity and isoforms

IAA is a common product of tryptophan metabolism by bacteria including those that stimulate plant growth and fix nitrogen. Several pathways have been proposed to exist in bacteria. One of the most studied is the IPyA pathway, in which the Trp is converted to IPyA by the α-keto-glutarate-dependent transaminases, followed by decarboxylation by IPDC enzyme to indole-3-acetaldehyde that is further oxidized to IAA [2,15].

In this work, we report the determination of AAT activity and detection after non-denaturing and denaturing PAGE from crude cell-free extracts of nine N_2-fixing bacterial strains (Table 1). Enzymatic activity, specific activity, and number of isoforms of the nine crude cell-free extracts used in this study are shown in Table 2. The specific activity determined by the method of Diamondstone [22] varies among strains, ranging from 0.084 to 0.404 $\mu mol\,min^{-1}\,mg^{-1}$ of *p*-hydroxyphenyl pyruvate (*p*HPP) produced in reaction using tyrosine, and α-ketoglutarate as substrates. *P. stutzeri* A15 value was the highest and *Gluconacetobacter* strains values were the lowest (Table 2).

After non-denaturing PAGE, aromatic amino acid aminotransferase activity was detected in all strains assessed. Table 2 and Fig. 1 show the number of AAT isoforms observed for each strain: *P. stutzeri* A19 three bands; *Azospirillum* strains two bands, and *Gluconacetobacter* strains one band. The bands differed in size on the gel (Fig. 1). Whatever the aromatic amino acid used in the AAT reaction, the activity (represented by the band) was detected in the same position on the gel. Omitting Trp, or any other aromatic amino acid from the transamination mixture completely eliminated the bands in the gel (data not shown). The number of the isoforms observed after non-denaturing PAGE varied among strains, but the profile for each was similar regardless of the aromatic amino acid used in the transamination reaction (Fig. 1). This confirms that the aminotransferases detected in this study are non-specific, as reported by others [4,19]. In addition, these observations were validated by the enzyme activity determined according Diamodstone [22], where the lowest activities correspond to strains with only one aminotransferase (Table 2).

All *Azospirillum* strains studied here showed two isoforms, including *A. lipoferum* USA5a with molecular

Table 2
Enzymatic activity and number of isoforms detected after PAGE

Strain	Enzyme units[a]	Specific activity[b]	Native-PAGE isoforms				SDS–PAGE			
			Tyr	Trp	Phe	His	Tyr	Trp	Phe	His
Sp7	0.772	0.271 ± 0.035	2	2	2	1	1	1	1	nd
Sp245	0.817	0.255 ± 0.024	2	2	2	2	1	1	1	nd
UAP14	1.093	0.290 ± 0.012	2	2	2	2	1	1	1	nd
R07	0.810	0.244 ± 0.052	2	2	2	1	nd	nd	nd	nd
USA5a	0.855	0.261 ± 0.084	2	2	2	2	1	1	nd	nd
A15	1.564	0.404 ± 0.028	3	3	3	3	1	1	nd	nd
PAL5	0.418	0.109 ± 0.007	1	1	1	1	nd	nd	nd	nd
CFN-Ca54^T	0.527	0.139 ± 0.023	1	1	1	1	nd	nd	nd	nd
CFN-Cf55^T	0.338	0.084 ± 0.023	1	1	1	1	nd	nd	nd	nd

nd, not detected.
[a] Expressed as μmol min^{-1} of product *p*-hydroxyphenyl pyruvate (pHPP) produced in reaction, using tyrosine as substrate.
[b] Expressed as μmol min^{-1} mg^{-1}, mean value of three determinations and standard deviation.

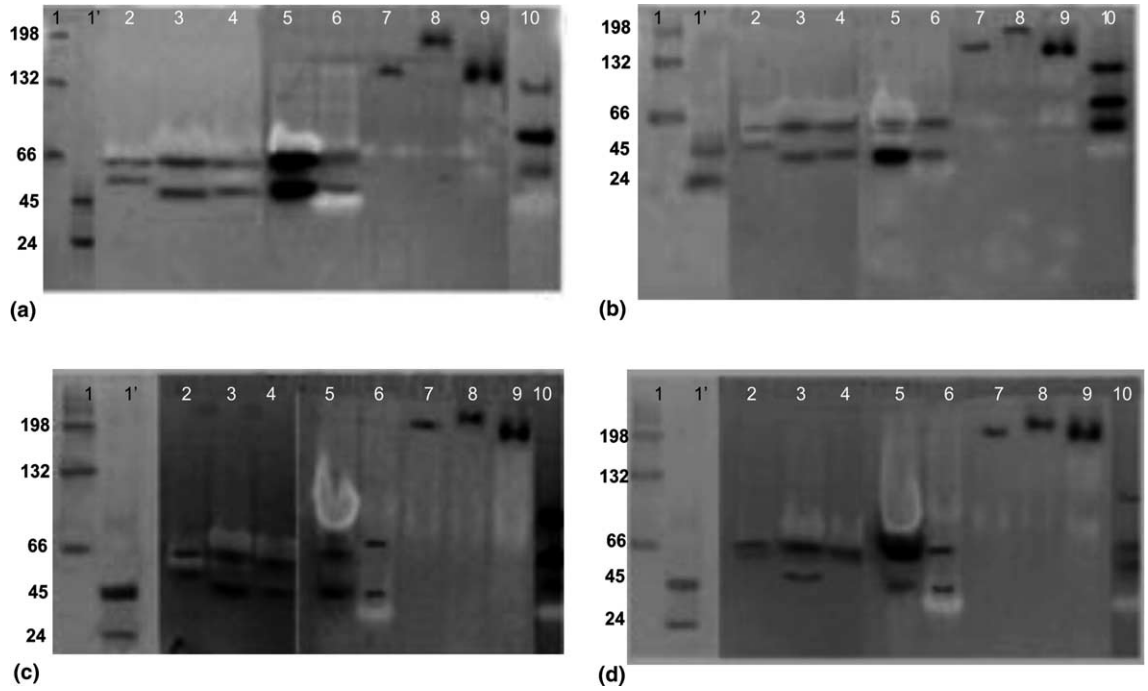

Fig. 1. Zymogram of AATs in non-denaturating PAGE conditions. (a–d) Gel stained for AAT activity, using Trp, tyrosine, phenyalanine, and histidine as amino acid donor substrates, respectively. Lane 1, and 1′ M; lane 2, Sp7; lane 3, Sp245; lane 4, RO7; lane 5, UAP14; lane 6, USA5a; lane 7, PAL5; lane 8, CFNCa54^T; lane 9, CFNCf55^T and lane10, A15. Reaction conditions were described in Materials and methods. Molecular mass markers (M): Bovine serum albumin, 66 kDa monomer; 133 dimer and 198 trimer; ovoalbumin, 45 kDa; and trypsinogen, 24 kDa.

mass (MMr) estimated of 44 and 66 kDa, respectively. This is in contrast to, *A. lipoferum* ATCC 29708, which presents four aminotransferases [19]. *P. stutzeri* A15 showed three isoforms with MMr of 46, 68, and 86 kDa, respectively. In the case of the three *Gluconacetobacter* strains studied here, showed only one isoform which MMr is much more higher than those of *P. stutzeri* A15, and *Azospirillum* strains. It has been described that AATs are homodimeric proteins with native MMr ranging from 40 to 100 kDa [18,26].

Aminotransferase activity could also be detected in gels after SDS–PAGE by the elimination of two components routinely employed in the assay: PMSF and β-mercaptoethanol. Since PMSF has the potential to covalently modified and/or alter the activity of proteases proteins, it can be toxic for proteases under certain conditions [27]. Covalent associations between proteins units can be preserved by including β-mercaptoethanol from the sample buffer. In the absence of this reducing agent, the intra- and inter-chain disulfide bonds of the

sample proteins remain intact [28]. During the adjustment of our method, AAT activity in situ was not observed, when PMSF was included in the crude extract, although it was possible to measure it in the enzymatic activity assay. Likewise, when β-mercaptoethanol was included in the sample buffer, reagents in the AAT reaction mixture precipitated.

After SDS–PAGE the AAT activity was detected in *P. stutzeri* A15 and *Azospirillum* strains but not in *Gluconacetobacter* strains (Table 2). However, when histidine was used as substrate, no aminotransferase activity was observed (data not shown). Fig. 2(a) shows the proteins profiles for different strains after Comassie brilliant staining. Fig. 2(b) shows AAT activity by the different strains after exposure of the gel in the transamination mixture at 37 °C in the dark. When Trp was omitted from the reactions mixture, AAT activity was not detected. The enzymatic activity preserved in *P. stutzeri* A15 belongs to isoform of 86 kDa. In *A. brasilense* Sp7, UAP14, 245, and *A. lipoferum* USA 5a strains, the isoform detected is that of 66 kDa; whereas in *A. brasilense* R07 the isoform corresponds to 44 kDa. *Gluconacetobacter* strains even their higher MMr isoforms (compared with the other strains, see Fig. 1), probed to be more susceptible to the denaturing process as no signal was observed in the gel. These data show that AATs have differential stability to SDS, and raising the question of particular structural features of AAT of *Gluconacetobacter* strains [21].

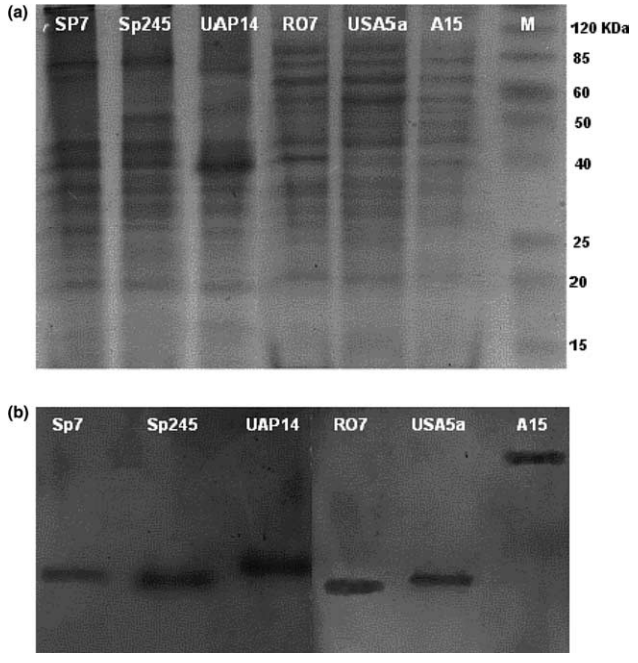

Fig. 2. Zymogram of AAT in PAGE–SDS. (a) Gel stained with Coomassie brilliant blue. M, Molecular mass standards, BenchMark™ Life technologies. (b) Gel stained for AAT activity using tyrosine as substrate, as was described in Materials and methods.

3.2. Indolic compounds and IAA production

Considering that aromatic amino acid aminotransferases have been proposed to be implicated in the biosynthesis of IAA, total indoles and IAA were analyzed by a colorimetric method, and by HPLC, respectively; results are presented in Table 3. To compare the indolic production without Trp as precursor, four strains were assessed without Trp addition to the growth medium (*P. stutzeri* A16, *G. diazotrophicus* PAL 5, *G. azotocaptans* CFN-Ca54^T and *G. johannae* CFN-Cf55^T). Fig. 3(a) shows comparative results of total indole production with and without tryptophan in the culture medium.

To validate the auxinic production of the strains assessed in this study, HPLC analysis was performed to determine IAA production. In all cases, it was possible to measure IAA levels, although with different values among bacterial species and within the same genus. Results are presented in Table 3 and Fig. 3(b), where IAA production was assessed with or without Trp in the culture medium.

In general, all the bacterial strains assayed here produced IAA, but *Azospirillum* strains showed higher values of total indoles, *A. brasilense* UAP14 having the highest levels (27.362 μg/mg protein). Recently, it was communicated by Radwan et al. [29] that several strains of *Azospirillum* produced different amounts of indole compounds, with *A. brasilense* among the four species of *Azospirillum* with the highest levels (including *A. lipoferum*). This is in agreement with our results; however, the colorimetric approach did not correlate with values obtained by HPLC. As observed in Table 3, a high levels of total indoles does not mean high levels of IAA (e.g., CFN-Ca45^T, CFN-Cf55^T, and USA5a strains), and vice versa (e.g., UAP14 and R07 strains). The auxine-like substances are detected using the Salkowski reagent, which is a simple and fast assay for rapid screening of indolic compound production, but no correlation is observed with levels of IAA determined by HPLC as it has also been evaluated by others [1,3].

Although *P. stutzeri* A15 presented three aminotransferases isoforms and the highest level of enzyme activity (Table 2), the production of total indolic compounds, and IAA in cultures with or without Trp addition was low. We can speculate that, in this strain the IPyA pathway is the unique route for IAA biosynthesis (considering the existence of pathogenic species within this genus); since the indole-3-acetamide pathway was reported as a trait for pathogens but not for beneficial plant growth-promoting bacteria, where the IPyA route prevails [30,31].

Regarding the *Gluconacetobacter* strains studied here, they presented only one aminotransferase. Although *Gluconacetobacter* strains exhibited low enzyme specific activities (0.084–0.139 $\mu mol\,min^{-1}\,mg^{-1}$), their cultures

Table 3
Total indoles (μg/mg protein) and IAA (μg/mg protein) production

Strain	Total indoles[a] (Trp^+)	Total indoles[b] (Trp^-)	IAA[a](Trp^+)	IAA[b](Trp^-)
Sp7	20.327 ± 1.241	N.D.	16.564 ± 6.342	N.D.
Sp245	22.016 ± 5.193	N.D.	22.455 ± 4.836	N.D.
UAP14	27.362 ± 3.394	N.D.	38.286 ± 5.139	N.D.
RO7	19.899 ± 13.521	N.D.	21.343 ± 5.812	N.D.
USA5a	17.271 ± 1.186	N.D.	2.680 ± 0.421	N.D.
A15	4.373 ± 1.373	nd	1.032 ± 0.690	0.475 ± 0.085
PAL5	7.719 ± 0.708	0.206 ± 0.04	2.910 ± 1.888	1.033 ± 0.143
CFNCa54^T	14.915 ± 1.565	0.307 ± 0.02	1.491 ± 0.779	0.719 ± 0.179
CFNCf55^T	12.463 ± 1.917	0.580 ± 0.04	2.251 ± 0.286	1.833 ± 0.809

Trp^+, supplemented with tryptophan; Trp^-, without tryptophan supplementation, N.D., not determined and nd, not detected.
[a] Mean value of five replications and standard deviation.
[b] Mean value of three replications and standard deviation. Indole compounds determined by Salkowski reaction; IAA determined by HPLC.

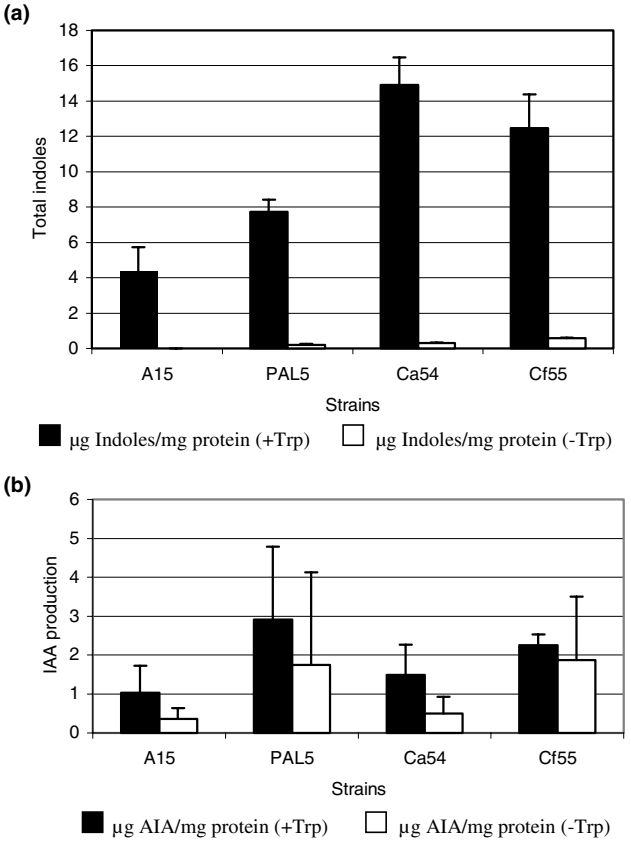

Fig. 3. Total indoles determined by a colorimetric method (a) and IAA production detected by HPLC analysis (b) in four diazotrophic strains, assayed for IAA production with and without supplementation of Trp in the growth culture media.

supplemented or not with Trp, produced IAA (Figs. 3(a) and (b)).

The IAA production was already reported for *G. diazotrophicus* PAL5, indeed our result matches well with those previously obtained by Fuentes et al. [3] and Bastían et al. [7]; but not for *Gluconacetobacter azotocaptans* CFN-Ca54^T and *G. johannae* CFN-Cf55^T. These three strains reduced total indoles production in the absence of Trp, but still produced measurable concentrations of these compounds by the colorimetric assay (Fig. 3(a)). When using HPLC analysis to determine IAA concentration, a similar effect was observed in *G. diazotrophicus* PAL5 and *G. azotocaptans* CFN-Ca54^T but, in *G. johannae* CFN-Cf55^T did not exhibit a marked difference in the absence or presence of Trp (1.868–2.251 μg IAA/mg protein; see Fig. 3(b)). The amounts of IAA obtained depended on the species, and strains, as well as on the condition of their cultivation such as: presence of Trp, oxygenation, pH and growth phase [2,27,29,33]. Addition of Trp to culture media strongly stimulated the release of IAA, which showed a rise at the stationary phase [3,10,11,16]. This is in favor for the existence of Trp-dependent route(s) [15,16]. The strain *G. johannae* CFN-Cf55^T produced active physiologically levels of IAA even in the absence of added Trp. Therefore, *G. johannae* CFN-Cf55^T may be a good candidate for IAA synthesis by a Trp-independent route [15], even though biosynthesize the AAT.

In 2001, Sevilla et al. [32] observed that, a *nifH*$^-$ mutant of *G. diazotrophicus* PAL5 could still stimulate plant growth, in inoculation experiments on sugarcane plants, suggesting the implication of auxin production. Considering that IAA is active in submicrogram amounts in a range of bioassays and is associated with a variety of physiological processes [33], IAA produced by *G. azotocaptans* CFN-Ca54^T (5.30 μM) and *G. johannae* CFN-Cf55^T (24.51 μM) could have a positive growth-promoting effect on plants.

Acknowledgements

We are thankful to Dr. Claudine Elmerich for the gift of *P. stutzeri* A15, and Dr. Luis Ernesto Fuentes for the gift of *G. azotocaptans* CFN-Ca54^T and *G. johannae* CFN-Cf55^T. This study was partially supported by a grant of CONACyT-BUAP II-85G01; R.O.P. was a post-doctoral fellow of BUAP, A.R.M. holds a CONACyT fellowship.

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