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Original Article

Molecular and physiological analysis of indole-3-acetic acid degradation in *Bradyrhizobium japonicum* E109

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ABSTRACT

Bradyrhizobium japonicum E109 is a bacterium widely used for inoculants production in Argentina. It is known for its ability to produce several phytohormones and degrade indole-3-acetic acid (IAA). The genome sequence of *B. japonicum* E109 was recently analyzed and it showed the presence of genes related to the synthesis of IAA by indole-3-acetonitrile, indole-3-acetamide and tryptamine pathways. Nevertheless, *B. japonicum* E109 is not able to produce IAA and instead has the ability to degrade this hormone under saprophytic culture conditions. This work aimed to study the molecular and physiological features of IAA degradation and identify the genes responsible of this activity. In *B. japonicum* E109 we identified two sequences coding for a putative 3-phenylpropionate dioxygenase (subunits α and β) responsible for the IAA degradation that were homologous to the canonical cluster of *iac*C and *iac*D of *Pseudomonas putida* 1290. These genes form a separate cluster together with three additional genes with unknown functions. The degradation activity was found to be constitutively expressed in *B. japonicum* E109. As products of IAA degradation, we identified two compounds, 3-indoleacetic acid 2,3-oxide and 2-(2-hydroperoxy-3-hydroxyindolin-3-yl) acetic acid. Our report proposes, for the first time, a model for IAA degradation in *Bradyrhizobium*.

1. Introduction

The auxins are a group of phytohormones characterized by their ability to induce plant growth and to reproduce the physiological effects of the naturally occurring indole-3-acetic acid (IAA). The IAA metabolism in higher plants, fungi and bacteria comprises several mechanisms, such as the biosynthesis, degradation, catabolism (i.e. oxidation and assimilation), conjugation and hydrolysis of auxin conjugates [1], which globally regulate the IAA levels in these biological systems. *Bradyrhizobium* is an alphaproteobacterium with the ability to produce IAA and this capacity has been demonstrated *in vitro* and *in planta* after soybean inoculation [2–4]. Thimann [5] proposed that auxins play an important role in the ontogeny of the root nodules during the rhizobia-legume symbiosis, as many studies have indicated that a change in the concentration of this phytohormone is a pre-requisite for nodule

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organogenesis [6]. Nielsen et al. [7] were the first to report that *Bradyrhizobium diazoefficiens* USDA110 (formerly *Bradyrhizobium japonicum* USDA110) has the capacity to degrade indole-3-acetic acid (IAA) and Egebo et al. [8] suggested that this reaction might be dependent on oxygen availability in the culture medium. Jensen et al. [9] isolated metabolites of IAA degradation in *B. diazoefficiens* USDA110 and the identified products suggested a new metabolic pathway that included anthranilic acid. However, they could not confirm this pathway or identify the gene(s) or enzyme(s) responsible for such capacity.

Two sets of canonical genes have been characterized for IAA catabolism in bacteria: i) the *iac* (indole 3-acetic acid catabolism) gene cluster identified in *Pseudomonas putida* 1290 [10] and others bacteria, which codes for an aerobic pathway through which IAA is transformed into catechol; the *iaa* (indole 3-acetic acid degradation) gene cluster identified in *Aromatoleum aromaticum* EbN1 [11], which underlies the anaerobic conversion of IAA into 2-aminobenzoyl-CoA. *P. putida* 1290 has the ability to degrade IAA as the sole source of carbon and energy (catabolism). This process is carried out by the *iac* locus, which consists of 10 genes coding for enzymes with a metabolic activity for both indole and aromatics molecules, as well as proteins with regulatory functions [10]. At present, there is no information about the full set of processes involved in the IAA catabolism in *B. japonicum*, its homeostatic control, or the effects on the symbiotic interaction with soybean. *B. japonicum* E109 has been one of the most used strains for the production of soybean inoculants in Argentina for 40 years [12]. The genome sequence of *B. japonicum* E109 has revealed the presence of genes involved in several plant growth promoting mechanisms and in particular in the biosynthesis of the phytohormone IAA [13]. The genome analysis of E109 revealed the existence of three putative pathways for IAA biosynthesis: indole-3-pyruvate (IPyA), indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM) [14]. However, the LC-MS-MS analysis did not show significant amounts of the hormone in liquid culture media, suggesting that *B. japonicum* E109 is not able to produce IAA, but on the contrary it has the ability to degrade IAA under *in vitro* conditions [15]. In the same report, we also confirmed the ability of *B. japonicum* E109 to degrade other natural and synthetic auxins, such as indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA). Exogenous IAA induced an increase in the production of biomass and exopolysaccharide in liquid culture media of *B. japonicum* E109, leading to a modification of its symbiotic behavior with soybean plants.

The aim of this work was to analyze the molecular and physiological features of IAA degradation in *B. japonicum* E109 in order to identify the responsible genes and the produced metabolites, and to outline a putative degradation pathway in this bacterium.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains and their characteristics are summarized in Table 1. *B. japonicum* E109 (*Bj*E109) was provided by the IMYZA-INTA, Buenos Aires (Argentina); *Bradyrhizobium elkanii* SEMIA5019 (*Be*SEMIA5019) was obtained from Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA) in Brazil; *B. diazoefficiens* USDA110 wild type (*Bd*USDA110) and *B. diazoefficiens* USDA110 deficient in *nrg*C gene named *B. diazoefficiens* 8620 or USDA110 spc4 (*Bd*USDA110-Δ*iac*A) were provided by Prof. Dr. Hans-Martin Fischer of Institute of Microbiology in Zürich, Switzerland. Despite the product of *ngr*C (NrgC, accession number AAG61032) has been previously associated with nitrogen fixation [16], *in silico* analysis showed that *ngr*C sequence has homology with that of *iac*A of *P. putida* 1290, related with IAA degradation [10] and *ido*A of *Pseudomonas alcaligenes* PA-10 related with indigo production [17]. Thus this strain was named as *Bd*USDA110Δ*iac*A in Table 1 and along the manuscript. Bacteria were inoculated in 250 ml flasks containing 100 ml of Yeast Extract Mannitol (YEM) culture medium [21] modified by the addition of 10 mg l^{-1} L-tryptophan (Trp) as the main precursor for IAA biosynthesis [22] and antibiotics according to the strain (Table 1). Cultures were incubated at 30 °C with orbital shaking (180 rpm) until exponential growth phase was reached. The purity of the cultures was checked routinely on YEM agar [21] and TSA plating (Trypticase Soybean Agar) at 10% (v/v).

2.2. IAA degradation by Bradyrhizobium

We compared the IAA degradation activity in three strains belonging to different species of *Bradyrhizobium* according to Torres et al. [15]. In particular, 100 μl aliquots obtained from exponential growth phase cultures of *Bj*E109, *Be*SEMIA5019 and *Bd*USDA110 were individually inoculated in 250 ml flasks containing 100 ml of YEM culture medium [21] modified by the addition L-Trp as described before. Cultures were incubated at 30 °C with orbital shaking (180 rpm) until early exponential growth phase (OD_{595} ~ 0.6), and were divided into 5 ml aliquots which were dispensed into 10 ml sterile borosilicate tubes. After 15 min of incubation conditions at 30 °C, 200 μl of 1 mg ml^{-1} IAA solution (or equivalent volume of sterilized water) were separately added into the tubes containing the cultures of each strain to obtain a final concentration ≈ 40 μg ml^{-1} (38.5 μg ml^{-1}) IAA per tube. This concentration was selected because it is similar to that produced by several rhizobacteria, including the phytostimulatory model *Azospirillum brasilense* [22]. To account for the non-biological degradation of the compound the same volume of IAA solution was added to tubes containing 5 ml of non-inoculated YEM medium. Tubes were incubated at 30 °C with orbital shaking (180 rpm) and samples were taken at T0 (5–10 min after IAA induction) and every 2 h to measure the IAA concentration (μg.ml^{-1}).

2.3. Quantification of IAA

Quantification of IAA was performed by spectrophotometry [23] and confirmed by HPLC according to Torres et al. [15]. Briefly, aliquots of 500 μl of bacterial culture were centrifuged at 11.300×*g* for 10 min. Subsequently, 250 μl of supernatant was filtered (0.2 μm), mixed with 250 μl of Salkowski's reagent (7.9 M H_2SO_4 and 12.5 gl^{-1} $FeCl_3$) and gently shaken in inverted position at least 10 times. Samples were incubated in the dark for 30 min and the absorbance at 530 nm was measured. An aliquot of filtered supernatants was also injected with a final volume of 20 μl in an HPLC Waters 600-MS device (Waters Inc., USA) equipped with an U6K injector and C18 reverse phase column (Purospher STAR RP C-18, 3 mm, Lichrocart 55-4) heated at 30 °C, coupled to a system with UV-VIS Waters 486 detector (Waters Inc., USA) set at 265 nm. The elution was performed with a mixture of ethanol: acetic acid: water (Et–OH/H–Ac/H20) (12: 1: 87) as mobile phase at a flow rate of 1 ml min^{-1} at 30 °C. The retention time for IAA was 10.1–10.3 min and it was previously identified using an appropriate standard solution of IAA (Sigma Aldrich, Germany). Quantification was performed by integration of the peak area corresponding to the retention time (RT) using an integration software (Waters Inc. USA). The IAA concentration was expressed in μg.$ml.^{-1}$

2.4. Analysis of constitutive or inducible character of IAA degradation

We evaluated whether the degradation of IAA was triggered by its own addition (inducible) or it depended on bacterial growth

Table 1
Strains, plasmids and primers.

Strains, plasmid and primers	Relevant characteristics	Reference or source
Strains		
B. japonicum E109 (*Bj*E109)	degrade IAA	WDCM31[a]
B. diazoefficiens USDA110 (*Bd*USDA110)	degrade IAA	[18]
B. japonicum E109 Δ*iac*C (*Bj*E109Δ*iac*C)	unable to degrade IAA	This study
B. diazoefficiens USDA110 Δ*iac*C (*Bd*USDA110Δ*iac*C)	unable to degrade IAA	This study
B. diazoefficiens USDA110 spc4 (*Bd*USDA110Δ*iac*A)	degrade IAA	[16]
B. elkanii SEMIA 5019 (*Be*SEMIA5019)	produce IAA, unable to degrade IAA	EMBRAPA-CNPAB
B. japonicum E109 Δ*iac*C/pJN105 *iac*C	degrade IAA	This study
Cupriavidus pinatubonensis JMP134	unable to use IAA as a sole carbon and energy source	[35]
Plasmids		
pK18mobSacB	*lacZα* Km^r *sac*B	[19]
pK18Sac::*iacCBd*	*lacZα* Kmr sacB carrying the internal fragment of *iac*C from *Bd*	This study
pK18Sac::Δ*iacCBd*	*lacZα* Kmr sacB carrying the internal fragment of *iac*C from *Bd* deleted in SalI sites	This study
pK18SacB::*iacCBj*	*lacZα* Kmr sacB -*iac*C carrying the internal fragment of *iac*C from *Bj*	This study
pK18Sac::Δ*iacCBj*	*lacZα* Kmr sacB -*iac*C carrying the internal fragment of *iacC* from *Bj* deleted in SalI sites	This study
pJN105 (pBBR)	Gm^r	[20]
pBS1	broad host range gateway destination vector, *ara*C-P_{BAD}, Gm^R	[48]
Primers		
FW*iac*CE (EcoRI)	AAAAgaattcGCTGGGTCTATGTCGGGC	This study
RV*iac*CH (Hind III)	AAAaagcttGACCTGCCACTGCATCGT	This study
FWext	TCCTCAGCGACGACGAGA	This study
RVext	TTCAGGAGCAGCAGGTCC	This study

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(constitutive). For that, *Bj*E109 was grown until late exponential growth phase ($OD_{595} = 1.2$), then a 50 ml aliquot was centrifuged at 8000 rpm for 15 min, and the cell-free supernatant was filtered (0.2 μm). The resultant filtrate was divided into 5 ml aliquots and transferred into 10 ml sterile borosilicate tubes for IAA induction. The treatments of the supernatants were the following: (1) addition of IAA solution to obtain a final concentration ≈ 40 μg ml^{-1} IAA per tube (sE109); (2) addition of IAA to heat denatured supernatants (15 min at 90 °C) to obtain a final concentration ≈ 40 μg ml^{-1} IAA per tube (sE109φ) and (3) addition of 200 μl of sterile water to supernatants, and (4) addition of 200 μl of sterile water to denatured supernatants. Tubes were incubated as previously described and 500 μl of the mixtures were sampled every 2 h to measure IAA concentration (μg.ml^{-1}).

2.5. Analysis of cellular or extracellular character of IAA degradation

*Bj*E109 was grown as described before, but 10 ml aliquots obtained from exponential growth phase cultures were centrifuged at 12,000×*g* for 20 min (4 °C). The supernatants were used as control treatment for IAA degradation assays (extracellular feature) as described before. The pellets were washed twice and resuspended in 10 ml of 20 mM Tris−HCl buffer pH 8.0 containing 1 mM EDTA, 10 mM $MgCl_2$, 12.5% (v/v) glycerol, 0.1% (v/v) Triton, and the complete mini EDTA-free protease inhibitor cocktail (Sigma−Aldrich, Germany). The cells were disrupted (lysed) by sonic treatment (M.S.E. 150 W) for 210 s (in 30 s period), centrifuged and the cellular debris discarded. The supernatants were used for IAA degradation assays (cellular feature).

2.6. Evaluation of IAA degradation kinetics

We assessed the kinetics of IAA degradation in both cell-free supernatants and non-centrifuged cell cultures of *Bj*E109.

In the case of cell cultures, we evaluated the addition of different concentrations of exogenous IAA to *Bj*E109 cultures during exponential growth phase in order to estimate the maximum quantity of IAA that this metabolically active microorganism is able to degrade. To achieve this, cell cultures were incubated as previously described until early exponential growth phase (OD_{595} ~0.6) and divided into 5 ml aliquots, which were added into 10 ml sterile borosilicate tubes. After 15 min of stabilization at 30 °C with orbital shaking (180 rpm), 200 μl of IAA solution or equivalent volume of sterilized water was alternatively added into the tubes containing *Bj*E109 to obtain a final concentration ≈ 20, 40, 80, 120 and 160 μg ml^{-1} of IAA per tube. Control treatments using uninoculated YEM medium modified by the addition of IAA solutions were also performed. Tubes were incubated at 30 °C with orbital shaking (180 rpm), and 500 μl of the mixture was sampled after 15 min (T0), 8, 12, 24 and 32 h of incubation to measure IAA concentration (μg.ml^{-1}). The viability of *Bj*E109 cells was evaluated after 32 h of IAA addition by direct plate counting on YEM agar, according to Vincent [21].

In the case of the cell-free supernatants, *Bj*E109 cultures were incubated at 30 °C with orbital shaking (180 rpm) until early exponential (EE) (OD_{595} ~0.6) or late exponential (LE) (OD_{595} ~1.2) growth phase. A 50 ml aliquot obtained from these cultures was centrifuged at 8000 rpm for 15 min, filtered (0.2 μm) to obtain a cell free supernatant and divided in 5 ml aliquot in borosilicate tubes for IAA-treatment as described before. The treatments carried out were the following: (1) addition of pure IAA solution to EE supernatant to obtain a final concentration ≈ 40 μg ml^{-1} IAA per tube; (2) addition of IAA to LE supernatant to obtain a final concentration ≈ 40 μg ml^{-1} IAA per tube. Treatments were performed in triplicate. Control treatments using uninoculated YEM medium modified by the addition ≈40 μg ml^{-1} IAA solutions were also carried out. Tubes were incubated as previously described, and 500 μl of the mixture was sampled every 2 h to measure IAA concentration (μg.ml^{-1}).

2.7. Analysis of IAA degradation pathway

2.7.1. Analysis in silico

Based on the canonical metabolic pathway described in *P. putida* 1290 by Leveau and Gerards [10] for the IAA degradation, we analyzed the presence of similar proteins by sequence comparison in the *Bj*E109 genome. For bioinformatics analysis, the following tools (T) and databases were used: Blast (T) [24], KEGG [25], RAST

[26], T-coffee (T) [27], Smart [28], MaGe [29], UniProt [30] and InterPro [31].

2.7.2. Construction of Δiac*C* mutants

The general cloning procedures were performed according to Sambrook et al. [32] with minor modifications. The deletions in the *iac*C gene in both strains of *Bradyrhizobium* were carried out through an unmarked in-frame deletion using the pK18mobSacB plasmid carrying the counter-selection gene *sac*B, as described by Mongiardini et al. [33]. Given the sequences similarity between the predicted *iac*C genes (AJA64126.1 in E109 or blr3400 in USDA110), the PCR products were amplified with the same set of primers, FW*iac*CE and RV*iac*CH (Table 1). The fragments were gel-purified and cloned at the restriction sites *Eco*RI/*Hind*III into the pK18mobSacB [19] giving the plasmids, pK18mobSacB::*iac*C*Bd* and pK18mobSacB::*iac*C*Bj* carrying the *Bd*USDA110fragment and the *Bj*E109 fragment respectively. Each plasmid was *Sal*I digested which released a 468 bp fragment from the insert cloned before as exemplify for *Bj*E109 in Fig. S1. Then, each plasmid was re-ligated giving the final construction, pK18mobSacB::Δ*iac*C*Bd* and pK18mobSacB::Δ*iac*C*Bj*. These plasmids were transferred by biparental conjugations to the corresponding wild-type strain according to Quelas et al. [34] and simple-crossover transconjugants (cointegrate) were selected by Km resistance in YEM media agar and the double crossover recombination was induced by plating the transconjugants in YEM agar plates supplemented with 10% (w/v) sucrose. These sucrose resistant clones, that resolve the recombinant plasmid and that are Km sensitive, were checked by PCR to dissociate between the mutant and the wild-type genotype. The mutants obtained carry an in-frame deletion in each *iac*C gene and were named *Bj*E109Δ*iac*C and *Bd*USDA110Δ*iac*C. All the plasmids and constructions are listed in Table 1.

2.7.3. Restoration of the IAA degradation capacity in Δiac*C* mutants

The restored phenotype of the mutant *Bj*E109Δ*iac*C was obtained using competent cells of *Escherichia coli* S-17 containing the pJN105 (pBBR) plasmid [20] with the *iac*C cloned fragment inducible by L-arabinose. *E. coli* S-17 was grown in LB medium modified by the addition of 30 μg ml^{-1} gentamicin (Gm), and *Bj*E109 was grown in PSY medium modified by the addition of L-arabinose [47]. For conjugation, *E. coli* S-17 culture was diluted a hundred times in LB medium without antibiotics and grown at 30 °C with orbital shaking (180 rpm) until OD_{595} ~0.8–1. Subsequently, a mix with 900 μl of E109 culture during exponential growth phase with 400 μl of S-17 culture was made. The culture mix was centrifuged for 8 min at 3000 rpm and the pellet was re-suspended and placed in a plate containing PSY agar medium with L-arabinose. Plates were incubated at 30 °C for 48 h. Colonies were re-suspended and homogenized into 1 ml of saline solution. A 150 μl aliquot of conjugation homogenate was placed in YEM medium with 50 μg ml^{-1} Gm and plates were incubated in the conditions previously mentioned for 72–96 h. The restored phenotype of *Bj*E109 named *Bj*E109Δ*iac*C/pJN105*iac*C was evaluated in comparison with *Bj*E109 (wild type) and *Bj*E109Δ*iac*C as control treatments.

2.8. IAA biosynthesis and degradation in Bj*E109,* Bd*USDA110 and their mutants*

We evaluated both degradation and biosynthesis of IAA by the *Bj*E109 and *Bd*USDA110 wild type strains and their mutants *Bj*E109Δ*iac*C, *Bd*USDA110Δ*iac*A and *Bd*USDA110Δ*iac*C. Cultures of each strain were incubated until early exponential growth phase (OD_{595} ~ 0.6), and treated with IAA solution to obtain a final concentration ≈ 40 μg ml^{-1} IAA as described before. A control treatment using uninoculated YEM medium, but modified by addition of pure IAA was also considered. Tubes were incubated at 30 °C with orbital shaking (180 rpm), and 500 μl of the mixture was taken every 2 h incubation to measure IAA concentration (μg.ml^{-1}). In the case of the IAA biosynthesis, 250 ml flasks containing 100 ml of YEM culture medium modified by addition of 10 mg l^{-1} L-trp were inoculated with 10% (v/v) *Bj*E109 cultures in early exponential growth phase (OD_{595}~0.6) or its mutant *Bj*E109Δ*iac*C. Cultures were incubated as previously described and after 12 h and 24 h of incubation samples were taken to measure cell growth (OD_{595}) and IAA concentration (μg.ml^{-1}).

2.9. Identification of metabolites produced by IAA degradation

The identification of IAA degradation metabolites was achieved by analyzing the expression of the *Bj*E109 genes *iac*C, *iac*D or *iac*CD in the heterologous strain *Cupriavidus pinatubonensis* JMP134 (Table 1) under the control of the AraBAD (P_{BAD}) promoter in the pBS1 plasmid [48]. This strain lacks *iac* gene sequences and is unable to use IAA as a sole carbon and energy source which allow its use as an appropriate heterologous host for higher expression of *iac* genes. An empty pBS1 plasmid (control) and pBS1 derivatives containing *iac*C, *iac*D or *iac*CD were independently electroporated in *C. pinatubonensis* JMP134 and selected in LB medium plus Gm (30 μg ml^{-1}). For the expression of *iac* genes driven by the heterologous P_{BAD} promoter, these derivatives were exposed to 5 mM L-arabinose [35]. Exponential growth cultures of *C. pinatubonensis* JMP134 of 100 ml final volume were exposed to a final concentration of 40–160 μg ml^{-1} IAA during 24 h and then analyzed for metabolites identification. The organic fraction of the culture medium was extracted by triplicate using an equal volume of ethyl acetate. Then, the organic layer was dried over anhydrous Na_2SO_4 and filtered, and the solvent was removed in vacuum. The resultant mixture of products was subjected to a spectroscopic analysis. The NMR (nuclear magnetic resonance) spectra were recorded on a Bruker Advance III HD 400 at 400 MHz for ^{1}H and 100 MHz for ^{13}C. NMR spectra were recorded in DMSO-*d6* (dimethyl sulfoxide), using the solvent signal as reference. The chemical shifts are expressed in ppm (δ scale) downfield from tetramethylsilane (TMS) and coupling constant values (J) are given in Hertz. The mass spectra were determined in TQ 4500 triple-quadrupole mass spectrometer coupled with electrospray ionization (ESI) source and were operated in the negative ion mode. The IAA degradation metabolites identified by expression of *iac* genes in *C. pinatubonensis* JMP134 were evaluated by spectroscopic and spectrometric analysis in late exponential cultures of *Bj*E109 obtained from YEM culture media exposed during 24 h to ≈40 μg ml^{-1} IAA. The analytical procedure was the same previously described.

2.10. Bradyrhizobium-*soybean symbiosis in* Bj*E109,* Bd*USDA110 and their mutants*

Cultures of *Bj*E109, *Bd*USDA110 and *iac*C mutants, grown as previously described in section 2.1, were used to inoculate soybean seeds cv. Don Mario 4800 with a 3 ml kg^{-1} dose (Note: this dose is normally used under field conditions by most of the agricultural companies). The nodulation pattern was individually evaluated on both inoculated and non-inoculated (control) seeds according to Burton et al. [36] with some modifications. A triplicate of 9 soybean seeds (n = 27) were sown in three separate plastic pots (300 ml volume capacity) containing vermiculite as a solid substrate, irrigated with nitrogen-deficient sterile N-free Hoagland's solution (25% v/v) [37]. The seedlings were maintained for 21 days in a growth chamber at 30/20 °C and 80% relative humidity with a 16/8 h day/night photoperiod. At the end of the experiment, the

following parameters were measured: (1) number of nodules on the main root per plant, (2) number of nodules on the secondary roots per plant, (3) number of nodules per plant, following Burton et al. [36], and (4) shoot and root dry weight.

2.11. Statistical analyses

Treatments were performed in triplicate from three independent experiments. Values shown represent mean ± standard deviation (SD). Results of IAA degradation were analyzed by the Kruskal–Wallis non-parametric test, while results obtained from soybean symbiosis assays were analyzed by ANOVA followed by a Tukey's *post hoc* analysis at $p < 0.05$. Analyses and graphs were performed using the PRISM V 4.0 statistical package for Windows (GraphPad Software, San Diego, CA USA).

3. Results

3.1. IAA degradation by Bradyrhizobium

We measured the evolution of IAA concentration (μg.ml^{-1}) along time in exponential cultures of *Bj*E109, *Bd*USDA110 and *Be*SEMIA5019 modified by the exogenous addition of the hormone.

The concentration of IAA in cultures of *Bj*E109 and *Bd*USDA110, modified by the addition of the hormone, decreased as a function of time (Fig. 1A). A 50% decrease of in the IAA concentration was observed 8 h after addition, whereas it was no longer detected in culture media after 24 h of incubation. Despite both *Bj*E109 and *Bd*USDA110 strains were able at 24 h after addition to degrade all the IAA, the degradation rate in *Bd*USDA110 (6.570 ± 0.2754 μg ml^{-1} h^{-1}) was faster than *Bj*E109 (5.655 ± 0.4524 μg ml^{-1} h^{-1}) between 4 and 8 h after addition. On the other hand, *Be*SEMIA5019 was unable to degrade IAA and the hormone concentration was maintained along the experiment and even found to be slightly increased after 24 h of incubation, presumably as a result of the ability of this bacterium to synthesize this molecule [15]. In the case of uninoculated YEM culture media, the IAA concentration (μg.ml^{-1}) did not change over time, probably because the culture medium did not affect the stability of the molecule.

To elucidate the nature of the molecule responsible for IAA degradation in *Bj*E109, we measured the IAA concentration (μg.ml^{-1}) in supernatants (sE109) and heat denatured supernatants (sE109φ). For that, around 40 μg ml^{-1} IAA was added to each treatment. A significant decrease in the IAA concentration (μg.ml^{-1}) was observed 4 h after addition of the hormone in sE109, while a complete absence of the molecule was found after 8 h of incubation (Fig. 1B). IAA concentration in sE109φ did not change over time. No IAA degradation was observed in disrupted cells (lysates cells) indicating the extracellular feature of the activity. Under our experimental conditions, results suggest the molecule responsible for IAA degradation should be released into the culture medium (extracellular feature) before IAA addition (constitutive feature). The loss of activity in the supernatants following heat denaturation suggested an enzyme candidate.

3.2. Evaluation of the kinetics for IAA degradation by Bj*E109*

We evaluated the IAA degradation kinetics of *Bj*E109 by the use of two different approaches. In the first one, we estimated the maximum concentration of IAA that could be degraded by these bacteria, using the whole exponential cultures of *Bj*E109. Results of these experiments are summarized in Fig. 2. In the second approach, we used the supernatants obtained from both early (EE) and late exponential (LE) growth phases of *Bj*E109 cultures and these results are summarized in Fig. 3.

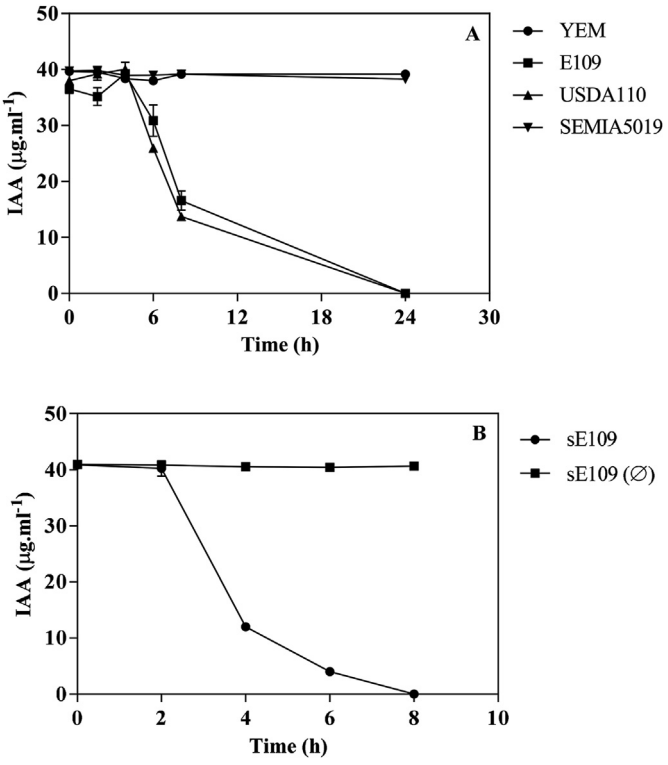

Fig. 1. Evolution of IAA concentration (μg.ml^{-1}) measured along time (h) in **(A)** YEM (Yeast Extract Mannitol) culture medium, *Bj*E109, *Bd*USDA110 and *Be*SEMIA5019 cultures modified by the exogenous addition of IAA to a final concentration of 40 μg ml^{-1}. **(B)** Supernatants and heat denatured supernatant of *Bj*E109 modified by the exogenous addition of IAA to a final concentration of 40 μg ml^{-1}. Values shown are mean ± SD (n = 3). Where not shown, the SD was smaller than the symbol.

Fig. 2 shows that *Bj*E109 cultures under exponential growth phase were able to fully degrade up to ≈80 μg ml^{-1} of exogenous IAA after 24 h of treatment. However, at higher concentrations of the hormone (i.e. around 120 and 160 μg ml^{-1}), there was a reduction in the degradation capacity: about 50% of the initial concentration was measured at the end of the experiment, clearly showing a saturation kinetics after 8 h of IAA treatment over 80 μg ml^{-1}. The viability of the *Bj*E109 was measured at 24, 36 and 48 h incubation by direct plate counting on YEM agar and no significant variations between treatments were recorded.

Fig. 3 shows that supernatants of *Bj*E109 cultures obtained from both early (EE) and late exponential (LE) growth phases had different kinetics for IAA degradation after 12 h of IAA treatment. The supernatants obtained from the late exponential (LE) growth phase of *Bj*E109 cultures degrade the IAA more rapidly than the ones obtained from the early exponential (EE) growth phase. This behavior is probably due to the fact that enzyme biosynthesis has kinetics similar to that of a primary metabolite produced during bacterial growth, in which the accumulation of the enzyme into the culture medium increases the degradation capacity of IAA per unit of time.

3.3. IAA degradation is encoded in the genome of B. japonicum*E109*

Based on the metabolic pathway described in *P. putida* 1290 by Leveau and Gerards [10] for the IAA degradation, we checked for the presence of similar sequences in the *Bj*E109 genome. The results of the *in silico* analyses are showed in Table S1. We found homologous sequences for all the proteins related to the *iac* cluster of *P. putida* 1290 in the *Bj*E109 genome; however, they are not located

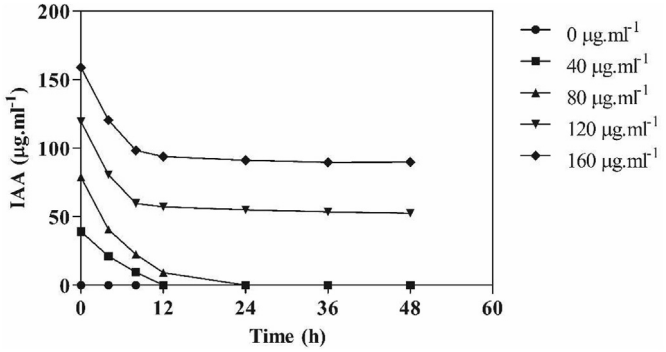

Fig. 2. IAA concentration (μg.ml^{-1}) measured along time (h) in *Bj*E109 cultures modified by the exogenous addition of IAA to a final concentration of 0, 20, 40, 80, 120 and 160 μg ml^{-1} of IAA (μg.ml^{-1}) per tube. Values shown are mean ± SD (n = 3). Where not shown, the SD was smaller than the symbol.

in a unique cluster of genes but distributed along the genome in a random way. The protein sequence with highest amino acid similarity is the one homologue to *iac*A, which codes for an acyl-CoA dehydrogenase in *Bj*E109. The protein encoded by the *iac*A gene has been previously reported in *Bd*USDA110 as NrgC, which is regulated by NifA during the *Bradyrhizobium*-soybean interaction [16]. We assessed the IAA degradation by *Bd*USDA110Δ*iac*A mutant [16] and fully discarded that *iac*A expression product would participate in the IAA degradation process (Fig. 4).

The wild type strain degraded 100% of the 40 μg ml^{-1} IAA added in the culture medium before 24 h of incubation. This behavior was similar to that of the mutant *Bd*USDA110Δ*iac*A. In our experimental conditions the gene *iac*A was not involved in IAA catabolism, thus this result allowed us to abandon the hypothesis about *iac*A as the main responsible for IAA degradation. In the case of the Δ*iac*C, the results showed that the mutant strain could not degrade the hormone and this fact indicates that the product of *iac*C is required for IAA degradation in *Bd*USDA110.

3.3.1. *The* iac*CDF cluster of* Bj*E109*

The *in silico* analysis of the *Bj*E109 genome shows that *iac*C codes for an oxidoreductase 3-phenylpropionate dioxygenase (α-subunit), according to the Blast-p tool of NCBI and RAST database (Fig. S2). The *iac*C is flanked by *iac*D, which codes for a 3-phenylpropionate dioxygenase (β-subunit) and *iac*F, which codes for a ferredoxin and grouped with two additional genes encoding for long chain fatty acid CoA ligase and an oxidoreductase, forming

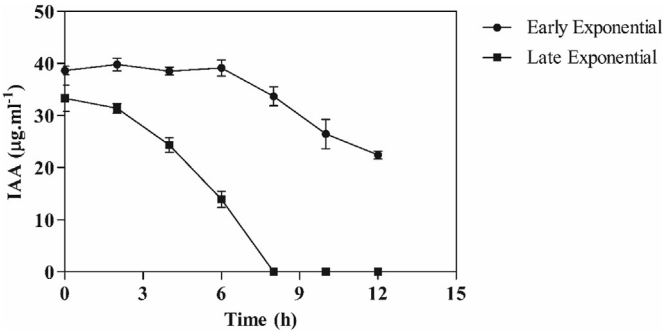

Fig. 3. IAA concentration (μg.ml^{-1}) measured along time (h) in supernatants of *Bj*E109 cultures obtained from early exponential (EE) or late exponential (LE) growth phase modified by the addition of an IAA solution to a final concentration of 40 μg ml^{-1}. Values shown are mean ± SD (n = 3). Where not shown, the SD was smaller than the symbol.

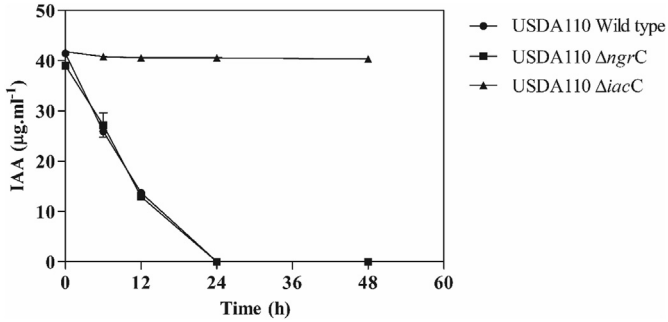

Fig. 4. IAA concentration (μg.ml^{-1}) measured along time (h) in *Bd*USDA110 wild type, *Bd*USDA110Δ*iac*A and *Bd*USDA110Δ*iac*C mutant modified by exogenous addition of IAA to a final concentration of 40 μg ml^{-1}. Values shown are mean ± SD (n = 3). Where not shown, the SD was smaller than the symbol.

a separate cluster in the genome of *Bj*E109 (Fig. S2). We performed a complementary analysis of the IacC sequence by SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) and established its tentative extracellular localization by the presence of a TAT [Tat/SP] signal peptide, responsible for the protein translocation. Finally, the *iac*C sequence of *Bj*E109 was compared with similar sequences obtained from other strains belonging to different species of the genus *Bradyrhizobium*. In the case of those strains belonging to the same species as *Bj*E109, the *iac*C gene identity was higher than 97%, while in other species the identity decreased by 95–91% (Table S2) with *Bradyrhizobium yuanmingense* and *B. diazoefficiens* as the closest to *B. japonicum*.

3.4. *IAA degradation by* B. japonicum *E109 and E109*Δiac*C mutant*

Taking into account the *in silico* analysis, we decided to consider the *iac*C of *Bj*E109 as the target gene for mutagenesis procedure. The main objective of this procedure was to obtain the *Bj*E109Δ*iac*C mutant and to evaluate the capacity of such strain to degrade IAA in comparison with the wild type one.

Fig. 5 shows that *Bj*E109Δ*iac*C mutant was unable to degrade IAA when the molecule was exogenously added into the culture medium, even for a long period of 48 h incubation. On the contrary, the strain *Bj*E109 degraded 100% of the ≈40 μg ml^{-1} IAA in the culture medium before 24 h of incubation. To confirm that the observed phenotype was due to the mutation in the *iac*C, we evaluated the complemented strain *Bj*E109Δ*iac*C/pJN105*iac*C. The capacity to degrade IAA was resumed: after IAA addition a 28% IAA degradation (10.96 μg ml^{-1}) in the first 24 h, a 72% after 48 h (28.4 μg ml^{-1}) and a 90% after 72 h (34.1 μg ml^{-1}) was measured

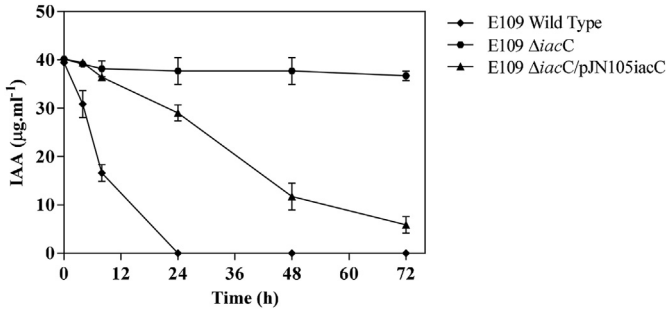

Fig. 5. IAA concentration (μg.ml^{-1}) measured along time (h) in *Bj*E109 wild type and *Bj*E109Δ*iac*C mutant modified by exogenous addition of IAA to a final concentration of 40 μg ml^{-1}. Values shown are mean ± SD (n = 3). Where not shown, the SD was smaller than the symbol.

(Fig. 5). On the other hand, the IAA concentration did not change along the experiment ($\approx$40 μg ml^{-1}) in the case of the *Bj*E109*Δiac*C mutant, in contrast to the *Bj*E109 strain, for which IAA degradation was 100% at 24 h after IAA-treatment. These results allowed us to confirm that *iac*C codes for the putative enzyme responsible for IAA degradation in this bacterium. Experiments of heterologous expression in *C. pinatubonensis* JMP134 showed that the product of *iac*D alone and the control with empty vector were not able to degrade $\approx$40 μg ml^{-1} IAA in comparison with the product of *iac*C and *iac*CD genes, which were able to degrade this molecule in less than 24 h incubation.

3.5. Evaluation of IAA biosynthesis in the BjE109ΔiacC

In a previous report using liquid chromatography–multiple reaction monitoring-mass spectrometry (LC-MRM-MS), we found a negligible amount of IAA (0.67 pmol ml^{-1}) in supernatants of *Bj*E109. However, the molecule did not accumulate into the culture medium at a significant concentration to be detected by HPLC, we thus concluded that this strain was unable to produce IAA [15]. In this study, no significant amount of IAA was detected by HPLC for both wild type and the mutant *Bj*E109*Δiac*C. The mutation in the *iac*C gene led to the bacterium losing the ability to degrade IAA; however, the mutant did not release a detectable amount of the hormone in the culture media. The results obtained in this paper (under defined experimental conditions), in addition to those previously published [15] and our particular experience modifying a broad range of experimental conditions (i.e. culture media, incubation time, temperature, etc.) to evaluate the bacterial capacity to biosynthesize IAA (data not shown), allow us to conclude that the *Bj*E109 has no significant activity in any of the proposed pathways for IAA biosynthesis and thus it cannot synthesize it in significant amounts.

3.6. Metabolites of IAA catabolism in BjE109

The metabolites deriving from the *iac*CD expression in *C. pinatubonensis* JMP134 in presence of IAA were analyzed by different spectroscopic and spectrometric techniques (^{1}H NMR, ^{13}C NMR, DEPT, MS) (Fig. S3, S4 and S5) and confirmed in *Bj*E109 cultures modified by the presence of the hormone. The ^{1}H NMR spectrum analyses indicated that the product of degradation of IAA contained two metabolites in equal proportions with a structural similarity. Chemically, both metabolites showed an intact phenyl ring and a signal concordant with CH group between 5 and 6 ppm. This result suggests that the pyrrole ring of the IAA molecule was saturated at positions C2–C3. On the other hand, the presence of signals corresponding to aliphatic hydrogen atoms between 2 and 3 ppm indicates that a methylene group adjacent to the carboxylic group presents both hydrogens with a different magnetic environment, suggesting that the pyrrole ring was modified in both metabolites in relation to the precursor IAA**.** The ^{13}C NMR spectrum analysis indicated that both metabolites have a carbonyl group with a similar magnetic environment, reinforcing the idea that both products have very similar identities. Also, the joint analysis of the ^{13}C NMR and DEPT spectra indicated the presence of one carbonyl group, three quaternary carbon atoms, five CH groups and one methylene group for each of the molecules. The number and type of carbon atoms finding in metabolites are equal to the presents in the precursor (IAA); however, the displacement of one of the CH groups and of one of the quaternary carbons accounts for an aliphatic rather than aromatic character for these two carbon atoms. The MS analysis indicated the presence of two compounds with a mass of 190.8 (A) and 224.9 (C) g mol^{-1}, respectively (Fig S3, S4, S5 and Fig. 6). The IAA molecule has an exact mass of 175.1 g mol^{-1}. Mass difference of 190.8 g mol^{-1} between IAA and the metabolite A is equal to the mass of an oxygen atom. This experimental data together with NMR evidences allow us to postulate that the

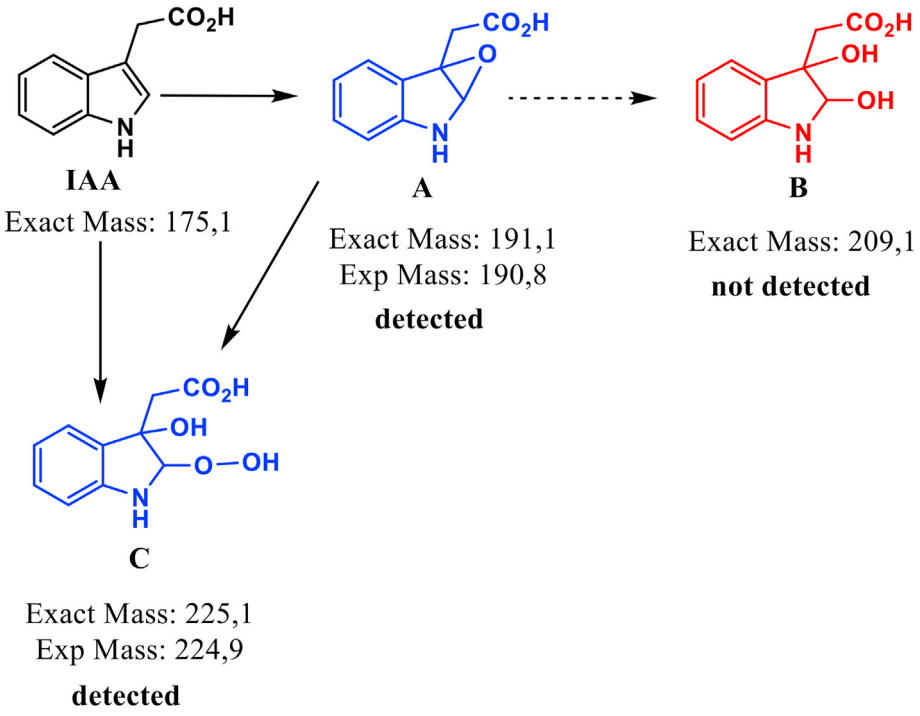

Fig. 6. Metabolic products identified after heterologous expression of the *iac*CD construct in *C. pinatubonensis* JMP134. The figure represents the putative degradation pathway in *Bj*E109 in relation of the identified metabolites (blue color) and the ones expected (red color) in our experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Effects of inoculation with *Bj*E109, *Bd*USDA110 and *iac*C mutants on soybean seedlings growth and nodulation. Different letters represent significant differences according to Tukey test $p < 0.05$. Numbers in **bold** represent the difference percentages between mutants and wild type strain.

	MRN	SRN	RN	SDW	RDW
Control	nd	nd	nd	0.21 ± 0.007^{d}	0.048 ± 0.009^{d}
E109	11.68 ± 0.29^{b}	9.14 ± 0.62^{a}	20.83 ± 0.61^{b}	0.33 ± 0.008^{a}	0.073 ± 0.004^{b}
E109 Δ*iac*C	8.68 ± 0.38^{c}	7.64 ± 0.83^{ab}	16.32 ± 0.91^{c}	0.27 ± 0.009^{b}	0.057 ± 0.011^{c}
	−25.6%	**−16.4%**	**−21.6%**	**−18.05%**	**−21.9%**
USDA110	22.66 ± 0.3^{a}	3.22 ± 0.71^{b}	25.88 ± 0.34^{a}	0.29 ± 0.004^{b}	0.080 ± 0.007^{a}
USDA110 Δ*iac*C	12.94 ± 0.47^{b}	2.09 ± 0.57^{b}	15.04 ± 0.52^{c}	0.23 ± 0.003^{c}	0.072 ± 0.015^{b}
	−42.8%	**−35.0%**	**−40.4%**	**−20.6%**	**−8.75%**

References: **MRN**: Main root nodules.plant^{-1}; **SRN**: Secondary root nodules.plant^{-1}; **RN**: Root nodules.plant^{-1}; **SDW**: Shoot dry weight.plant^{-1}; **RDW**: Root dry weight.plant^{-1} (36). nd: not determined.

molecule A is one of the metabolites isolated from IAA degradation by E109. However, the presence of the metabolite C (224.9 g mol^{-1}) in the degradation product was interesting, as from a chemical point of view the logical sequence would be the opening of the epoxide group in A by a molecule of water to obtain the molecule B (209.7 g mol^{-1}). Nevertheless, MS did not show any compound B with a 209 g.mol^{-}1. Summarizing, analyses by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry suggested that purified compounds produced by IAA degradation in *Bj*E109 are 3-indoleacetic acid 2,3-oxide or 2-(1a,2-dihydro-6bH-oxireno [2,3-b] indol-6b-yl) acetic acid, according to IUPAC (A) and 2-(2-hydroperoxy-3-hydroxyindolin-3-yl) acetic acid (C).

3.7. Bradyrhizobium-*soybean symbiosis in* Bj*E109,* Bd*USDA110 and their mutants*

Table 2 shows the growth and nodulation of soybean seedlings inoculated with *Bj*E109, *Bd*USDA110 and their *iac*C mutants.

Plant growth and nodulation increased in soybean plants obtained from inoculated seeds as compared to those uninoculated, in which no nodule was observed. In the case of inoculated seeds, nodulation as MRN (main root nodules), SRN (secondary root nodules) and RN (root nodules) increased in plants treated with the wild type strain in comparison with *iac*C mutants. The MRN, SRN and RN of *Bj*E109 inoculated plants increased by 25.6, 16.4 and 21.6% respectively, in comparison with *Bj*E109*Δiac*C. In the case of *Bd*USDA110, plants inoculated with this strain increased by 42.8; 35.0 and 40.4% respectively in comparison with the mutant. Shoot dry weight increased by 18.0% and 20.6% in plants inoculated with *Bj*E109 and *Bd*USDA110 respectively, in comparison with the ones inoculated with the *iac*C mutants. Root dry weight increased by 21.9% and 8.7% in plants inoculated with wild type strains in comparison with mutants.

4. Discussion

Minamisawa and Fukai [38] observed, for the first time, that some strains of *B. japonicum* were unable to accumulate IAA in the culture media. They suggested that such strains were unable to produce IAA or degraded the molecule after its biosynthesis. At the same time, Egebo et al. [8] confirmed that *Bd*USDA110 degraded IAA under an oxygen-dependent reaction and suggested that IAA is oxidized to aminobenzoyl-acetic acid and anthranilic acid by a putative tryptophan 2,3-dioxygenase. A different pathway for IAA degradation, starting with an oxidation of IAA to anthranilic acid, was proposed by Jensen et al. [9]. They reported the production of dioxindole-3-acetic acid, dioxindole, isatin, 2-aminophenyl glyoxylic acid (isatinic acid) and anthranilic acid as metabolites related to IAA catabolism in *Bd*USDA110. Olesen and Jochimsen [39] suggested that both an isatin reductase and isatin amidohydrolase were responsible of IAA degradation in *B. japonicum*. However, these metabolites or the related enzymes were not identified in this or later reports [40]. A transcriptional analysis of *Bd*USDA110 after exogenous treatment with 1 mM of IAA did not reveal a potential catabolic pathway of IAA or any enzymes involved [41]. Recently, we confirmed the non-assimilative degradation of both natural and synthetic auxins in *Bj*E109 cultures after 24 h exposition [15]. In the present work we showed two new features about the IAA degradation in *Bj*E109. The IAA degradation capacity is preexisting to the exogenous addition of the molecule in the culture medium and the degradation capacity occurs in the extracellular environment. The denaturation by heat of *Bj*E109 supernatants led to the complete loss of IAA degradation capacity, supporting the existence of a preexisting (constitutive) and extracellular enzyme as responsible.

Two different clusters for IAA catabolism have been characterized in eubacteria, the *iac* cluster identified in the alphaproteobacteria *P. putida* 1290 [10] and the *iaa* cluster identified in the betaproteobacteria *A. aromaticum* EbN1 [11]. In the case of *P. putida* 1290, which uses IAA as a sole source of carbon and energy, it contains a cluster of 10 genes [10]. The canonical cluster of *P. putida* 1290 is well conserved and named the *iac*ABCDEFGRHI gene cluster. The *iac*A encodes the acyl-CoA dehydrogenase, which would start the IAA degradation attacking the indole ring of IAA, thus generating 2-hydroxy-IAA. The *iac*B encodes a conserved hypothetic protein with unknown functions. The *iac*C and *iac*D genes encode the alpha and beta subunits of Rieske (2Fe−2S) domain containing aromatic ring hydroxylating dioxygenase, which is involved in 3-hydroxy-2-oxo-IAA conversion to catechol. The *iac*E encodes a short-chain dehydrogenase/reductase, which is involved in the conversion of 2-hydroxy-IAA to 3-hydroxy-2-oxo-IAA. The *iac*F encodes for ferredoxin and the *iac*G encodes a flavin reductase domain protein. This protein, in *Paraburkholderia phytofirmans* PsJN, might provide reduced flavins to the IacA protein [35]. The *iac*H and *iac*I genes, which encode for a putative Glu-tRNA amidotransferase and conserved hypothetical protein respectively, both have unknown functions. The *iac*R encodes for transcriptional regulator of the MarR family [42,43]. *P. putida* 1290 carries two copies each of the *iac*C, *iac*D and *iac*F genes [44]. In the case of *Bj*E109, the *iac*C gene forms a separate cluster together with *iac*D, *iac*F and two additional genes (Fig. S2). Based on this fact, we constructed the *Bj*E109Δ*iac*C mutant and observed the full loss of IAA degradation capacity We then confirm the role of 3-phenylpropionate dioxygenase (α-subunit) in the IAA degradation process.

Both, *P. putida* 1290 and *Caballeronia glathei* DSM 50014 utilize IAA as a sole source of carbon and energy and the final product of this pathway is catechol [10,45]. The first reaction is catalyzed by IacA, which converts IAA to 2-hydroxy-IAA (2-oxindole-3-acetic acid) [42] or 2,3-dihydroxy-indoline-3-acetic acid, according to Sadauskas et al. [45]. Then, IacE catalyzes the conversion of these metabolites to 3-hydroxy-2-oxindole-3-acetic acid (dioxindole-3-acetic acid). The conversion of dioxindole-3-acetic acid to the

final product, catechol, involves multiple reactions but mainly are carried out by IacC and IacD. Both *Bj*E109 cultures and heterologous expression of *iac*C or *iac*CD genes in *C. pinatubonensis* JMP134 during IAA degradation allowed us to identify 3-indoleacetic acid 2,3-oxide and 2-(2-hydroperoxy-3-hydroxyindolin-3-yl) acetic acid. These metabolites were not produced in any of the pathways previously described in both *Bd*USDA110 and *P. putida* 1290.

Bradyrhizobium sp. has been extensively studied because of its symbiotic association with legumes and its biological nitrogen fixation capacity. *Bj*E109 is broadly used to inoculate soybean seeds because it has the ability to increase the performance of the legumes under agronomic conditions [46]. In a previous report, we observed that addition of IAA to *Bj*E109 cultures increased the viable cell recovery from soybean seeds after inoculation, in comparison with the untreated control [15]. In similar experiments, Donati et al. [41] examined the effect of pretreatment with IAA on the ability of *Bd*USDA110 to nodulate soybean roots, but in this report, none of the pretreatments affected the number of nodules, nodule weight, or plant weight. In our experiments *Bj*E109 and *Bd*USDA110 were able to increase the number of nodules and dry weight of shoots and roots in soybean plants in comparison with the mutants *iac*C, unable to degrade the phytohormone. These parameters were remarkably higher in *Bd*USDA110 in comparison to plants inoculated with *Bj*E109. Summarizing, the inability of the *iac*C mutants to degrade the hormone determined a change in their symbiotic behavior: the establishment of the symbiosis at the level of the nodules number and their location in the roots, as well as the growth of the inoculated plant, were negatively affected. This behavior could be partially due to the change in the mutant's abilities related to: (a) the survival on soybean seeds after inoculation; (b) the colonization of roots after germination or (c) the formation of active nodules during symbiosis process. This issue should be addressed in a future work.

In this study, we have presented new molecular and physiological evidences related to the IAA degradation in *B. japonicum* and we have proposed a putative model/pathway for IAA degradation in this bacterium.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2021.103814.

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