# Journal Pre-proof

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

Daniela Torres, Elías Mongiardini, Florencia Donadío, Raúl Donoso, Gonzalo Recabarren-Gajardo, José Gualpa, Stijn Spaepen, Roberto Defez, Gastón Lopez, Carmen Bianco, Fabricio Cassán

1 **Molecular and physiological analysis of indole-3-acetic acid degradation in**

2 ***Bradyrhizobium japonicum* E109**

3

4 **Daniela Torres[a], Elías Mongiardini[b], Florencia Donadío[a], Raúl Donoso[c,d], Gonzalo**

5 **Recabarren-Gajardo[e,f], José Gualpa[a], Stijn Spaepen[g], Roberto Defez[h], Gastón**

6 **Lopez[a,1], Carmen Bianco [h,1], Fabricio Cassán[a,1]**

7

8 [a]Instituto de Investigaciones Agrobiotecnológicas (INIAB). Universidad Nacional de Río

9 Cuarto, Córdoba Argentina

10 [b]Laboratorio de Interacciones Rizobios y Soja. Instituto de Biotecnología y Biología

11 Molecular. Facultad de Ciencias Exactas. Universidad Nacional de La Plata, La Plata,

12 Argentina

13 [c]Programa Institucional de Fomento a la Investigación, Desarrollo e Innovación (PIDi),

14 Universidad Tecnológica Metropolitana, Santiago, Chile

15 [d]Center of Applied Ecology and Sustainability (CAPES), Santiago, Chile

16 [e]Bioactive Heterocycles Synthesis Laboratory, BHSL, Departamento de Farmacia,

17 Facultad de Química y de Farmacia, Pontificia Universidad Católica de Chile, Santiago,

18 Chile

19 [f]Centro Interdisciplinario de Neurociencias, Pontificia Universidad Católica de Chile,

20 Santiago, Chile

21 [g]Katholieke Universiteit Leuven. Leuven, Belgium.

22 [h]Institute of Biosciences and Bioresources, National Research Council of Italy, UOS

23 Naples, Italy

24

25 *Correspondence: fcassan@exa.unrc.edu.ar; Tel.: +54-9-358-4676-532

26 [1] Cassán Fabricio, Carmen Bianco and López Gastón have contributed equally to this work.

27  E-mail addresses: dtorres@exa.unrc.edu.ar (Daniela Torres), Elías Mongiardini

28  (mongiardini@biol.unlp.edu.ar), fdonadio@exa.unrc.edu.ar (Florencia Donadío),

29  radonoso@uai.cl (Raúl Donoso), grecabarren@uc.cl (Gonzalo Recabarren-Gajardo),

30  jgualpa@exa.unrc.edu.ar (Jose Gualpa), glopez@exa.unrc.edu.ar (Gastón Lopez),

31  roberto.defez@ibbr.cnr.it (Roberto Defez), carmen.bianco@ibbr.cnr.it (Carmen Bianco),

32  fcassan@exa.unrc.edu.ar (Fabricio Cassán).

33

34

35  **Abstract**

36  *Bradyrhizobium japonicum* E109 is a bacterium widely used for inoculants production in

37  Argentina. It is known for its ability to produce several phytohormones and degrade

38  indole-3-acetic acid (IAA). The genome sequence of *B. japonicum* E109 was recently

39  analyzed and it showed the presence of genes related to the synthesis of IAA by

40  indole-3-acetonitrile, indole-3-acetamide and tryptamine pathways. Nevertheless, *B.*

41  *japonicum* E109 is not able to produce IAA and instead has the ability to degrade this

42  hormone under saprophytic culture conditions. This work aimed to study the molecular

43  and physiological features of IAA degradation and identify the genes responsible of this

44  activity. In *B. japonicum* E109 we identified two sequences coding for a putative

45  3-phenylpropionate dioxygenase (subunits α and β) responsible for the IAA degradation

46  that were homologous to the canonical cluster of *iac*C and *iac*D of *P. putida* 1290. These

47  genes form a separate cluster together with three additional genes with unknown

48  functions. The degradation activity was found to be constitutively expressed in *B.*

49  *japonicum* E109. As products of IAA degradation, we identified two compounds,

50  3-indoleacetic acid 2,3-oxide and 2-(2-hydroperoxy-3-hydroxyindolin-3-yl) acetic acid.

51  Our report proposes, for the first time, a model for IAA degradation in *Bradyrhizobium.*

52

53  **Keywords:** *B. japonicum*; indole-3-acetic acid; IAA*;* 3-phenylpropionate dioxygenase*;*

54  3-indoleacetic acid 2*;*3-Oxide*;* 2-(2-hydroperoxy-3-hydroxyindolin-3-yl) acetic acid

55

56  **1. Introduction**

57       The auxins are a group of phytohormones characterized by their ability to induce

58  plant growth and to reproduce the physiological effects of the naturally occurring

59  indole-3-acetic acid (IAA). The IAA metabolism in higher plants, fungi and bacteria

60  comprises several mechanisms, such as the biosynthesis, degradation, catabolism (i.e.

61  oxidation and assimilation), conjugation and hydrolysis of auxin conjugates [1], which

62  globally regulate the IAA levels in these biological systems. *Bradyrhizobium* is an

63  alphaproteobacterium with the ability to produce IAA and this capacity has been

64  demonstrated *in vitro* and *in planta* after soybean inoculation [2-4]. Thimann [5]

65  proposed that auxins play an important role in the ontogeny of the root nodules during the

66  rhizobia-legume symbiosis, as many studies have indicated that a change in the

67  concentration of this phytohormone is a pre-requisite for nodule organogenesis [6].

68  Nielsen et al. [7] were the first to report that *B. diazoefficiens* USDA110 (formerly *B.*

69  *japonicum* USDA110) has the capacity to degrade indole-3-acetic acid (IAA) and Egebo

70  et al. [8] suggested that this reaction might be dependent on oxygen availability in the

71  culture medium. Jensen et al. [9] isolated metabolites of IAA degradation in *B.*

72  *diazoefficiens* USDA110 and the identified products suggested a new metabolic pathway

73  that included anthranilic acid. However, they could not confirm this pathway or identify

74  the gene(s) or enzyme(s) responsible for such capacity.

75       Two sets of canonical genes have been characterized for IAA catabolism in bacteria:

76  i) the *iac* (indole 3-acetic acid catabolism) gene cluster identified in *P. putida* 1290 [10]

77 and others bacteria, which codes for an aerobic pathway through which IAA is

78 transformed into catechol; the *iaa* (indole 3-acetic acid degradation) gene cluster

79 identified in *Aromatoleum aromaticum* EbN1 [11], which underlies the anaerobic

80 conversion of IAA into 2-aminobenzoyl-CoA. *P. putida* 1290 has the ability to degrade

81 IAA as the sole source of carbon and energy (catabolism). This process is carried out by

82 the *iac* locus, which consists of 10 genes coding for enzymes with a metabolic activity for

83 both indole and aromatics molecules, as well as proteins with regulatory functions [10].

84 At present, there is no information about the full set of processes involved in the IAA

85 catabolism in *B. japonicum*, its homeostatic control, or the effects on the symbiotic

86 interaction with soybean. *B. japonicum* E109 has been one of the most used strains for the

87 production of soybean inoculants in Argentina for 40 years [12]. The genome sequence of

88 *B. japonicum* E109 has revealed the presence of genes involved in several plant growth

89 promoting mechanisms and in particular in the biosynthesis of the phytohormone IAA

90 [13]. The genome analysis of E109 revealed the existence of three putative pathways for

91 IAA biosynthesis: indole-3-pyruvate (IPyA), indole-3-acetonitrile (IAN) and

92 indole-3-acetamide (IAM) [14]. However, the LC-MS-MS analysis did not show

93 significant amounts of the hormone in liquid culture media, suggesting that *B. japonicum*

94 E109 is not able to produce IAA, but on the contrary it has the ability to degrade IAA

95 under *in vitro* conditions [15]. In the same report, we also confirmed the ability of *B.*

96 *japonicum* E109 to degrade other natural and synthetic auxins, such as indole-3-butyric

97 acid (IBA) or α-naphthalene acetic acid (NAA). Exogenous IAA induced an increase in

98 the production of biomass and exopolysaccharide in liquid culture media of *B. japonicum*

99 E109, leading to a modification of its symbiotic behavior with soybean plants.

100    The aim of this work was to analyze the molecular and physiological features of IAA

101    degradation in *B. japonicum* E109 in order to identify the responsible genes and the

102    produced metabolites, and to outline a putative degradation pathway in this bacterium.

103

104    **2. Materials and Methods**

105    **2.1 Bacterial strains and culture conditions**

106    The strains and their characteristics are summarized in Table 1. *B. japonicum* E109

107    (*Bj*E109) was provided by the IMYZA-INTA, Buenos Aires (Argentina); *B. elkanii*

108    SEMIA5019 (*Be*SEMIA5019) was obtained from Empresa Brasileira de Pesquisa

109    Agropecuaria (EMBRAPA) in Brazil; *B. diazoefficiens* USDA110 wild type

110    (*Bd*USDA110) and *B. diazoefficiens* USDA110 deficient in *nrg*C gene named *B.*

111    *diazoefficiens* 8620 or USDA110 spc4 (*Bd*USDA110Δ*iac*A) were provided by Prof. Dr.

112    Hans-Martin Fischer of Institute of Microbiology in Zürich, Switzerland. Despite the

113    product of *ngr*C (NrgC, accession number AAG61032) has been previously associated

114    with nitrogen fixation [16], *in silico* analysis showed that *ngr*C sequence has homology

115    with that of *iac*A of *P. putida* 1290, related with IAA degradation [10] and *ido*A of *P.*

116    *alcaligenes* PA-10 related with indigo production [17]. Thus this strain was named as

117    *Bd*USDA110Δ*iac*A in Table 1 and along the manuscript. Bacteria were inoculated in

118    250 ml flasks containing 100 ml of Yeast Extract Mannitol (YEM) culture medium [21]

119    modified by the addition of 10 mg.l$^{-1}$ L-tryptophan (Trp) as the main precursor for IAA

120    biosynthesis [22] and antibiotics according to the strain (Table 1). Cultures were

121    incubated at 30°C with orbital shaking (180 rpm) until exponential growth phase was

122    reached. The purity of the cultures was checked routinely on YEM agar [21] and TSA

123    plating (Trypticase Soybean Agar) at 10% (v/v).

124

125    **Table 1**

126

127    **2.2 IAA degradation by *Bradyrhizobium***

128        We compared the IAA degradation activity in three strains belonging to different

129    species of *Bradyrhizobium* according to Torres et al. [15]. In particular, 100 µl aliquots

130    obtained from exponential growth phase cultures of *Bj*E109, *Be*SEMIA5019 and

131    *Bd*USDA110 were individually inoculated in 250 ml flasks containing 100 ml of YEM

132    culture medium [21] modified by the addition L-Trp as described before. Cultures were

133    incubated at 30°C with orbital shaking (180 rpm) until early exponential growth phase

134    ($OD_{595} \approx 0.6$), and were divided into 5 ml aliquots which were dispensed into 10 ml sterile

135    borosilicate tubes. After 15 min of incubation conditions at 30°C, 200 µl of 1 mg.ml$^{-1}$

136    IAA solution (or equivalent volume of sterilized water) were separately added into the

137    tubes containing the cultures of each strain to obtain a final concentration $\approx$ 40 µg.ml$^{-1}$

138    (38.5 µg.ml$^{-1}$) IAA per tube. This concentration was selected because it is similar to that

139    produced by several rhizobacteria, including the phytostimulatory model *Azospirillum*

140    *brasilense* [22]. To account for the non-biological degradation of the compound the same

141    volume of IAA solution was added to tubes containing 5 ml of non-inoculated YEM

142    medium. Tubes were incubated at 30°C with orbital shaking (180 rpm) and samples were

143    taken at T0 (5-10 min after IAA induction) and every 2 h to measure the IAA

144    concentration (µg.ml$^{-1}$).

145

146  **2.3 Quantification of IAA**

147  Quantification of IAA was performed by spectrophotometry [23] and confirmed by

148  HPLC according to Torres et al. [15]. Briefly, aliquots of 500 µl of bacterial culture were

149  centrifuged at 11.300 x g for 10 min. Subsequently, 250 µl of supernatant was filtered

150  (0.2 µm), mixed with 250 µl of Salkowski´s reagent (7.9 M $H_2SO_4$ and 12.5 gl$^{-1}$ $FeCl_3$)

151  and gently shaken in inverted position at least 10 times. Samples were incubated in the

152  dark for 30 min and the absorbance at 530 nm was measured. An aliquot of filtered

153  supernatants was also injected with a final volume of 20 µl in an HPLC Waters 600-MS

154  device (Waters Inc., USA) equipped with an U6K injector and C18 reverse phase column

155  (Purospher STAR RP C-18, 3 mm, Lichrocart 55-4) heated at 30 °C, coupled to a system

156  with UV-VIS Waters 486 detector (Waters Inc., USA) set at 265 nm. The elution was

157  performed with a mixture of ethanol: acetic acid: water (Et-OH/H-Ac/H20) (12: 1: 87) as

158  mobile phase at a flow rate of 1 ml.min$^{-1}$ at 30 °C. The retention time for IAA was

159  10.1-10.3 minutes and it was previously identified using an appropriate standard solution

160  of IAA (Sigma Aldrich, Germany). Quantification was performed by integration of the

161  peak area corresponding to the retention time (RT) using an integration software (Waters

162  Inc. USA). The IAA concentration was expressed in µg.ml$^{-1}$

163

164  **2.4 Analysis of constitutive or inducible character of IAA degradation**

165  We evaluated whether the degradation of IAA was triggered by its own addition

166  (inducible) or it depended on bacterial growth (constitutive). For that, *Bj*E109 was grown

167  until late exponential growth phase ($OD_{595}$=1.2), then a 50 ml aliquot was centrifuged at

168  8000 rpm for 15 min, and the cell-free supernatant was filtered (0.2 µm). The resultant

169  filtrate was divided into 5 ml aliquots and transferred into 10 ml sterile borosilicate tubes

170  for IAA induction. The treatments of the supernatants were the following: (1) addition of

171      IAA solution to obtain a final concentration $\approx$ 40 µg.ml$^{-1}$ IAA per tube (sE109); (2)

172      addition of IAA to heat denatured supernatants (15 min at 90 °C) to obtain a final

173      concentration $\approx$ 40 µg.ml$^{-1}$ IAA per tube (sE109$\phi$) and (3) addition of 200 µl of sterile

174      water to supernatants, and (4) addition of 200 µl of sterile water to denatured

175      supernatants. Tubes were incubated as previously described and 500 µl of the mixtures

176      were sampled every 2 h to measure IAA concentration (µg.ml$^{-1}$).

177

178      **2.5. Analysis of cellular or extracellular character of IAA degradation**

179      *Bj*E109 was grown as described before, but 10 ml aliquots obtained from exponential

180      growth phase cultures were centrifuged at 12,000 x g for 20 min (4 °C). The

181      supernatants were used as control treatment for IAA degradation assays (extracellular

182      feature) as described before. The pellets were washed twice and resuspended in 10 ml of

183      20 mM Tris–HCl buffer pH 8.0 containing 1 mM EDTA, 10 mM MgCl$_2$, 12.5 % (v/v)

184      glycerol, 0.1% (v/v) Triton, and the complete mini EDTA-free protease inhibitor

185      cocktail (Sigma-Aldrich, Germany). The cells were disrupted (lysed) by sonic treatment

186      (M.S.E. 150 W) for 210 s (in 30 s period), centrifuged and the cellular debris discarded.

187      The supernatants were used for IAA degradation assays (cellular feature).

188

189      **2.6 Evaluation of IAA degradation kinetics**

190      We assessed the kinetics of IAA degradation in both cell-free supernatants and

191      non-centrifuged cell cultures of *Bj*E109.

192      In the case of cell cultures, we evaluated the addition of different concentrations of

193      exogenous IAA to *Bj*E109 cultures during exponential growth phase in order to estimate

194     the maximum quantity of IAA that this metabolically active microorganism is able to

195     degrade. To achieve this, cell cultures were incubated as previously described until early

196     exponential growth phase ($OD_{595} \approx 0.6$) and divided into 5 ml aliquots, which were added

197     into 10 ml sterile borosilicate tubes. After 15 min of stabilization at 30°C with orbital

198     shaking (180 rpm), 200 µl of IAA solution or equivalent volume of sterilized water was

199     alternatively added into the tubes containing *Bj*E109 to obtain a final concentration ≈ 20,

200     40, 80, 120 and 160 µg.ml$^{-1}$ of IAA per tube. Control treatments using uninoculated YEM

201     medium modified by the addition of IAA solutions were also performed. Tubes were

202     incubated at 30°C with orbital shaking (180 rpm), and 500 µl of the mixture was sampled

203     after 15 min (T0), 8, 12, 24 and 32 h of incubation to measure IAA concentration

204     (µg.ml$^{-1}$). The viability of *Bj*E109 cells was evaluated after 32 h of IAA addition by direct

205     plate counting on YEM agar, according to Vincent [21].

206         In the case of the cell-free supernatants, *Bj*E109 cultures were incubated at 30°C with

207     orbital shaking (180 rpm) until early exponential (EE) ($OD_{595} \approx 0.6$) or late exponential

208     (LE) ($OD_{595} \approx 1.2$) growth phase. A 50 ml aliquot obtained from these cultures was

209     centrifuged at 8000 rpm for 15 min, filtered (0.2 µm) to obtain a cell free supernatant and

210     divided in 5 ml aliquot in borosilicate tubes for IAA-treatment as described before. The

211     treatments carried out were the following: (1) addition of pure IAA solution to EE

212     supernatant to obtain a final concentration ≈ 40 µg.ml$^{-1}$ IAA per tube; (2) addition of IAA

213     to LE supernatant to obtain a final concentration ≈ 40 µg.ml$^{-1}$ IAA per tube. Treatments

214     were performed in triplicate. Control treatments using uninoculated YEM medium

215     modified by the addition ≈ 40 µg.ml$^{-1}$ IAA solutions were also carried out. Tubes were

216     incubated as previously described, and 500 µl of the mixture was sampled every 2 h to

217     measure IAA concentration (µg.ml$^{-1}$).

218

219 **2.7 Analysis of IAA degradation pathway**

220 **2.7.1 Analysis *in silico***

221    Based on the canonical metabolic pathway described in *P. putida* 1290 by Leveau

222 and Gerards [10] for the IAA degradation, we analyzed the presence of similar proteins

223 by sequence comparison in the *Bj*E109 genome. For bioinformatics analysis, the

224 following tools (T) and databases were used: Blast (T) [24], KEGG [25], RAST [26],

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

226

227 **2.7.2 Construction of Δ*iac*C mutants**

228    The general cloning procedures were performed according to Sambrook et al. [32]

229 with minor modifications. The deletions in the *iac*C gene in both strains of

230 *Bradyrhizobium* were carried out through an unmarked in-frame deletion using the

231 pK18mobSacB plasmid carrying the counter-selection gene *sac*B, as described by

232 Mongiardini et al. [33]. Given the sequences similarity between the predicted *iac*C genes

233 (AJA64126.1 in E109 or blr3400 in USDA110), the PCR products were amplified with

234 the same set of primers, FW*iac*CE and RV*iac*CH (Table 1). The fragments were

235 gel-purified and cloned at the restriction sites *EcoR*I/*Hind*III into the pK18mobSacB [19]

236 giving the plasmids, pK18mobSacB::*iac*C*Bd* and pK18mobSacB::*iac*C*Bj* carrying the

237 *Bd*USDA110fragment and the *Bj*E109 fragment respectively. Each plasmid was *Sal*I

238 digested which released a 468 bp fragment from the insert cloned before as exemplify for

239 *Bj*E109 in the Fig. S1. Then, each plasmid was re-ligated giving the final construction,

240 pK18mobSacB::Δ*iac*C*Bd*    and    pK18mobSacB::Δ*iac*C*Bj*.    These    plasmids    were

241 transferred by bi-parental conjugations to the corresponding wild-type strain according to

242 Quelas et al. [34] and simple-crossover transconjugants (cointegrate) were selected by

243 Km resistance in YEM media agar and the double crossover recombination was induced

244  by plating the transconjugants in YEM agar plates supplemented with 10% (w/v) sucrose.

245  These sucrose resistant clones, that resolve the recombinant plasmid and that are Km

246  sensitive, were checked by PCR to dissociate between the mutant and the wild-type

247  genotype. The mutants obtained carry an in-frame deletion in each *iac*C gene and were

248  named *Bj*E109Δ*iac*C and *Bd*USDA110Δ*iac*C. All the plasmids and constructions are

249  listed in the Table 1.

250

251  **2.7.3 Restoration of the IAA degradation capacity in Δ*iac*C mutants**

252  The restored phenotype of the mutant *Bj*E109Δ*iac*C was obtained using competent

253  cells of *Escherichia coli* S-17 containing the pJN105 (pBBR) plasmid [20] with the *iac*C

254  cloned fragment inducible by L-arabinose. *E. coli* S-17 was grown in LB medium

255  modified by the addition of 30 µg.ml$^{-1}$ gentamicin (Gm), and *Bj*E109 was grown in PSY

256  medium modified by the addition of L-arabinose [47]. For conjugation, *E. coli* S-17

257  culture was diluted a hundred times in LB medium without antibiotics and grown at 30°C

258  with orbital shaking (180 rpm) until OD$_{595}$≈0.8-1. Subsequently, a mix with 900 µl of

259  E109 culture during exponential growth phase with 400 µl of S-17 culture was made. The

260  culture mix was centrifuged for 8 minutes at 3000 rpm and the pellet was re-suspended

261  and placed in a plate containing PSY agar medium with L-arabinose. Plates were

262  incubated at 30°C for 48 h. Colonies were re-suspended and homogenized into 1 ml of

263  saline solution. A 150 µl aliquot of conjugation homogenate was placed in YEM medium

264  with 50 µg.ml$^{-1}$ Gm and plates were incubated in the conditions previously mentioned for

265  72-96 h. The restored phenotype of *Bj*E109 named *Bj*E109Δ*iac*C/pJN105*iac*C was

266  evaluated in comparison with *Bj*E109 (wild type) and *Bj*E109Δ*iac*C as control

267  treatments.

268

**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 ($OD_{595} \approx 0.6$), and treated with IAA solution to obtain a final concentration $\approx$ 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 ($OD_{595} \approx 0.6$) 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

294    exposed to 5 mM L-arabinose [35]. Exponential growth cultures of *C. pinatubonensis*

295    JMP134 of 100 ml final volume were exposed to a final concentration of 40-160 $\mu$g.ml$^{-1}$

296    IAA during 24 h and then analyzed for metabolites identification. The organic fraction of

297    the culture medium was extracted by triplicate using an equal volume of ethyl acetate.

298    Then, the organic layer was dried over anhydrous $Na_2SO_4$ and filtered, and the solvent

299    was removed in vacuum. The resultant mixture of products was subjected to a

300    spectroscopic analysis. The NMR (nuclear magnetic resonance) spectra were recorded on

301    a Bruker Advance III HD 400 at 400 MHz for $^1$H and 100 MHz for $^{13}$C. NMR spectra

302    were recorded in DMSO-*d6* (dimethyl sulfoxide), using the solvent signal as reference.

303    The chemical shifts are expressed in ppm ($\delta$ scale) downfield from tetramethylsilane

304    (TMS) and coupling constant values (*J*) are given in Hertz. The mass spectra were

305    determined in TQ 4500 triple-quadrupole mass spectrometer coupled with electrospray

306    ionization (ESI) source and were operated in the negative ion mode. The IAA degradation

307    metabolites identified by expression of *iac* genes in *C. pinatubonensis* JMP134 were

308    evaluated by spectroscopic and spectrometric analysis in late exponential cultures of

309    *Bj*E109 obtained from YEM culture media exposed during 24 h to $\approx$ 40 $\mu$g.ml$^{-1}$ IAA. The

310    analytical procedure was the same previously described.

311

312    **2.10 *Bradyrhizobium*-soybean symbiosis in *Bj*E109, *Bd*USDA110 and their mutants**

313        Cultures of *Bj*E109, *Bd*USDA110 and *iac*C mutants, grown as previously described

314    in section 2.1, were used to inoculate soybean seeds cv. Don Mario 4800 with a 3 ml.kg$^{-1}$

315    dose (Note: this dose is normally used under field conditions by most of the agricultural

316    companies). The nodulation pattern was individually evaluated on both inoculated and

317    non-inoculated (control) seeds according to Burton et al. [36] with some modifications.

318 A triplicate of 9 soybean seeds (n=27) were sown in three separate plastic pots (300 ml

319 volume capacity) containing vermiculite as a solid substrate, irrigated with

320 nitrogen-deficient sterile N-free Hoagland's solution (25% v/v) [37]. The seedlings were

321 maintained for 21 days in a growth chamber at 30/20 °C and 80% relative humidity with

322 a 16/8 h day/night photoperiod. At the end of the experiment, the following parameters

323 were measured: (1) number of nodules on the main root per plant, (2) number of nodules

324 on the secondary roots per plant, (3) number of nodules per plant, following Burton et al.

325 [36], and (4) shoot and root dry weight.

326

327 **2.11 Statistical analyses**

328　　Treatments were performed in triplicate from three independent experiments. Values

329 shown represent mean ± standard deviation (SD). Results of IAA degradation were

330 analyzed by the Kruskal-Wallis non-parametric test, while results obtained from soybean

331 symbiosis assays were analyzed by ANOVA followed by a Tukey's *post hoc* analysis at

332 $p < 0.05$. Analyses and graphs were performed using the PRISM V 4.0 statistical package

333 for Windows (GraphPad Software, San Diego, CA USA).

334

335 **3. Results**

336 **3.1 IAA degradation by *Bradyrhizobium***

337　　We measured the evolution of IAA concentration ($\mu g.ml^{-1}$) along time in

338 exponential cultures of *Bj*E109, *Bd*USDA110 and *Be*SEMIA5019 modified by the

339 exogenous addition of the hormone.

340

341 **Fig. 1**

342

343    The concentration of IAA in cultures of *Bj*E109 and *Bd*USDA110, modified by the

344 addition of the hormone, decreased as a function of time (Fig. 1A). A 50% decrease of

345 in the IAA concentration was observed 8 h after addition, whereas it was no longer

346 detected in culture media after 24 h of incubation. Despite both *Bj*E109 and

347 *Bd*USDA110 strains were able at 24 h after addition to degrade all the IAA, the

348 degradation rate in *Bd*USDA110 ($6.570 \pm 0.2754$ µg.ml$^{-1}$.h$^{-1}$) was faster than *Bj*E109

349 ($5.655 \pm 0.4524$ µg.ml$^{-1}$.h$^{-1}$) between 4 and 8 h after addition. On the other hand,

350 *Be*SEMIA5019 was unable to degrade IAA and the hormone concentration was

351 maintained along the experiment and even found to be slightly increased after 24 h of

352 incubation, presumably as a result of the ability of this bacterium to synthesize this

353 molecule [15]. In the case of uninoculated YEM culture media, the IAA concentration

354 (µg.ml$^{-1}$) did not change over time, probably because the culture medium did not affect

355 the stability of the molecule.

356    To elucidate the nature of the molecule responsible for IAA degradation in *Bj*E109,

357 we measured the IAA concentration (µg.ml$^{-1}$) in supernatants (sE109) and heat

358 denatured supernatants (sE109ϕ). For that, around 40 µg.ml$^{-1}$ IAA was added to each

359 treatment. A significant decrease in the IAA concentration (µg.ml$^{-1}$) was observed 4 h

360 after addition of the hormone in sE109, while a complete absence of the molecule was

361 found after 8 h of incubation (Fig.1B). IAA concentration in sE109φ did not change

362 over time. No IAA degradation was observed in disrupted cells (lysates cells) indicating

363 the extracellular feature of the activity. Under our experimental conditions, results

364 suggest the molecule responsible for IAA degradation should be released into the

365 culture medium (extracellular feature) before IAA addition (constitutive feature). The

366 loss of activity in the supernatants following heat denaturation suggested an enzyme

367 candidate.

368

369 **3.2 Evaluation of the kinetics for IAA degradation by *Bj*E109**

370      We evaluated the IAA degradation kinetics of *Bj*E109 by the use of two different

371 approaches. In the first one, we estimated the maximum concentration of IAA that could

372 be degraded by these bacteria, using the whole exponential cultures of *Bj*E109. Results

373 of these experiments are summarized in Fig. 2. In the second approach, we used the

374 supernatants obtained from both early (EE) and late exponential (LE) growth phases of

375 *Bj*E109 cultures and these results are summarized in Fig.3.

376

377 **Fig.2**

378

379      Fig.2 shows that *Bj*E109 cultures under exponential growth phase were able to fully

380 degrade up to $\approx 80$ μg.ml$^{-1}$ of exogenous IAA after 24 h of treatment. However, at

381 higher concentrations of the hormone (i.e. around 120 and 160 μg.ml$^{-1}$), there was a

382    reduction in the degradation capacity: about 50% of the initial concentration was

383    measured at the end of the experiment, clearly showing a saturation kinetics after 8 h of

384    IAA treatment over 80 μg.ml$^{-1}$. The viability of the *Bj*E109 was measured at 24, 36 and

385    48 h incubation by direct plate counting on YEM agar and no significant variations

386    between treatments were recorded.

387

388    **Fig.3**

389

390        Fig.3 shows that supernatants of *Bj*E109 cultures obtained from both early (EE) and

391    late exponential (LE) growth phases had different kinetics for IAA degradation after 12

392    h of IAA treatment. The supernatants obtained from the late exponential (LE) growth

393    phase of *Bj*E109 cultures degrade the IAA more rapidly than the ones obtained from the

394    early exponential (EE) growth phase. This behavior is probably due to the fact that

395    enzyme biosynthesis has kinetics similar to that of a primary metabolite produced

396    during bacterial growth, in which the accumulation of the enzyme into the culture

397    medium increases the degradation capacity of IAA per unit of time.

398

399    **3.3. IAA degradation is encoded in the genome of *B. japonicum*E109**

400        Based on the metabolic pathway described in *P. putida* 1290 by Leveau and

401    Gerards [10] for the IAA degradation, we checked for the presence of similar sequences

402    in the *Bj*E109 genome. The results of the *in silico* analyses are showed in Table S1. We

403    found homologous sequences for all the proteins related to the *iac* cluster of *P. putida*

404    1290 in the *Bj*E109 genome; however, they are not located in a unique cluster of genes

405    but distributed along the genome in a random way. The protein sequence with highest

406    amino acid similarity is the one homologue to *iac*A, which codes for an acyl-CoA

407    dehydrogenase in *Bj*E109. The protein encoded by the *iac*A gene has been previously

408    reported in *Bd*USDA110 as NrgC, which is regulated by NifA during the

409    *Bradyrhizobium*-soybean interaction [16]. We assessed the IAA degradation by

410    *Bd*USDA110Δ*iac*A mutant [16] and fully discarded that *iac*A expression product would

411    participate in the IAA degradation process (Fig. 4).

412

413    **Fig. 4.**

414

415        The wild type strain degraded 100% of the 40 μg.ml$^{-1}$ IAA added in the culture

416    medium before 24 h of incubation. This behavior was similar to that of the mutant

417    *Bd*USDA110Δ*iac*A. In our experimental conditions the gene *iac*A was not involved in

418    IAA catabolism, thus this result allowed us to abandon the hypothesis about *iac*A as the

419    main responsible for IAA degradation. In the case of the Δ*iac*C, the results showed that

420    the mutant strain could not degrade the hormone and this fact indicates that the product

421    of *iac*C is required for IAA degradation in *Bd*USDA110.

422

423    **3.3.1 The *iac*CDF cluster of *Bj*E109**

424    The *in silico* analysis of the *Bj*E109 genome shows that *iac*C codes for an

425    oxidoreductase 3-phenylpropionate dioxygenase (α-subunit), according to the Blast-p

426    tool of NCBI and RAST database (Fig. S2). The *iac*C is flanked by *iac*D, which codes

427    for a 3-phenylpropionate dioxygenase (β-subunit) and *iac*F, which codes for a

428    ferredoxin and grouped with two additional genes encoding for long chain fatty acid

429    CoA ligase and an oxidoreductase, forming a separate cluster in the genome of *Bj*E109

430    (Fig. S2). We performed a complementary analysis of the IacC sequence by SignalP-5.0

431    (http://www.cbs.dtu.dk/services/SignalP/) and established its tentative extracellular

432    localization by the presence of a TAT [Tat/SP] signal peptide, responsible for the

433    protein translocation. Finally, the *iac*C sequence of *Bj*E109 was compared with similar

434    sequences obtained from other strains belonging to different species of the genus

435    *Bradyrhizobium*. In the case of those strains belonging to the same species as *Bj*E109, the

436    *iac*C gene identity was higher than 97%, while in other species the identity decreased by

437    95-91% (Table S2) with *B. yuanmingense* and *B. diazoefficiens* as the closest to *B.*

438    *japonicum*.

439

440    **3.4 IAA degradation by *B. japonicum* E109 and E109Δ*iac*C mutant**

441    Taking into account the *in silico* analysis, we decided to consider the *iac*C of

442    *Bj*E109 as the target gene for mutagenesis procedure. The main objective of this

443    procedure was to obtain the *Bj*E109Δ*iac*C mutant and to evaluate the capacity of such

444    strain to degrade IAA in comparison with the wild type one.

445

446     **Fig.5**.

447

448          Fig.5 shows that *Bj*E109Δ*iac*C mutant was unable to degrade IAA when the

449     molecule was exogenously added into the culture medium, even for a long period of 48

450     h incubation. On the contrary, the strain *Bj*E109 degraded 100% of the ≈ 40 µg.ml$^{-1}$

451     IAA in the culture medium before 24 h of incubation. To confirm that the observed

452     phenotype was due to the mutation in the *iac*C, we evaluated the complemented strain

453     *Bj*E109Δ*iac*C/pJN105*iac*C. The capacity to degrade IAA was resumed: after IAA

454     addition a 28% IAA degradation (10.96 µg.ml$^{-1}$) in the first 24 h, a 72% after 48 h (28.4

455     µg.ml$^{-1}$) and a 90% after 72 h (34.1 µg.ml$^{-1}$) was measured (Fig. 5). On the other hand,

456     the IAA concentration did not change along the experiment (≈ 40 µg.ml$^{-1}$) in the case of

457     the *Bj*E109Δ*iac*C mutant, in contrast to the *Bj*E109 strain, for which IAA degradation

458     was 100% at 24 h after IAA-treatment. These results allowed us to confirm that *iac*C

459     codes for the putative enzyme responsible for IAA degradation in this bacterium.

460     Experiments of heterologous expression in *C. pinatubonensis* JMP134 showed that the

461     product of *iac*D alone and the control with empty vector were not able to degrade ≈ 40

462     µg.ml$^{-1}$ IAA in comparison with the product of *iac*C and *iac*CD genes, which were able

463     to degrade this molecule in less than 24 h incubation.

464

465     **3.5 Evaluation of IAA biosynthesis in the *Bj*E109Δ*iac*C**

466    In a previous report using liquid chromatography-multiple reaction

467    monitoring-mass spectrometry (LC-MRM-MS), we found a negligible amount of IAA

468    (0.67 pmol.ml$^{-1}$) in supernatants of *Bj*E109. However, the molecule did not accumulate

469    into the culture medium at a significant concentration to be detected by HPLC, we thus

470    concluded that this strain was unable to produce IAA [15]. In this study, no significant

471    amount of IAA was detected by HPLC for both wild type and the mutant *Bj*E109Δ*iac*C.

472    The mutation in the *iac*C gene led to the bacterium losing the ability to degrade IAA;

473    however, the mutant did not release a detectable amount of the hormone in the culture

474    media. The results obtained in this paper (under defined experimental conditions), in

475    addition to those previously published [15] and our particular experience modifying a

476    broad range of experimental conditions (i.e. culture media, incubation time,

477    temperature, etc.) to evaluate the bacterial capacity to biosynthesize IAA (data not

478    shown), allow us to conclude that the *Bj*E109 has no significant activity in any of the

479    proposed pathways for IAA biosynthesis and thus it cannot synthesize it in significant

480    amounts.

481

482    **3.6 Metabolites of IAA catabolism in *Bj*E109**

483    The metabolites deriving from the *iac*CD expression in *C. pinatubonensis* JMP134

484    in presence of IAA were analyzed by different spectroscopic and spectrometric

485    techniques ($^1$H-NMR, $^{13}$C-NMR, DEPT, MS) (Fig. S3, S4 and S5) and confirmed in

486    *Bj*E109 cultures modified by the presence of the hormone. The $^1$H-NMR spectrum

487     analyses indicated that the product of degradation of IAA contained two metabolites in

488     equal proportions with a structural similarity. Chemically, both metabolites showed an

489     intact phenyl ring and a signal concordant with CH group between 5-6 ppm. This result

490     suggests that the pyrrole ring of the IAA molecule was saturated at positions C2-C3. On

491     the other hand, the presence of signals corresponding to aliphatic hydrogen atoms

492     between 2-3 ppm indicates that a methylene group adjacent to the carboxylic group

493     presents both hydrogens with a different magnetic environment, suggesting that the

494     pyrrole ring was modified in both metabolites in relation to the precursor IAA**.** The

495     $^{13}$C-NMR spectrum analysis indicated that both metabolites have a carbonyl group with

496     a similar magnetic environment, reinforcing the idea that both products have very

497     similar identities. Also, the joint analysis of the $^{13}$C-NMR and DEPT spectra indicated

498     the presence of one carbonyl group, three quaternary carbon atoms, five CH groups and

499     one methylene group for each of the molecules. The number and type of carbon atoms

500     finding in metabolites are equal to the presents in the precursor (IAA); however, the

501     displacement of one of the CH groups and of one of the quaternary carbons accounts for

502     an aliphatic rather than aromatic character for these two carbon atoms. The MS analysis

503     indicated the presence of two compounds with a mass of 190.8 (A) and 224.9 (C)

504     g.mol$^{-1}$, respectively (Fig S3, S4, S5 and Fig. 6). The IAA molecule has an exact mass

505     of 175.1 g.mol$^{-1}$. Mass difference of 190.8 g.mol$^{-1}$ between IAA and the metabolite A is

506     equal to the mass of an oxygen atom. This experimental data together with NMR

507     evidences allow us to postulate that the molecule A is one of the metabolites isolated

508  from IAA degradation by E109. However, the presence of the metabolite C (224.9

509  g.mol$^{-1}$) in the degradation product was interesting, as from a chemical point of view the

510  logical sequence would be the opening of the epoxide group in A by a molecule of

511  water to obtain the molecule B (209.7 g.mol$^{-1}$). Nevertheless, MS did not show any

512  compound B with a 209 g.mol$^{-1}$. Summarizing, analyses by $^1$H and $^{13}$C NMR

513  spectroscopy and mass spectrometry suggested that purified compounds produced by

514  IAA    degradation    in    *Bj*E109    are    3-indoleacetic    acid    2,3-oxide    or

515  2-(1a,2-dihydro-6bH-oxireno [2,3-b] indol-6b-yl) acetic acid, according to IUPAC (A)

516  and 2-(2-hydroperoxy-3-hydroxyindolin-3-yl) acetic acid (C).

517

518  **Fig.6**.

519

520  **3.7 *Bradyrhizobium*-soybean symbiosis in *Bj*E109, *Bd*USDA110 and their mutants**

521     Table 2 shows the growth and nodulation of soybean seedlings inoculated with

522  *Bj*E109, *Bd*USDA110 and their *iac*C mutants.

523

524  **Table 2**

525

526     Plant growth and nodulation increased in soybean plants obtained from inoculated

527  seeds as compared to those uninoculated, in which no nodule was observed. In the case

528  of inoculated seeds, nodulation as MRN (main root nodules), SRN (secondary root

529    nodules) and RN (root nodules) increased in plants treated with the wild type strain in

530    comparison with *iac*C mutants. The MRN, SRN and RN of *Bj*E109 inoculated plants

531    increased by 25.6, 16.4 and 21.6% respectively, in comparison with *Bj*E109Δ*iac*C. In

532    the case of *Bd*USDA110, plants inoculated with this strain increased by 42.8; 35.0 and

533    40.4% respectively in comparison with the mutant. Shoot dry weight increased by

534    18.0% and 20.6% in plants inoculated with *Bj*E109 and *Bd*USDA110 respectively, in

535    comparison with the ones inoculated with the *iac*C mutants. Root dry weight increased

536    by 21.9% and 8.7% in plants inoculated with wild type strains in comparison with

537    mutants.

538

539    **4. Discussion**

540        Minamisawa and Fukai [38] observed, for the first time, that some strains of *B.*

541    *japonicum* were unable to accumulate IAA in the culture media. They suggested that

542    such strains were unable to produce IAA or degraded the molecule after its biosynthesis.

543    At the same time, Egebo et al. [8] confirmed that *Bd*USDA110 degraded IAA under an

544    oxygen-dependent reaction and suggested that IAA is oxidized to aminobenzoyl-acetic

545    acid and anthranilic acid by a putative tryptophan 2,3-dioxygenase. A different pathway

546    for IAA degradation, starting with an oxidation of IAA to anthranilic acid, was proposed

547    by Jensen et al. [9]. They reported the production of dioxindole-3-acetic acid,

548    dioxindole, isatin, 2-aminophenyl glyoxylic acid (isatinic acid) and anthranilic acid as

549    metabolites related to IAA catabolism in *Bd*USDA110. Olesen and Jochimsen [39]

550   suggested that both an isatin reductase and isatin amidohydrolase were responsible of

551   IAA degradation in *B. japonicum*. However, these metabolites or the related enzymes

552   were not identified in this or later reports [40]. A transcriptional analysis of

553   *Bd*USDA110 after exogenous treatment with 1 mM of IAA did not reveal a potential

554   catabolic pathway of IAA or any enzymes involved [41]. Recently, we confirmed the

555   non-assimilative degradation of both natural and synthetic auxins in *Bj*E109 cultures

556   after 24 h exposition [15]. In the present work we showed two new features about the

557   IAA degradation in *Bj*E109. The IAA degradation capacity is preexisting to the

558   exogenous addition of the molecule in the culture medium and the degradation capacity

559   occurs in the extracellular environment. The denaturation by heat of *Bj*E109

560   supernatants led to the complete loss of IAA degradation capacity, supporting the

561   existence of a preexisting (constitutive) and extracellular enzyme as responsible.

562       Two different clusters for IAA catabolism have been characterized in eubacteria,

563   the *iac* cluster identified in the alphaproteobacteria *P. putida* 1290 [10] and the *iaa*

564   cluster identified in the betaproteobacteria *A. aromaticum* EbN1 [11]. In the case of *P.*

565   *putida* 1290, which uses IAA as a sole source of carbon and energy, it contains a cluster

566   of 10 genes [10]. The canonical cluster of *P. putida* 1290 is well conserved and named

567   the *iac*ABCDEFGRHI gene cluster. The *iac*A encodes the acyl-CoA dehydrogenase,

568   which would start the IAA degradation attacking the indole ring of IAA, thus generating

569   2-hydroxy-IAA. The *iac*B encodes a conserved hypothetic protein with unknown

570   functions. The *iac*C and *iac*D genes encode the alpha and beta subunits of Rieske (2Fe–

571    2S) domain containing aromatic ring hydroxylating dioxygenase, which is involved in

572    3-hydroxy-2-oxo-IAA conversion to catechol. The *iac*E encodes a short-chain

573    dehydrogenase/reductase, which is involved in the conversion of 2-hydroxy-IAA to

574    3-hydroxy-2-oxo-IAA. The *iac*F encodes for ferredoxin and the *iac*G encodes a flavin

575    reductase domain protein. This protein, in *P. phytofirmans* PsJN, might provide reduced

576    flavins to the IacA protein [35]. The *iac*H and *iac*I genes, which encode for a putative

577    Glu-tRNA amidotransferase and conserved hypothetical protein respectively, both have

578    unknown functions. The *iac*R encodes for transcriptional regulator of the MarR family

579    [42-43]. *P. putida* 1290 carries two copies each of the *iac*C, *iac*D and *iac*F genes [44]. In

580    the case of *Bj*E109, the *iac*C gene forms a separate cluster together with *iac*D, *iac*F and

581    two additional genes (Fig. S2). Based on this fact, we constructed the *Bj*E109Δ*iac*C

582    mutant and observed the full loss of IAA degradation capacity We then confirm the role

583    of 3-phenylpropionate dioxygenase (α-subunit) in the IAA degradation process.

584        Both, *P. putida* 1290 and *Caballeronia glathei* DSM 50014 utilize IAA as a sole

585    source of carbon and energy and the final product of this pathway is catechol [10;45].

586    The first reaction is catalyzed by IacA, which converts IAA to 2-hydroxy-IAA

587    (2-oxindole-3-acetic acid) [42] or 2,3-dihydroxy-indoline-3-acetic acid, according to

588    Sadauskas et al. [45]. Then, IacE catalyzes the conversion of these metabolites to

589    3-hydroxy-2-oxindole-3-acetic acid (dioxindole-3-acetic acid). The conversion of

590    dioxindole-3-acetic acid to the final product, catechol, involves multiple reactions but

591    mainly are carried out by IacC and IacD. Both *Bj*E109 cultures and heterologous

592  expression of *iac*C or *iac*CD genes in *C. pinatubonensis* JMP134 during IAA

593  degradation allowed us to identify 3-indoleacetic acid 2,3-oxide and

594  2-(2-hydroperoxy-3-hydroxyindolin-3-yl) acetic acid. These metabolites were not

595  produced in any of the pathways previously described in both *Bd*USDA110 and *P.*

596  *putida* 1290.

597      *Bradyrhizobium* sp. has been extensively studied because of its symbiotic

598  association with legumes and its biological nitrogen fixation capacity. *Bj*E109 is broadly

599  used to inoculate soybean seeds because it has the ability to increase the performance of

600  the legumes under agronomic conditions [46]. In a previous report, we observed that

601  addition of IAA to *Bj*E109 cultures increased the viable cell recovery from soybean

602  seeds after inoculation, in comparison with the untreated control [15]. In similar

603  experiments, Donati et al. [41] examined the effect of pretreatment with IAA on the

604  ability of *Bd*USDA110 to nodulate soybean roots, but in this report, none of the

605  pretreatments affected the number of nodules, nodule weight, or plant weight. In our

606  experiments *Bj*E109 and *Bd*USDA110 were able to increase the number of nodules and

607  dry weight of shoots and roots in soybean plants in comparison with the mutants *iac*C,

608  unable to degrade the phytohormone. These parameters were remarkably higher in

609  *Bd*USDA110 in comparison to plants inoculated with *Bj*E109. Summarizing, the

610  inability of the *iac*C mutants to degrade the hormone determined a change in their

611  symbiotic behavior: the establishment of the symbiosis at the level of the nodules

612  number and their location in the roots, as well as the growth of the inoculated plant, were

613 negatively affected. This behavior could be partially due to the change in the mutant's

614 abilities related to: (a) the survival on soybean seeds after inoculation; (b) the

615 colonization of roots after germination or (c) the formation of active nodules during

616 symbiosis process. This issue should be addressed in a future work.

617     In this study, we have presented new molecular and physiological evidences related

618 to the IAA degradation in *B. japonicum* and we have proposed a putative model/pathway

619 for IAA degradation in this bacterium.

620

621 **Conflicts of Interest**

622 The authors declare no conflict of interest

623

624 **Acknowledgments**

635

**References**

1. Glick BR, Patten C, Holguin G, Penrose D. Auxin production. *In* Biochemical and genetic mechanisms used by plant growth promoting bacteria, Imperial College Press, London, England, 1999, p 86-133.

2. Sekine M, Ichikawa T, Kuga N, Kobayashi M, Sakurai A, Syōno K. Detection of the IAA biosynthetic pathway from tryptophan via indole-3-acetamide in *Bradyrhizobium* spp. Plant Cell Physiol 1988; 29(5):867-874. https://doi.org/10.1093/oxfordjournals.pcp.a077574

3. Sekine M, Watanabe K, Syōno K. Molecular cloning of a gene for indole-3-acetamide hydrolase from *Bradyrhizobium japonicum*. J Bacteriol 1989; 171(3):1718-1724. https://doi.org/10.1128/jb.171.3.1718-1724.1989

4. Kaneshiro T, Kwolek WF. Stimulated nodulation of soybeans by *Rhizobium japonicum* mutant (B-14075) that catabolizes the conversion of tryptophan to indol-3yl-acetic acid. Plant Sci 1985; 42(3):141-146. https://doi.org/10.1016/0168-9452(85)90119-0

5. Thimann KV. On the physiology of the formation of nodules on legume roots. Proc Natl Acad Sci USA 1936; 22(8):511-514.https://doi.org/10.1073/pnas.22.8.511

6. Taller B, Sturtevant D. Modification of cytokinin production in *Bradyrhizobium* cultures. J Cell Biochem Suppl 1989; 12(Part C) 274.

655   7. Nielsen SV, Egebo LA, Jochimsen B. *Bradyrhizobial* indoleacetic acid metabolism

656      and its significance for root nodule development. *In* Molecular Genetics of

657      Plant-Microbe Interactions APS Press St Paul MN, 1988, 151-152.

658   8. Egebo LA, Nielsen SV, Jochimsen BU. Oxygen-dependent catabolism of

659      indole-3-acetic acid in *Bradyrhizobium japonicum*. J Bacteriol 1991; 173(15):

660      4897-4901. https://doi.org/10.1128/jb.173.15.4897-4901.1991

661   9. Jensen JB, Egsgaard H, Van Onckelen H, Jochimsen BU. Catabolism of

662      indole-3-acetic acid and 4-and 5-chloroindole-3-acetic acid in *Bradyrhizobium*

663      *japonicum*.          J          Bacteriol          1995;          177(20):5762-5766.

664      https://doi.org/10.1128/jb.177.20.5762-5766.1995

665   10. Leveau JH, Gerards S. Discovery of a bacterial gene cluster for catabolism of the

666      plant hormone indole 3-acetic acid. FEMS MicrobiolEcol 2008; 65(2):238-250.

667      https://doi.org/10.1111/j.1574-6941.2008.00436.x

668   11. Ebenau-Jehle C, Thomas M, Scharf G, Kockelkorn D, Knapp B, Schühle K, et al.

669      Anaerobic metabolism of indoleacetate. J Bacteriol 2012; 194:2894–2903.

670      https://doi.org/10.1128/JB.00250-12

671   12. Piccinetti C, Arias N, Ventimiglia L, Díaz Zorita M, Murua L, Sánchez H, et al.

672      Positive effects of inoculation of soybean on nodulation BNF and the parameters of

673      crop production. *In* Microbiología agrícola: un aporte de la investigación en

674      Argentina, 2nd ed, Albanesi AS (ed) Magna Publicaciones, Tucumán, Argentina,

675      2013, 283–297.

676    13. Torres D, Revale S, Obando M, Maroniche G, Paris G, Perticari A, et al. Genome

677        sequence of *Bradyrhizobium japonicum* E109, one of the most agronomically used

678        nitrogen-fixing    rhizobacteria    in    Argentina.    Genome    Announc    2015;

679        3(1):e01566-14. https://doi.org/10.1128/genomeA.01566-14

680    14. Costacurta A, Vanderleyden J. Synthesis of phytohormones by plant-associated

681        bacteria.        Crit        rev        microbiol        1995;        21(1):1-18.

682        https://doi.org/10.3109/10408419509113531

683    15. Torres D, Benavidez I, Donadío F, Mongiardini E, Rosas S, Spaepen S, et al. New

684        insights into auxin metabolism in *Bradyrhizobium japonicum*. Res microbial 2018;

685        169(6):313-323. https://doi.org/10.1016/j.resmic.2018.04.002

686    16. Nienaber  A,  Huber  A,  Göttfert  M,  Hennecke  H,  Fischer  H.  Three  new

687        NifA-regulated genes in the *Bradyrhizobium japonicum* symbiotic gene region

688        discovered  by  competitive  DNA-RNA  hybridization.  J  Bacteriol  2000;

689        82(6):1472-1480. https://doi.org/10.1128/JB.182.6.1472-1480.2000

690    17. Alemayehu D, Gordon LM, O'Mahony MM, O'Leary ND, Dobson ADW. Cloning

691        and functional analysis by gene disruption of a novel gene involved in indigo

692        production and fluoranthene metabolism in *Pseudomonas alcaligenes* PA-10.

693        FEMS        Microbiol        Lett        2004;        239(2):285-293.

694        https://doi.org/10.1016/j.femsle.2004.08.046

695    18. Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, et al.

696        Complete   genomic   sequence   of   nitrogen-fixing   symbiotic   bacterium

697    *Bradyrhizobium    japonicum*    USDA110.    DNA    Res    2002;    9(6):189-197.

698    https://doi.org/10.1093/dnares/9.6.189

699    19. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. Small

700    mobilizable    multi-purpose    cloning    vectors    derived    from    the    *Escherichia coli*

701    plasmids pK18 and pK19: selection of defined deletions in the chromosome of

702    *Corynebacterium    glutamicum*.    Gene    1994;    145    (1):69–73.

703    https://doi.org/10.1016/0378-1119(94)90324-7 ANTES 30

704    20. Newman JR, Fuqua C. Broad-host-range expression vectors that carry the

705    L-arabinose-inducible *Escherichia coli ara*BAD promoter and the *ara*C regulator.

706    Gene 1999; 227(2):197-203. https://doi.org/10.1016/S0378-1119(98)00601-5

707    21. Vincent JA. 1970. Manual for the Practical Study of Root-nodul Bacteria. Int Biol

708    Program Handb, Blackwell, Oxford. https://doi.org/10.1093/nar/gki106

709    22. Cassán F, Vanderleyden J, Spaepen S. Physiological and agronomical aspects of

710    phytohormone production by model plant-growth-promoting rhizobacteria (PGPR)

711    belonging to the genus *Azospirillum*. J Plant Growth Regul 2014; 33(2):440-459.

712    https://doi.org/10.1007/s00344-013-9362-4

713    23. Glickmann E, Dessaux Y. A critical examination of the specificity of the salkowski

714    reagent    for    indolic    compounds    produced    by    phytopathogenic    bacteria.    Appl

715    Environ    Microbiol    1995;    61(2):793-796.

716    https://doi.org/10.1128/AEM.61.2.793-796.1995

717     24. Madden T, Busby B, Ye J. Reply to the paper: Misunderstood parameters of NCBI

718          BLAST impacts the correctness of bioinformatics workflows. Bioinformatics 2019;

719          35(15):2699-2700. https://doi.org/10.1093/bioinformatics/bty1026

720     25. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and

721          interpretation of large-scale molecular data sets. Nucleic Acids Res 2012;

722          40(D1):109-114. https://doi.org/10.1093/nar/gkr988

723     26. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST

724          Server: rapid annotations using subsystems technology. BMC Genom 2008;

725          9(1):75. https://doi.org/10.1186/1471-2164-9-75

726     27. Notredame C, Higgins D, Heringa J. T-Coffee: A novel method for fast and

727          accurate multiple sequence alignment. J Mol Biol 2000; 302(1):205-217.

728          https://doi.org/10.1006/jmbi.2000.4042

729     28. Letunic I, Bork P. 20 years of the SMART protein domain annotation resource.

730          Nucleic Acids Res 2018; 46(1):493-496. https://doi.org/10.1093/nar/gkx922

731     29. Vallenet D, Labarre L, Rouy Z, Barbe V, Bocs S, Cruveiller S, et al. MaGe: a

732          microbial genome annotation system supported by synteny results. Nucleic Acids

733          Res 2006; 34(1):53-65. https://doi.org/10.1093/nar/gkj406

734     30. Apweiler R, Bairoch A, Wu CH, Barker WC, Boeckmann B, Ferro S, et al.

735          UniProt: the universal protein knowledgebase. Nucleic Acids Res 2004;

736          32(1):115-119. https://doi.org/10.1093/nar/gkh131

737   31. Mulder N, Apweiler R, Attwood T, Bairoch A, Bateman A, Binns D, et al.

738        InterPro, progress and status in 2005. Nucleic Acids Res 2005; 33(1):201-205.

739        https://doi.org/10.1093/nar/gki106

740   32. Sambrook J, Fritsch E, Maniatis T. Molecular cloning: a laboratory manual, ed 2,

741        Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 1989.

742   33. Mongiardini E, Quelas J, Dardis C, Althabegoiti M, Lodeiro A. Transcriptional

743        control of the lateral-flagellar genes of *Bradyrhizobium diazoefficiens*. J Bacteriol

744        2017; 199(15): e00253-17. https://doi.org/10.1128/JB.00253-17

745   34. Quelas JI, Mongiardini EJ, Casabuono A, López-García SL, Althabegoiti MJ,

746        Covelli JM, et al. Lack of galactose or galacturonic acid in *Bradyrhizobium*

747        *japonicum* USDA110 exopolysaccharide leads to different symbiotic responses in

748        soybean.     Mol     Plant-Microbe     Interact     2010;     23(12):1592-1604.

749        https://doi.org/10.1094/MPMI-05-10-0122

750   35. Donoso R, Leiva-Novoa P, Zúñiga A, Timmermann T, Recabarren-Gajardo G,

751        González B. Biochemical and genetic bases of indole-3-acetic acid (auxin

752        phytohormone) degradation by the plant-growth-promoting rhizobacterium

753        *Paraburkholderia phytofirmans* PsJN. Appl Environ Microbiol 2016; 83(1).

754        https://doi.org/10.1128/AEM.01991-16

755   36. Burton J, Martinez C, Curley R. Methods of testing and suggested standards for

756        legume inoculants and preinoculated seeds. Milwaukee, Wisconsin, USA, Nitragin

757        Sales Corp, 1972.

758    37. Hoagland D, Broyer T. General nature of the process of salt accumulation by roots

759         with description of experimental methods. Plant Physiol 1936; 11:471e507.

760         https://doi.org/10.1104/pp.11.3.471.

761    38. Minamisawa K, Fukai K. Production of indole-3-acetic acid by *Bradyrhizobium*

762         *japonicum*: a correlation with genotype grouping and rhizobitoxine production.

763         Plant            Cell            Physiol            1991;            32(1):1-9.

764         https://doi.org/10.1093/oxfordjournals.pcp.a078042

765    39. Olesen MR, Jochimsen BU. Identification of enzymes involved in indole-3-acetic

766         acid degradation. Current Issues in Symbiotic Nitrogen Fixation. Springer,

767         Dordrecht, 1996, 143-149. https://doi.org/10.1007/978-94-011-5700-1_20

768    40. Jarabo-Lorenzo A, Perez-Galdona R, Vega-Hernandez M, Trujillo J, Leon-Barrios

769         M. Indole-3-acetic acid catabolism by bacteria belonging to the *Bradyrhizobium*

770         genus. In Biological Nitrogen Fixation for the 21st Centur*y*, Current Plant Science

771         and Biotechnology in Agriculture, Elmerich C, Kondorosi A, Newton WE (eds),

772         Springer,         Dordrecht,         1998,         vol.         31,         484-484.

773         https://doi.org/10.1007/978-94-011-5159-7_299

774    41. Donati A, Lee H, Leveau J, Chang W. Effects of indole-3-acetic acid on the

775         transcriptional activities and stress tolerance of *Bradyrhizobium japonicum*. PloS

776         one 2013; 8(10). https://doi.org/10.1371/journal.pone.0076559

777    42. Scott J, Greenhut I, Leveau J. Functional characterization of the bacterial *iac* genes

778        for degradation of the plant hormone indole-3-acetic acid. J Chem Ecol 2013;

779        39(7):942-951. https://doi.org/10.1007/s10886-013-0324-x

780    43. Shu HY, Lin LC, Lin TK, Chen HP, Yang HH, Peng KC, et al. Transcriptional

781        regulation of the *iac* locus from *Acinetobacter baumannii* by the phytohormone

782        indole-3-acetic  acid.  Anton  Leeuw  Int  J  G  2015;  107(5):1237-1247.

783        https://doi.org/10.1007/s10482-015-0417-3

784    44. Laird  T,  Leveau  J.  Finished  Genome  Sequence  of  the  Indole-3-Acetic

785        Acid-Catabolizing Bacterium *Pseudomonas putida* 1290. Microbiol. Resour.

786        Announc 2019; 8(28), https://doi.org/10.1128/MRA.00519-19.

787    45. Sadauskas  M,  Statkevičiūtė  R,  Vaitekūnas  J,  Meškys  R.  Bioconversion  of

788        Biologically  Active  Indole  Derivatives  with  Indole-3-Acetic  Acid-Degrading

789        Enzymes  from  *Caballeronia glathei*  DSM50014.  Biomolecules  2020;  10(4).

790        https://doi.org/10.3390/biom10040663

791    46. Collino D, Salvagiotti F, Perticari A, Piccinetti C, Ovando G, Urquiaga S, et al.

792        Biological nitrogen fixation in soybean in Argentina: relationships with crop, soil,

793        and    meteorological    factors.    Plant    Soil    2015;    392:239–252

794        https://doi.org/10.1007/s11104-015-2459-8

795    47. Mengucci F, Dardis C, Mongiardini E, Althabegoiti M, Partridge J, Kojima S, et al.

796        Characterization of FliL Proteins in *Bradyrhizobium diazoefficiens*: Lateral FliL

797    Supports Swimming Motility, and Subpolar FliL Modulates the Lateral Flagellar

798    System. J Bacteriol 2020; 202(5):e00708-19. https://doi.org/10.1128/JB.00708-19

799    48. Bronstein P, Marrichi M, Cartinhour S, Schneider D, De Lisa M. 2005.

800    Identification of a twin-arginine translocation system in *Pseudomonas syringae* pv.

801    Tomat DC3000 and its contribution to pathogenicity and fitness. J. Bacteriol.

802    187:8450-8461. https://jb.asm.org/content/187/24/8450

803

**804    Legends to figures**

805    **Fig. 1.** Evolution of IAA concentration ($\mu$g.ml$^{-1}$) measured along time (h) in **(A)** YEM

806    (Yeast Extract Mannitol) culture medium, *Bj*E109, *Bd*USDA110 and *Be*SEMIA5019

807    cultures modified by the exogenous addition of IAA to a final concentration of 40

808    $\mu$g.ml$^{-1}$. **(B)** Supernatants and heat denatured supernatant of *Bj*E109 modified by the

809    exogenous addition of IAA to a final concentration of 40 $\mu$g.ml$^{-1}$. Values shown are

810    mean $\pm$ SD (n = 3). Where not shown, the SD was smaller than the symbol.

811

812    **Fig. 2**. IAA concentration ($\mu$g.ml$^{-1}$) measured along time (h) in *Bj*E109 cultures

813    modified by the exogenous addition of IAA to a final concentration of 0, 20, 40, 80, 120

814    and 160 $\mu$g.ml$^{-1}$ of IAA ($\mu$g.ml$^{-1}$) per tube. Values shown are mean $\pm$ SD (n = 3). Where

815    not shown, the SD was smaller than the symbol.

816

817    **Fig. 3**. IAA concentration ($\mu$g.ml$^{-1}$) measured along time (h) in supernatants of *Bj*E109

818    cultures obtained from early exponential (EE) or late exponential (LE) growth phase

819    modified by the addition of an IAA solution to a final concentration of 40 $\mu$g.ml$^{-1}$.

820    Values shown are mean $\pm$ SD (n = 3). Where not shown, the SD was smaller than the

821    symbol.

822

823    **Fig. 4.** IAA concentration ($\mu$g.ml$^{-1}$) measured along time (h) in *Bd*USDA110 wild type,

824    *Bd*USDA110$\Delta iac$A and *Bd*USDA110$\Delta iac$C mutant modified by exogenous addition of

825    IAA to a final concentration of 40 $\mu$g.ml$^{-1}$. Values shown are mean $\pm$ SD (n = 3). Where

826    not shown, the SD was smaller than the symbol.

827

828    **Fig. 5**. IAA concentration ($\mu$g.ml$^{-1}$) measured along time (h) in *Bj*E109 wild type and

829    *Bj*E109$\Delta iac$C mutant modified by exogenous addition of IAA to a final concentration of

830    40 $\mu$g.ml$^{-1}$. Values shown are mean $\pm$ SD (n = 3). Where not shown, the SD was smaller

831    than the symbol.

832

833    **Fig. 6**. Metabolic products identified after heterologous expression of the *iac*CD

834    construct in *C. pinatubonensis* JMP134. The figure represents the putative degradation

835    pathway in *Bj*E109 in relation of the identified metabolites (blue color) and the ones

836    expected (red color) in our experiments.

1     **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 | *lacZ*α Km$^r$ *sac*B | [19] |
| pK18Sac::*iac*C*Bd* | *lacZ*α Kmr sacB carrying the internal fragment of *iac*C from *Bd* | This study |
| pK18Sac::Δ*iac*C*Bd* | *lacZ*α Kmr sacB carrying the internal fragment of *iac*C from *Bd* deleted in SalI sites | This study |
| pK18SacB::*iac*C*Bj* | *lacZ*α Kmr sacB -*iac*C carrying the internal fragment of *iac*C from *Bj* | This study |
| pK18Sac::Δ*iac*C*Bj* | *lacZ*α Kmr sacB -*iac*C carrying the internal fragment of *iac*C 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 |

2    [a] IMYZA-Instituto de Microbiología y Zoología Agrícola. Castelar. Argentina

**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 | $0.21\pm0.007^d$ | $0.048\pm0.009^d$ |
| **E109** | $11.68\pm0.29^b$ | $9.14\pm0.62^a$ | $20.83\pm0.61^b$ | $0.33\pm0.008^a$ | $0.073\pm0.004^b$ |
| **E109 Δ*iac*C** | $8.68\pm0.38^c$ | $7.64\pm0.83^{ab}$ | $16.32\pm0.91^c$ | $0.27\pm0.009^b$ | $0.057\pm0.011^c$ |
| | **-25.6%** | **-16.4%** | **-21.6%** | **-18.05%** | **-21.9%** |
| **USDA110** | $22.66\pm0.3^a$ | $3.22\pm0.71^b$ | $25.88\pm0.34^a$ | $0.29\pm0.004^b$ | $0.080\pm0.007^a$ |
| **USDA110 Δ*iac*C** | $12.94\pm0.47^b$ | $2.09\pm0.57^b$ | $15.04\pm0.52^c$ | $0.23\pm0.003^c$ | $0.072\pm0.015^b$ |
| | **-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.

**Figure 1**
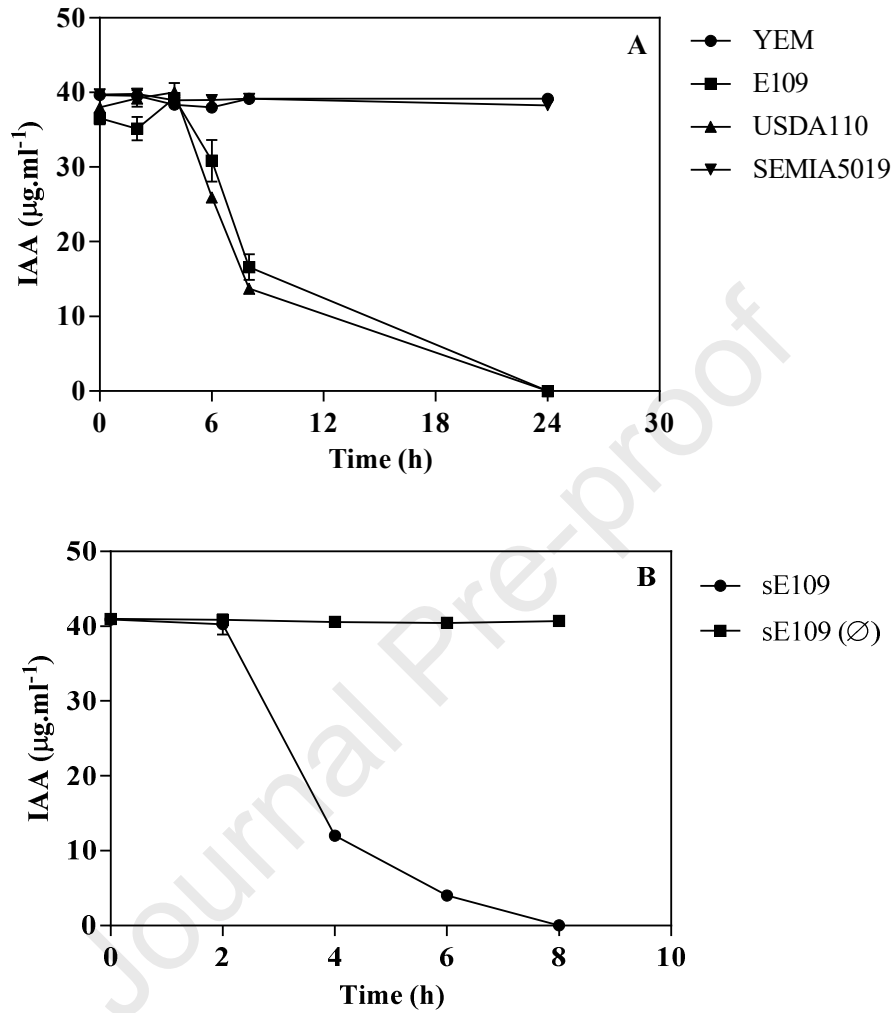
**Figure 2**
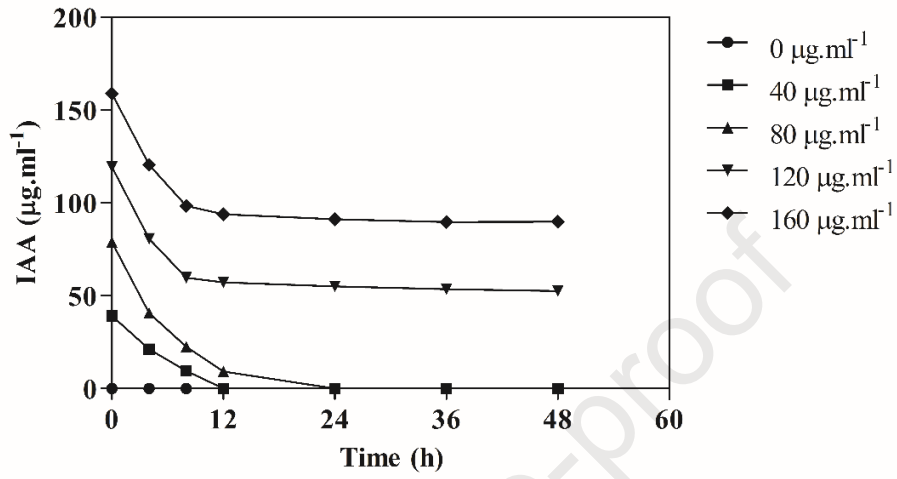
**Figure 3**
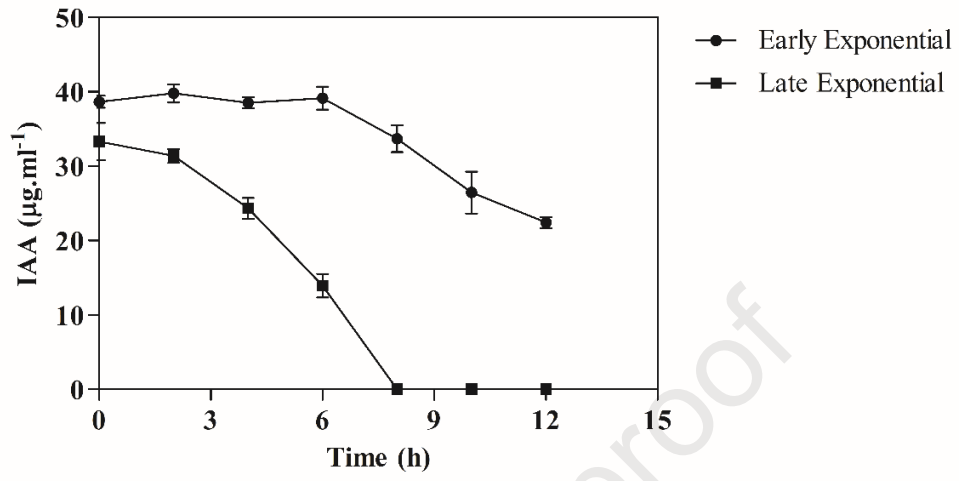
**Figure 4**

**Figure 5**

**Figure 6**



**IAA**
Exact Mass: 175,1

**A**
Exact Mass: 191,1
Exp Mass: 190,8
**detected**

**B**
Exact Mass: 209,1
**not detected**

**C**
Exact Mass: 225,1
Exp Mass: 224,9
**detected**