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Mención Genética Molecular y Microbiología

SYNTHESIS AND DEGRADATION OF INDOLE-3-ACETIC ACID BY *BURKHOLDERIA PHYTOFIRMANS* PsJN

Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al Grado de Doctor en Ciencias con mención en Genética Molecular y Microbiología.

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"Donde hay educación no hay distinción de clases"
Confucio.

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ABBREVIATIONS

4-HPA	4-hydroxyphenylacetic acid
ACC	1- aminocyclopropane-1-carboxylic acid
bp	Base pair
BHR	Broad host range
Bz	Benzoic acid
CFU	Colony forming unit
FW	Fresh weight
GC-MS	Gas chromatography-Mass spectrometry
GFP	Green fluorescent protein
Gm	Gentamicin
GOI	Gene of interest
HK	Housekeeping
HPLC-UV	High performance liquid chromatography-Ultraviolet
IAA	Indole-3-acetic acid
Km	Kanamycin
LB	Luria Bertani
LTTR	LysR-type transcriptional regulator
MFS	Major facilitator superfamily
MS	Murashige and Skoog
OD600	Optical density at 600 nm
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria

RR	Response regulator protein
RT	Reverse transcription
SDRs	Short chain dehydrogenase/reductases
SK	Sensor kinase
Sp	Spectinomycin
WT	Wild type

RESUMEN

Se ha descrito que las rizobacterias promotoras del crecimiento de plantas (PGPR) usan múltiples mecanismos para producir su efecto benéfico sobre las plantas, tal como la síntesis de la fitohormona auxina ácido indol-3-acético (IAA). Se han propuesto varias vías de biosíntesis del IAA en bacterias, con triptófano como su principal precursor. Por otro lado, se han reportado bacterias capaces de utilizar IAA como única fuente de carbono y energía, asociando esta habilidad con los genes *iac*, aunque la función y regulación de estos genes es escasamente comprendida. Además, es sabido que las plantas usan múltiples mecanismos para alcanzar la homeostasis de auxinas, incluyendo su biosíntesis y catabolismo. En este contexto, se ha propuesto que las bacterias que poseen simultáneamente genes relacionados a síntesis y degradación de IAA podrían controlar las asociaciones planta-bacteria. Este sería el caso de la β-proteobacteria *Burkholderia phytofirmans* PsJN, capaz de establecer interacciones benéficas con plantas, debido a que posee habilidades de biosíntesis y degradación de IAA. Se determinó que la cepa PsJN produciría IAA mediante al menos tres vías funcionales diferentes de biosíntesis dependientes de triptófano. Además, se observó que la cepa PsJN usa IAA como única fuente de carbono y energía empleando los genes *iac* para este propósito, y se propusieron los pasos en que cada gen estaría involucrado en la degradación de IAA. Asimismo, se reportó que *iacA*, *iacF* e *iacG* presentan redundancia funcional y revelamos algunas pistas sobre la regulación de los genes *iac*. Adicionalmente, se determinó que la degradación de IAA juega un papel en la promoción de crecimiento de plantas. Por otra parte, se observó que la degradación de IAA a través de los genes *iac* fue reprimida por la presencia de triptófano, pero, sorprendentemente, cuando el metabolismo de triptófano está activado el

IAA es degradado a través de una vía independiente de los genes *iac*. En conclusión, estos resultados sugieren que existe una compleja regulación en el metabolismo de IAA en *B. phytofirmans* PsJN.

ABSTRACT

It has been described that plant-growth promoting rhizobacteria (PGPR) use multiple mechanisms to produce its effects on plants, such as synthesis of phytohormones as the auxin indole-3-acetic acid (IAA). Several pathways have been proposed for IAA biosynthesis in bacteria, with tryptophan as the main precursor. On the other hand, it has been reported that bacteria are able to use IAA as sole carbon and energy source, with *iac* genes encoding this ability, although the function and regulation of these genes are poorly understood. Furthermore, it is known that plants use multiple mechanisms to achieve auxin homeostasis, including biosynthesis and catabolism. In this context, it has been proposed that bacteria that simultaneously possess genes related to the synthesis and degradation of IAA may control plant-bacteria associations. This would be the case of the β-proteobacterium *Burkholderia phytofirmans* PsJN, able to establish positive interactions with plants, which possesses IAA biosynthesis and degradation abilities. We determined that strain PsJN produces IAA by a tryptophan-dependent way, through at least three different functional biosynthetic pathways. We have also shown that strain PsJN uses IAA as the sole carbon and energy source employing *iac* genes, and have proposed steps in IAA degradation in which each *iac* gene is involved. We also reported that *iacA, iacF* and *iacG* genes present functional redundancy and provided some hints on *iac* gene regulation. Furthermore, we determined that IAA degradation plays a role in plant-growth-promoting traits. Additionally, we observed that IAA degradation by *iac* genes was repressed by tryptophan but, surprisingly, when tryptophan metabolism was activated IAA is degraded in an *iac*-independent way. Taken together, these results show a complex regulation of IAA turnover in this PGPR.

GENERAL INTRODUCTION

IAA belongs to a class of plant hormones called auxins, which play an important role in plant growth and development (Gray, 2004; Teale *et al*, 2006; Benjamins and Scheres, 2008; Vanneste and Friml, 2009); therefore, plants tightly control IAA production (Ljung, 2013). Remarkably, some microorganisms are also able to produce this auxin (Costacurta and Vanderleyden 1995; Patten and Glick, 1996), and bacterial IAA formation has been frequently linked to their interaction with plants (Spaepen and Vanderleyden, 2011). Furthermore, it is well known that IAA production contributes to virulence of phytopathogen strains, such as *Agrobacterium tumefaciens*, *Pseudomonas savastanoi*, *P. syringae* or *Pantoea agglomerans* (Comai and Kosuge, 1982; Liu *et al,* 1982; Chalupowicz *et al*, 2013; Mutka *et al*, 2013), which produce high levels of IAA and induce tumors or galls in plants. In contrast, it has been reported that beneficial bacteria are able to produce IAA at lower levels, which are not detrimental to plants, and conversely exert a phytostimulating effect (Spaepen *et al*, 2007a; Spaepen and Vanderleyden, 2011). Phytohormone production in this type of bacteria, such as *Azospirillum brasilense*, *Bradyrhizobium japonicum, Enterobacter cloacae* or *Pseudomonas putida* (Dobbelaere *et al*, 1999; Saleh and Glick, 2001; Patten and Glick, 2002a; Boiero *et al*, 2007), has been considered as a mechanism for plant-growth promotion. Bacteria employ several strategies for IAA biosynthesis with tryptophan as main precursor (Spaepen and Vanderleyden, 2011), although tryptophan-independent pathways for IAA synthesis have been also reported, but enzymes involved in these pathways remain uncharacterized (Prinsen *et al*, 1993; Idris *et al*, 2007). Five main tryptophan-dependent IAA biosynthesis pathways have been reported, whose names are based on some of the intermediates generated: indole-3-

acetamide (Comai and Kosuge, 1982; Clark *et al*, 1993), indole-3-pyruvate (Koga *et al*, 1991a; Costacurta *et al*, 1994; Brandl and Lindow, 1996), indole-3-acetonitrile (Kobayashi *et al*, 1993; Howden *et al*, 2009), tryptamine (Perley and Stowe, 1966; Carreño-López *et al*, 2000) and tryptophan side-chain oxidase pathways (Oberhänsli *et al*, 1991) (Figure 1). Some bacteria harbor more than one of these biosynthetic pathways (Prinsen *et al*, 1993; Manulis *et al*, 1998; Carreño-López *et al*, 2000), suggesting that auxin synthesis is important for these plant-interacting microorganisms.

On the other hand, it has been described that a substantial diversity of microorganisms isolated from soils or plants possess the ability to transform IAA (Proctor, 1958; Libbert and Risch, 1969; Strzelczyk *et al*, 1973). Complete degradation of this auxin has been reported in *Pseudomonas* (Leveau and Lindow, 2005), *Bradyrhizobium* (Egebo *et al*, 1991; Jensen *et al*, 1995), *Burkholderia*, *Rhodococcus* and *Sphingomonas* genera (Leveau and Gerards, 2008). Different routes for aerobic IAA degradation have been proposed in bacteria, specifically in *Bradyrhizobium* (Figure 2) (Egebo *et al*, 1991; Jensen *et al*, 1995), and also an anaerobic IAA pathway was proposed in *Azoarcus evansii* (Ebenau-Jehle *et al*, 2012). Also, Leveau and Gerards (2008) described in *P. putida* 1290, a cluster of genes (named *iac*) related to the aerobic catabolism of IAA. The biochemical and genetic characterization of this IAA degradation pathway and its gene regulation is still poorly understood, although Scott *et al* (2013) have recently proposed two putative intermediates (Figure 2) and carried out an initial functional analysis of some *iac* genes.

It is known that plants use multiple mechanisms for auxin homeostasis, including control of its biosynthesis and catabolism (Ljun *et al*, 2002; Tromas and Perrot-Rechenmann, 2010). Remarkably, some bacteria possess simultaneously genes for IAA biosynthesis and degradation, which should be part of control mechanisms of IAA

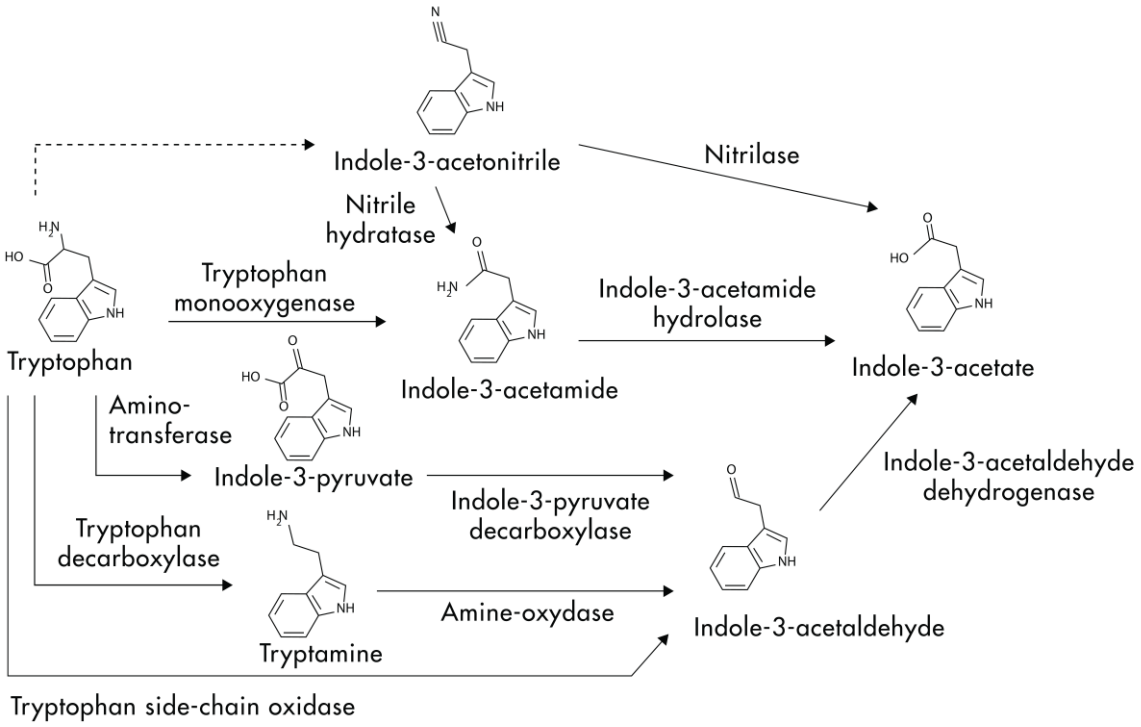

Figure 1: Tryptophan-dependent pathways of bacterial indole-3-acetic acid (IAA) synthesis (Spaepen and Vanderleyden, 2011). Dashed lines indicate more than one step.

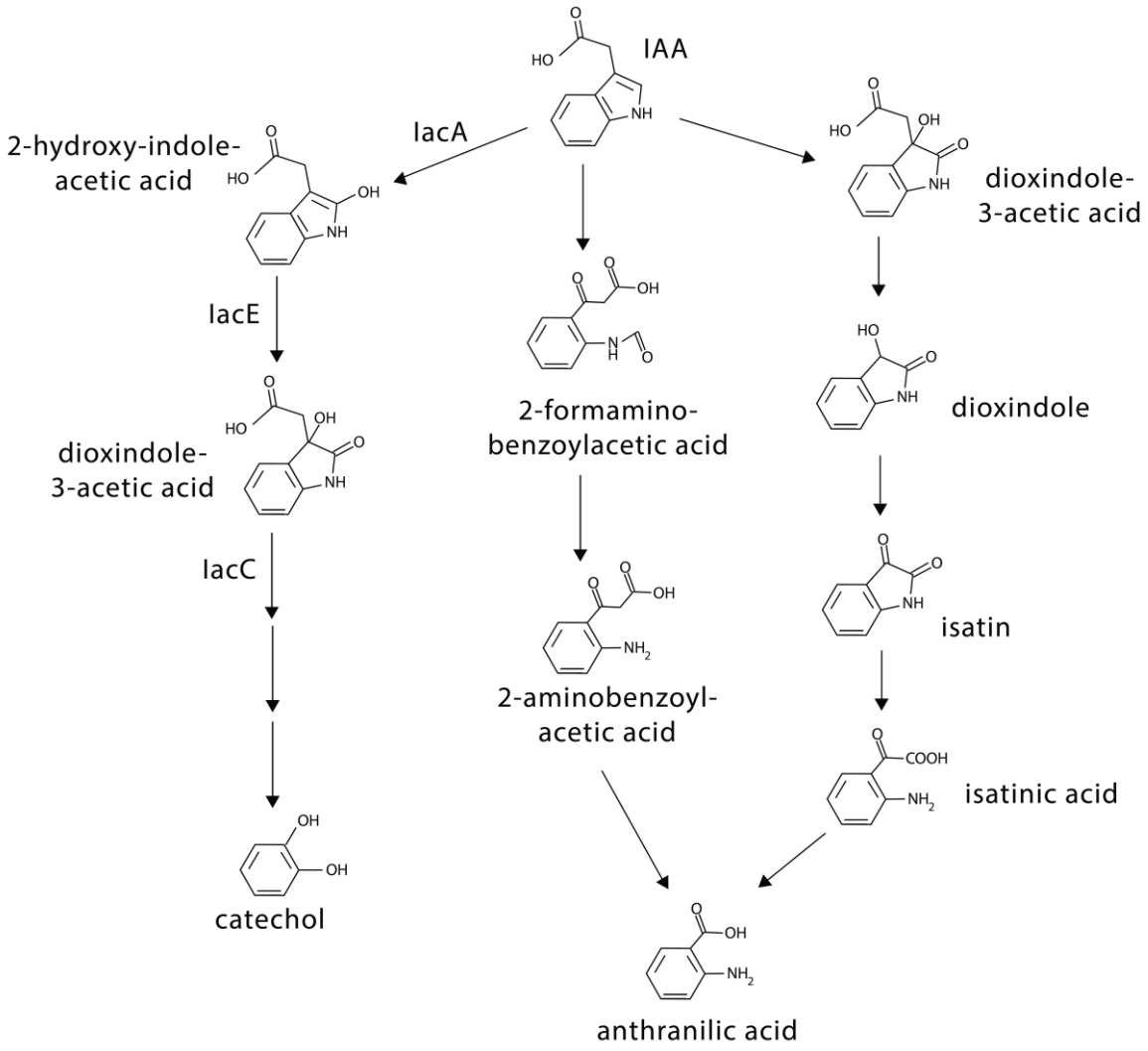

Figure 2: Proposed indole-3-acetic acid (IAA) degradation pathways for *Bradyrhizobium japonicum*, channeling IAA to anthranilic acid (Egebo *et al*, 1991; Jensen *et al*, 1995). Recently, Scott *et al* (2013) proposed two intermediates and steps in which are involved *iacA*-, *iacE*- and *iacC*-encoding products in *Pseudomonas putida*, channeling IAA to catechol.

levels in plant-bacteria associations. In this context, the β-proteobacterium *Burkholderia phytofirmans* PsJN (Mitter *et al*, 2013) is interesting because is able to establish beneficial interactions with plants at the rhizospheric and the endophytic level, promoting growth of agronomically important crops such as tomato, potato and grape (Frommel *et al*, 1991; Nowak *et al*, 1995; Pillay and Nowak, 1997; Bensalim *et al*, 1998; Ait Barka *et al*, 2000, 2002; Compant *et al*, 2005), and also the model plant *Arabidopsis thaliana* (Zúñiga *et al*, 2013). Studies on plant-growth promotion by rhizobacteria in different plants have been recently reported. As example, production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Sun *et al*, 2009), degradation of IAA and signaling via acyl-homoserine lactones (Zúñiga *et al*, 2013) play significant roles in the interactions of strain PsJN with plants. The role of IAA degradation in plant growth promotion is part of the results of this thesis work and will be explained below. In previous reports, it have been described that *B. phytofirmans* PsJN has the ability to produce IAA (Sun *et al*, 2009), encoding at least two putative IAA biosynthesis routes (Weilharter *et al*, 2011), but additional studies are clearly required to understand IAA synthesis in this strain. Additionally, strain PsJN possesses a cluster of *iac* genes related to the IAA degradation pathway reported in *P. putida* 1290 (Leveau and Gerards, 2008). These data revealed that strain PsJN could possess simultaneously abilities of synthesis and degradation of IAA, raising questions such as: are functional these pathways? How strain PsJN is coordinating the processes of IAA synthesis and degradation? Do these processes have some relation to the growth-promoting abilities of *B. phytofirmans*?, among others.

Based on the preceding elements the hypothesis of this thesis was:

There is interplay between IAA biosynthesis and degradation in the PGPR B. phytofirmans PsJN, to control IAA concentration in bacteria-plant systems, due to certain auxin levels could exert benefical effects on B. phytofirmans / plant host interactions.

The overall objective of this thesis was to study the biosynthesis of IAA in *B. phytofirmans* PsJN, analyzing posible ways to IAA production, also to evaluate IAA degradation, linking this processe with *iac* genes, additionally, exploring the relation of IAA levels with plant growth promoting mechanisms in *B. phytofirmans* PsJN and determining a possible interplay between biosynthesis and degradation processess.

The specific objectives of this thesis were:

1. Assess capability of IAA biosynthesis in *B. phytofirmans* PsJN cultures, evaluating ability to transform tryptophan and intermediates tryptophan-related in IAA.
2. Evaluate functionality, participation and regulation of *iac* genes of *B. phytofirmans* PsJN in catabolism of IAA.
3. Investigate the role of an *iac* gene mutant, unable to use IAA as sole carbon and energy source, in plant growth promoting abilities.
4. Determine if exist an interplay between tryptophan-dependent IAA biosynthesis and catabolism of IAA by *iac* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacteria and plasmids are listed in Table 1. *B. phytofirmans* and its derivatives were grown at 30°C in mineral salts medium (Dorn *et al*, 1974), supplemented with 2.5 or 5 mM benzoate (Bz); 2.5 mM IAA; 2.5 mM tryptophan; 5 or 10 mM fructose and 10 mM succinate, plus the appropriate antibiotics: kanamycin (Km, 50 μg ml^{-1}), gentamycin (Gm, 30 μg ml^{-1}) or spectinomycin (Sp, 100 μg ml^{-1}). *Cupriavidus pinatubonensis* JMP134 and its derivatives were grown under the same conditions. *Escherichia coli* Mach (Invitrogen, Carlsbad, CA, USA) was grown at 37°C in Luria–Bertani (LB). Growth was measured by optical density at 600 nm (OD600) in a GeneQuant 1300 spectrophotometer (GE Healthcare, Piscataway, NJ, USA) or EON Microplate spectrophotometer (Biotek, Winooski, VT, USA). At least three replicates were performed for each growth measurement.

Chromosomal disruption of gene sequences in *B. phytofirmans* PsJN

Internal fragments of *iac* and associated genes (*iacA, iacA2, iacB, iacC, iacD, iacE, iacF, iacG, iacG2, iacH, iacI, iacS, iacR1, iacR2, iacR3, iacT1, iacT2, catA*) were amplified by PCR, using the primer pairs listed in Table 2. The PCR products were cloned using the pCR2.1-TOPO system (Invitrogen) to generate plasmids listed in Table 1. For single mutant construction, suicidal pCR2.1*iac* o pCR2.1*cat* plasmids were electroporated in *B. phytofirmans* PsJN to get one-recombination-event disruption of the target gene, obtaining *B. phytofirmans iac* or *catA* mutants (Table 1), which were selected on LB agar containing 50 μg ml^{-1} Km.

Table 1: Bacterial strains and plasmids used in this thesis

Strain or plasmid	Relevant phenotype and/or genotype[a]	Source or reference[b]
***B. phytofirmans* strains**		
PsJN	IAA^+ Bz^+ fructose$^+$ succinate$^+$ tryptophan$^+$	Sessitsch *et al*, 2005
PsJNΔ*iacA*	IAA^- Km^R	This study
PsJNΔ*iacB*	IAA^- Km^R	This study
PsJNΔ*iacC*	IAA^- Km^R	This study
PsJNΔ*iacD*	IAA^- Km^R	This study
PsJNΔ*iacE*	IAA^- Km^R	This study
PsJNΔ*iacF*	IAA^- Km^R	This study
PsJNΔ*iacG*	IAA^+ Km^R	This study
PsJNΔ*iacH*	IAA^- Km^R	This study
PsJNΔ*iacI*	IAA^- Km^R	This study
PsJNΔ*iacA2*	IAA^+ Km^R	This study
PsJNΔ*iacG2*	IAA^+ Km^R	This study
PsJNΔ*iacT1*	IAA^+ Km^R	This study
PsJNΔ*iacT2*	IAA^+ Km^R	This study
PsJNΔ*iacS*	IAA^+ Km^R	This study
PsJNΔ*iacR1*	IAA^- Km^R	This study
PsJNΔ*iacR2*	IAA^- Km^R	This study
PsJNΔ*iacR3*	IAA^+ Km^R	This study
PsJNΔ*catA*	IAA^- Bz^+ Km^R	This study
PsJNΔ*kynA*	IAA^+ tryptophan$^-$ Km^R	This study
Other strains		
C. pinatubonensis JMP134	IAA^- fructose$^+$	DSMZ
E. coli Mach™	ΔrecA1398 endA1 tonA Φ80ΔlacM15 ΔlacX74 hsdR(r_K^- m_K^+)	Invitrogen

Table 1. Continued.

Strain or plasmid	Relevant phenotype and/or genotype[a]	Source or reference[b]
Plasmids		
pCR2.1-TOPO	Suicide vector in *B. phytofirmans* PsJN, Ap^R Km^R	Invitrogen
pCR2.1 *iac*[c]	pCR2.1 derivatives with internal fragment of *iac* genes, Ap^R Km^R	This study
pCR2.1 *catA*	pCR2.1 derivative with internal fragment of *catA* gene, Ap^R Km^R	This study
pCR2.1 *kynA*	pCR2.1 derivative with internal fragment of *kynA* gene, Ap^R Km^R	This study
pKGWP0	BHR *lacZ* transcriptional fusion vector, Sp^R	Marín *et al*, 2010
pIacABIHE-*lacZ*	$P_{iacABIHE}$, pKGWP0 derivative, Sp^R	This study
pIacCD-*lacZ*	P_{iacCD}, pKGWP0 derivative, Sp^R	This study
pIacF-*lacZ*	P_{iacF}, pKGWP0 derivative, Sp^R	This study
PIacG-*lacZ*	P_{iacG}, pKGWP0 derivative, Sp^R	This study
PcatBAC-*lacZ*	P_{catBAC}, pKGWP0 derivative, Sp^R	This study
pBS1	BHR gateway destination vector, *araC*-P_{BAD}, Gm^R	Bronstein *et al*, 2005
pBS1*iacABIHECDGF*	*iacABIHECDGF*-expressing pBS1 derivative, Gm^R	This study
pBS1*iac*ΔA	*iacBIHECDGF*-expressing pBS1 derivative, Gm^R	This study
pBS1*iac*ΔB	*iacAIHECDGF*-expressing pBS1 derivative, Gm^R	This study
pBS1*iac*ΔCD	*iacABIHEGF*-expressing pBS1 derivative, Gm^R	This study
pBS1*iac*ΔE	*iacABIHCDGF*-expressing pBS1 derivative, Gm^R	This study
pBS1*iac*ΔF	*iacABIHECDG*-expressing pBS1 derivative, Gm^R	This study
pBS1*iac*ΔG	*iacABIHECDF*-expressing pBS1 derivative, Gm^R	This study
pBS1*iac*ΔH	*iacABIECDGF*-expressing pBS1 derivative, Gm^R	This study
pBS1*iac*ΔI	*iacABHECDGF*-expressing pBS1 derivative, Gm^R	This study

[a] IAA^+, Bz^+, ability to grow on indole-3-acetate and benzoate, respectively; Ap^R, Gm^R, Km^R and Sp^R, resistance to ampicillin, gentamicin, kanamycin, and spectynomicin respectively; BHR, broad host range. [b]DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. [c]Internal fragments of *iacA*, *iacB*, *iacC*, *iacD*, *iacE*, *iacF*, *iacG*, *iacH*, *iacI*, *iacA2*, *iacG2*, *iacT1*, *iacT2*, *iacS*, *iacR1*, *iacR2* and *iacR3* cloned in pCR2.1 plasmid, to obtain their respective derivatives.

Table 2: Primer pairs used in this thesis.

Primer name	Sequence (5'→3')	Primer name	Sequence (5'→3')
Inactivation of genes			
Mut iacAFw	GCCCAGTTTCTCGACATGAT	Mut iacARv	GTCGTCTGCAACGAACTGGT
Mut iacBFw	CGAACAGATCGGCGAAGT	Mut iacBRv	CATAGCGCCACAGGTTGTATT
Mut iacCFw	GGTCA ACGTCTTGCA GAACC	Mut iacCRv	GTTTCGTCGTCGATCGATTT
Mut iacDFw	GACTATCGCGAGTGGCTCA	Mut iacDRv	CTTGCAAAACTGATGCGATG
Mut iacEFw	AAGTCGTCTCCGTGATGGAC	Mut iacERv	GATCACCGCCTTCAGTTTGT
Mut iacFFw	AACAGGAGCGCTTCGTATTG	Mut iacFRv	GCAAACCGTTCGAGATGAAT
Mut iacGFw	TCGACGTCAAACAGCAATTC	Mut iacGRv	ACCGAAGCGTTCTTCCATC
Mut iacHFw	ATCGGATTGAAGCCCACTTT	Mut iacHRv	CGAGGTCTCGACATTGATGA
Mut iacIFw	GAAATTCTCGCGATGCTCAC	Mut iacIRv	GAAAGTGCGTGATGAACCAG
Mut iacA2Fw	GTCGAGAACTGGAACGTGGT	Mut iacA2Rv	CAGTTGGCTTCTGCTTTCG
Mut iacG2Fw	CCAGCTACGTGCATGACATC	Mut iacG2Rv	AATCTGCGCAAACATGACC
Mut iacT1Fw	GGTGGAAAGCTTCATGTCGT	Mut iacT1Rv	GAGGAACACCAGCATTGGTT
Mut iacT2Fw	AGCATTCTGCTGATTCACGA	Mut iacT2Rv	TTGAGAACGCGAAGAAGATG
Mut iacSFw	TCAGCGTGGAAGACACACTC	Mut iacSRv	GTTGTAACGTCCGGCGTATT
Mut iacR1Fw	GTGCAAGCGGAGTTGAATC	Mut iacR1Rv	CTTCACGCTGATCGACAGAT
Mut iacR2Fw	CGCTGGAACTGTCGACTATC	Mut iacR2Rv	GAAACGCCTTCAAACTCAGC
Mut iacR3Fw	CAAAGCGTGCATCAGATTGT	Mut iacR3Rv	CGCTCGTTCTCCAAGGTAAG
Mut catAFw	TCTGGGTTTCGAGCATTTTC	Mut catARv	CGGAAGCTGTATTTGCCTTC
Mut kynAFw	TGAAGCTCGCGTTGTATGAG	Mut kynARv	TCCGATAAACCTCCAACCAG
RT-PCR analysis			
iacA intFw	GCCCAGTTTCTCGACATGAT	iacA intRv	AGATTTCCGCTTGTGTATGC
iacC intFw	GATGGTTATGAAGGCGTGGT	iacC intRv	GGCTCGATCTCCTGATTGAA
iacF intFw	CTGATGCGATTCATCTCGAA	iacF intRv	ATGGTCGTGTCCTTGTCCAC
iacG intFw	GCGACTTTCCAGAGCAACC	iacG intRv	ACCGAAGCGTTCTTCCATC

Table 2. Continued.

Primer name	Sequence (5'→3')	Primer name	Sequence (5'→3')
iacA2 intFw	GTCGAGAACTGGAACGTGGT	iacA2 intRv	CAGTTGGCTTCTGCTTTCG
catA intFw	ACGTACAACGGACCTTCGAT	catA intRv	TCTGGGTTTCGAGCATTTTC
catA2 intFw	TTCAACAAGCTCGGACAGG	catA2 intRv	ACATACAGCGGACCTTCGAT
kynA intFw	TGAAGCTCGCGTTGTATGAG	kynA intRv	TCCGATAAACCTCCAACCAG
rpS7 intFw	CGAACAGATCCAGACCAAGG	rpS7 intRv	ACTTCTCGCTGCGCTTCTT
16S intFw	CGGGCTAGAGTATGGCAGAG	16S intRv	CGTGCATGAGCGTCAGTATT
Transcriptional fusions			
PiacABIHE Fw	GCGCGGTACCCAGGCGGTGAAAG TCTTCTT	PiacABIHE Rv	TGTCCTCGAGGGTGAGGTATCTCCTTG ATTCG
PiacCD Fw	GCGCGGTACCGAATGAAAAGCGG GTCGTC	PiacCD Rv	TCGCCTCGAGCTTGTGTAGGTGGGGG TGGT
PiacF Fw	TAGAGGTACCGATTTGAATGTTC CGACTGGA	piacF Rv	CGTTCTCGAGGGTTCTTTCTATGAGCT TGC
PiacG Fw	TTATGGTACCCGAAGGCTGGGAC ATGGT	PiacG Rv	ATTTCTCGAGGATTCCTCGGGCAGGT GT
PcatBAC Fw	GTTTGGTACCTTCCTCGATCTGCT GGATCT	PcatBAC Rv	ATTCCTCGAGCTCCTGAACTGCCTGAC CTG
***iac* gene expression**			
1 (iacABIHE Fw)	TTGGGCTAGCGAATTCCTGCTTAC GTTTCGATCCAACTTGC	2 (iacABIHE Rv)	GTGGTCGATGAAGCGAAGTCGTCAGC GCATGAAGAGTCC
3 (iacCD Fw)	GACTTCGCTTCATCGACCAC	4 (iacCD Rv)	CAGGTGTCGAGTGGTTTGATTTCGAA AAGATCAAAGCAGGAA
5 (iacG Fw)	AATCAAACCACTCGACACCTG	6 (iacG Rv)	GGTTGTGCGTCAAGGATAAGCCACTG TGCGTAATCAATGC
7 (iacF Fw)	CTTATCCTTGACGCACAACC	8 (iacF Rv)	GTAATACGACTCACTATAGGAAGAAA AGGCGCTTGTGAAA
9 (pBS1 Fw)	CCTATAGTGAGTCGTATTAC	10 (pBS1 Rv)	GCAGGAATTCGCTAGCCCAA
11 (iacB Fw)	TTGGGCTAGCGAATTCCTGCCTAT CCGCACAACCAGGATT	12 (iacA Rv)	AATCCTGGTTGTGCGGATAG
13 (iacI Fw)	CTATCCGCACAACCAGGATTCTT GAGCGTTTCGGAACAAC	14 (iacH Rv)	GTGGTCGATGAAGCGAAGTCCCACTC CACTTCGAAATAACG

Table 2. Continued

Primer name	**Sequence (5'→3')**	**Primer name**	**Sequence (5'→3')**
15 (iacE Rv)	CAGGTGTCGAGTGGTTTGATTATGCCGCTTTGGTGTTGT	16 (iacG Rv)	GTAATACGACTCACTATAGGCCACTGTGCGTAATCAATGC
17 (iacF Fw2)	TTCCTGCTTTGATCTTTTCGACTTATCCTTGACGCACAACC	18 (iacI Rv)	CCTATCGACAGCGTCTGGA
19 (iacE Fw)	TCCAGACGCTGTCGATAGGGCCGTTATTTCGAAGTGGAG	20 (iacB Rv)	TCCTGGTTCGCGTTCATT
21 (iacH Fw)	AATGAACGCGAACCAGGAAGGTCAACGCAAGGAAACC		

Detection of transcripts by qRT-PCR

Cells of *B. phytofirmans* were grown on fructose (2.5 mM), Bz (2.5 mM) and IAA (2.5 mM), as the sole carbon and energy source. Then, total RNA was obtained from 4 ml of mid-phase cells, using RNA protect bacteria reagent and RNeasy Mini Kit (QIAGEN, Chatsworth, CA, USA). The RNA was quantified using an EON Microplate Spectrophotometer (Biotek, Winooski, VT, USA) and treated with TURBO DNase Kit (Ambion, Austin, TX, USA) to remove DNA contamination. The RT-PCR was performed using ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA) with 1 µg of RNA in 20 µl reactions.

Real time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and the Eco Real-Time PCR detection system (Illumina, San Diego, CA, USA). The PCR mixture (15 µl) contained 3.0 µl of template cDNA (diluted 1:10) and 0.2 µM of each primer. Amplification was performed under the following conditions: 95°C for 10 min; followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s; followed by a melting cycle from 55 to 95°C. Relative gene expression calculations were conducted as described in the software manufacturer's instructions: an accurate ratio between the expression of the gene of interest (GOI) and the housekeeping (HK) gene was calculated according to the following expression $2^{-(\Delta CtGOI-\Delta CtHK)}$. Then, gene expression levels were normalized to the average value of the gene expressions determined in the fructose treatment. 16S rRNA (Bphyt_R0071) was used as HK in this assay. For qRT-PCR analysis, primer pairs for *iacA* (Bphyt_2161), *iacC* (Bphyt_2156), *iacF* (Bphyt_2150), *iacG* (Bphyt_2167), *iacA2* (Bphyt_6911), *catA* (Bphyt_2152), *catA2* (Bphyt_1590), *rpS7* (Bphyt_3648) and *kynA* (Bphyt_3229) genes

were used (Table 2). All experiments were performed in three biological and two technical replicates.

Construction of *lacZ* reporter fusions

Putative promoter regions were fused to *lacZ* gene of pKGWP0 (Marín *et al*, 2010). PCR products comprising approximately 200 bp of region upstream of the translational starts of the *iacABIHE, iacCD, iacF, iacG or catBCA* genes were obtained, using primers listed in Table 2. The amplified DNA fragments were cloned into *Xho*I-*Kpn*I restriction site of pKGWP0 forming pIacABIHE-lacZ, pIacCD-lacZ, pIacF-lacZ, pIacG-lacZ and pCatBAC-lacZ, and transferred into *B. phytofirmans* and its derivatives, and selected in minimal medium supplemented with 100 μg ml^{-1} Sp. β-galactosidase assays were performed according to standard protocols (Miller, 1972), after 5 h of incubation using IAA, indole, indole-3-acetamide, indole-3-pyruvate, tryptophan, anthranilate or Bz (0.25 mM) as inducers.

Construction of plasmid derivatives expressing *iac* genes

PCR products comprising *iacABIHE* (primer pair 1-2), *iacCD* (3-4), *iacG* (5-6), *iacF* (7-8) genes, and pBS1 plasmid (9-10) (Bronstein *et al*, 2005) were obtained. All fragments were amplified using primer pairs listed in Table 2. These primer pairs contain a 20 bp terminal sequence that overlap each other, which were joined into a covalently sealed molecule in a one-step isothermal reaction, as described by Gibson *et al* (2009), to get pBS1-*iacABIHECDGF* plasmid that contains the complete *iac* cluster under the control of an arabinose-inducible promoter. Also, PCR products lacking one or two genes were amplified using primers listed in Table 2: *iacBIHE* (11-2), *iacCD* (3-4), *iacG* (5-6) and

iacF (7-8) genes, to obtain the *iac*ΔA derivative; *iacA* (1-12), *iacIHE* (13-2), *iacCD* (3-4), *iacG* (5-6) and *iacF* (7-8) genes, the *iac*ΔB derivative; *iacABIHE* (1-15), *iacG* (5-6) and *iacF* (7-8) genes, the *iac*ΔCD derivative; *iacABIH* (1-14), *iacCD* (3-4), *iacG* (5-6) and *iacF* (7-8) genes, the *iac*ΔE derivative; *iacABIHE* (1-2), *iacCD* (3-4) and *iacG* (5-16) genes, the *iac*ΔF derivative; *iacABIHE* (1-2), *iacCD* (3-4) and *iacF* (17-8) genes, the *iac*ΔG derivative; *iacABI* (1-18), *iacE* (19-2), *iacCD* (3-4), *iacG* (5-6) and *iacF* (7-8) genes, the *iac*ΔH derivative; *iacAB* (1-20), *iacHE* (21-2), *iacCD* (3-4), *iacG* (5-6) and *iacF* (7-8) genes, the *iac*ΔI derivative. All fragments containing the 20 bp terminal sequence that overlap each other were ligated to pBS1 plasmid (9-10) using Gibson assembly method (Gibson *et al*, 2009), to get plasmids that contain *iac* cluster deficient in one or two genes, under the control of AraBAD promoter in pBS1 (Bronstein *et al*, 2005). Finally, the recombinant plasmids were electroporated in *C. pinatubonensis* JMP134 and selected in LB medium supplemented with Gm (30 μg/ml).

Colorimetric detection of IAA synthesis

Bacterial cells were grown with 5 mM of fructose, Bz, tryptophan or 10 mM of succinate at OD600 ~ 1, which corresponds to the stationary phase of growth. At this point, bacterial cells were incubated with 2.5 mM tryptophan (as inducer of IAA synthesis); then, IAA synthesis was measured on supernatants of cultures using Salkowski reagent as described previously (Glickmann and Dessaux 1995). Three replicates were performed for each IAA synthesis measurement.

Preparation of bacterial inoculants for plant colonization

For plant colonization and growth effect experiments, *B. phytofirmans* PsJN and its mutant derivative strain PsJNΔ*iacC* were routinely grown in Dorn mineral salts medium (Dorn *et al*, 1974) with 10 mM fructose in an orbital shaker (150 rpm), for 24 h at 30°C. Cell suspensions from each inoculum were then obtained and adjusted to approximately 10^8 CFU/ml, as determined by plate counting. Then, 10^4 CFU/ml of each strain were homogeneously inoculated on 1% agar plates containing Murashige and Skoog (MS) basal salt mixture (Sigma, St. Louis, USA).

Plant growth.

A. thaliana ecotype Col-0 was used. Seeds were surface sterilized with 50% (vol/vol) commercial chlorine bleach for 7 min and washed three times in sterile distilled water. Then, seeds were kept at 4°C for two days in the absence of light to produce stratification. After that, seeds were sown in sterile plastic Petri dishes with 1% agar plates containing MS basal salt mixture (Sigma, St. Louis, USA) inoculated or not with bacteria. Eight seeds were sown in each plate and five plates were used for each treatment: control without bacteria, wild type and *iacC* mutant strain. To perform exogenous IAA degradation assays, seeds were germinated and grown for two weeks on plates with MS medium containing 0.001 μM or 1 μM of IAA inoculated or not with either wild type or *iacC* gene mutant strains. Plates were placed vertically, sealed with parafilm and arranged in a completely randomized design. The plant growth chamber was run with a cycle of 12 h of light and 12 h of darkness and a temperature of 22 ± 2°C. Three biological replicates were carried out.

Determination of rhizospheric and endophytic bacterial colonization.

For rhizospheric colonization tests, three weeks old plants were removed from inoculated MS media agar and were washed in phosphate buffer solution, with vortex agitation. Extracted liquid material was serially diluted with Dorn mineral salts medium before plating on Dorn medium plates supplemented with Bz as the sole carbon and energy source. The CFU/mg FW were determined after 48 h of incubation at 30°C. For endophytic colonization tests, three weeks old plantlets inoculated with GFP-labeled PsJN strains were removed from the agar plates, and surface sterilized with 70% ethanol for 1 min, followed by 1% commercial chlorine bleach and a 0.01% Tween 20 solution for 1 min, and then washed three times in sterile distilled water (adapted from Compant *et al*, 2005). Plating the distilled water from a final wash on R2A medium routinely controlled sterility on these plants. Then, the sterilized plant material was macerated in sterile mortars and the disrupted tissue was resuspended in 1 ml of sterile 50 mM phosphate buffer to obtain an aqueous extract. CFU/mg FW were determined by serial dilutions of these extracts in R2A agar plates after 48 h of incubation at 30°C and examined under UV light using an Optical Epi-fluorescence Nikon Eclipse 50i microscope (Nikon, Japan). Experiments were conducted with six plants analyzed for each treatment, in two biological replicates.

Determination of the plant growth parameters.

Twenty-five plantlets from each inoculated treatment, as well as twenty-five non-inoculated plantlets, were analyzed. Root length and lateral roots number were measured directly in harvested plants, and fresh weight was recorded as previously described (Compant *et al*, 2005; Sessitsch *et al*, 2005). The chlorophyll contents were also determined following a published procedure (Porra et al. 1989). Chlorophyll was extracted

from leaves of *A. thaliana* in *N,N*-9-dimethylformamide for 24 h at 4°C in the dark, and chlorophyll a and chlorophyll b concentrations were measured simultaneously by spectrophotometry. For root hair number measurements, 1 cm distance segments from root tip of primary root were analyzed using light microscopy.

Analytical methods

The presence of IAA and intermediates was determined by high-performance liquid chromatography using cell-free supernatants from cells grown on this compound. Samples (20 µl) were obtained at different times of the growth curve and injected into a Hitachi LaChrom Elite liquid chromatograph equipped with a Kromasil 100-3.5 C18 4.6 µm diameter column. A methanol-H_2O (40:60) mixture containing 0.1% (vol/vol) phosphoric acid was used as the solvent, at a flow rate of 1 ml min^{-1}. The column effluent was monitored at 210 nm. Retention times for IAA, catechol, compound 1, compound 2, and compound 3, were 6.75, 2.95, 2.77, 2.04, and 1.60, respectively.

Chemicals

IAA, indole, indole-3-pyruvate, indole-3-acetamide, indole-3-acetonitrile and tryptamine were purchased to Sigma-Aldrich (Steinheim, Germany). Bz, fructose, succinic acid, tryptophan, anthranilic acid and L-arabinose were purchased to Merck (Darmstadt, Germany).

Statistical analysis.

Data for promoter activities, plant growth parameters and population density (CFU/mg FW) were statistically analyzed using one-way analysis of variance. The

chlorophyll contents and CFU/mg FW were subjected to logarithmic transformation before data analysis. When analysis of variance showed significant treatment effects, the Tukey's HSD (Honestly Significant Difference; $p < 0.05$) test was applied to make comparisons between treatments.

RESULTS

IAA synthesis by *B. phytofirmans* PsJN

Although Sun *et al* (2009) reported that strain PsJN was able to synthesize IAA in detectable amounts, additional research is required about this potentially relevant bacterial feature, in the context of plant-PGPR interactions. To evaluate in more detail IAA biosynthesis in *B. phytofirmans* PsJN, we performed batch growth cultures of this strain on different compounds: a sugar, fructose; a dicarboxylic acid, succinate; and an aromatic acid, benzoate (Bz), as the sole carbon and energy sources. Detection and quantification of the production of IAA, using a colorimetric method with Salkowski's reagent (Glickmann and Dessaux, 1995), was carried out at the stationary phase, once the carbon source was completely consumed and biomass reached OD600 approx. 1.0 (~$1x10^9$ cells/ml). Results indicated that wild type strain is unable to produce IAA at detectable levels (detection limit, ~2 μM) with these carbon sources (data not shown). As previous reports mentioned that IAA production occurs in the presence of the amino acid tryptophan in several bacterial strains (Spaepen and Vanderleyden, 2011), we evaluated whether the presence of this amino acid in the growth medium stimulates IAA production in strain PsJN. Effectively, significant amounts of IAA were detected in the supernatants of cultures of strain PsJN 1 h after exposure to (2.5 mM) tryptophan, in all conditions tested; (strain grown in fructose, 47.4 ± 9.8 μM; succinate, 17.0 ± 8.9 μM; Bz, 26.3 ± 7.7 μM), indicating that IAA biosynthesis in this bacterium is tryptophan-dependent. Subsequently, IAA production was determined at different tryptophan concentrations in cultures with fructose as growth substrate, chosen because produces higher IAA levels. As low as 0.1 mM tryptophan allowed significant IAA synthesis (16.9 ± 0.6 μM IAA). IAA synthesis reached a plateau at

tryptophan concentrations of 1.0 mM (44.2 ± 9.6 μM IAA) (Fig. 3A). Synthesis of IAA begins quickly (5-10 min) after addition of tryptophan (data not shown), suggesting that proteins involved in production of IAA are already present in the grown cells when tryptophan is supplied. Synthesis of IAA is not related to the growth phase, as it is observed with other bacteria such as *A. brasilense*, *P. putida* or *E. cloacae*, which high levels of IAA accumulated in culture media after entrance into the stationary phase (Vande Broek *et al*, 1999; Patten and Glick, 2002b; Saleh and Glick, 2001), because IAA was detected in the supernatant of *B. phytofirmans* cultures exposed to tryptophan at different growth stages (data not shown). The production of IAA showed a maximum at 8 h in fructose, succinate and Bz (Fig. 3B), but a decrease to below detection levels was observed after 24 h (Fig. 3B), suggesting that IAA is degraded or internalized after its synthesis.

Tryptophan-dependent IAA synthesis pathways in *B. phytofirmans* PsJN

As mentioned before, several tryptophan-dependent IAA synthesis pathways have been reported in bacteria: indole-3-acetamide, indole-3-pyruvate, indole-3-acetonitrile, tryptamine and tryptophan side-chain oxidase pathways (Fig. 1), although no protein or gene sequence has been reported for tryptophan side-chain oxidase pathway to date (Oberhänsli *et al* 1991; Spaepen and Vanderleyden, 2011).

To determine which IAA synthesis pathway(s) is(are) functional in *B. phytofirmans* PsJN, we exogenously supplied available key intermediates in fructose-grown cells and follow IAA production. Results shown in Table 3 indicate that strain PsJN transformed

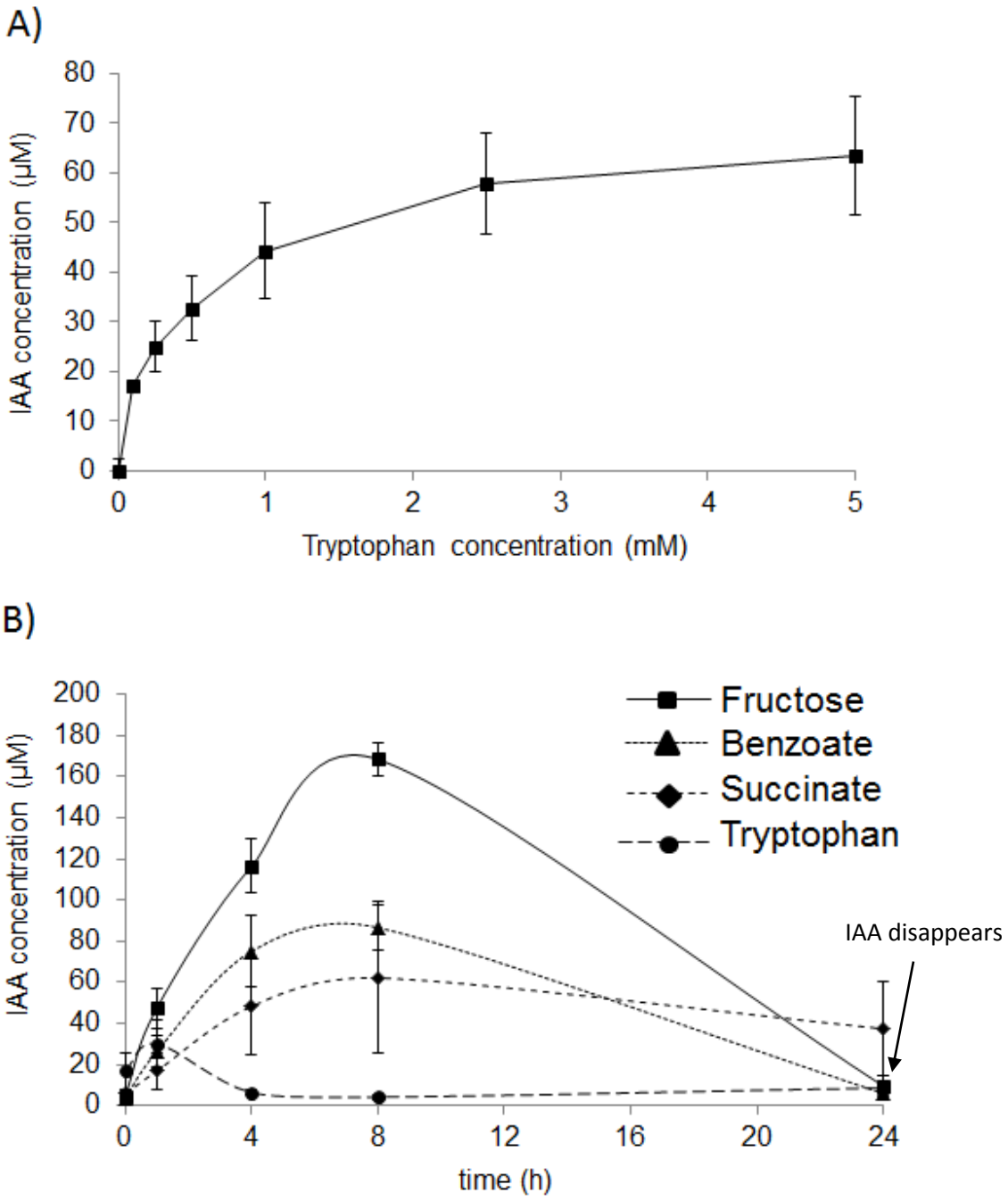

Figure 3: Production and subsequent disappearance of indole-3-acetic acid (IAA) by *Burkholderia phytofirmans* PsJN. A) IAA production in strain PsJN exposed to increasing tryptophan concentrations after 1 h at stationary phase. B) IAA levels produced at different times by the wild type strain after growth on different carbon sources; 2.5 mM tryptophan was added at 0 h. Vertical bars represent standard deviations from the mean.

Table 3. Assay of tryptamine, indole-3-acetamide, indole-3-acetonitrile and indole-3-pyruvate transformation into indole-3-acetic acid (IAA) by cells of *Burkholderia phytofirmans* PsJN.

Triptamine		
time (h)	*B .phytofirmans* PsJN	Control
0	-	-
2	-	-
20	+ (red)	-
40	-	-
indole-3-acetamide		
time (h)	*B .phytofirmans* PsJN	Control
0	+ (violet)	+ (violet)
2	+ (violet)	+ (violet)
20	+ (red)	+ (violet)
40	-	+ (violet)
indole-3-acetonitrile		
time (h)	*B .phytofirmans* PsJN	Control
0	-	-
2	-	-
20	-	-
40	-	-
indole-3-pyruvate		
time (h)	*B .phytofirmans* PsJN	Control
0	+ (red)	+ (red)
2	+ (red)	+ (red)
20	-	+ (red)
40	-	+ (red)

Bacterial cells were grown on 10 mM of fructose to stationary phase of growth. At this point, bacterial cells were incubated with 0.25 mM indole-3-acetamide, indole-3-pyruvate, indole-3-acetonitrile or tryptamine, and samples were taken at different times; then, IAA synthesis was measured on supernatants of cultures using Salkowski reagent as described previously (Glickmann and Dessaux, 1995). Three replicates were performed for each IAA synthesis measurement.

+ = Positive detection of indole-3-acetic (or indole-3-pyruvate), red color; or indole-3-acetamide, violet color.

- = No detection.

indole-3-acetamide (violet color) and tryptamine (no color) into IAA (red color) transiently. In turn, indole-3-pyruvate (red color) was removed and indole-3-acetonitrile remained untouched, suggesting that at least three IAA synthesis pathways could be functional in strain PsJN (indole-3-acetamide, trytamine and indole-3-pyruvate). To determine the gene(s) responsible(s) for IAA production in *B. phytofirmans* PsJN, a BLASTP analysis (Altschul *et al*, 1997) was performed using sequences from proteins with confirmed biochemical function in tryptophan-dependent IAA biosynthesis.

To search in strain PsJN genome for proteins involved in indole-3-acetamide pathway (Fig. 1), we used as probe protein sequences of tryptophan monooxygenase (IaaM) and indole-3-acetamide hydrolase (IaaH) from *P. savastanoi* (Yamada *et al*, 1985), *A. tumefaciens* (Bonnard *et al*, 1991) or *B. japonicum* (Sekine *et al*, 1989). Remarkably, IaaM proteins homologous were not present in *B. phytofirmans* PsJN, although more than fifteen IaaH-like protein sequences were found (amino acid identities ranging 25-40%). For indole-3-pyruvate pathway (Fig. 1), we used protein sequences of the key enzyme indole-3-pyruvate decarboxylase (IpdC) described in *A. brasilense* (Costacurta *et al*, 1994), *Enterobacter cloacae* (Koga *et al*, 1991b), *Methylobacterium extorquens* (Fedorov *et al*, 2010) and *Paenibacillus polymyxa* (Phi *et al*, 2008). We found more than thirteen IpdC-like protein sequences in strain PsJN genome, but with low amino acid identities (ranging 20-35%). Furthermore, we used amine oxidase of *Klebsiella aerogenes* with confirmed biochemical function (Sugino *et al*, 1992) to make the genome search for tryptamine pathway related-genes (Fig. 1). We found a single gene (Bphyt_1997, named *maoA*) with low amino acid identity (33%), which is clustered with a putative indole-3-acetaldehyde dehydrogenase. Although indole-3-acetonitrile remained untouched in the assay shown in Table 3, we still searched for proteins involved in the related pathway (Fig.1). We used

nitrilase gene reported in *Alcaligenes faecalis* (Kobayashi *et al*, 1993) and *A. thaliana* (Bartel and Fink, 1994); six nitrilase-related genes were found (25-35% of amino acid identity).

Growth of *B. phytofirmans* PsJN on IAA.

The tryptophan-dependent IAA production and subsequent disappearance (Fig. 3B), and the transient IAA formation from indole-derivatives found with cells of strain PsJN (Table 3), raises the possibility that *B. phytofirmans* may degrade IAA. To evaluate this possibility, growth tests in minimal medium cultures with exogenously supplied auxin (2.5 mM) were carried out. Results showed that strain PsJN uses IAA as the sole carbon and energy source, and also as the sole nitrogen source (Fig. 4). Growth on IAA showed an unusually long lag phase of approximately 20 h (Fig 4), unlike the growth on other compounds, e. g. aromatic compound Bz (lag phase approx. 5 h).

Functional IAA degradation genes in *B. phytofirmans* PsJN.

We examined if genes involved in auxin catabolism described in a previous report (Leveau and Gerards, 2008), have homologues in *B. phytofirmans* PsJN. Ten *iac* genes from *P. putida* 1290: *iacA, iacB, iacC, iacD, iacE, iacF, iacG, iacH, iacI* and *iacR* genes have been involved in IAA catabolism (Leveau and Gerards, 2008; Scott *et al*, 2013; Fig. 5). *iac* genes were also found in *B. phytofirmans* PsJN, although with different gene synteny (Leveau and Gerards, 2008; Fig. 5), with amino acid identities ranging from 38 to 62% (Table 4). Only *iacR* gene, a potential transcriptional repressor belonging to MarR family (Scott *et al*, 2013) is absent in the genome of strain PsJN, whereas genes *iacA, iacF*

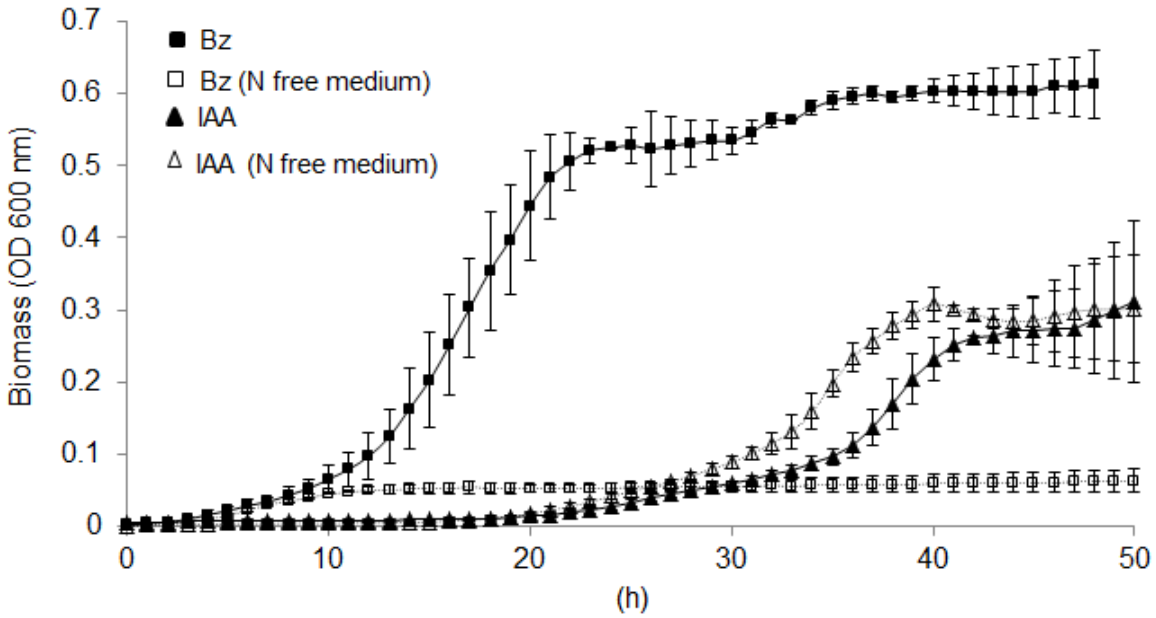

Figure 4: Growth of *Burkholderia phytofirmans* on indole-3-acetic acid (IAA) and benzoate as sole carbon, nitrogen and energy source. Three replicates were performed for growth measurements. Vertical bars represent standard deviation from the mean.

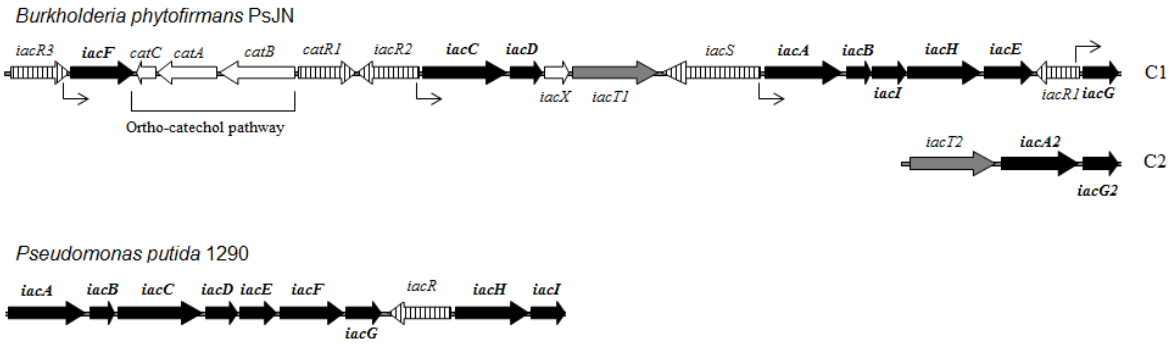

Figure 5: Gene clusters putatively involved in indole-3-acetic acid (IAA) catabolism in *Burkholderia phytofirmans* PsJN and *Pseudomonas putida* 1290. Black boxes indicate putative encoding-genes directly involved in IAA degradation, grey boxes indicate probable transporters of IAA and dashed boxes indicate putative regulators of IAA degradation genes. Genes involved in catechol catabolism are shown in brackets. Arrows indicate promoter sequences from *iac* genes cloned to construct *lacZ* transcriptional fusions. C1, located in chromosome 1; C2, located in chromosome 2.

Table 4. *iac* genes of *Pseudomonas putida* 1290 present in *Burkholderia phytofirmans* PsJN.

***P. putida* 1290 gene; Accession number; Automatic annotation**	***B. phytofirmans* PsJN locus tag**	**Amino acid identity**
iacA; ABY62757; Acyl-CoA dehydrogenase-like	Bphyt_2161, Bphyt_6911	56%, 49%
iacB; ABY62758; Conserved hypothetical protein	Bphyt_2162	57%
iacC; ABY62759; Aromatic ring hydroxylating dioxygenase	Bphyt_2156	62%
iacD; ABY62760; Aromatic ring hydroxylating dioxygenase	Bphyt_2157	44%
iacE; ABY62761; Short-chain dehydrogenase/reductase	Bphyt_2165	52%
iacF; ABY62762; Ferredoxin	Bphyt_2150, Bphyt_4243	41%, 38%
iacG; ABY62763; Flavin reductase	Bphyt_5028, Bphyt_2167, Bphyt_6112	53%, 50%, 47%
iacH; ABY62765; Glu-tRNA amidotransferase	Bphyt_2164	52%
iacI; ABY62766; Conserved hypothetical protein	Bphyt_2163	44%
iacR; ABY62764; Transcriptional regulator MarR family	absent	NA

NA: not apply

Automatic annotation; *iac* genes of *P. putida* 1290 have highest BLASTP similarity to sequenced strain *P. putida* GB-1, which possess *iac* genes automatic annotated (Leveau and Gerards, 2008).

and *iacG* have more than one copy (Table 4).

The *iac* genes in the genome of strain PsJN, presumably involved in the IAA degradation pathway (see below), are close to other genes that possibly play regulatory and transporter roles in degradation of IAA (Fig. 5). They are a two component system, a histidine kinase signal transducer and a LuxR family protein receptor, provisionally named *iacS* and *iacR1,* respectively, which are near to *iacA* and *iacG* genes; two transcriptional regulators belonging to LysR family, provisionally named *iacR2* and *iacR3,* which are near to *iacC* and *iacF* gene, respectively; and a transporter-related gene belonging to the major facilitator superfamily (MFS), named *iacT1*.

To test if these *iac* genes are involved in IAA degradation by strain PsJN, the corresponding mutants were generated for each *iac* gene and growth tests were performed with IAA as the sole carbon and energy source. These mutants also included the putative IAA gene regulators and the IAA transporter gene not reported in *P. putida* (*iacR1*, *iacR2*, *iacR3* and *iacT1*) (Fig. 5). With the exception of the *iacG* gene, the putative regulatory genes *iacR3* and *iacS*, and the putative transporter *iacT* gene, all other *iac* genes were essential for growth of strain PsJN and IAA degradation, as no growth of such mutant strains on IAA was detected, even if bacteria were cultured for seven days (Table 5). Growth on IAA was recovered in the corresponding mutants when the mutated gene was introduced to complement PsJN derivatives. In the case of the *iacG* and *iacS* gene mutants, growth was partially impaired (Table 5), suggesting that the function of *iacG* and *iacS* gene is replaced by other gene function in strain PsJN. Remarkably, *iacA*, *iacG* and *iacT* genes possess homologues in the genome (referred as *iacA2*, *iacG2* and *iacT2*) which are clustered altogether (Fig. 5). Single mutants of these genes have no influence in growth on IAA as its behavior is quite similar to the wild type strain (data not shown), although

Table 5. Growth of strain PsJN and its derivative mutants in indole-3-acetic acid (IAA) or benzoate (Bz).

Strain	IAA	Bz
WT	+	++
ΔiacA	-	++
ΔiacB	-	++
ΔiacC	-	++
ΔiacD	-	++
ΔiacE	-	++
ΔiacF	-	++
ΔiacG	+/-	++
ΔiacH	-	++
ΔiacI	-	++
ΔiacR1	-	++
ΔiacR2	-	++
ΔiacR3	+	++
ΔiacS	+/-	++
ΔiacT1	+	++
ΔcatA	-	++

++, growth concluded at 24 h; +, growth concluded at 48h; +/-, growth concluded at 60h; -, no growth at 7 days.

its participation in IAA metabolism cannot presently be discarded.

Participation of *ortho* catechol degradation genes in IAA catabolism.

Based on mutant strains related to catechol degradation through *ortho*-cleavage pathway that turned brown IAA plates (Leveau and Gerards, 2008), and cell extracts of IAA-grown *P. putida* 1290 that showed elevated levels of catechol 1,2-dioxygenase activity (Leveau and Lindow, 2005), Leveau and Gerards (2008) proposed that IAA catabolism in *P. putida* produces catechol as intermediate, possibility that was recently confirmed by Scott *et al* (2013), through GC-MS analysis. Interestingly, the genome of *B. phytofirmans* PsJN has *ortho* catechol pathway genes named *catA*, *catB* and *catC*, encoding catechol 1,2-dioxygenase, muconate cycloisomerase and muconolactone isomerase, respectively, close to or as part of the *iac* cluster (Fig. 5). These genes encode conversion of catechol via *cis,cis*-muconic acid to 3-oxoadipate enol-lactone (Harwood and Parales, 1996).

To evaluate if catechol effectively is an intermediate in IAA degradation in strain PsJN, a *catA* mutant was generated. As in Leveau and Gerards (2008), strain PsJN *catA* gene mutant turned brown IAA plates (data not shown), suggesting accumulation and posterior polymerization of catechol. More relevant, this *catA* mutant is unable to grow on IAA (Table 3), strongly indicating the involvement of this function on IAA degradation. Remarkably, a second catechol 1,2-dioxygenase (named *catA2*) is present in the strain PsJN genome, clustered with Bz 1,2-dioxygenase genes, and therefore, involved in Bz catabolism. The *catA2* gene seems not to be involved in IAA degradation because *catA* gene mutant completely lost this ability, and consistently, grows on Bz (Table 5).

IAA dependent *iac* and *cat* gene expression.

To further evaluate functionality of *iac* genes, a quantitative real-time PCR analysis of RNA extracted from mid growth phase cells of *B. phytofirmans* PsJN grown on IAA, fructose or Bz as the sole carbon and energy source was performed. Results showed that mRNA levels of *iacA*, *iacC*, *iacF* and *iacG* genes in IAA-grown cells were at least two orders of magnitude higher than in cells grown on fructose or Bz (Fig. 6). Interestingly, mRNA levels of redundant *iacA2* gene was also increased in IAA-grown cells (Fig. 6), suggesting a functional role in IAA degradation. On the other hand, *catA* mRNA levels in IAA-grown cells was about 500 times higher than in fructose-grown cells (Fig. 6), suggesting that this catechol 1,2-dioxygenase is strongly induced during growth with IAA. In contrast *catA2* mRNA was not increased in IAA-grown cells but it was induced in Bz-grown cells (Fig. 6).

Promoter activity of *iac* genes

The presence of a set of putative regulators of the IAA pathway (*iacS*, *iacR1*, *iacR2*), made interesting to study possible inducers of *iac* gene promoters. We performed ß-galactosidase transcriptional fussion assays with promoters of *iacABIHE*, *iacCD*, *iacF*, *iacG* and *catBAC* genes (see Fig. 5), in a background in which single *iac* genes were mutated, therefore, some IAA degradation intermediates accumulated. Firstly, we checked *iac* promoters in the wild type strain, and we found that all *iac* gene promoters were induced in the presence of IAA (Fig. 7A). We also determined that indole, indole-3-pyruvate, indole-3-acetamide, tryptophan, anthranilate or Bz did not induce *iacABIHE* or *iacCD* promoter activity (data not shown).

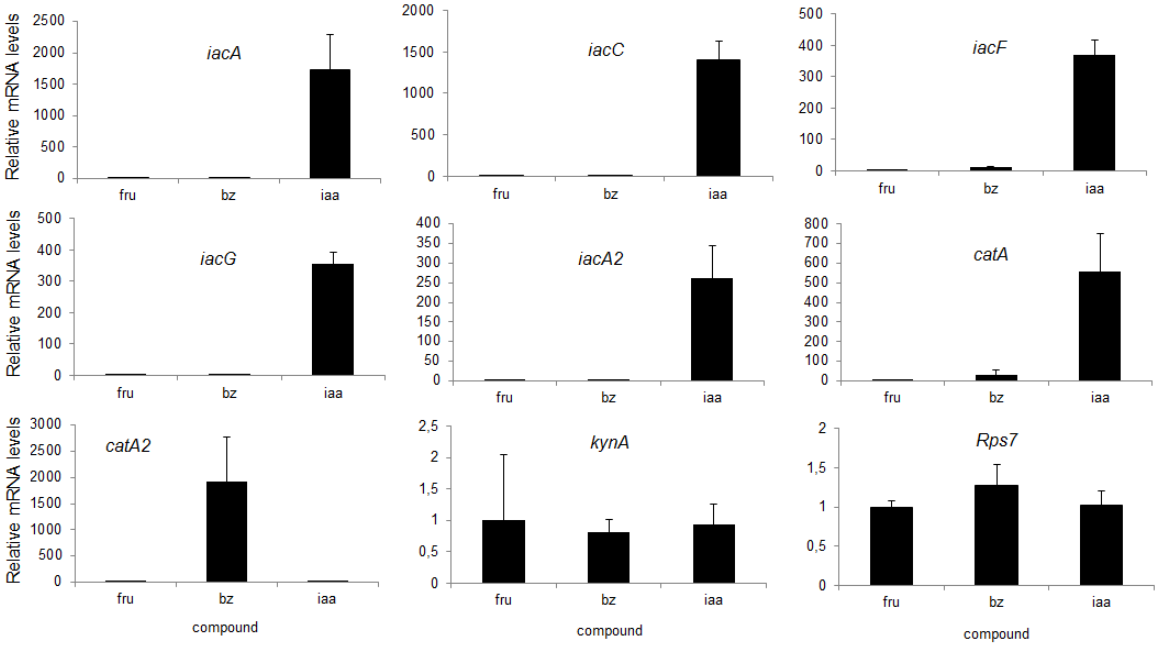

Figure 6: mRNA increased levels of *iac* and *cat* genes from *Burkholderia phytofirmans* exposed to indole-3-acetic acid (IAA). Real-time PCR analysis of *iac* and *cat* genes in cells grown in fructose (fru), benzoate (bz) or IAA (iaa) as the sole carbon and energy source. Ribosomal protein S7 (*rpS7*) and tryptophan dioxygenase (*kynA*) genes were used as control genes. mRNA levels were normalized to the average value of the mRNA level in fructose treatment. 16S rRNA was used as housekeeping. Note different scales. All experiments were performed in three biological and two technical replicates. Vertical bars represent standard deviations from the mean.

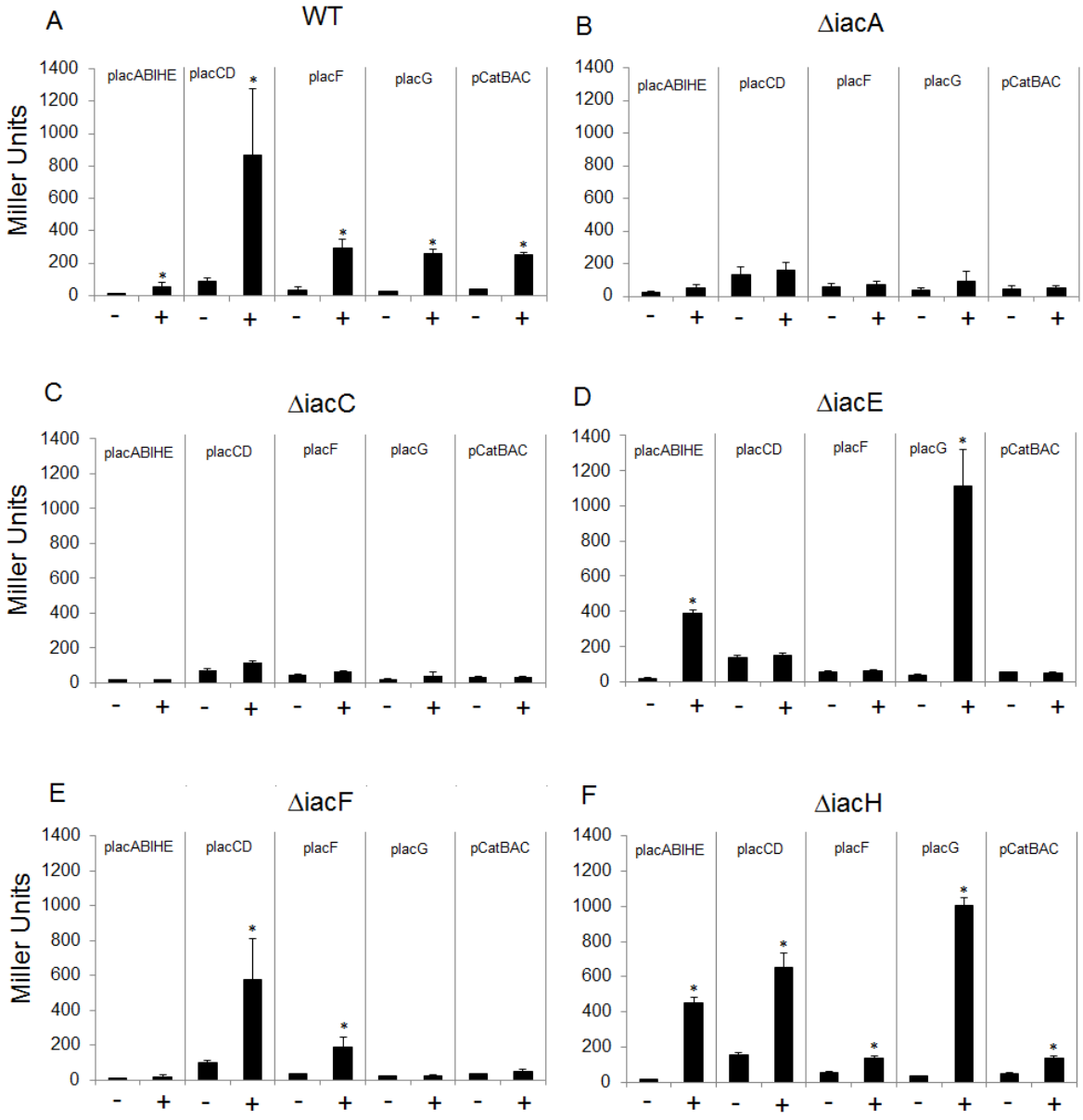

Figure 7: Promoter activity of *iac* and *cat* genes exposed to indole3-acetic acid (IAA) in *B. phytofirmans* PsJN. β-galactosidase activity levels obtained from transcriptional fusions of *iacABIHE*, *iacCD*, *iacF*, *iacG* and *catBAC* promoters exposed to IAA in A) WT or its derivative strains, B) Δ*iacA*, C) Δ*iacC*, D) Δ*iacE*, E) Δ*iacF* and F) Δ*iacH*. +, IAA 0.25 mM; -, water. Vertical bars represent standard deviation from the mean. Asterisk indicates statistical significance (one-way analysis of variance, $p < 0.05$).

No promoters in the *iacA* mutant were induced with IAA (Fig 7B), indicating that auxin *per se* is not the inducer molecule of *iac* promoters. The *iacE* mutant showed induction of *iacABIHE* and *iacG* gene promoters (Fig. 7D), suggesting that the inducer was produced before the IacE-catalized step. In the case of the *iacF* mutant, the *iacC* and *iacF* gene promoters were induced (Fig. 7E). Surprisingly, none of the promoters were induced in the *iacC* mutant (Fig. 7C), suggesting that IAA in this mutant is quickly transformed to an intermediate with non-induction capacity. Finally, all promoters were induced in the *iacH* mutant (Fig. 7F), suggesting that all the intermediates that are inducers are produced before the pathway step catalyzed by the *iacH* gene.

Resting cells IAA degradation by *B. phytofirmans* PsJN and *iac* mutants.

To determine IAA consumption and accumulation of intermediates in *iac* mutants, resting cells assays were performed and the supernatants were analyzed through HPLC-UV. Results showed that wild type strain completely consumed IAA in 36-40 h (Fig. 8), whereas *iacB*, *iacC*, *iacE*, *iacH* and *iacI* gene mutants were completely unable to transform IAA, or were very slow consuming IAA, since even after 6 days of incubation 85-100% of the substrate remained intact (Fig. 8). The *iacG* mutant fully degraded IAA as the wild type strain (Fig. 8), supporting the notion that the activity encoded by *iacG* gene is redundant. In turn, *iacA* and *iacF* gene mutants completely degraded IAA although degradation was completed in 6 days (Fig. 8), indicating that *B. phytofirmans* has additional functions (Table 4) that replace these mutated genes. No IAA intermediates were detected using the resting cells approach.

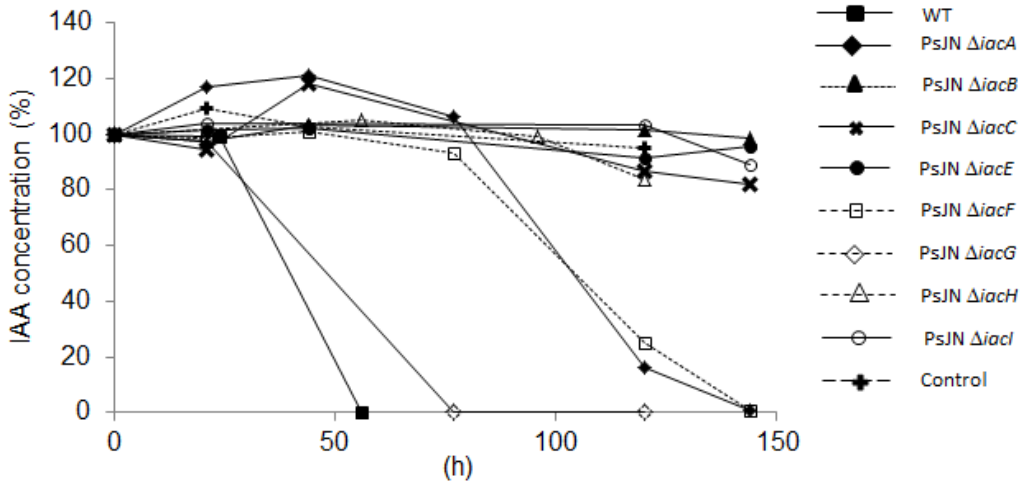

Figure 8: Indole-3-acetic acid (IAA) degradation by resting cells of *Burkholderia phytofirmans* and selected *iac* genes catabolic mutants. IAA concentration is shown as percent of initial concentration (2.5 mM).

Expression of the *iac* genes cluster in a heterologous strain

A different approach was chosen to detect some IAA degradation intermediates. The *iac* genes cluster (*iacABIHECDGF*) of strain PsJN, presumably encoding all the enzymes involved in IAA degradation, was cloned and introduced into the heterologous bacterial host, taxonomically and metabolically related to *B. phytofirmans*, *C. pinatubonensis* JMP134 (Pérez-Pantoja *et al*, 2008; Lykidis *et al*, 2010), which is unable to use IAA as sole carbon and energy source and lacks *iac* genes (data not shown). Strain JMP134 derivative carrying this *iac* cluster turned brown IAA plates (data not shown), indicating IAA degradation and catechol accumulation, as in the *catA* mutant PsJN strain. Resting cells assays of this derivative, using 1 mM IAA, demonstrated IAA consumption and transient catechol production (data not shown). Interestingly, an additional intermediate was detected in HPLC-UV profiles (designated compound 2, Fig. 9A), and not removed even after longer (43 h) incubations of the cells from this strain JMP134 derivative (data not shown). When strain PsJN IAA-grown cells were exposed to the supernatant containing compound 2, complete removal of this compound was observed (data not shown), indicating that an additional *iac* gene function is missing in the *iacABIHECDGF* cluster. Additionally, incomplete *iac* gene clusters lacking one or two *iac* genes were cloned in *C. pinatubonensis* to help elucidation of the steps in IAA degradation pathway encoded by each *iac* gene. We generated *iac* gene cluster plasmids without *iacA* (*iac*ΔA), *iacB* (*iac*ΔB), *iacCD* (*iac*ΔCD), *iacE* (*iac*ΔE), *iacF* (*iac*ΔF), *iacG* (*iac*ΔG), *iacH* (*iac*ΔH) or *iacI* (*iac*ΔI) and were cloned in *C. pinatubonensis* JMP134. Then, derivatives carrying plasmids with incomplete *iac* clusters were incubated with 1 mM IAA and supernatants analyzed at different times by HPLC-UV. As expected, strain JMP134 *iac*ΔA, was unable to degrade

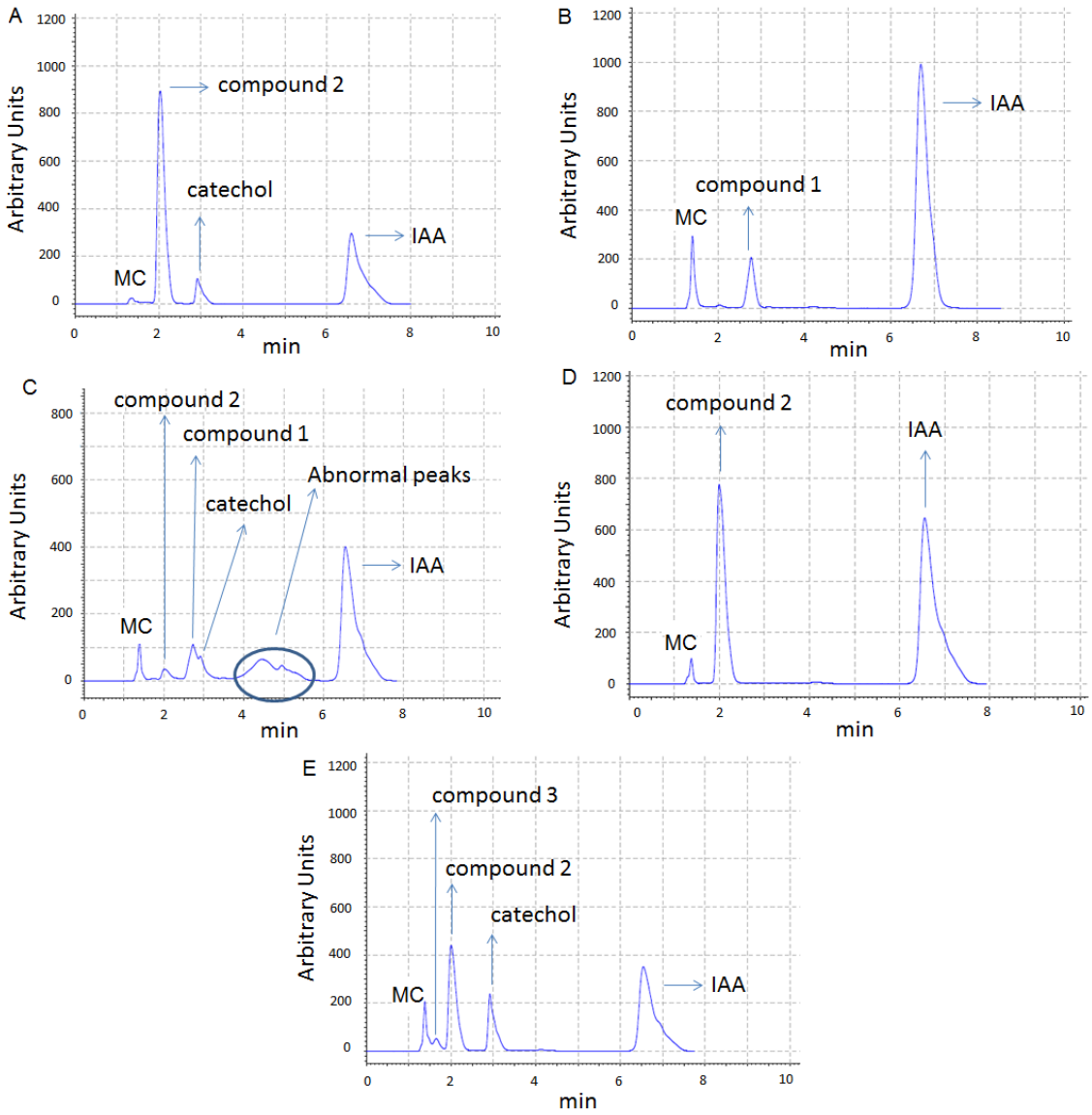

Figure 9. HPLC-UV chromatograms of supernatants from cultures of derivatives of *Cupriavidus pinatubonensis* JMP134 harboring different *iac* gene constructs. A) *iacABIHECDGF*, B) *iac*ΔE, C) *iac*ΔB, D) *iac*ΔCD and E) *iac*ΔH exposed to indole-3-acetic acid (IAA) 1 mM for 1 h. MC, a signal found in all chromatograms, even in controls without bacteria.

IAA (data not shown), suggesting that *iacA* gene encodes the first step in the IAA degradation pathway. Strain JMP134 (*iac*ΔG) was able to degrade IAA, although more slowly as more than the half of IAA was still present after 10 h of incubation (data not shown). These results suggest that *iacA* and *iacG* genes work together or in consecutive steps and participate in the initial attack on IAA. Strain JMP134 (*iac*ΔE) completely removed IAA from the medium after 10 h, accumulating in the first hour a single intermediate designated compound 1 (Fig. 9B). Compound 2 was not present in this supernatant, suggesting that the *iacE* gene encoded product is formed in an IAA catabolic pathway step before compound 2 is produced. Strain JMP134 (*iac*ΔB) degraded IAA completely, and accumulated compound 1, minor amounts of compound 2, catechol and two other abnormal peaks (Fig. 9C). The latter two were also detected with strain JMP134 (*iac*ΔE) cells at 10 h (data not shown), suggesting that product of *iacB* gene could be acting together with the product of the *iacE* gene, possibly as an auxiliary enzyme. Cells of *C. pinatubonensis* JMP134 *iac*ΔCD, *iac*ΔF and *iac*ΔI completely degraded IAA and accumulated high amounts of compound 2 (Fig. 9D; data not shown). These results indicate that *iacCDFI* genes possibly were involved in the same step, probably rate limiting. Finally, strain JMP134 (*iac*ΔH) fully degraded IAA, and accumulated three different intermediates, one of them would be compound 2, other designated compound 3 and catechol (Fig. 9E), suggesting that the *iacH* gene encoded product could be involved in the last step: IAA transformation to catechol.

Effect of an IAA degradation mutant of *B. phytofirmans* on growth promotion and colonization of *A. thaliana.*

To study if IAA degradation by *B. phytofirmans* PsJN has a role on plant growth promotion, the *iacC* gene mutant unable to grow on IAA (Table 5) was analyzed. After three weeks of inoculation of a gnotobiotic *A. thaliana* culture with the *iacC* gene mutant, no differences were observed in number of root hairs, chlorophyll content, and number of lateral roots with respect to plants inoculated with the wild type PsJN (Fig. 10A). However, the growth promotion effects of the wild type strain on fresh weight and primary root lengths were not observed with the *iacC* gene mutant (Fig. 10A), indicating that degradation of IAA by *B. phytofirmans* PsJN is required for full plant growth promotion. Neither the rhizospheric (9.4±0.1 log CFU/mg FW, vs. 9.84±0.01 log CFU/mg FW) nor the endophytic (6.8±0.6 log CFU/mg FW vs. 7.9±0.5 log CFU/mg FW) colonization levels in *A. thaliana* were affected in this mutant compared with the wild type PsJN.

To further study the effect of IAA degradation on growth promotion by *B. phytofirmans*, the abilities of wild type strain and the *iacC* gene mutant to abolish the effects of exogenously added IAA on root development were compared. Two concentrations of IAA were exogenously added when seeds were placed on plates. A sharp decrease in primary root lengths was verified at 1 μM IAA (Fig. 10B). The inoculation with the wild type strain completely prevented the primary root length inhibition effect of such concentration of IAA, whereas the *iacC* gene mutant only partially reverted such inhibition (Fig. 10B).

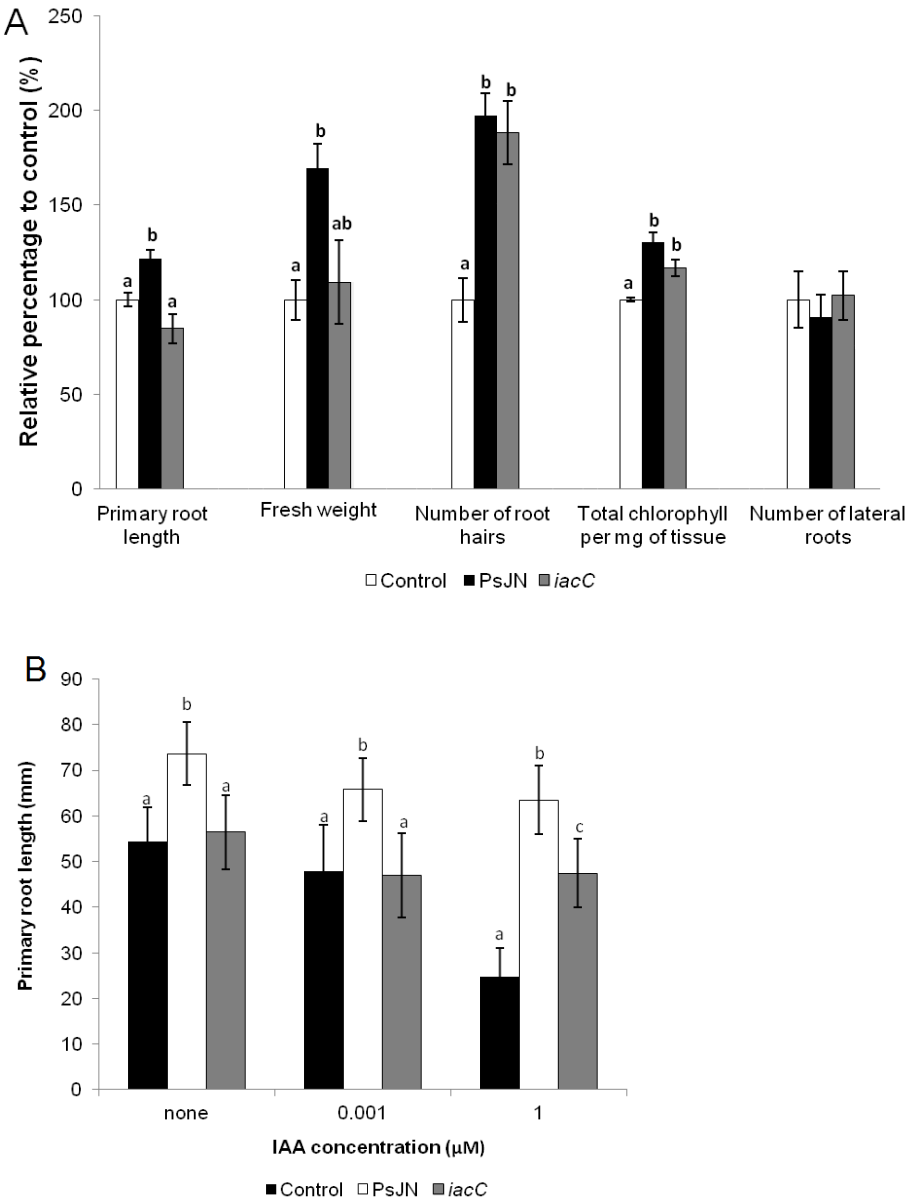

Figure 10. Indole-3-acetic acid (IAA) degradation plays a role in plant growth promotion of *Arabidopsis thaliana*. A) Plant growth and metabolic parameters in gnotobiotic *Arabidopsis thaliana* col-0 culture systems and B) Effect of exogenous addition of IAA on the elongation of primary roots inoculated with *Burkholderia phytofirmans* PsJN, *iacC* mutants or non-inoculated (control). Growth parameters were measured three weeks and ten days after inoculation for A) or B), respectively. The bars show mean percentage values with respect to control plants, and the error bars indicate standard deviations from average of three biological replicates experiments for each treatment. Different letters indicate statistically significant differences between treatments (One way ANOVA Tukey's HSD tests; $p < 0.05$).

Interplay between biosynthesis and degradation of IAA

As mentioned above, when *B. phytofirmans* PsJN is exposed to tryptophan, it synthesizes IAA and after 24 h, IAA disappears from supernatant (Fig. 3B). To test if IAA disappearance is linked to the *iac* genes mediated degradation, we carried out the same test with the *iacC* gene mutant. As expected, this mutant synthesized IAA when exposed to tryptophan (Fig. 11A), but unexpectedly, as in the wild type strain (Fig. 11A), was still showing the decrease in IAA levels after 24 h (Fig. 11A), despite this mutant was unable to degrade exogenously added IAA (Fig. 11B). Similar results were found for *iacA* gene and the *iacT1* mutant (data not shown), indicating that an alternative way of IAA removal is taken place in such experimental conditions. To clarify this point, a *B. phytofirmans* tryptophan 2,3-dioxygenase (*kynA*) gene mutant was constructed. As the first step in degradation of tryptophan is blocked, this mutant is unable to use tryptophan as the sole carbon and energy source, but is still able to degrade exogenously added IAA (Fig. 11B). Interestingly, disappearance of IAA after 48 h (Fig. 11A) did not occur in the *kynA* gene mutant, suggesting that tryptophan dioxygenase activity was in some way involved in IAA disappearance produced by biosynthesis in the wild type and in the *iacC* mutant. When the *kynA* gene mutant was exposed to equimolar mixtures of IAA-tryptophan, auxin utilization resulted impaired (Fig. 12), suggesting that *iac* genes are not responsible for degrading IAA generated from tryptophan, and possibly there is an alternative tryptophan dependent pathway of IAA degradation. To explore such possibility strain PsJN was grown on tryptophan as the sole carbon and energy source. Once the growth substrate was completely consumed and OD600 was about 1.0, additional tryptophan was added to induce IAA

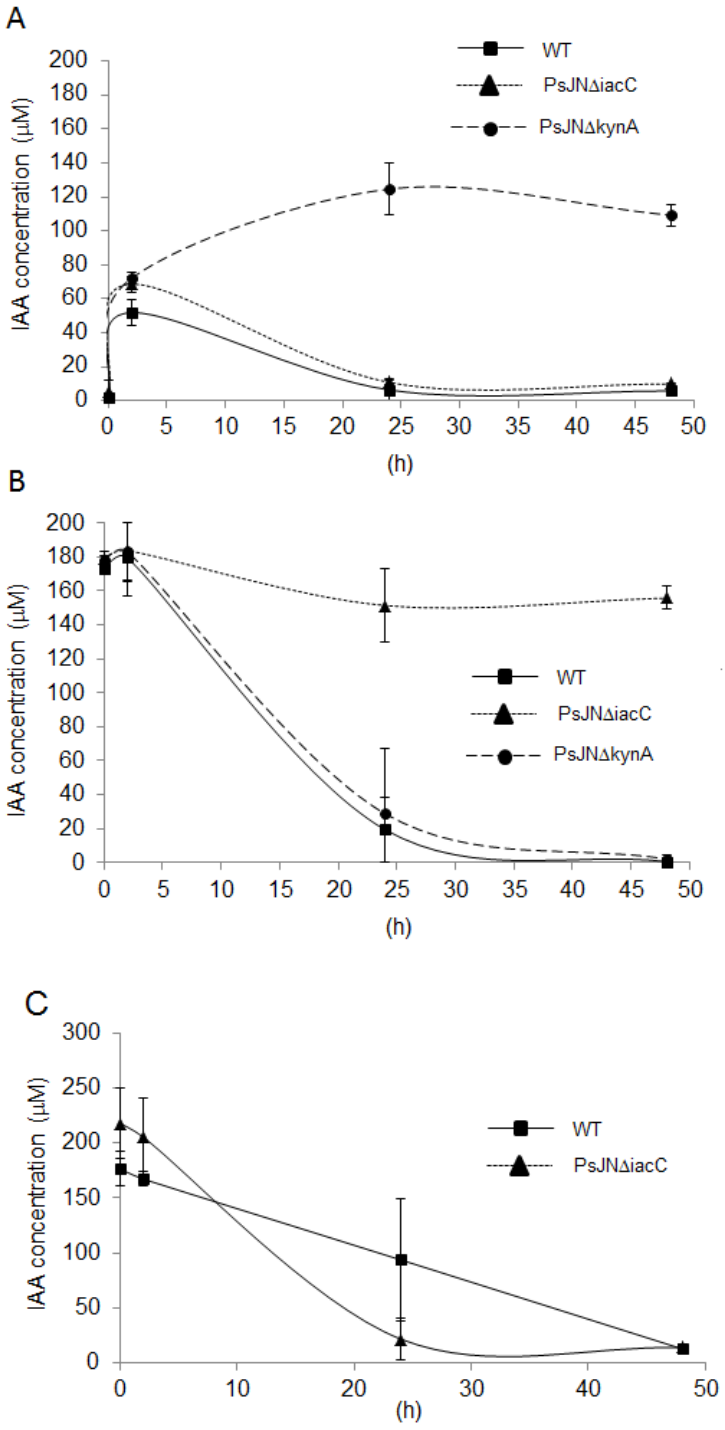

Figure 11: Interplay between indol-3-acetic acid (IAA) biosynthesis and a putative alternative IAA degradation pathway in *Burkholderia phytofirmans*. Strains were evaluated for A) tryptophan-dependent IAA biosynthesis, and degradation of exogenously added IAA in cells grown in B) fructose, or C) tryptophan. Three replicates were performed for growth measurement. Vertical bars represent standard deviation from the mean.

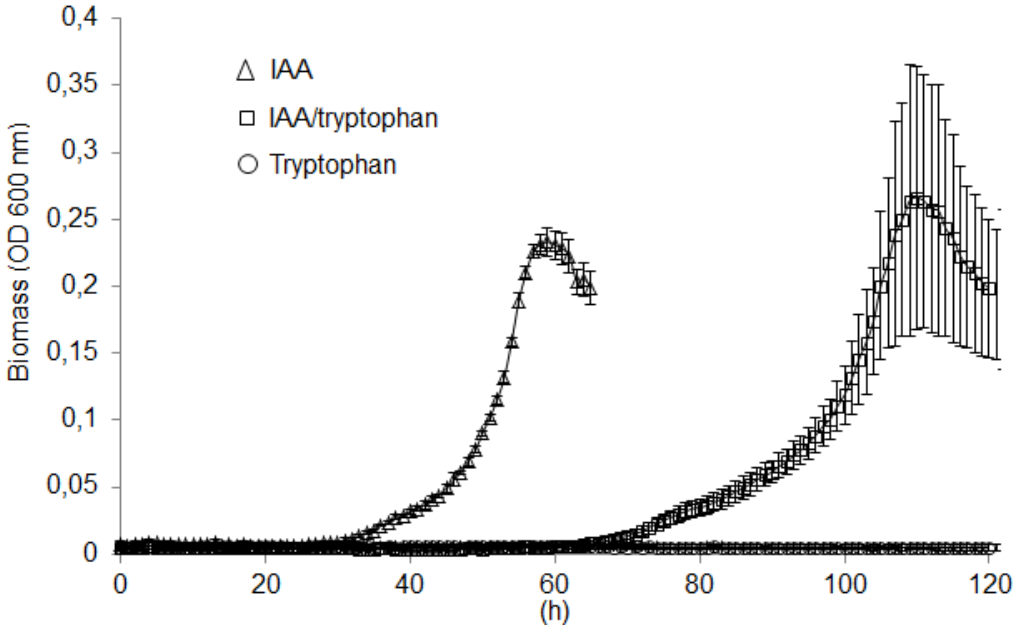

Figure 12: Tryptophan mediated delay of indole-3-acetic acid (IAA) consumption in strain PsJNΔ*kynA*. Growth of strain PsJNΔ*kynA* on indole-3-acetic acid (IAA) (open triangles), tryptophan (open circles) and IAA/tryptophan mixtures (open squares) as the sole carbon and energy source. IAA, 2.5 mM; tryptophan, 2.5 mM. Three replicates were performed for growth measurements. Vertical bars represent standard deviation from the mean.

synthesis. IAA production (30.2 ± 11.9 μM) after one hour was quite similar to other growth substrates tested (Fig. 3B). However, IAA completely disappeared from supernatant after 4 h (Fig. 3B), in contrast with other growth substrates in which complete removal of IAA was observed only after 24 h (Fig 3B), indicating tryptophan metabolism accelerates IAA disappearance. Remarkably, when the *iacC* mutant was grown on tryptophan as the sole carbon and energy source, and then IAA was exogenously added, this mutant degraded IAA (Fig. 11C), unlike to when the *iacC* mutant is grown on fructose (Fig. 11B), suggesting again an alternative IAA pathway dependent of tryptophan metabolism.

DISCUSSION

Biosynthesis of IAA in *B. phytofirmans* PsJN

IAA biosynthesis is widespread and it is related to diverse effects on plants (Patten and Glick, 1996; Spaepen *et al*, 2007a; Spaepen and Vanderleyden, 2011; Bhattacharyya and Jha, 2012). Nevertheless, it is known that IAA produced by PGPR enhances development of the host plant root system increasing nutrient absorption and establishment in soil (Barbieri *et al*, 1986; Dobbelaere *et al*, 1999; Patten and Glick, 2002a). Consequently, a better manipulation of the positive effects of PGPR on plants could be reached if IAA production is completely understood. We selected for this study the PGPR *B. phytofirmans* PsJN because it increases yields of agronomically important crops such as tomato, potato and grape (Frommel *et al*, 1991; Nowak *et al*, 1995; Pillay and Nowak, 1997; Bensalim *et al*, 1998; Ait Barka *et al*, 2000, 2002; Compant *et al*, 2005). Although IAA production in strain PsJN has been demonstrated (Sun *et al*, 2009), it is still poorly understood. In this thesis, we clearly demonstrated that strain PsJN is able to synthesize IAA in a tryptophan-dependent way, which might reflect that tryptophan acts as inducer or modulator, and/or provides some IAA precursor. These possibilities are plausible because IAA production at different tryptophan concentrations saturates quickly, and it is also triggered at low tryptophan levels.

It has been described that diverse factors influence bacterial IAA biosynthesis, such as pH, carbon starvation or osmotic stress, also suggesting IAA would be a signal molecule in plant-bacteria interactions (Spaepen *et al*, 2007a; Spaepen and Vanderleyden, 2011). Increased IAA synthesis has been reported in *Pseudomonas* and *Enterobacter* when stationary phase sigma factor RpoS is overexpressed (Saleh and Glick, 2001; Patten and

Glick, 2002b). The expression of the IAA biosynthesis *ipdC* gene in *A. brasilense* increases in slightly acid pH or carbon limitation in stationary phase of growth (Ona *et al*, 2005; Vande Broek *et al*, 1999, 2005). Also, *P. agglomerans ipdC* gene expression increases when bacteria is under osmotic stress but it is not responsive to pH changes (Brandl and Lindow, 1997), suggesting that IAA enhanced production is related to factors usually present in plant-related environments in which these bacteria thrive, such as acidic pH characteristic of rhizospheric soils colonized by *A. brasilense*, or variable water availability in leave surfaces where the epiphyte bacteria *P. agglomerans* proliferates (Brandl *et al*, 2001). In addition, plant extracts or exudates components, such as flavonoids, increase IAA synthesis (Prinsen *et al* 1991; Costacurta *et al*, 1998), and IAA has extensive transcriptional effects on *A. brasilense*, suggesting that auxin may be part of plant-bacteria molecular signaling (Van Puyvelde *et al*, 2011). We observed that strain PsJN synthesizes IAA in all growth phases, and possibly it is not regulated by stationary phase conditions, but the study of the effect of other conditions found in *B. phytofirmans* PsJN niches (both rizhospheric and endophytic), as well as the putative role as molecular signal, is still incomplete.

As the results of this thesis clearly show that IAA biosynthesis in strain PsJN requires tryptophan, the genetic determinants encoding tryptophan dependent-pathways were searched for. The results indicate that the indole-3-acetamide pathway is not complete although the strain transforms indole-3-acetamide to IAA (Table 3), which might be explained by the presence of an undetected gene sequence with low amino acid identity to the used probe, or by other biochemical steps. With respect to the indole-3-pyruvate pathway, although the precursor was transformed, we were unable to define a precise gene sequence, as about thirteen IpdC-like protein sequences were present in strain PsJN. In contrast, we found *maoA* gene from the tryptamine pathway, whose third step is shared

with the final steps of indole-3-pyruvate and tryptophan side-chain oxidase pathways (Fig. 1). Interestingly, *maoA* gene is located close to a putative indole-3-acetaldehyde dehydrogenase gene, suggesting an interesting target for further research on this pathway in strain PsJN. We found six nitrilase-related genes in strain PsJN, but with low amino acid identity, and because indole-3-acetonitrile remained intact in the resting cells assay (Table 3), we believe that the indole-3-acetonitrile pathway is not present in *B. phytofirmans*. Additional research (e. g. evaluate different experimental conditions) is required to completely discard the presence of this pathway in strain PsJN. Finally, the tryptophan side-chain oxidase pathway cannot be discarded, as no gene has been described to date (Spaepen and Vanderleyden, 2011).

The results from this thesis also indicate that the *B. phytofirmans* PsJN genome could possess high levels of gene redundancy in putative IAA biosynthesis, suggesting their function is relevant for this bacterium. The fast IAA production detected after tryptophan addition to cells, suggests that genes involved in IAA biosynthesis are already expressed in strain PsJN and may belong to general metabolism or other functions not related to IAA biosynthesis. Interestingly, *ipdC* gene of *A. brasilense* is also involved in synthesis of phenylacetic acid, a weak auxin with antimicrobial activity (Sommers *et al*, 2005). Phenylacetic and other synthetic auxins can induce *ipdC* gene expression in a concentration range similar to that of IAA (Vande Broek *et al*, 1999; Sommers *et al*, 2005), suggesting that indole-3-pyruvate decarboxylase activity of *ipdC*-encoded product can be explained by the structural similarity between IAA and phenylacetic acid (Spaepen *et al*, 2007b). In *E. cloacae* UW5, a regulatory protein (TyrR) related to transport and aromatic amino acid metabolism, is involved in *ipdC* expression in response to tryptophan, phenylalanine and

tyrosine, suggesting that *ipdC* gene has a broader function in amino acid metabolism (Ryu and Patten, 2008), instead to be specific for IAA synthesis.

IAA degradation pathway in *B. phytofirmans* PsJN

This thesis work makes contributions to the genetic and biochemistry of the IAA catabolic pathway. Leveau and Gerards (2008) described an *iac* gene cluster involved in IAA degradation in *P. putida* 1290, also present in some Actinobacteria and α-, β- and γ-Proteobacteria, and very recently (Scott *et al*, 2013), provided some hints on putative IAA catabolism intermediates and some *iac* gene functions.

This thesis reports that the PGPR β-proteobacterium *B. phytofirmans* PsJN harbors an *iac* gene cluster although with a different gene sinteny with respect to *P. putida* 1290 (Leveau and Gerards, 2008; Fig. 5). The functionality of the *iac* genes found in *B. phytofirmans* is supported by the lack of IAA growth and degradation in several *iac* gene mutants (Table 5, Fig. 8), and the responsiveness of *iac* gene promoters to IAA (Figs. 6 and 7). The genetic context of *iac* genes in *B. phytofirmans* is more complex than that observed in *P. putida* (Fig. 5): the presence of *cat* genes homologues, several putative regulator genes and transporter genes. This feature suggests that regulation of *iac* gene expression in *B. phytofirmans* is complex, with transcriptional units that are not expressed coordinately, giving raise to accumulation of toxic intermediates and other metabolic problems. Growth of strain PsJN shows an unusually long lag phase (Fig. 4), compared with strain 1290, which may be explained by inefficient induction, and/or formation of toxic intermediates. Catechol accumulation, a toxic intermediary preventing growth (Schweigert *et al*, 2001;

Pérez-Pantoja *et al*, 2003), was reported by Scott *et al* (2013), and also detected in this work (Fig. 9).

Although still incomplete, available information allows proposing a possible order of *iac* gene encoded products in the IAA catabolic pathway. The fact that strain JMP134 *iac*ΔA derivative was unable to remove IAA, unlike all the other JMP134 *iac*Δ derivatives, clearly indicates that the *iacA* gene encoded enzyme of strain PsJN participates in the first step of the IAA degradation pathway (Fig. 13), as was also proposed by Lin *et al* (2012) and Scott *et al* (2013) in *Acinetobacter baumannii* ATCC 19606 and *P. putida* 1290, respectively. *E. coli* colonies carrying the *iacA* gene from *P. putida* 1290 turned blue color LB plates (Gerards and Leveau, 2008) and blue color development is indole-dependent (Scott *et al*, 2013), indicating that *iacA* gene encoded product was responsible of indole hydroxylation to produce indoxyl, which in the presence of oxygen produces indigo (blue color), thus suggesting that IacA has an indole hydroxylase activity. Sequence analysis predicted that IacA belongs to the acyl-CoA dehydrogenase family which are flavoproteins (Ghisla and Thorpe, 2004; Kim and Miura, 2004; Lin *et al*, 2012), although IacA lacks the conserved FAD-binding sequence motifs identified in dehydrogenases (Dym and Eisenberg, 2001). The conversion of indole into indigo was demonstrated for the *iacA* gene encoded enzyme from *A. baumannii* ATCC 19606, whose reaction was absolutely dependent on NADH and FAD (Lin *et al*, 2012), supporting its classification as a flavoprotein. Scott *et al* (2013) proposed that IacA protein was able to hydroxylate IAA to 2-hydroxy-indole-3-acetic acid (Fig. 2).

Strain JMP134 *iac*ΔG derivative removes IAA very slowly, suggesting that participates in the first step of IAA degradation, together with the *iacA* gene. Its role seems

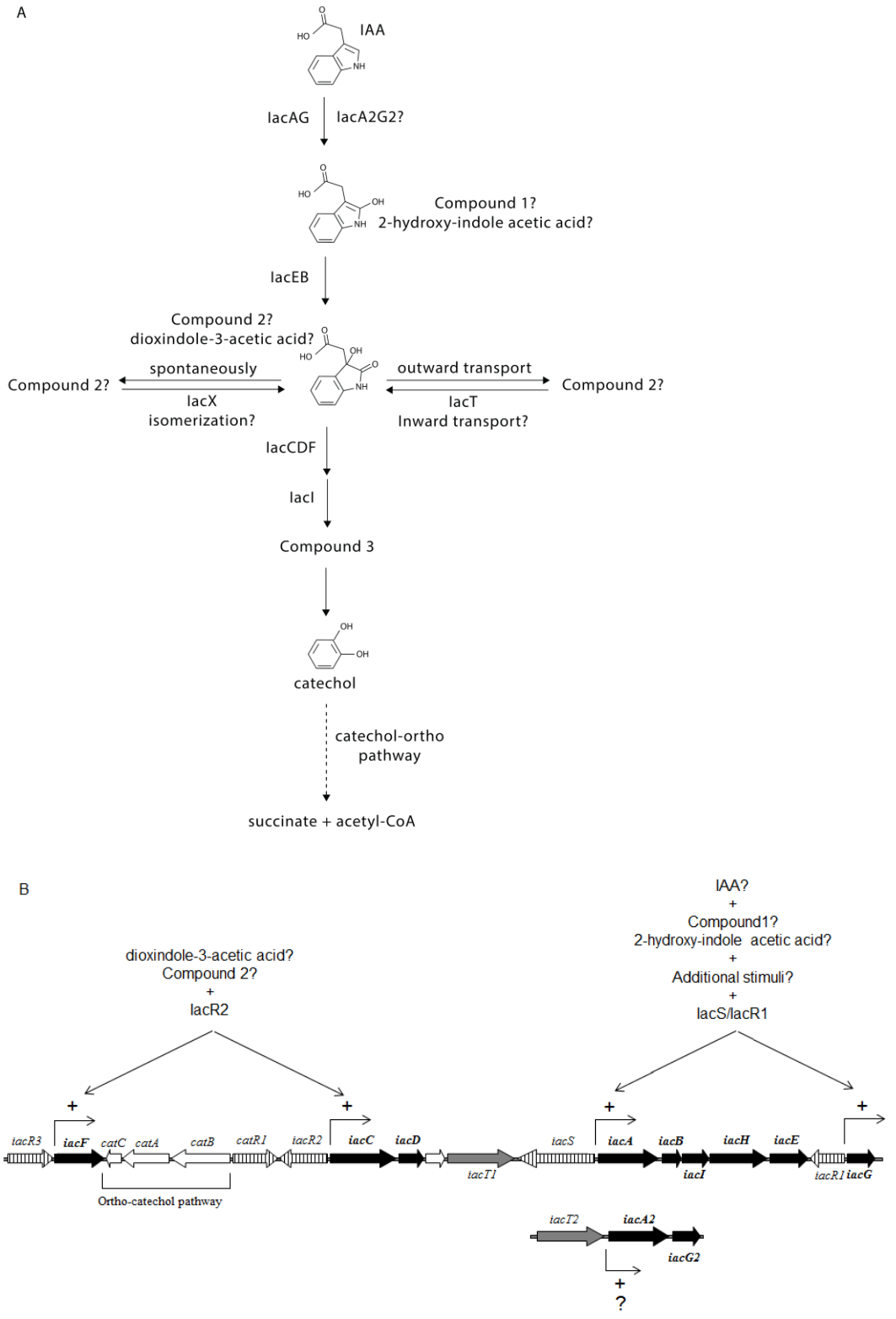

Figure 13. Proposed indole-3-acetic acid (IAA) degradation pathway and regulation in *Burkholderia phytofirmans* PsJN. See section "IAA degradation pathway in *B. phytofirmans* PsJN" for further details.

to be accessory because it is not essential in *B. phytofirmans* (Table 5), and may be partially replaced in the heterologous strain JMP134. The non-essential characteristic is supported by the results of BLASTP analysis showing that *iacG* gene has at least two equivalents in the genome of strain PsJN (Table 4). The accessory role is supported by the fact that this gene has the best hit (56% of amino acid identity) with the 4-hydroxyphenylacetate (4-HPA) 3-monooxygenase reductase component (*hpaC* gene) of *E. coli* W, a protein with an experimentally verified function that, along with the oxygenase component of 4-HPA 3-monooxygenase (*hpaB*), catalyzes the introduction of hydroxyl group into the benzene nucleus of 4-HPA forming 3,4-dihydroxyphenylacetate (Prieto and Garcia, 1994; Galán *et al*, 2000). Interestingly, HpaC is able to reduce FAD to $FADH_2$, which dissociates from the enzyme, and later is captured by HpaB to hydroxylate 4-HPA. Therefore, direct interaction between HpaB and HpaC is not critical and, theoretically, any flavin reductase present in the host cell would be able to replace HpaC role (Galán *et al*, 2000). HpaC catalyzes reduction of free flavins by NADH (Galán *et al*, 2000), allowing proposal that IacG provides reduced flavins to IacA, which is directly involved in the first step of IAA pathway (Fig. 13).

Concerning the second step of the IAA pathway, Scott *et al* (2013) proposed that the *iacE* gene encoded enzyme was involved in formation of 3-hydroxy-2-oxindole-3-acetic acid (dioxindole-3-acetic acid) from 2-hydroxy-indole-3-acetic acid. Sequence analysis predicted that the *iacE* gene product of strain PsJN belongs to the short-chain dehydrogenases/reductases (SDRs) family, characterized by a Rossmann-fold scaffold and a NAD(P)(H) binding region (Kavanagh *et al*, 2008; Persson and Kallberg, 2013). IacE from strain PsJN is a classical SDR member that is typically about 250 residues long, with specific cofactor (TGxxx[AG]xG) and active site (YxxxK) sequence motifs, with substrate

and reaction spectrum comprising NAD(P)(H)-dependent oxidoreduction of hydroxy/keto groups (Kavanagh *et al*, 2008). Strain JMP134 *iac*ΔE derivative was able to completely degrade IAA, and based on the early accumulation of compound 1, but not compound 2, we suggests that IacE participates after IacAG, in the second step of IAA pathway in *B. phytofirmans* (Fig. 13), similar to that proposed by Scott *et al* (2013). However, production of dioxindole-3-acetic acid requires an hydroxylation step (Fig. 2), as proposed by Scott *et al* (2013), which should be not supported by a classical dehydrogenase activity (Kavanagh *et al*, 2008), suggesting that this conversion requires additional steps or enzymatic activities, putatively encoded by other *iac* genes. This possibility is consistent with the fact that strain JMP134 *iac*ΔB, accumulates compound 1, and also compound 2 and catechol. If these latter two are downstream intermediates mean that further metabolism of compound 1 is occurring, and the role of IacB would be as a coupling or auxiliary protein of IacE (Fig. 13). Unfortunately, as sequence analysis showed that IacB has no conserved domains, no function could be predicted from primary structure and thus was annotated as a hypothetical protein by Leveau and Gerards (2008).

Scott *et al* (2013) proposed that the third step in IAA pathway involved *iacC* gene in *P. putida* 1290, because dioxindole-3-acetic acid was accumulated in strain carrying *iac* genes cluster with an interrupted *iacC* gene. This compound was also found in cells carrying the complete *iac* gene cluster, suggesting this step is rate-limiting in the IAA pathway from *P. putida* (Scott *et al*, 2013). The strain JMP134 *iac*ΔCD derivative only accumulates high levels of compound 2, suggesting that these genes are involved in the third step of IAA pathway (Fig. 13), similar to that proposed by Scott *et al* (2013) in *P. putida* 1290. *iacC* and *iacD* gene products seem to be subunits of the same enzyme, an

aromatic ring hydroxylating dioxygenase (Leveau and Gerards, 2008; Scott *et al*, 2013). Sequence analysis showed that the *iacC* gene product of *B. phytofirmans* PsJN belongs to the Rieske non-heme iron oxygenase family (Ferraro *et al*, 2005; Kweon *et al*, 2008), whereas the *iacD* gene product belong to the beta subunit of ring hydroxylating dioxygenases, which has a similar structure to NTF-2 and scytalone dehydratase (Lundqvist *et al*, 1994; Bullock *et al*, 1996). Rieske non-heme iron oxygenase systems use non-heme Fe(II) to catalyze addition of hydroxyl groups to the aromatic ring, an initial step in oxidative degradation of aromatic compounds (Ferraro *et al*, 2005; Kweon *et al*, 2008), employing an electron transport chain to use reductive power of NAD(P)H and activate molecular oxygen (Ferraro *et al*, 2005). Some oxygenase components contain also a β-subunit, but with a function solely structural, although some reports suggest that can influence substrate binding in some oxygenases (Hurtubise *et al*, 1998). Alpha subunits are the catalytic components, carrying an N-terminal domain, which binds a Rieske-like [2Fe-2S] that accepts electrons from a reductase or ferredoxin and passes them to a C-terminal catalytic domain which binds the non-heme Fe(II) for catalysis (Ferraro et al, 2005). In addition, Rieske non-heme iron oxygenase systems are constituted by additional components involved in movement of an electron from NAD(P)H to O_2: a reductase, a ferredoxin (only in three component systems) and the previously indicated oxygenase (with oligomers α_3 or $\alpha_3\beta_3$) (Ferraro *et al*, 2005, Kweon *et al*, 2008). In this context, sequence analysis of the *iacF* gene revealed that belongs to ferredoxin-NADP reductase family (Aliverti *et al*, 2008), which contains a FAD or FMN-binding domain, a NADH-binding domain and a plant type [2Fe–2S] cluster domain, catalyzing transfer of reducing equivalents between $NADP^+$/NADPH pair and the oxygenase component (Ferraro *et al*,

2005; Aliverti *et al*, 2008). Consistently, the *iacF* gene deficiency accumulates compound 2, suggesting that *iacF* is the reductase component of aromatic ring hydroxylating dioxygenase, whereas the *iacC* and *iacD* genes are the oxygenase components (alpha and beta subunits, respectively), thus performing the third step of IAA pathway (Fig. 13). Remarkably, similar to the effect of the absence of *iacCD* or *iacF* genes in the constructed *iac* cluster, the lack of the *iacI* gene also accumulates compound 2, suggesting a role of this gene in the third step of IAA degradation. Sequence analysis of IacI shows that no function can be predicted from primary sequence, although belongs to the SnoaL-4 superfamily, which is a family of proteins that shares the SnoaL fold, mainly represented by polyketide cyclases which catalyze ring closure steps in the biosynthesis of polyketide antibiotics (Sultana *et al*, 2004; Kallio *et al*, 2006). Thus, is possibly that IacI may participate in the same or an additional step after that catalyzed by IacCDF (Fig. 13), but this possibility needs more biochemical and genetic research.

The strain JMP134 *iacABIHECDGF* derivative accumulates catechol, and compound 2, the latter remaining intact after 2 days of incubation, indicating that this derivative was unable to channel it to catechol, despite having all *iac* genes. This suggests that strain JMP134 *iacABIHECDGF* derivative lacks a function related to compound 2 turnover that is present in *B. phytofirmans*, which was corroborated by the fast removal of compound 2 after incubation of the corresponding supernatant with wild type strain PsJN cells. A possible candidate for the missing function in the *iacABIHECDGF* cluster is the *iacT* gene present in the *iac* cluster of strain PsJN (Fig. 5), encoding a putative inward transporter that would allow internalization of the intermediate to reassume metabolism (Fig. 13). Another possible candidate is *iacX* gene, close to the *iacCD* genes and possibly in the same transcriptional unit (Fig. 5), encoding a protein belonging to a tautomerase

superfamily (Poelarends *et al*, 2008), and putatively catalizing isomerism between two molecules, which would be required for further metabolism (Fig. 13).

Sequence analysis indicated that *iacH*-encoded product of *P. putida* was related to amidases/amidohydrolases (Leveau and Gerards, 2008), as corroborated in analysis of the sequence of the strain PsJN homologue. However, IacH is distant from amidases with demonstrated function, and because amidases are able to cleave amide, ester or nitrile bonds (Clarke and Drew, 1988; Cilia *et al*, 2005; Pollmann *et al*, 2006), which could be the substrate of this enzyme is not clear. Strain JMP134 *iac*ΔH derivative accumulated compound 2, but also catechol, and also compound 3, suggesting that the IacH-catalyzed step occurs after the third step (Fig. 13). Production of catechol could be explained by an unspecific *iacH*-like activity present in this heterologous strain, which is an extraordinary aromatic degrader (Pérez-Pantoja *et al*, 2008; Lykidis *et al*, 2010) or that the substrate of the *iacH* gene product spontaneously generates catechol, although more slowly than in strain carrying the *iacH* gene.

iacA, *iacF* and *iacG* genes have homologous in the strain PsJN genome (Table 4). Our results suggest that part of the *iac* genes redundancy could be functional, because resting cells assays with *iacA*, *iacF* and *iacG* mutants degraded IAA entirely (Fig. 8). As mentioned above, *iacG* gene has two homologues in strain PsJN genome (Table 4) and they are possible candidates for additional *iacG* gene encoded activity as putative flavin reductases. Furthermore, it was observed that one *iacG* homologue is clustered with an additional copy of the *iacA* gene (named *iacG2 and iacA2*, respectively; Fig. 5), raising the possibility that they work together as an extra *iacAG* genes encoded protein (Fig. 13). Also, we observed that *iacA2* gene was induced in cells grown on IAA (Fig. 6), indicating a

possible functional role in IAA metabolism, although the *iacA2* gene mutant has no differences in IAA degradation in comparison with the wild type strain. In several bacteria, the presence of a second gene usually is a selective advantage, such as two copies of (chloro)catechol 1,2-dioxygenase avoid accumulation of toxic (chloro)catechol intermediates (Pérez-Pantoja *et al*, 2003). Alternatively, two gene copies associated to different gene contexts avoid toxic or dead-end metabolites production, such as in strains *C. pinatubonensis* JMP134 and *C. metallidurans* CH34 that harbour two copies of phenol monooxygenases, one copy associated to catechol 1,2-dioxygenase and the other to catechol 2,3-dioxygenase (Pérez-Pantoja *et al*, 2012), and depend on substrate (phenol or methylphenols) whether are channeled by *ortho* (catechol 1,2-dioxygenase) or *meta* (catechol 2,3-dioxygenase) cleavage to avoid posterior dead-end or highly toxic intermediates formation (Catelani *et al*, 1971; Knackmuss *et al*, 1976; Pieper *et al*, 1989). In addition, substrate specificity is not necessarily determined by amino acid sequence, as it was observed in the interconversion of ACC deaminase and D-cysteine desulfhydrase, by altering two amino acid residues at the same positions within the active site of ACC deaminase, resulting in two different enzymatic activities (Todorovic and Glick, 2008). We suggest that apparently redundant *iacA2* gene encoded function, the more probable candidate to replace the *iacA* gene function in the respective mutant, would be act on indole-3-acetic acid derivatives and extend spectrum of substrates catalyzed by the IAA pathway. This would be the reason why *iacA2* gene could not be an efficient substitute of *iacA* gene, as degradation of IAA was completed after six days (Fig. 8). To replace putative function of the *iacF* gene product as a ferredoxin-NADP reductase, there is also a homologue in strain PsJN genome (Table 4), although it is clustered with a protein that belongs to Rieske family (data not shown) and possibly acts as reductase component in a

specific Rieske non-heme iron oxygenase system. Further research is required to confirm putative roles of *iacA*, *iacF* and *iacG* gene copies in IAA metabolism.

The *iac* cluster of *P. putida* 1290 contains a potential transcriptional repressor belonging to MarR family, named *iacR* (Scott *et al*, 2013), which is absent in the genome of strain PsJN. However, the *iac* genes cluster in strain PsJN contains additional genes that may play a role in regulation of IAA catabolism (Fig. 5): a two-component system (histidine kinase signal transducer and a LuxR family protein receptor, named *iacS* and *iacR1*, respectively, Fig. 5) and two LysR-like regulators (named *iacR2* and *iacR3*, Fig. 5). The basic two-component system contains a sensor kinase (SK) and a response regulator protein (RR). In response to environmental stimuli, SK must first autophosphorylate in a His residue, and then the phosphoryl group from SK-P is passed to an Asp residue in RR, which phosphorylation serves to control its ability to either bind its target DNA sequence or interact with other components of the transcription machinery (Chang and Stewart, 1998; Yamada and Shiro, 2008). A classic example is SK osmosensor EnvZ, which in response to changes in osmolarity, autophosphorylates and then the phosphate group is transferred to RR transcription factor OmpR, affecting the promoter interactions of the OmpR DNA-binding module, which regulates the transcription of two porin genes, *ompF* and *ompC* (Forst and Roberts, 1994; Pratt and Silhavy, 1995). On the other hand, LysR-type transcriptional regulator (LTTR) family is a well-characterized group of transcriptional regulators, highly conserved and ubiquitous among bacteria (Maddocks and Oyston, 2008). Usually, LTTRs act as either activators or repressors of often divergently transcribed single or operon genes, and have been involved in metabolism, cell division, quorum sensing, virulence, motility, nitrogen fixation, oxidative stress responses, toxin production, attachment, secretion, among others (Kovacikova and Skorupski, 1999; Deghmane *et al*,

2000; Cao *et al*, 2001; Kim *et al*, 2004; Lu *et al*, 2007; Sperandio *et al*, 2007). Co-inducers are important for the function of LTTRs and frequently appear to contribute to a feedback loop in which a product or intermediate of a given metabolic/synthesis pathway acts as the co-inducer necessary for transcriptional activation or repression (Maddocks and Oyston, 2008). Classical LysR regulator involved in aromatic metabolism is *catR*, related to Bz/catechol catabolism, that binds the intermediate *cis*,*cis*-muconate and induces *cat* genes (Parsek *et al*, 1994). In IAA catabolism, it was demonstrated that *iacR1* and *iacR2* are essential genes, and lack of *iacS* gene impair catabolism of IAA (Table 4), confirming their participation in IAA degradation as positive regulators. In contrast, the absence of *iacR3* gene did not affect IAA degradation (Table 4), suggesting either that this gene does not participate, it is a negative regulator in this pathway, or its function is expressed in other experimental conditions. Based on their location in the *iac* cluster (Fig. 5), family characteristics and promoter activities in mutant strains (Fig. 7), we suggest that the *iacS/iacR1* pair is a SK/RR two-component system, and probably *iacS* senses externally added IAA and/or the IacAG produced catabolite, autophosphorylates and then phosphorylates *iacR1*, which positively interacts with *iacABIHE* and *iacG* promoters (Fig. 13). The latter is supported by results showing that the *iacE* gene mutant was able to induce these promoters in the presence of IAA, but not the *iacCD* gene and *iacF* gene promoters (Fig. 7B and 7D). Interestingly, Leveau and Gerards (2008) found that mutants in a gene with similarity to the SK component of the two-component system CbrAB of *P. aeruginosa* PAO1 were unable to use IAA. CbrAB inactivated derivatives in strain PAO1 were unable to grow on several N-substrates, suggesting that CbrAB controls expression of several catabolic pathways in response to changing intracellular C:N ratios (Nishijyo *et al*, 2001), raising the interesting question if *iacS*/*iacR1* pair in strain PsJN is able to sense similar

conditions. We also propose that *iacR2* gene is a classical LysR transcriptional regulator that is able to induce transcription of *iacCD* and *iacF* genes, using as co-inducer an intermediate in IAA degradation, possibly a compound generated by the *iacE* gene-encoded product (Fig. 13). This is supported by the fact that *iacA* and *iacE* gene mutants are unable to induce promoter activity of *iacCD* and *iacF* genes (Fig. 7D), but the *iacF* gene mutant is able to induce *iacCD* and *iacF* gene promoters (Fig. 7E). Surprisingly, the *iacC* gene mutant is unable to induce any promoter (Fig. 7C), although should have the same pattern that *iacF* gene mutant, if they are part of the same enzyme system. A possible explanation is that a mutation in *iacC* gene could exert a polar effect on the *iacX* or *iacT* gene (Fig. 5), and assuming that turnover of compound 2 is controlled by the *iacX* and/or *iacT1* gene (see above), the inducer was not produced and/or inward transported and thus unable to induce *iac* genes. Further research is needed to clarify this point.

A transporter-related gene was found neighboring *iacCD* genes, named *iacT1* (Fig. 5), and a second transporter homolog to the first one was found clustered with the *iacA2* and *iacG2* genes (named *iacT2*; Fig. 5), suggesting that *iacT* genes encode transporters of IAA-related molecules. Database searches predict that both *iacT* genes belong to the major MFS family of transporters, which are single-polypeptide secondary carriers capable of transporting small solutes in response to chemiosmotic ion gradients (Pao *et al*, 1998; Reddy *et al*, 2012). *iacT* genes specifically belong to the metabolite:H^+ symporter family related to the shikimate transporter encoded by the *shiA* gene of *E. coli* (~40% amino acid identity) (Whipp *et al*, 1998). *IacT1* and *IacT2* mutants of *B. phytofirmans* PsJN grow with similar rates as the wild type strain, indicating that these genes not participate or their function is redundant in IAA transport. Because of the hydrophobic nature of the aromatic

ring of IAA it is possible that, in cells exposed to high concentrations of auxin, this molecule moves across the plasmatic membrane without requiring a carrier.

Despite the fact that several aspects of IAA degradation by strain PsJN are still unclear, here we report that this property is relevant in plants as an IAA degradation mutant (unable to use IAA as sole carbon and energy source) is not effective in promoting plant growth traits by strain PsJN and in preventing adverse effects of exogenously added IAA. It has been described that root growth can be stimulated or inhibited depending on the concentration of IAA produced by bacteria (Persello-Cartieux *et al*, 2001; López-Bucio *et al*, 2007). In plants inoculated with the *iacC* gene mutant, IAA levels in roots should be higher than in roots inoculated with the wild type PsJN strain, because this mutant cannot degrade IAA and thus the plant would be more susceptible to primary root shortening. Leveau and Lindow (2005) have described similar effects in radish plants inoculated with *P. putida* 1290.

Interplay between synthesis and degradation of IAA

We showed that IAA biosynthesis in *B. phytofirmans* PsJN is followed by removal from the culture medium (Fig. 3B), which would be explained by degradation and/ or inward transport. This raises the question if this phenomenon is linked to *iac* genes. Surprisingly, in the *iacC* mutant unable to degrade IAA molecule exogenously added (Fig. 11B), removal of the tryptophan-dependent synthesized IAA is still produced (Fig. 11A). Similar results were obtained for *iacA* and *iacT1* mutant (data not shown), suggesting that IAA disappear was not a result of IAA transformation or inward transport by IacA or IacT1, respectively, indicating that an *iac* genes-independent, alternative way of IAA removal is taking place in these experimental conditions. In this context, a tryptophan

dioxygenase (*kynA*) mutant, unable to use tryptophan as sole carbon and energy source, exposed to IAA-tryptophan equimolar mixtures grows very poorly on IAA (Fig. 12), suggesting potential catabolite repression by tryptophan of the *iac* genes mediated IAA degradation. Several examples that binary aromatic compounds mixtures are hierarchically degraded have been reported, with the (preferred) substrate that is catabolized first in the mixture delaying degradation of the second mixture component (Nichols and Harwood, 1995; Ampe *et al*, 1998; Brzostowicz *et al*, 2003; Choi *et al*, 2007; Zhan *et al*, 2009, Donoso *et al*, 2011). Although further research is needed to understand the mechanism behind tryptophan stop/delay in IAA utilization, which could be related to transcriptional control, involving global and/or specific regulators preventing mRNA synthesis, analogous to catabolite control protein A in *Bacillus subtilis* or cyclic AMP receptor protein in *Escherichia coli* (Görke and Stülke, 2008), or RNA-binding proteins that acts post-transcriptionally, such as catabolite repression control protein of *Acinetobacter baylyi* or *P. putida* (Zimmermann et al, 2009; Rojo, 2010), or allosteric regulation and/or competitive inhibition of repressor molecule (tryptophan) on enzymes that act in IAA pathway, analogous to carbon catabolite repression of Bz on 4-hydroxybenzoate catabolism in *C. pinatubonensis* (Donoso et al, 2011), we propose that IAA degradation by *iac* genes is repressed by tryptophan, and thus tryptophan-dependent IAA biosynthesis is not a futile process. In addition, it is interesting to note that in *kynA* mutant stops degradation of biosynthesized IAA (Fig. 11A), but this mutant degrades exogenously added IAA (Fig. 11B). This provides a clue about IAA removal, because KynA could be responsible for IAA disappearance in the wild type and the *iacC* mutant (Fig. 11A), using this molecule as substrate. Egebo *et al* (1991) hypothesized that IAA degradation in *B. japonicum* (Fig. 2) is started by an enzyme that catalyzes an oxygen-consuming opening of the indole ring

analogous to the one catalyzed by tryptophan 2,3-dioxygenase (Thackray *et al*, 2008). To explore this possibility, the *iacC* gene mutant was grown on tryptophan as the sole carbon and energy source, to induce enzymes involved in tryptophan catabolism, and later incubated with exogenously added IAA (Fig. 11C). Finally, it was observed IAA elimination in these experimental conditions, suggesting that tryptophan catabolism (via kynurenine) and specifically KynA possibly are involved in IAA removal when *iac* genes are repressed by tryptophan. These results show a complex regulation of IAA turnover and a close relationship with tryptophan metabolism (Figure 14).

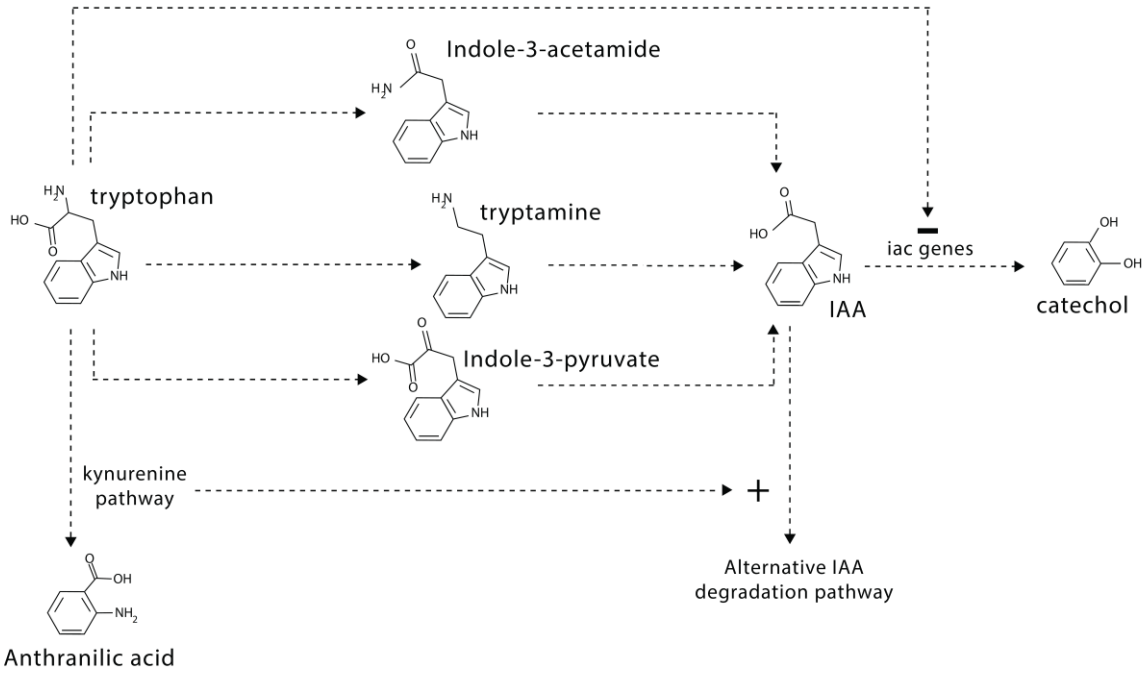

Figure 14. Interplay between tryptophan and IAA metabolism. See section "Interplay between synthesis and degradation of IAA" for further details.

CONCLUSIONS

** *Burkholderia phytofirmans* PsJN produces IAA by a tryptophan-dependent way, through at least three potential biosynthetic pathways.

** Strain PsJN grows on IAA as the sole carbon and energy source employing *iac* genes, with *iacAG*, *iacEB*, *iacCDFI* and *iacH*-encoding products catalyzing consecutive steps in IAA degradation pathway, respectively.

** *iac* genes possibly are regulated by a system of two component (*iacS*/*iacR1*) and a LysR-like protein (*iacR2*), and *iacA, iacF* and *iacG* genes present functional redundancy.

** IAA levels would play a role in the plant-growth-promotion of Arabidopsis thaliana, and because *iacC* mutant is unable to degrade IAA, it would influences levels of auxin around roots in comparison with wild type, working as a mechanism of plant-growth-promotion in *B. phytofirmans* PsJN.

** Apparently, tryptophan catabolism activates IAA degradation by an *iac*-genes independent way.

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