RESEARCH ARTICLE

Discovery of a bacterial gene cluster for catabolism of the plant hormone indole 3-acetic acid

Johan H.J. Leveau & Saskia Gerards

Netherlands Institute of Ecology (NIOO-KNAW), Heteren, The Netherlands

Correspondence: Johan H.J. Leveau, Netherlands Institute of Ecology (NIOO-KNAW), Boterhoeksestraat 48, 6666 GA Heteren, The Netherlands. Tel.: +31 26 479 1306; fax: +31 26 472 3227; e-mail j.leveau@nioo.knaw.nl

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Abstract

The isolation and annotation of an 8994-bp DNA fragment from *Pseudomonas putida* 1290, which conferred upon *P. putida* KT2440 the ability to utilize the plant hormone indole 3-acetic acid (IAA) as a sole source of carbon and energy, is described. This *iac* locus (for indole 3-acetic acid catabolism) was identified through analysis of a plasposon mutant of *P. putida* 1290 that was no longer able to grow on IAA or indole 3-acetaldehyde and was unable to protect radish roots from stunting by exogenously added IAA. The *iac* locus consisted of 10 genes with coding similarity to enzymes acting on indole or amidated aromatics and to proteins with regulatory or unknown function. Highly similar *iac* gene clusters were identified in the genomes of 22 bacterial species. Five of these, i.e. *P. putida* GB-1, *Marinomonas* sp. MWYL1, *Burkholderia* sp. 383, *Sphingomonas wittichii* RW1 and *Rhodococcus* sp. RHA1, were tested to confirm that bacteria with IAA-degrading ability have representatives in the *Alpha*-, *Beta*- and *Gammaproteobacteria* and in the *Actinobacteria*. In *P. putida* 1290, *cat* and *pca* genes were found to be essential to IAA-degradation, suggesting that IAA is channeled via catechol into the β-ketoadipate pathway. Also contributing to the IAA degrading phenotype were genes involved in tricarboxylate cycling, gluconeogenesis, and carbon/nitrogen sensing.

Introduction

Indole 3-acetic acid (IAA; Fig. 1) is a readily biodegradable compound. IAA-destroying activity has been demonstrated in soils under conditions that favor bacterial growth (Proctor, 1958; Riviere *et al.*, 1966; Chandramohan & Mahadevan, 1968; Martens & Frankenberger, 1993). Rapid degradation of IAA has also been observed in plant tissue preparations, where it has often been attributed to natural bacterial contaminants (Andreae & van Ysselstein, 1960; Bouillenne-Walrand *et al.*, 1963; Tomaszewski & Thimann, 1966; Wichner, 1968). Bacterial isolates with the ability to destroy IAA have been recovered from various environments, including air (Tsubokura *et al.*, 1961; Mino, 1970), soil (Proctor, 1958; Gieg *et al.*, 1996) and plant material (Libbert *et al.*, 1966; Libbert & Risch, 1969; Strzelczyk *et al.*, 1973; Leveau & Lindow, 2005; Gravel *et al.*, 2007).

The existence of this IAA-degrading phenotype can be explained in two, not mutually exclusive, ways. On the one hand, IAA is just one of many compounds that bacteria have adapted to as sources of carbon and/or nitrogen. Complete mineralization of IAA and growth on IAA have been reported for representatives of the genera *Pseudomonas* (Gieg *et al.*, 1996; Leveau & Lindow, 2005), *Arthrobacter* (Mino, 1970), *Alcaligenes* (Claus & Kutzner, 1983) and *Bradyrhizobium* (Jarabo-Lorenzo *et al.*, 1998). For these bacteria, plants and many plant-associated microorganisms (Costacurta & Vanderleyden, 1995; Patten & Glick, 1996; Spaepen *et al.*, 2007) represent natural sources of IAA. On the other hand, IAA is not just any compound: as a growth hormone, it has an essential biological function in plants, governing many physiological processes (Teale *et al.*, 2006). Thus, mineralization of IAA, or transformation into a biologically inactive form, offers bacteria the potential for manipulation of IAA-related plant activities. An example from the fungal world is *Marasmius perniciosus*, which causes witches' broom disease on cacao, a condition that is characterized by an imbalance in IAA levels resulting from IAA-oxidizing enzymes produced by the fungus (Krupasagar & Sequeira, 1969). A functionally homologous

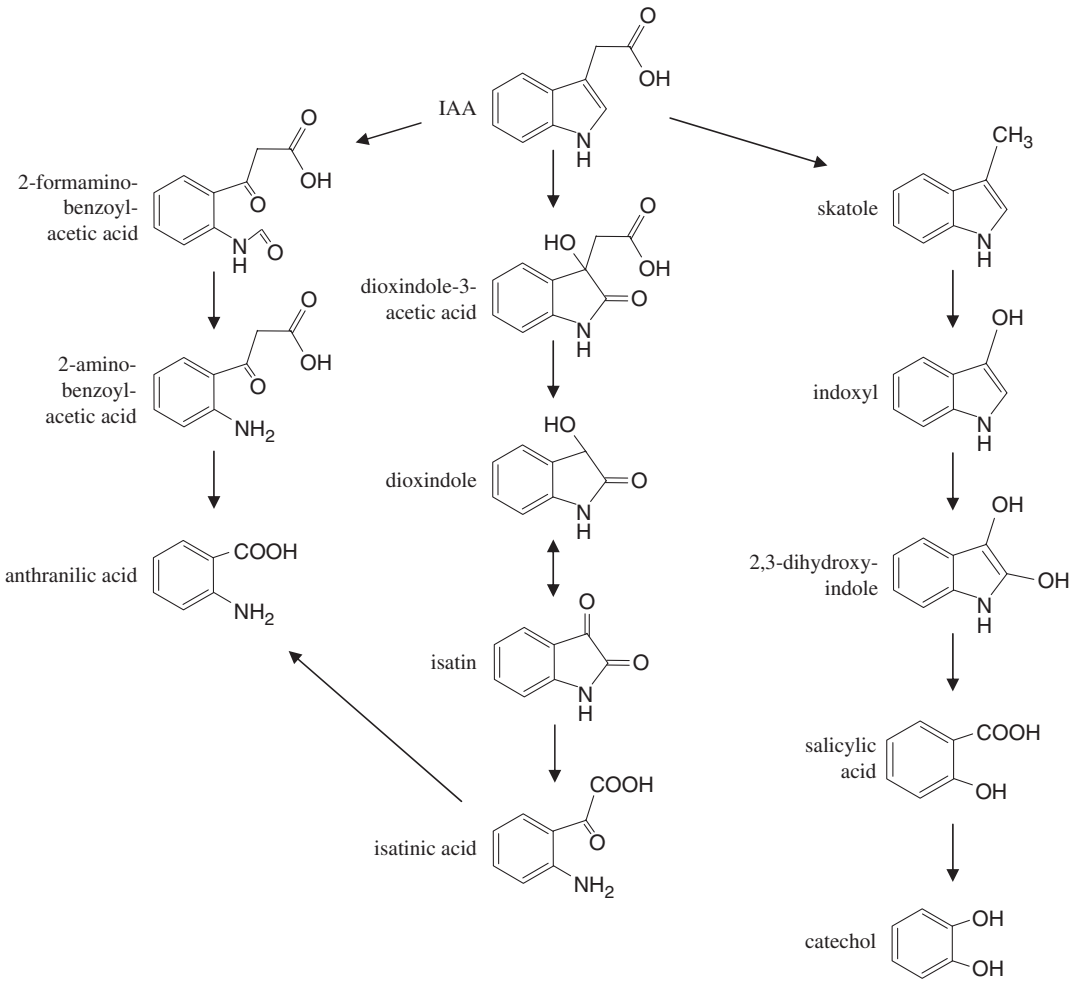

Fig. 1. Proposed pathways for bacterial transformation and mineralization of IAA. Arrows represent published IAA degradation pathway reactions, as discussed in the Introduction.

phenomenon is the ability of *Pseudomonas savastanoi* to convert IAA to the biologically inactive IAA–lysine (Kuo & Kosuge, 1969). This bacterium is also one of many that produce IAA and can thus sabotage plant physiology by adding to the endogenous IAA pool in plants (Costacurta & Vanderleyden, 1995; Patten & Glick, 1996; Spaepen *et al*., 2007).

Several pathways have been suggested for the bacterial mineralization or transformation of IAA (Fig. 1). Tsubokura *et al*. (1961) described a bacterium isolated from air that attacks IAA by splitting the indole ring to produce 2-formaminobenzoylacetic acid. *Bradyrhizobium japonicum* metabolizes IAA via dioxindole-3-acetic acid, dioxindole, isatin, isatinic acid and anthranilic acid (Jensen *et al*., 1995). This pathway overlaps partially with the mineralization of IAA by an *Alcaligenes* sp. via isatin, anthranilic acid and gentisate (Claus & Kutzner, 1983). Decarboxylation of IAA to skatole has been reported for several bacteria (Yokoyama *et al*., 1977; Deslandes *et al*., 2001), including a *Pseudomonas* sp. from soil, that converts skatole to catechol (Proctor, 1958). The latter is a confirmed intermediate in the mineralization of IAA by *Pseudomonas putida* 1290 (Leveau & Lindow, 2005), *Pseudomonas* sp. LD2 (Gieg *et al*., 1996) and an *Arthrobacter* species (Mino, 1970). For the two *Pseudomonas* species, it was shown that catechol is ortho-cleaved to *cis*,*cis*-muconic acid and channeled into the β-ketoadipate pathway (Harwood & Parales, 1996). Other types of bacterial biotransformation of IAA are (1) transformation to indole-3-methanol by *Rhizobium phaseoli* (Ernstsen *et al*., 1987) and (2) conjugation, as exemplified by the production of IAA-lysine by *Pseudomonas savastanoi* (Kuo & Kosuge, 1969).

Surprisingly, almost nothing is known about the genetics that underlie the IAA degradative pathways described above. Except for the *iaaL* gene from *P. savastanoi*, encoding the IAA-lysine synthase (Glass & Kosuge, 1986), no other genes

have been linked to IAA-catabolic enzyme activities. This lack of knowledge has seriously hampered studies of the biology, ecology, and evolution of the bacterial IAA degradative phenotype. Without DNA sequences, no tools exist to assess and quantify the occurrence, diversity, and activity of IAA-degradative genes in natural bacterial populations. Furthermore, the unavailability of IAA degradative genes has held back exploitation of such genes in the study of IAA functioning in plants, which actually owes much of its progress to bacterial genes encoding the synthesis of IAA (Hedden & Phillips, 2000).

In the current study, the authors' aim was to be the first to identify and describe bacterial genes involved in the biotransformation and mineralization of IAA. For this, *Pseudomonas putida* 1290 was chosen as a model strain (Leveau & Lindow, 2005). This bacterium was shown previously to use IAA efficiently as the sole source of carbon, nitrogen, and energy. Here, it is described how a combination of plasposon mutagenesis and functional complementation was used to identify a gene locus in *P. putida* 1290 that carries genes encoding IAA degradative activity.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Pseudomonas* strains were grown at 30 °C on King's B (KB) medium (King *et al.*, 1954) or on M9 minimal medium (Sambrook *et al.*, 1989) supplemented with one of the following carbon sources at a final concentration of 5 mM: IAA (Sigma-Aldrich, Steinheim, Germany), indole 3-acetaldehyde-sodium bisulfite addition compound, (Sigma-Aldrich), indole 3-acetamide (Sigma-Aldrich), benzoate (Sigma-Aldrich) or glucose. *Escherichia coli* strains were grown at 37 °C on Luria–Bertani (LB) medium. Where applicable, antibiotics were used at the following final concentrations: chloramphenicol, 12.5 μg mL^{-1} ($Cm_{12.5}$); kanamycin, 50 μg mL^{-1} (Km_{50}); and rifampicin, 40 μg mL^{-1} (Rif_{40}).

Construction and screening of a *P. putida* 1290 plasposon library

Plasposon pTn*Mod*-KmO*lacZ* (Table 1) was used to create a mutant library of *P. putida* 1290R. To this end, equal amounts of exponentially growing cells of *P. putida* 1290R, *E. coli* DH5α (pTn*Mod*-KmO*lacZ*), and helper strain *E. coli* DH5α (pRK2013) were mixed, spotted onto a KB plate, and incubated at 30 °C for 24 h. Cells were then recovered from the plate, resuspended in 10% glycerol, and plated onto M9 medium containing 5 mM glucose, Rif_{40}, and Km_{50}. Of the resulting *P. putida* 1290R::KmO*lacZ* colonies, 4416 were transferred to fresh M9 glucose Rif_{40} Km_{50} plates, and then streaked onto M9 plates containing Km_{50} and either IAA or benzoate. Mutants unable to grow on IAA or benzoate were selected for further study. Their genomic DNA was isolated using an Ultra Clean Soil DNA Kit (Mobio, Carlsbad, CA), digested with PstI (Amersham Life Sciences, Piscataway, NJ), religated with T4 DNA ligase (New England Biolabs, Beverly, MA), and used to electrotransform *E. coli* EP-Max 10B cells with a Gene Pulser Xcell Microbial System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. From the resulting transformants, plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen Benelux, Venlo, the Netherlands) and used as template DNA in a sequencing reaction (BaseClear, Leiden, the Netherlands) with primers LAC or PP1 (Table 1). Thus, for each mutant the nucleotide sequence of the DNA surrounding the plasposon insertion site was obtained. These sequences, between 228 and 1008 bp in length, were analyzed by LASERGENE software (DNASTAR, Madison, WI) and used as a query in BLAST searches at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Screening of a large-insert DNA library from *P. putida* 1290 genomic DNA

A CopyControl Fosmid Library Production Kit was used (Epicentre, Madison, WI) to construct a large-insert DNA library of *P. putida* 1290 genomic DNA as described earlier for *Collimonas fungivorans* Ter331 (Leveau *et al.*, 2004). This library consisted of 3072 *E. coli* EPI300 clones, each carrying a random ~38-kb DNA fragment of the *P. putida* 1290 genome in vector pCC1FOS (Table 1). A subset of 768 clones, grown overnight in eight microtiterplates containing 150 μL LB $Cm_{12.5}$ well^{-1}, was screened by PCR for DNA sequences containing the *iacH* gene (see below) as follows: cells from every row of each microtiterplate were pooled resulting in 64 pools of 12 clones. Pooled cells were pelleted by centrifugation, resuspended in milli-Q water, incubated at 99 °C for 10 min, and centrifuged again. The supernatant was diluted 30× in milli-Q water and 4.5 μL was used as template DNA in a 15-μL PCR reaction containing 7.5 μL ABsolute QPCR SYBR Green Mix (ABGene, Hamburg, Germany), 2.7 μL of a 2.5 μM *iacH* locus-specific primer set (Table 1), and 0.3 μL milli-Q water. PCR reactions were set up with a CAS-1200 liquid handling robot (Corbett Research, Sydney, Australia). PCRs were run in a Rotor-Gene 3000 (Corbett Research) using the following settings: 15 min at 95 °C, and then 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Positive pools were identified as those that produced the same fluorescent signal as the positive control (i.e. genomic DNA of *P. putida* 1290). Positive pools were split into individual clones for a second round of PCR. Individual positive clones were analyzed further by

Table 1. Strains, plasmids, and primers used in this study

Strain	Description	Relevant phenotype	References or sources
Pseudomonas putida 1290	Able to use IAA, but also benzoate and glucose, as sole source of carbon and energy	IAA^+ $benzoate^+$ $glucose^+$	Leveau & Lindow (2005)
Pseudomonas putida 1290R	Spontaneous rifampicin-resistant derivative of *P. putida* 1290	IAA^+ $benzoate^+$ $glucose^+$ Rif^r	Leveau & Lindow (2005)
Pseudomonas putida KT2440	Well-characterized soil bacterium; genome sequence available; not able to use IAA as carbon and energy source; obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany)	IAA^- $benzoate^+$ $glucose^+$	Nelson *et al.* (2002)
Escherichia coli DH5α	Host for plasmids pCR4Blunt-TOPO and pRK2013	Km^s	Sambrook *et al.* (1989)
Escherichia coli EPI300	Host for large-insert-containing derivatives of fosmid pCC1FOS	Cm^s	Epicentre (Madison, WI)
Escherichia coli EP-Max 10B	Host for rescued plasposons and insertion-flanking DNA fragments	Km^s	Bio-Rad (Hercules, CA)

Plasmid	Description	Antibiotic resistance	References or sources
pTn*Mod*-KmO*lacZ*	Plasposon; suicide mini-Tn*5* derivative used for transposon mutagenesis of *P. putida* 1290; allows for rescue cloning of insertion-flanking DNA sequences	Km_{50}	Dennis & Zylstra (1998), Leveau & Lindow (2005)
pCC1FOS	Fosmid used for construction of large-insert library of *P. putida* 1290 DNA in *E. coli* EPI300	$Cm_{12.5}$	Epicentre (Madison, WI)
pCR4Blunt-TOPO	Plasmid used for DNA (sub)cloning	Km_{50}	Invitrogen (Carlsbad, TX)
pRK2013	Used as helper plasmid in triparental mating	Km_{50}	Figurski & Helinski (1979)
pCC1FOS9	pCC1FOS containing genomic fragment of *P. putida* 1290; confers IAA^+ phenotype to *P. putida* KT2440	$Cm_{12.5}$	This paper
pCC1FOS10	pCC1FOS containing genomic fragment of *P. putida* 1290; confers IAA^+ phenotype to *P. putida* KT2440	$Cm_{12.5}$	This paper
pCC1FOS11	pCC1FOS containing genomic fragment of *P. putida* 1290; confers IAA^+ phenotype to *P. putida* KT2440	$Cm_{12.5}$	This paper
pCC1FOS12	pCC1FOS containing genomic fragment of *P. putida* 1290; confers IAA^+ phenotype to *P. putida* KT2440	$Cm_{12.5}$	This paper
pCC1FOS15	pCC1FOS containing genomic fragment of *P. putida* 1290; contains *cat* locus	$Cm_{12.5}$	This paper
pCC1FOS11Eco	pCC1FOS11 digested with EcoRI and religated	$Cm_{12.5}$	This paper
pCC1FOS11EcoMlu	pCC1FOS11Eco digested with MluI and religated	$Cm_{12.5}$	This paper
pCC1FOS11EcoXho	pCC1FOS11Eco digested with XhoI and religated	$Cm_{12.5}$	This paper
pCC1FOS11EcoPsP	pCC1FOS11Eco digested with PspXI and religated	$Cm_{12.5}$	This paper
pCC1FOS11EcoAsc	pCC1FOS11Eco digested with AscI and religated	$Cm_{12.5}$	This paper
pCC1FOS11EcoAscplus	AscI/EcoRI digested pCC1FOS11EcoAsc ligated to 1.2-kb AscI/EcoRI fragment of pO4-H06	$Cm_{12.5}$	This paper
pO4-H06	3.7-kb genomic fragment of *P. putida* 1290 containing ORF 18 in pCR4Blunt-TOPO	Km_{50}	This paper

Primer	Description	Sequence (5′–3′)
LAC	Sequencing primer	CGACGGCCAGTGAATTCCC
PP1	Sequencing primer	TGCATGGCGCGCCGGCGA
iacH1	PCR amplification of *iacH* locus	CCGAACACGCCGCAGCAACA
iacH2	PCR primer	CGACTTCAGCCTGGGGACCGATAC
MR	Sequencing primer	GGATAACAATTTCACACAGG
MF-20	Sequencing primer	GTAAA CGACGGCCAG

$10\times$ dilution of o/n cultures in LB $Cm_{12.5}$ supplemented with $1\times$ induction solution and incubation for 5 h at 37 °C and 300 r.p.m. Five milliliters of this culture were used in a QIAprep Spin Miniprep Kit protocol (Qiagen). The resulting fosmid DNAs were used for restriction analysis with EcoRI, BamHI and/or HindIII (New England Biolabs, Beverly, MA), and as DNA templates in end-sequencing reactions using primers MR and MF-20 (Table 1).

Electrotransformation of *P. putida* strains

Fosmids were introduced into *P. putida* by electrotransformation as follows: an overnight KB culture was diluted 100 × into fresh KB medium and incubated at 30 °C for 3.5 h, placed on ice for 1 h, and centrifuged for 5 min at 4147 ***g*** at 4 °C. Cells were resuspended and washed three times in an equal volume, half volume, and quarter volume, respectively, of ice-cold 10% glycerol. After the final wash, cells were concentrated 50-fold in 10% glycerol. An aliquot of 100 μL was mixed with 8 μL of plasmid or fosmid DNA, transferred to a 0.2-cm cuvette, and electroporated with a Gene Pulser Xcell Microbial System (Bio-Rad) using the settings suggested for *P. aeruginosa* (i.e. 25 μF, 200 Ω and 2500 V). After addition of 1 mL SOC (Sambrook *et al.*, 1989), cells were incubated for 3 h shaking at 30 °C, and plated on appropriate media.

Root elongation assays and IAA measurements

The ability of wild-type *P. putida* 1290 and selected plasposon-mutant derivatives to rescue radish roots (variety French Breakfast) from IAA-induced growth inhibition was tested in a root assay as described previously (Leveau & Lindow, 2005). Root lengths were analyzed in STATISTICA (StatSoft, Tulsa, OK) by a standard *t*-test after passing Levene's test for homogeneity of variance. In supernatants of cultures that were incubated in the presence of IAA, IAA concentrations were measured using Salkowski reagent as described previously (Leveau & Lindow, 2005).

DNA sequencing and annotation

DNA preparations of four fosmids (i.e. pCC1FOS9, −10, −11 and −12) conferring an IAA-degradative (IAA^+) phenotype to *P. putida* KT2440 were pooled and sent to Macrogen (Seoul, Korea), where two libraries were constructed: (1) a small-insert library of 384 clones in plasmid pCR4Blunt-TOPO, and (2) a 96-clone library of transposon-treated fosmids each carrying a random insertion of TN < rOri/kan2 > . DNA sequences were obtained from both ends of each cloned insert in the pCR4Blunt-TOPO library or from both regions flanking each TN < rOri/kan2 > transposon insertion. DNA sequence reads were assembled using Lasergene's SeqMan module (DNASTAR). FGENESB (SoftBerry, Mount Kisco, NY) and GenDB (Meyer *et al.*, 2003) were used to annotate the consensus DNA of the largest contig.

Results

Identification of an *iac* locus coding for indole 3-acetic acid catabolism

A total of 4416 plasposon mutants of *P. putida* 1290 were screened for reduced ability to grow on M9 minimal medium containing IAA as the sole source of carbon and energy. Twelve of such IAA^- mutants were identified. Only mutant 26E2 completely failed to produce biomass on M9 IAA plates; all others formed tiny microcolonies. The DNA sequence surrounding the plasposon insertion site in each of the IAA^- mutants was determined and analyzed by BLASTX (Table 2). Eleven mutants carried their plasposon in different ORFs with high coding similarity to genes identified previously in *Pseudomonas putida* strains. Mutant 26E2 carried the plasposon in an ORF provisionally called *iacH*, with coding similarity to several amidases from plants and bacteria, but without obvious homologs in any *Pseudomonas* species, with the exception of *P. putida* GB-1 (Corstjens *et al.*, 1992; unpublished genome sequence with accession number NZ_AAXR00000000).

Using *iacH*-specific primers (Table 1), four *iacH*-carrying fosmid clones were identified by PCR in a large-insert library of *P. putida* 1290 genomic DNA. Restriction analysis of these fosmids pCC1FOS9, −10, −11 and −12 revealed that their inserts overlapped over a region of *c.* 16 kb (not shown). *Pseudomonas putida* KT2440 (Nelson *et al.*, 2002), which is not able to utilize IAA as a carbon end energy source (Leveau & Lindow, 2005), was transformed with fosmid pCC1FOS9, −10, −11 or −12. In each case, transformants were obtained with the ability to grow on M9 IAA agar plates. In liquid M9 IAA medium, growth of *P. putida* KT2440 carrying pCC1FOS9, −10, −11 or −12 occurred at a rate μ_{max} of 0.51 h^{-1} and was characterized by concomitant and complete removal of IAA from the medium (Fig. 2). The same IAA^+ phenotype was observed after transformation of *P. putida* KT2440 with fosmid pCC1FOS11Eco, a truncated derivative of pCC1FOS11 carrying the fragment that is shared between pCC1FOS9, −10, −11 and −12 (Fig. 3). No IAA^+ transformants were obtained after transformation of KT2440 with only water or with negative control fosmid pCC1FOS15 (Table 1).

Shotgun DNA sequencing of pooled pCC1FOS9, −10, −11 and −12 revealed 39 ORFs on the largest assembled contig. ORFs 9 through 27 could be mapped to the DNA insert on pCC1FOS11Eco (Fig. 3). Transposon insertion analysis of pCC1FOS9-12 revealed that inactivation of ORFs 19, 21, 23, 24, 25 and 26 did not affect the IAA^+ phenotype, whereas insertions in ORFs 9, 11, 12, 13, 14, 17 and 18 abolished or reduced the ability to grow on IAA (Fig. 3). This was independently confirmed by testing deletion derivatives of pCC1FOS11Eco for their ability to confer an IAA^+ phenotype to *P. putida* KT2440 (Fig. 3). As only pCC1FOS11EcoAscPLUS scored positive, ORFs 19 through 27 seem dispensable for growth on IAA. From this, it was concluded that ORFs 9 through 18, collectively referred to as the *iac* locus, encode a pathway for indole 3-acetic acid catabolism. The DNA sequence of pCC1FOS11EcoAscPLUS carrying the 8994-bp *iac* locus has been

Table 2. BLASTX analysis of DNA sequences flanking plasposon insertion sites in IAA$^-$ mutants

IAA$^-$ mutant	Benzoate phenotype*	Putative function†	Most similar published gene product (identity score‡)	References
26E2	+	Amidase	Glu-tRNA amidotransferase of *Acinetobacter baumannii* ATCC 17978 (23/29)§	Smith *et al*. (2007)
47H11	+	Sensory box histidine kinase	MhaS of *P. putida* U (89/90)	Arias-Barrau *et al*. (2005)
45A5	+	Same as above	MhaS of *P. putida* U (311/319)	Arias-Barrau *et al*. (2005)
30E1	+	Transcriptional activator of *cat* gene expression	CatR of *P. putida* KT2440 (72/75)	Nelson *et al*. (2002), Jimenez *et al*. (2002)
39B11	+	Same as above	CatR of *P. putida* KT2440 (176/203)	Nelson *et al*. (2002), Jimenez *et al*. (2002)
64F5	+	Same as above	CatR of *P. putida* KT2440 (177/206)	Nelson *et al*. (2002), Jimenez *et al*. (2002)
52F8	+	Muconolactone isomerase	CatC of *P. putida* KT2440 (19/19)	Nelson *et al*. (2002), Jimenez *et al*. (2002)
39D2	−	Transcriptional activator of *pca* gene expression	PcaR of *P. putida* WCS358 (242/256)	Bertani *et al*. (2001)
40D5	−	3-carboxy-*cis*,*cis*-muconate cycloisomerase	PcaB of *P. putida* KT2440 (56/59)	Nelson *et al*. (2002), Jimenez *et al*. (2002)
44D7	−	Phosphoglycerate kinase	Pgk of *P. putida* KT2440 (193/201)	Nelson *et al*. (2002)
49F3	−	Malate : quinone oxidoreductase	Mqo-1 of *P. putida* KT2440 (94/94)	Nelson *et al*. (2002)
53D11	−	Same as above	Mqo-1 of *P. putida* KT2440 (229/239)	Nelson *et al*. (2002)

*Able (+) or unable (−) to grow on benzoate as sole carbon source.
†Based on BLASTX analysis of DNA sequences flanking the plasposon insertion site.
‡Identity score (*x*/*y*) is expressed as the number (*x*) of identical amino acids shared between query and subject over the length (*y*) of the comparison.
§The identity score was 27/29 with gene PputGB1DRAFT_3649 in the unpublished genome sequence of *P. putida* GB-1.

submitted to the DDBJ/EMBL/Genbank database (accession number EU 360594).

Within the *iac* locus, ORF18 (*iacI*) seemed absolutely required for growth utilization of IAA by *P. putida* 1290: the IAA$^-$ phenotype of mutant 26E2 (Fig. 4a), carrying a plasposon insertion in the *iacH* gene (ORF17), was fully restored after complementation with pCC1FOS11EcoASC-PLUS but not with pCC1FOS11EcoAsc (Fig. 3). Given the location and orientation of *iacI* relative to *iacH*, the IAA$^-$ phenotype of mutant 26E2 could be explained as a polar, negative effect on *iacI* expression as a result of the plasposon insertion in *iacH*.

It was found that mutant 26E2 was also affected in its ability to use indole-3-acetaldehyde as a carbon and energy source (Fig. 4b). Indole 3-acetamide could not be used as a growth substrate by either wild-type *P. putida* 1290 or mutant 26E2 (Fig. 4c). Radish root elongation assays revealed that of all the IAA$^-$ mutants, only 26E2 was significantly ($P = 0.00031$) affected in its ability to abolish the deleterious effect of IAA on root elongation (Table 3). The simplest explanation for this is to assume that *iacH* and/or *iacI* are involved in the early steps of the IAA degradation pathway. Mutants blocked further downstream in the degradation pathway (e.g. 39D2, 40D5, 52F8 and 64F5) would be expected to retain activity towards IAA and thus the ability to rescue roots from IAA toxicity (Table 3).

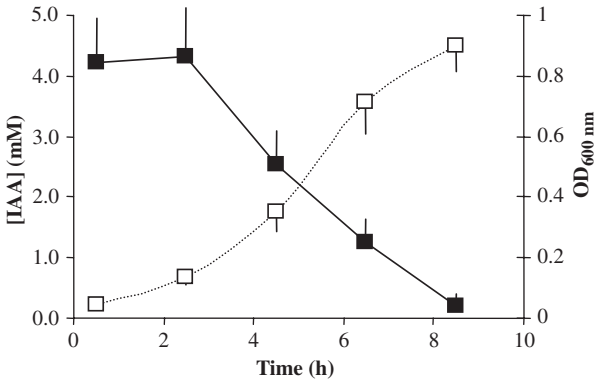

Fig. 2. Growth utilization of IAA by *Pseudomonas putida* KT2440 harboring pCC1FOS9, −10, −11 or −12. Each point shows the OD (□) or IAA concentration in the supernatant (■) averaged over M9 IAA cultures of *P. putida* KT2440 carrying pCC1FOS9, −10, −11 or −12.

Functional gene prediction of the *iac* locus and occurrence of *iac* gene clusters in other bacteria

The *iac* locus appeared to consist of three putative transcriptional units (Fig. 3): (1) an operon of ORFs 9–15 (provisionally named *iacA*, *iacB*, *iacC*, *iacD*, *iacE*, *iacF* and *iacG*), (2) an operon of ORFs 17 and 18 (*iacH* and *iacI*), and (3) a single gene (ORF16 or *iacR*) located upstream and transcribed divergently from ORF17. Table 4 lists the results of a

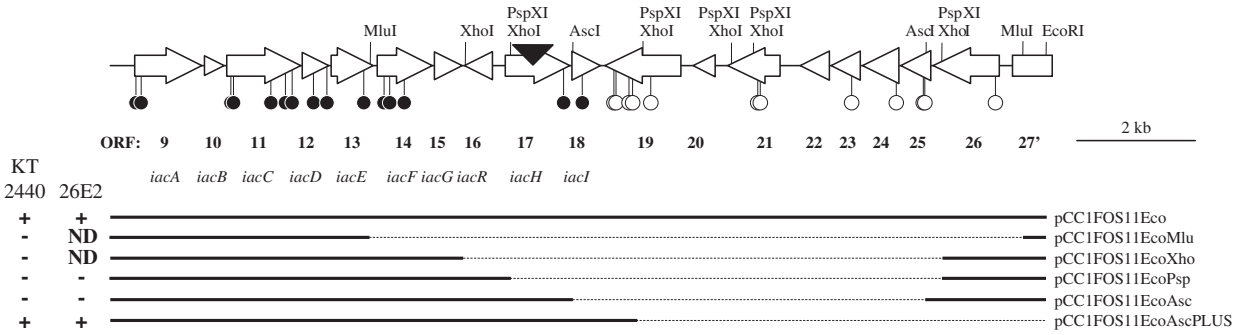

Fig. 3. Gene map of the 16-kb insert on fosmid library clone pCC1FOS11Eco. ORFs are numbered 9 through 27 and represented as open arrows. For some ORFs, their corresponding *iac* gene name is given (see Table 4). The relative locations of recognition sites for MluI, XhoI, PspXI, and AscI are indicated. The black arrow point in ORF17 marks the plasposon insertion site in *P. putida* 1290 mutant 26E2. Also indicated are the locations of transposon insertion sites on pCC1FOS9, −10, −11, or −12 and the effect of each insertion on the ability of these fosmid library clones to confer an IAA^+ phenotype to *Pseudomonas putida* KT2440. Closed circles indicate that this ability was abolished or reduced, and open circles indicate that there was no effect. The bottom part of the figure shows a set of deletion derivatives of pCC1FOS11Eco and their individual ability (+, yes; −, no) to bestow IAA growth utilization upon *P. putida* KT2440 or complement the IAA^- phenotype of *P. putida* 1290 mutant 26E2 (ND = not determined).

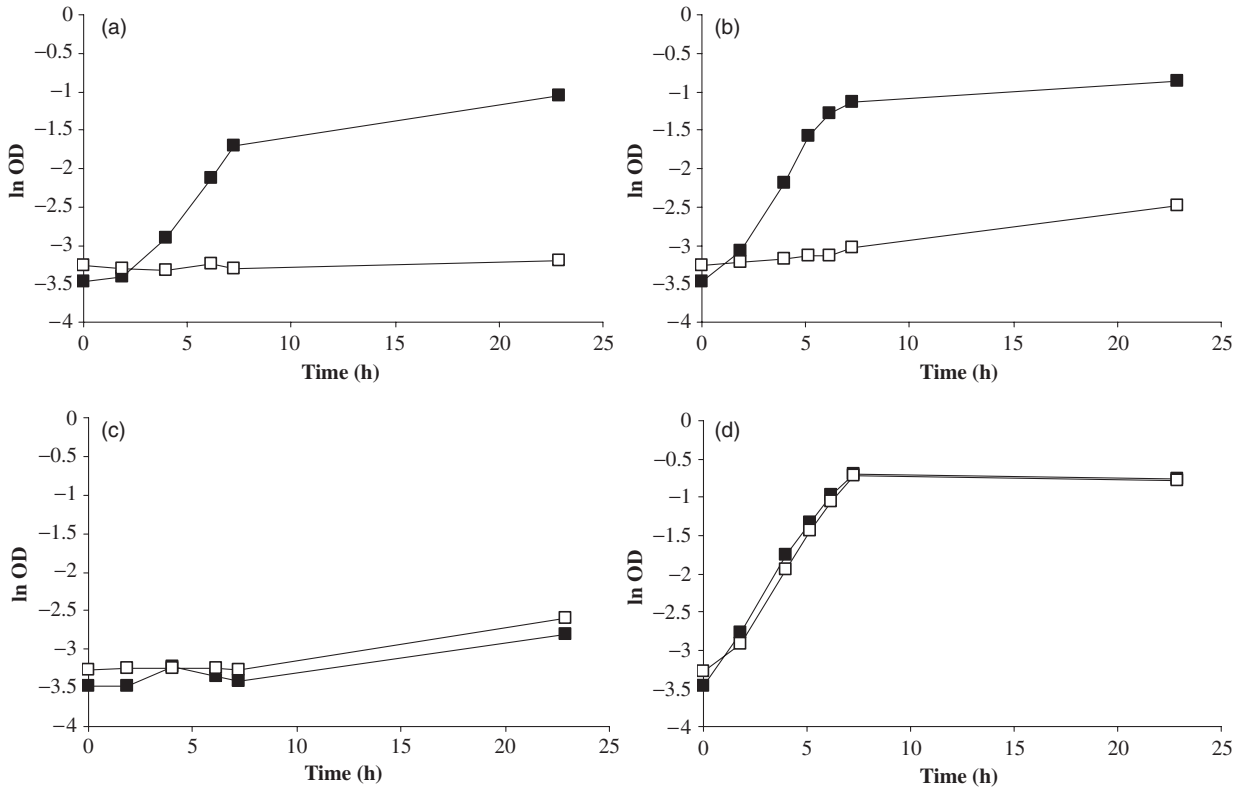

Fig. 4. Growth of *Pseudomonas putida* 1290 (filled squares) or mutant 26E2 (open squares) on M9 minimal medium supplemented with 5 mM IAA (a), indole 3-acetaldehyde (b), indole 3-acetamide (c), or glucose (control, d).

BLASTP analysis for each of the *iac* gene products. Among these, four genes, i.e. *iacA*, *iacC*, *iacD* and *iacH*, with substantial coding similarity to enzymes that have been shown to act on aromatic N-heterocyclic compounds, were identified (Table 4). For two genes in the *iac* locus, no function could be predicted based on sequence similarity. BLASTP analysis of the *iacB* and *iacI* gene products revealed 21 and 22 homologs for each, respectively, in Genbank. Annotated as (conserved) hypothetical proteins, these 43 homologs were encoded on 21 finished or draft bacterial

genome sequences (Fig. 5): three *Gammaproteobacteria*, five *Alphaproteobacteria*, ten *Burkholderia* species, and three species belonging to the class *Actinobacteria*, order *Actinomycetales*, suborder Corynebacterinae. On the near-complete genome sequence of uncultivated *Burkholderia* sp. SAR-1 (Venter *et al.* 2004), homologs of *iacB* and *iacI* were also identified (Fig. 5).

Strikingly, in all of the 22 genomes mentioned above, homologs of *iacB* and/or *iacI* were locally clustered with genes showing high coding similarity to at least five of the other genes from the *iac* locus in *P. putida* 1290 (Fig. 5). Synteny of *iac* genes was differentially conserved among these genomes. Most noticeable was the fully preserved gene order in *P. putida* GB-1, compared to *P. putida* strain 1290. Overall, the gene syntenies found in *Actinobacteria* and *Alpha-* and *Gammaproteobacteria* appeared more similar to each other than to those in the *Burkholderia* strains.

Based on the discovery of *iac*-like genes in other bacteria, several strains listed in Fig. 5 were tested for their ability to grow on IAA. Indeed, *P. putida* GB-1, *Burkholderia* sp. 383, *Sphingomonas wittichii* RW1, and *Rhodococcus* sp. RHA1 all produced biomass on M9 minimal medium containing 5 mM IAA as the sole source of carbon and energy. The same observation was made for *Marinomonas* sp. MWYL1 (M. Wexler, pers. commun.). These results confirmed that IAA utilizers have representatives in the *Alpha-*, *Beta-* and *Gammaproteobacteria* as well as among the high G+C Gram-positive bacteria.

Table 3. Ability of wild-type or mutant strains of *Pseudomonas putida* 1290 to abolish the effect of 1 mM exogenous IAA on radish root elongation

	Mean (± SD) radish root length (mm)		
Strain	− IAA	+IAA	*P*-value
None	86 ± 21	33 ± 22	0.00004
26E2	89 ± 15	57 ± 17	0.00031
53D11	73 ± 19	56 ± 17	0.06620
45A5	77 ± 17	60 ± 24	0.09007
1290R	68 ± 24	78 ± 22	0.32955
64F5	64 ± 25	71 ± 17	0.47302
52F8	78 ± 21	72 ± 23	0.50850
39D2	58 ± 26	52 ± 20	0.53064
40D5	80 ± 21	78 ± 17	0.89794

Involvement of *cat* and *pca* genes in the IAA degradation pathway

Based on sequence data from other plasposon mutants of *P. putida* 1290 (Table 2), it was assumed that the IAA

Table 4. ORF prediction for the *iac* locus on pCC1FOS11EcoAscPLUS

ORF; gene name	Length (bp)	Product size (aa)	Highest BLASTP similarity to; from; amino acid identity; *e*-value	Best BLAST hit with experimentally verified activity; from; amino acid identity (reference)
9; *iacA*	1170	389	Acyl-CoA dehydrogenase-like; *P. putida* GB-1; 95%; 0.0	IdoA: indole oxygenase; *Pseudomonas alcaligenes* PA-10; 42%; Alemayehu *et al.* (2004)
10; *iacB*	363	120	Conserved hypothetical protein; *P. putida* GB-1; 95%; 5e-63	None
11; *iacC*	1284	427	Aromatic ring hydroxylating dioxygenase, Rieske (2Fe–2S) protein, alpha subunit; *P. putida* GB-1; 95%; 0.0	TadA1: large subunit of terminal oxygenase component of aniline dioxygenase; *Delftia tsuruhatensis* AD9; 36%; Liang *et al.* (2005)
12; *iacD*	480	159	Aromatic ring hydroxylating dioxygenase, beta subunit; *P. putida* GB-1; 89%; 4e-79	AntB: small subunit of terminal oxygenase component of anthranilate 1,2-dioxygenase; *Pseudomonas resinovorans* CA10; 30%; Urata *et al.* (2004)
13; *iacE*	783	260	Short-chain dehydrogenase/reductase; *P. putida* GB-1; 91%; 2e-112	BbsD: 3-hydroxyacyl-CoA dehydrogenase; *Thauera aromatica* K172; 34%; Leuthner & Heider (2000)
14; *iacF*	960	319	Ferredoxin; *P. putida* GB-1; 88%; 1e-157	VanB: reductase component of vanillate-*O*-demethylase; *Pseudomonas fluorescens* BF13; 51%; Civolani *et al.* (2000)
15; *iacG*	588	195	Flavin reductase domain protein, FMN-binding; *P. putida* GB-1; 93%; 6e-84	HpaC: reductase component of 4-hydroxyphenylacetate 3-monooxygenase; *Escherichia coli*; 41%; Galán *et al.* (2000)
16; *iacR*	504	167	Transcriptional regulator, MarR family); *P. putida* GB-1; 88%; 3e-77	OhrR: organic peroxide-inducible negative regulator; *Xanthomonas campestris* pv. phaseoli; 30%; Sukchawalit *et al.* (2001)
17; *iacH*	1125	374	Glu-tRNA amidotransferase; *P. putida* GB-1; 89%; 2e-163	*At*AMI1; amidase; *Arabidopsis thaliana*; 33%; Pollmann *et al.* (2003)
18; *iacI*	540	179	Conserved hypothetical protein; *P. putida* GB-1; 90%; 9e-84	None

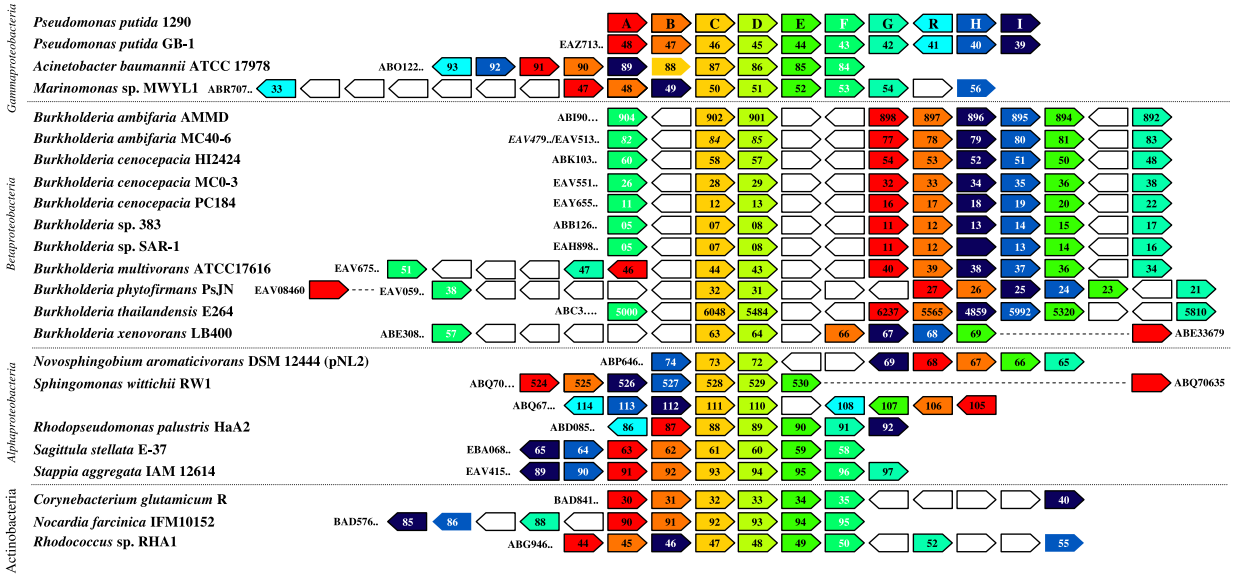

Fig. 5. Comparison of gene synteny of the *iac* locus in *Pseudomonas putida* 1290 with that of *iac*-type gene clusters in other bacterial genomes. The sizes of genes and intergenic spaces are not to scale. The orientation of each gene is indicated. Lettering of *iac* genes in *P. putida* 1290 is consistent with Table 4: for example, A stands for *iacA*. Homologs of *iac* genes in other bacteria are indicated by the same color and the accession numbers to their products are given in code, e.g. the IacB homolog of *Burkholderia* sp. 383 corresponds to accession number ABB12612. Genes lacking a black border code for homologs with a significantly lower degree of similarity to the corresponding *P. putida* 1290 *iac* gene product.

degradation pathway features catechol as a central metabolite. In three mutants (30E1, 39B11 and 64F5), the plasposon was inserted into a *catR*-like gene, which, in other pseudomonads, is required for the *catBCA*-encoded conversion of catechol via *cis,cis*-muconic acid to 3-oxoadipate enol-lactone (Harwood & Parales, 1996). Failure to express the *catBCA* operon in mutants 30E1, 39B11, and 64F5 would explain the brown coloring of IAA plates by these strains (not shown) as the accumulation of catechol. Another mutant, 52F8, carried a plasposon in the *catC* gene, which probably blocked the expression of downstream, located *catA* for catechol 1,2-dioxygenase, also explaining its brown color on IAA plates (not shown) as an accumulation of catechol. The apparent involvement of *cat* genes during IAA degradation is in agreement with the previous observation that cell extracts of IAA-grown *P. putida* 1290 cells showed induced levels of catechol 1,2-dioxygenase activity (Leveau & Lindow, 2005).

Five of the IAA$^-$ mutants (39D2, 40D5, 44D7, 49F3 and 53D11) also lacked the ability to grow on benzoate as the sole source of carbon and energy. This suggests a merger of the degradation pathways of IAA and benzoate at 3-oxoadipate enol-lactone, which is converted to acetyl-CoA and succinyl-CoA by the products of genes *pcaD*, *pcaIJ* and *pcaF*. In several *Pseudomonas* species, expression of these genes is positively regulated by the *pcaR* gene product. In mutant 39D2, a homolog of this gene was knocked out. The IAA$^-$ phenotype of another mutant, 40D5A, may be explained as a polar effect of a plasposon in *pcaB* on the downstream expression of *pcaD*.

Identification of other genes essential for IAA degradation

Products of the *pca* pathway, i.e. acetyl-CoA and succinyl-CoA, are typically fed into the tricarboxylic acid (TCA) cycle. It was hypothesized that malate : quinone oxidoreductase, encoded by the *mqo*-1 gene that is knocked out in mutants 49F3 and 53D11, is required for efficient TCA cycling, as has been suggested for other bacteria (Molenaar *et al.*, 2000). Furthermore, it was assumed that the *pgk* gene for phosphoglycerate kinase, which is disrupted in mutant 44D7, is necessary for gluconeogenesis during growth on IAA, based on the reported phenotypes of *pgk* mutants of *P. putida* strain A.3.12 (Aparicio *et al.*, 1971). Analysis of IAA$^-$ mutants 45A5 and 47H11 revealed a gene with high coding similarity to the sensor/histidine kinase component (Mascher *et al.*, 2006) of the two-component regulatory systems MhaSR in *P. putida* U (Arias-Barrau *et al.*, 2005) and CbrAB in *P. aeruginosa* PAO (Nishijyo *et al.*, 2001). Mutants of *P. aeruginosa* PAO1 that were inactivated in *cbrAB* were unable to grow on several N-substrates including arginine, ornithine, histidine, spermidine, putrescine and agmatine. It was suggested that CbrAB controls the expression of several catabolic pathways in response to changing intracellular C : N ratios (Nishijyo *et al.*, 2001).

Thus, their homolog in *P. putida* 1290, provisionally designated CbrA, is a protein possibly involved in IAA sensing.

Discussion

Given the fact that for two of the *iac* genes (i.e. *iacB* and *iacI*) no function could be predicted from sequence homology, and that at least one of them (i.e. *iacI*) is essential for IAA degradation, any prediction of the IAA-degradative pathway as it operates in *P. putida* 1290 remains speculative and needs further biochemical investigation. In preliminary experiments, it was observed that colonies of *E. coli* carrying the *iacA* gene from *P. putida 1290* turned a blue color on LB plates. This presumed production of indigo from indole has been linked to several genes related to *iacA* (Hart *et al.*, 1990; Kim & Oriel, 1995; Solaiman & Somkuti, 1996; Drewlo *et al.*, 2001; Alemayehu *et al.*, 2004; Choi *et al.*, 2004; Lim *et al.*, 2005), and targets IacA as a possible candidate for the initial attack on IAA. The contribution of *iacH* to the mineralization of IAA by *P. putida* 1290 remains enigmatic. The predicted IacH product carries the hallmarks of an EC 3.5 amidase/amidohydrolase (Chebrou *et al.*, 1996). However, together with its closest homologs in Fig. 5, it clearly clusters away from those amidases for which a function has been demonstrated. Among the latter, amino acid sequence is not necessarily a reliable predictor for substrate specificity, which can be altered by a single amino acid substitution (Okada *et al.*, 1983). Furthermore, many of these amidases have dual specificities, cleaving amide as well as ester and nitrile bonds (Patricelli & Cravatt, 2000; Pollmann *et al.*, 2003; Cilia *et al.*, 2005). Future studies will reveal the substrate(s) for IacH and ascertain whether these substrates and their products feature in the IAA mineralization pathway. It is unlikely that *iacH* is involved in the conversion of indole-3-acetamide (IAM) to IAA: if it were, it would be difficult to explain why *P. putida* 1290 cannot grow at the expense of IAM (Fig. 4c).

From Fig. 5, it is concluded that the distribution of *iac* genes is remarkably discontinuous within the bacterial tree. While the finished genome sequences of 81 *Alphaproteobacteria*, 144 *Gammaproteobacteria*, and 47 *Actinobacteria* are available to date (http://www.genomesonline.org), clusters of highly homologous *iac* genes were found in only three (DSM12444, RW1, HaA2), two (ATCC 17978, MWYL1), and three (R, IFM 10152, RHA1) of those genomes, respectively. As for the 49 finished Betaproteobacterial genomes, *iac* homologs were present only in six *Burkholderia* species (AMMD, HI2424, 383, E264, LB400, ATCC 17616) and notably absent in 10 other *Burkholderia* species (four *Burkholderia mallei*, four *Burkholderia pseudomallei*, *Burkholderia vietnamiensis* G4, and *Burkholderia cenocepacia* AU1054).

Having linked the *P. putida* 1290 phenotype of IAA catabolism to the *iac* gene locus, a significant contribution has been made towards assigning a putative biological function to several *iac* gene homologs in the public databases for which no function had yet been described. Future research efforts will be targeted towards establishing the functionality of these *iac* homologs and determining the significance of the observed differences in *iac* gene synteny in the different bacterial species (Fig. 5). Also, the link between IAA and *iac* allowed identification of several of the *iac*-bearing bacteria as previously unrecognized IAA degraders. Interestingly, these strains originated from environments known to be natural sources of IAA, such as the plant rhizosphere (*Marinomonas* sp. MWYL1) and soil (*Burkholderia* sp. 383, *Rhodococcus* sp. RHA1), but also from less obvious environments such as river water (*S. wittichii* RW1). Two of the bacterial species that were identified as carrying *iac* genes are clinical isolates, i.e. *N. farcinica* IFM 10152 and *Acinetobacter baumannii* ATCC 17978. Strains of *A. baumannii* are commonly isolated from hospitalized patients, where they are known to be able to infect, among other things, the urinary tract. Interestingly, urine is also a known source of IAA (Kogl *et al.*, 1934), which allows one to start speculating on the role of *iac* genes in the infection process of *A. baumannii* isolates.

Intriguingly, *B. japonicum* USDA 110, the only confirmed IAA degrader (Jarabo-Lorenzo *et al.*, 1998) for which a genome is available (Kaneko *et al.*, 2002), conspicuously lacks *iac* genes, except for a highly homologous *iacA* homolog. The gene product of the latter, NrgC (accession number AAG 61032), is mentioned in several studies as being regulated by nitrogen fixation regulator NifA (Nienaber *et al.*, 2000) and expressed in symbiosomes (Hoa *et al.*, 2004). However, this gene has not been linked previously to IAA, which, in the context of the authors' findings, seems to be an exciting avenue for further study, given (1) the stimulatory role of IAA in nodule formation by *B. japonicum* (Boiero *et al.*, 2007) and (2) the location of *nrgC* on a *nod*/*nif* symbiosis island (Gottfert *et al.*, 2001) with close proximity to genes encoding a nodulation protein (accession number AAG61031) and a nodulation formation efficiency protein (accession number AAG61037). The apparent absence of additional *iac* homologs from the *B. japonicum* USDA 110 genome furthermore suggests that this strain possesses genes other than *iac* homologs for IAA degradation.

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