Demonstration of the indolepyruvate decarboxylase gene homologue in different auxin-producing species of the *Enterobacteriaceae*

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Different *Enterobacteriaceae* were assayed for their ability to produce the plant hormone indole-3-acetate with the aim to study the distribution of the indole-3-pyruvate pathway, which is known to be involved in the production of indole-3-acetate in a root-associated *Enterobacter cloacae* strain. Other *E. cloacae* strains, and also *Enterobacter agglomerans* strains, *Pantoea agglomerans*, *Klebsiella aerogenes*, and *Klebsiella oxytoca* were found to convert tryptophan into indole-3-acetate. As it was also intended to identify the conserved regions of the indole-3-pyruvate decarboxylase, which is involved in producing indole-3-acetate in the *E. cloacae* strain, oligonucleotide primers were synthesized for different regions of the corresponding gene. One pair of these primers allowed us to amplify a segment of the predicted size by the polymerase chain reaction with DNA of the seven different *Enterobacteriaceae* that produce indole-3-acetate. Segments of five strains were cloned and sequenced. All sequences showed significant homology to the indole-3-pyruvate decarboxylase gene. As in addition a positive DNA–DNA hybridization signal was detected in the seven strains using the *E. cloacae* or *E. agglomerans* segments as a probe, indole-3-acetate biosynthesis is suggested to be catalyzed via the indole-3-pyruvate pathway not only in *E. cloacae* but also in the other soil-living *Enterobacteriaceae*. Conserved regions were detected in the indole-3-decarboxylase by alignment of the now-available five different partial sequences. These regions should enable identification of the gene in other bacterial families or even in plants.

Key words: indole-3-pyruvate decarboxylase, indole-3-acetic acid production, auxin, polymerase chain reaction, *Enterobacteriaceae*.

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Différentes *Enterobacteriaceae* ont été analysées quant à leur capacité de produire l'hormone végétale indole-3-acétate, dans le but d'étudier la distribution de la voie métabolique de l'indole-3-pyruvate; il est connu que cette voie est impliquée dans la production d'indole-3-acétate chez une souche d'*Enterobacter cloacae* associée à la racine. Il a été découvert que d'autres souches d'*Enterobacter cloacae*, mais aussi des souches d'*Enterobacter agglomerans*, de *Pantoea agglomerans*, de *Klebsiella aerogenes* et de *Klebsiella oxytoca* transforment le tryptophane en indole-3-acétate. Puisqu'il était aussi projeté d'identifier les régions conservées de l'indole-3-pyruvate décarboxylase, laquelle est impliquée dans la production d'indole-3-acétate chez la souche d'*E. cloacae*, des amorces d'oligonucléotides ont été synthétisées pour différentes régions du gène correspondant. Une paire de ces amorces a permis d'amplifier, par la réaction en chaîne de polymérisation, un segment de taille prévue avec l'ADN provenant des sept *Enterobacteriaceae* différentes productrices d'indole-3-acétate. Des segments de cinq souches ont été clonés et séquencés. Toutes les séquences ont montré une homologie significative au gène de l'indole-3-pyruvate décarboxylase. Puisqu'en plus, un signal positif d'hybridation ADN:ADN a été détecté dans les sept souches en utilisant les segments de l'*E. cloacae* ou de l'*E. agglomerans* comme sonde, la biosynthèse d'indole-3-acétate pourrait être catalysée par l'entremise de la voie de l'indole-3-pyruvate, non seulement chez l'*E. cloacae*, mais aussi chez les autres *Enterobacteriaceae* provenant du sol. Des régions conservées de l'indole-3-pyruvate décarboxylase ont été découvertes par l'alignement des cinq séquences partielles maintenant disponibles. Ces régions devraient aussi permettre l'identification du gène chez d'autres familles bactériennes ou même chez les plantes.

Mots clés : indole-3-pyruvate décarboxylase,.production d'acide indole-3-acétique, auxine, réaction en chaîne de polymérisation, *Enterobacteriaceae*.

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Many free-living, root-associated, or symbiotic bacteria produce the plant hormone indole-3-acetic acid (IAA). However, several pathways for the biosynthesis of this auxin have been described for different bacteria. IAA is synthesized from tryptophan (Trp) via indole-3-acetamide in phytopathogenic species, such as *Agrobacterium tumefaciens* and *Pseudomonas syringae* (Yamada et al. 1985; Klee et al. 1984). Indole-3-acetonitrile is an intermediate of IAA biosynthesis in *Alcaligenes faecalis* (Kobayashi et al. 1993). In *Pseudomonas fluorescens*, IAA is one of the products of a Trp side-chain oxidase (Oberhänsli et al. 1991). An indole-3-pyruvate decarboxylase (IPDC) is involved in IAA production in *Enterobacter cloacae* FERM BP-1529, which was isolated from the rhizosphere of cucumber in Japan (Koga et al. 1991). However, other *Enterobacter* species were found to be incapable of forming IAA (Koga et al. 1991). In plant growth promoting bacteria, like *Pseudomonas fluorescens*, *Azospirillum* spp., root-associated *Bacillus* species, and *Enterobacteriaceae*, the production of IAA is probably regulated, as IAA causes an increasement of isolated wheat root sections only in concentrations of 10^{-11} to 10^{-8} M (Libbert 1957) whereas in liquid cultures of the plant growth promoting bacterium *Azospirillum* sp., concentrations of up to 10^{-3} M IAA are reached, which inhibits growth of plant roots (Zimmer et al. 1988). Anthranilate is probably involved in the regulation of IAA biosynthesis in *Azospirillum brasilense* (Zimmer et al. 1991).

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TABLE 1. IAA and indole-3-lactate production by *Enterobacteriaceae*

	Concentration of indole-3-acetate in the culture (mg/L)	Concentration of indole-3-lactate in the culture (mg/L)
Enterobacter agglomerans 333	2.7±0.8	0.3±0.2
Enterobacter agglomerans 339	3.5±1.1	0.4±0.2
Enterobacter cloacae NCIMB 11461	2.5±0.8	0.5±0.3
Enterobacter cloacae NCIMB 11463	2.2±0.6	2.1±1.2
Pantoea agglomerans IMET 11328	3.3±1.2	1.6±0.7
Klebsiella aerogenes DSM 681	7.9±2.6	0.3±0.2
Klebsiella oxytoca DSM 3539	5.1±2.0	0.2±0.1
Escherichia coli K12	0.1±0.1	0.2±0.1

NOTE: The different strains were grown aerobically (30°C) in 10 mL of medium containing (in g/L): $MgSO_4 \cdot 7\ H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.02; $NaMoO_4 \cdot 2H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.01; NH_4Cl, 0.27; KH_2PO_4, 0.61; K_2HPO_4, 0.78; $FeSO_4 \cdot 7H_2O$, 0.0138; ethylenediaminetetraacetic acid, 0.0186; and glucose, 2.0. After 48 h, L-Trp was added (50 mg/L) and incubation was continued for 48 h. After centrifugation (5 min, 10 000 × *g*) the amount of IAA and indole-3-lactate was determined in the supernatant of the culture by HPLC (separation on a C-18 column, methanol – 1% H_3PO_4, 40:60 (v/v) as mobile phase, detection at 280 nm). Values are given as the mean ± standard deviation of three independently performed experiments. To verify the identity of the peaks the separation was also performed with ethanol – 5% acetic acid, 30:70 (v/v) as mobile phase (data not shown).

To identify the indole-3-pyruvate decarboxylase gene in distantly related bacterial families or even in plants it was intended to find conserved regions in the gene that might be used as oligonucleotide primers for polymerase chain reactions (PCR) or as a probe in DNA–DNA hybridization experiments. As sequence data were only available from *Enterobacter cloacae* FERM BP-1529 (Koga et al. 1991) it was necessary to isolate segments of the gene from several systematically related strains. To select strains for this purpose, the Trp-dependent IAA production of different *Enterobacteriaceae* was assayed. Whereas the soil-living strains converted about 1/20 to 1/7 of the added Trp into IAA in 48 h, only small amounts of IAA were measured in the culture of *Escherichia coli* K12, in which IAA formation is probably due to chemical degradation (Table 1). In all cases indole-3-lactate was also released (Table 1), which is reported to be reversibly formed from indole-3-pyruvate (Ernstsen et al. 1987; Garcia-Tabares et al. 1987), but the amount did not correlate with the concentration of IAA released.

Oligonucleotides were synthesized for eight regions of the polypeptide sequence of the published IPDC gene where degeneration of the genetic code was low (data not shown). PCR was performed with different combinations of oligonucleotide primers and DNA from different *Enterobacteriaceae* strains. In the case of oligonucleotide 1 (corresponding to amino acid positions 55–64 of the IPDC gene published by Koga et al. 1991: 5′AAC GGC (AG)TT GCC GGC AGC TA(CT) GC(GC) GA 3′) and oligonucleotide 2 (complementary to the coding DNA sequence of amino acid positions 407–416: 5′ GCC GCC GCC AGC GTG TAA CC(AG) AT(GC) GA(GC) CCC CA 3′), a 1-kb segment could be amplified in all strains except *Escherichia coli* K12 (Fig. 1A). As the 1-kb size corresponded to the distance between the oligonucleotide primers in the IPDC gene and as the segment could only be amplified in the IAA-producing strains, the amplification products were probably a segment of the IPDC gene in each case. As degenerated oligonucleotides were used in most of the assays, segments smaller than 1 kb were also amplified. The 1-kb amplification products of *Enterobacter cloacae* NCIMB 11461, *Enterobacter cloacae* NCIMB 11463, *Enterobacter agglomerans* 333, *Enterobacter agglomerans* 339, and *Klebsiella aerogenes* DSM 681 were either cloned directly into M13mp18 (see Zimmer 1993) or were cloned into pCR™II (TA Cloning™ System, Invitrogen Corporation, San Diego, Calif., U.S.A.). Hybridization with the labeled segments of *Enterobacter cloacae* NCIMB 11463, *Enterobacter agglomerans* 339, and *Klebsiella aerogenes* DSM 681 on a Southern blot of the separated PCR segments revealed strong signals only in the case of the 1-kb segment for seven of the strains but not for *Escherichia coli* K12 (Fig. 1B), indicating that the smaller segments were indeed unspecific amplification products. With these oligonucleotides and the given conditions, no segment of the expected size could be amplified from other soil-living bacteria (data not shown), such as *Agrobacterium tumefaciens* GMI9023, *Bradyrhizobium japonicum* DES 122, *Pseudomonas stutzeri* ZoBel, *Pseudomonas aeroginosa* DSM6195, and *Pseudomonas putida*, and also not from *Azospirillum brasilense* Sp7 in which the IPDC pathway was demonstrated (Bothe et al. 1994). To verify that the 1-kb segments originated from DNA of the seven strains a hybridization experiment was performed using a Southern blot of *Eco*R1-digested genomic DNA of the different *Enterobacteriaceae* and the labeled segment of *Enterobacter cloacae* NCIMB 11461 as a probe. It revealed positive signals in all strains where the PCR had led to amplification of the 1-kb segment (Fig. 2). The multiple bands in the case of *Enterobacter cloacae* NCIMB 11463 were probably due to an incomplete digestion. Even in the case of *Enterobacter agglomerans* 339 (Fig. 2, lane 3), where the DNA was almost undigested, the weak hybridization signal was comparable to that of *K. aerogenes* and *Enterobacter agglomerans* 333. As expected, there was absolutely no hybridization signal visible in the lane of complete *Eco*R1-digested DNA of *Escherichia coli* K12. Successful hybridization was also observed when the 1-kb segment of *Enterobacter agglomerans* 333 was used as a probe (data not shown). In this case strong hybridization signals were obtained with the digested DNA of *Enterobacter agglomerans* 333 and 339.

To verify the identity of the PCR products, the cloned segments in M13mp18 or the pCR™II fragments subcloned in M13mp18 and M13mp19 were sequenced (TAQuence™ sequencing kit, United States Biochemical Corporation, Cleveland, Ohio, U.S.A.). The complete DNA sequences of the

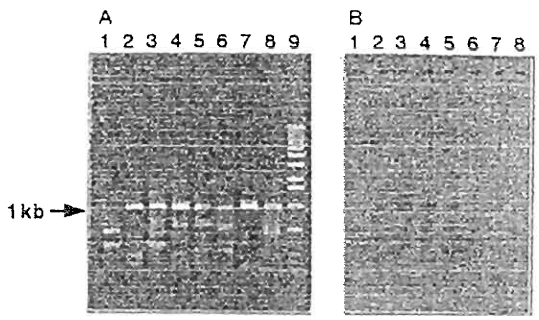

FIG. 1. Amplification of a 1-kb segment of the IPDC gene homologue from different *Enterobacteriaceae* by PCR. The reaction was performed in 30 cycles (30 s denaturation at 92°C, 30 s annealing at 48°C, and 60 s polymerization at 72°C) in a 50-μL volume containing *Taq* polymerase (Boehringer Mannheim, Germany), 2 U; 10× *Taq* polymerase buffer (Boehringer Mannheim), 5 μL; deoxynucleotides, 2.5 nmol of each; oligonucleotide 1, 50 pmol; oligonucleotide 2, 50 pmol; DNA, 1/100th of a resuspended single bacterial colony (1 mm diameter). After the reaction, 1/10 of each assay was separated on a 1.5% agarose gel (A). Lanes: 1, *Escherichia coli* K12; 2, *Enterobacter agglomerans* 333 (Kleeberger et al. 1983); 3, *Enterobacter agglomerans* 339 (Kleeberger et al. 1983); 4, *Enterobacter cloacae* NCIMB 11461; 5, *Enterobacter cloacae* NCIMB 11463; 6, *Pantoea agglomerans* IMET 11328 (Dr. S. Ruppel and Dr. C. Scholz, Zentrum für Agrarlandschaft und Landnutzungsforschung, D-15374 Müncheberg, Germany); 7, *Klebsiella aerogenes* DSM 681; 8, *Klebsiella oxytoca* DSM 3539; and 9, a 1-kb ladder (Gibco BRL, Life Technologies GmbH, Berlin, Germany) as size marker. One nanogram of the 1-kb IPDC gene insert of *Enterobacter cloacae* NCIMB 11463, *Enterobacter cloacae* 333, and *K. aerogenes* DSM 681 subcloned in pCR™ II was labeled independently by PCR in the assay described above supplemented by 2 nmol of digoxigenin-dUTP (Boehringer Mannheim). One tenth of the three PCR assays was mixed and was used as probe against a Southern blot of the separated PCR products (B). Hybridization was performed at 65°C overnight without formamide. The hybridized nitrocellulose filters (Hybond C, Amersham, Braunschweig) were coloured with a nonradioactive DNA labeling and detection kit (Boehringer Mannheim) for 2 h in the dark.

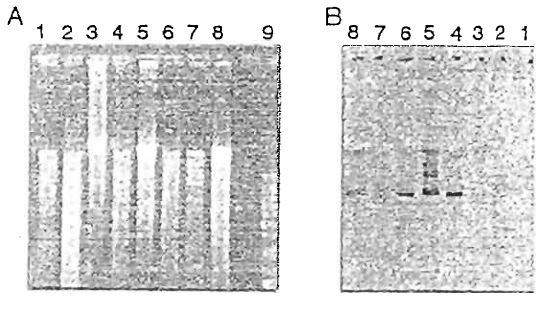

FIG. 2. DNA–DNA hybridization of the subcloned 1-kb IPDC gene segments against genomic DNA from different *Enterobacteriaceae*. One tenth of the PCR-labeled 1-kb IPDC segment of *Enterobacter cloacae* NCIMB 11463 was used as a probe against a Southern blot (B) of *Eco*RI-digested total DNA (~1000 ng) of the different *Enterobacteriaceae* (A) (for conditions see caption of Fig. 1). Lanes: 1, *Escherichia coli* K12; 2, *Enterobacter agglomerans* 333; 3, *Enterobacter agglomerans* 339; 4, *Enterobacter cloacae* NCIMB 11461; 5, *Enterobacter cloacae* NCIMB 11463; 6, *Pantoea agglomerans* IMET 11328; 7, *Klebsiella aerogenes* DSM 681; 8, *Klebsiella oxytoca* DSM 3539; and 9, a 1-kb ladder (Gibco BRL, Life Technologies GmbH, Berlin, Germany) as size marker. The most intense hybridization signals in lanes 4–8 corresponded to a size of about 8.5 kb.

1-kb segments of *Enterobacter agglomerans* 339 and *Enterobacter cloacae* NCIMB 11461 and the partially sequenced segments of *Enterobacter agglomerans* 333, *K. aerogenes* DSM 681, and *Enterobacter cloacae* NCIMB 11463 were submitted to the EMBL–GenBank–DDBJ data bases with the following accession numbers: X80714, X80715, X80713, X80716, and X80717, respectively. Homology was observed in all cases to the published sequence of the IPDC gene of *Enterobacter cloacae* FERM BP-1529 (Koga et al. 1991) with 56–67% identical bases (data not shown). Higher homology was observed for a pairwise alignment of *Enterobacter cloacae* NCIMB 11461 with *Enterobacter cloacae* NCIMB 11463, and for a pairwise alignment of *Enterobacter agglomerans* 333 with *Enterobacter agglomerans* 339, which was in agreement with the systematical relationship of these bacteria and correlated with the intensity of the hybridization signals (Fig. 2).

The deduced amino acid sequences of the sequenced regions of the six different *Enterobacteriaceae* strains were used to align the IPDC gene segments and fungal pyruvate decarboxylase sequences (Fig. 3). Fifty percent of the amino acid residues were identical in all partial IPDC sequences and a similarity of 65% was observed in all five sequences with respect to conserved amino acid exchanges (Fig. 3). The next strongest homology was detected with pyruvate decarboxylases of eukaryotic organisms that catalyze the decarboxylation of pyruvate in fungi. The strongly conserved regions of the IPDC sequences could also be partially recovered in the pyruvate decarboxylase sequences (Fig. 3). The IPDC from *Enterobacter cloacae* was reported to show this sequence similarity to the pyruvate decarboxylase of yeast (Koga et al. 1991). The alignment of the six partial sequences of bacterial indole-3-pyruvate decarboxylases and three fungal pyruvate decarboxylases now available from the EMBL data base establishes this similarity. However, the sequence comparison (Fig. 3) also showed that all IPDC sequences were more related to each other than to the pyruvate decarboxylase sequences. This is not surprising because eukaryotic sequences are often not very related to prokaryotic sequences and there are biochemical differences in the two enzymes. Whereas the pyruvate decarboxylase has a low specificity and affinity for pyruvate, the IPDC from *Enterobacter cloacae* has a high specificity and affinity for indole-3-pyruvate (Koga et al. 1992). The alignment of the indole-3-pyruvate decarboxylase and pyruvate decarboxylase sequences (Fig. 3) will permit regions specific for the IPDC gene to be selected, so that the two genes can be distinguished even in an organism that might possess both enzymes.

The homology of the PCR segments at the DNA level (data not shown) and in the deduced amino acid sequence (Fig. 3) is surprisingly low for such closely related bacteria. An explanation might be that IPDC is not essential for the survival of free-living bacteria, but plays a role in their interaction with plants. Therefore, the selection pressure might be low for this gene, allowing a high mutation rate.

Among the *Enterobacteriaceae*, *Enterobacter cloacae* was found to convert Trp into IAA by the IPDC pathway, whereas

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          **+******* +*+*******     *+ ***********+*  * +*   ++  +* **
EA333     NGIAGSYAEYVPVVHIVGAPALTSQRKGELLHHTLGDGEFSHFMRMSAPVSVAQASLT
EA339     NGIAGSYAEYVPVVHIVGAPRLTSQRKGELLHHTLGDGEFCHFMRMSAPVSVAQASLT
EC1529    NGIAGSYAEHVPVLHIVGAPGTAAQQRGELLHHTLGDGEFRHFYHMSEPITVAQAVLT
EC11461   NGVAGSYAEYVPLLHIVGAPCSGVQQRGELLHHTLGDGDFHHFYRMSEPVTAARAILT
EC11463   NGIAGSYAEYVPLLHIVGAPCSGVQQRGELLHHTLGDGDFHPFYRMSESVTAARAILT
KA681     NGIAGSYAEHIPVLHIVGAPSTGAQQPGELLHHTLGDGDFPSFARMTEQITS-QALLT
KMPDC     NGIAGSYAEHVGVLHVVGVPSISSQAKQLLLHHTLGNGDFTVFHRMSSNISETTAMIT
NCPDC     NGTGSAYAENLPLVLISGSPNTNDPSQYHILHHTLGHPDYTYQYEMAKKITCCAVAIP
SCPDC     NGIAGSYAEHVGVLHVVGVPSISSQAKQLLLH-TLGNGDFTVFHRMSANISETTAMIT
          ** ++++** + ++ + * *       +   ++ ***+ ++      **  ++      +

          +  **  *****+  ++   *****++*+**+    +    **          +
EA333     PE-NALAEIDRVIEEVMYHSRPGYLLLPSDVAALPVSTRAHALPARQPPFSPSSLEA
EA339     PE-NALAEIDRVIEEVMYSSRPGYLLLPSDVPALPVSTRAHALPARQPPFSPSSLEA
EC1529    EQ-NACYEIDRVLTTMLRERRPGYLMLPADVAKKAATPPVNALTHKQAHADSACLKA
EC11461   AQ-NACYEIDRVLEVMLMQSRPGYLMLPADVAKKPATPPVNALTTPPFPVNEACLNA
EC11463   AQ-NACYEIDRVLEVMLTQSRPGYLMLPADVAKKPATPPVNALTIPPFPVNEACLNA
KA681     AG-NAANEIDRVLRDMLTHHRPGYLIVPADVAR-AGTLPQPALRVEPPAVKPACRVL
KMPDC     DINSAPSEIDRCIRTTYISQRPVYLGLPANLVDLKVPASLLETPIDLSLKPNDPEAE
NCPDC     RAIDAPRLIDRALRAAILARKPCYIEIPTNLAG-ATCVRPGPISAITDPITSDKSAL
SCPDC     DICTPQAEIDRCIRTTYVTQRPVYLGLPANLVDLNVPAKLLQTPIDMSLKPNDAESE
              +   *** +        +* ** +*+++
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FIG. 3. Alignment of the indolepyruvate decarboxylase segments deduced from the 1-kb PCR segments and of pyruvate decarboxylase segments of fungi. Sequences are from *Enterobacter agglomerans* 333 (EA333, this work), *Enterobacter agglomerans* 339 (EA339, this work), *Enterobacter cloacae* FERM BP-1529 (EC1529, Koga et al. 1991), *Enterobacter cloacae* NCIMB 11461 (EC11461, this work), *Enterobacter cloacae* NCIMB 11463 (EC11463, this work), and *Klebsiella aerogenes* DSM 681 (KA681, this work). The first position of the sequences corresponds to amino acid position 55 of the IPDC of *Enterobacter cloacae* FERM BP-1529 (Koga et al. 1991). The pyruvate decarboxylase sequences are from *Neurospora crassa* (NCPDC, EMBL No. L09125, Alvarez et al. 1993), *Saccharomyces cerevisiae* (SCPDC, EMBL No. X04675, Kellermann et al. 1986), and *Kluyveromyces marxianus* (KMPDC, EMBL No. L09727, unpublished results). Identical and homologous residues in the IPDC segment are indicated by an asterisk or plus sign above the sequences, respectively. Identical and homologous residues in indolepyruvate decarboxylase and pyruvate decarboxylase segments are marked below the sequences.

Enterobacter aerogenes IAM 12348 and *Enterobacter agglomerans* JCM 1236 were described to be essentially unable to perform this conversion (Koga et al. 1991). Therefore, the ability to produce IAA and the enzyme involved seemed to be limited to the species *Enterobacter cloacae*. In contrast, the data presented here demonstrate that the capacity for Trp-dependent IAA production and the presence of an IPDC gene homologue are not unique to one strain of the genus *Enterobacter* but are also present in two other *Enterobacter cloacae* strains and two *Enterobacter agglomerans* strains. Moreover, this reaction and an IPDC gene segment could also be detected in members of other genera of soil-living, nitrogen-fixing enteric bacteria such as *Klebsiella oxytoca* DSM 3539 and *Pantoea agglomerans* IMET 11328 and are present even in the non-nitrogen-fixing bacterium *Klebsiella aerogenes* DSM 681. *Pantoea agglomerans* IMET 11328 isolated from wheat roots has been found to produce IAA and indole-3-lactate (S. Ruppel and C. Scholz, personal communication). The gene is not restricted to this strain and to the two *Enterobacter agglomerans* strains isolated from the rhizosphere of plants, but is also present in other free-living *Enterobacteriaceae*, which causes some doubt that the only role of IAA production is in the bacterial–plant interaction. The conversion of Trp into IAA by bacteria could have other functions such as decreasing the growth inhibition by high concentrations of Trp, an idea put forward for *Azospirillum* species (Bar and Okon 1992), or might serve to metabolize tryptophan as carbon source.

The data presented in this study show that it is possible to identify the IPDC gene in different organisms by the PCR technique, using carefully chosen oligonucleotide primers. The cloned gene segments can now be used as probes to detect the gene in other soil-living or root-associated bacteria such as *Azospirillum* species, in which the enzyme was postulated to be present because of the organism's ability to convert indole-3-pyruvate into IAA (Bothe et al. 1994). Alternatively, conserved regions can be found by comparison with the now-available six partial sequences that can be used as primers in PCR to amplify the gene from other plant growth promoting bacteria outside the *Enterobacteriaceae* or even from higher plants.

After submission of the manuscript, an IPDC gene from *Azospirillum brasilense* Sp245 was published (Costacurta et al. 1994). The homology of this sequence (27% identical residues at the amino acid level (data not shown)) with the *Enterobacter* and *Klebsiella* sequences is quite low, but the identified conserved regions (Fig. 3) were also present in the *Azospirillum* sequence.

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