

Aromatic Amino Acid-Dependent Expression of Indole-3-Pyruvate Decarboxylase Is Regulated by TyrR in *Enterobacter cloacae* UW5[∇]

R. Julie Ryu and Cheryl L. Patten*

Biology Department, University of New Brunswick, P.O. Box 4400, Fredericton, New Brunswick E3B 5A3, Canada

Received 8 June 2008/Accepted 22 August 2008

The plant growth-promoting rhizobacterium *Enterobacter cloacae* UW5 synthesizes the plant growth hormone indole-3-acetic acid (IAA) via the indole-3-pyruvate pathway utilizing the enzyme indole-3-pyruvate decarboxylase that is encoded by *ipdC*. In this bacterium, *ipdC* expression and IAA production occur in stationary phase and are induced by an exogenous source of tryptophan, conditions that are present in the rhizosphere. The aim of this study was to identify the regulatory protein that controls the expression of *ipdC*. We identified a sequence in the promoter region of *ipdC* that is highly similar to the recognition sequence for the *Escherichia coli* regulatory protein TyrR that regulates genes involved in aromatic amino acid transport and metabolism. Using a *tyrR* insertional mutant, we demonstrate that TyrR is required for IAA production and for induction of *ipdC* transcription. TyrR directly induces *ipdC* expression, as was determined by real-time quantitative reverse transcription-PCR, by *ipdC* promoter-driven reporter gene activity, and by electrophoretic mobility shift assays. Expression increases in response to tryptophan, phenylalanine, and tyrosine. This suggests that, in addition to its function in plant growth promotion, indolepyruvate decarboxylase may be important for aromatic amino acid uptake and/or metabolism.

Auxins are an important class of phytohormones that are essential for many aspects of plant growth and development, including organogenesis; tropic responses; cellular processes such as cell expansion, division, and differentiation; and gene regulation (1, 9, 21, 25, 60). The predominant natural auxin is indole-3-acetic acid (IAA) (8, 21, 60). In addition to synthesis in plant tissues, many plant-associated bacteria also produce and secrete IAA that can influence the health of host plants. Production of IAA by some phytopathogenic bacteria causes plant diseases such as gall tumor formation by *Agrobacterium tumefaciens*, *Erwinia herbicola* pv. *gypsophilae*, and *Pseudomonas syringae* pv. *savastanoi* and necrotic lesions caused by *Pseudomonas syringae* pv. *syringae* (15, 16, 35, 38, 42, 63). Loss of the ability to synthesize IAA, through mutagenesis, reduces the virulence of these pathogens (2, 16).

Paradoxically, IAA produced by plant growth-promoting rhizobacteria (PGPR) has been found to enhance host root system development. Plant roots colonized with the PGPR species *Azospirillum brasilense* Sp6, *Enterobacter cloacae* UW5, and *Pseudomonas putida* GR12-2 displayed increases in root hair formation, the number and length of lateral roots, and/or primary root length that were dependent on bacterial IAA production. Mutants that were unable to synthesize IAA did not increase root proliferation (7, 23, 45, 58, 65). Well-developed root systems are important for natural nutrient uptake and for anchoring plants in soil. The differences in the effect of IAA produced by these two groups of bacteria may be due to differences in the levels of IAA production in planta or other contributing factors (46, 57, 65).

A number of IAA biosynthetic pathways have been identified in bacteria, most requiring tryptophan as a precursor. Synthesis via the intermediates indole-3-acetamide or indole-3-pyruvate is widespread among IAA-producing bacteria. Most phytopathogens, such as *A. tumefaciens* and *P. syringae* pv. *savastanoi*, use the indole-3-acetamide pathway to synthesize IAA (32, 54), while the indole-3-pyruvate pathway is found in many PGPR species, including *A. brasilense* and *E. cloacae*, and in the nonpathogenic epiphytic bacterium *Erwinia herbicola* 299R (11, 17, 30, 45, 73). In the latter pathway, the precursor tryptophan is converted to indole-3-pyruvate by tryptophan transaminase, and indole-3-pyruvate is then converted to indole-3-acetaldehyde by indole-3-pyruvate decarboxylase (IPDC). IAA is produced after oxidation of indole-3-acetaldehyde by indole-3-acetaldehyde oxidase. The key enzyme in this pathway, IPDC, is encoded by *ipdC*, and elimination of *ipdC* abolishes IAA biosynthesis in *E. cloacae* UW5 and greatly reduces IAA production in *A. brasilense* and *E. herbicola* 299R (11, 14, 17, 45, 51).

Currently, regulation of IAA synthesis in bacteria is not completely understood at the molecular level, although it is clear that synthesis responds to environmental cues. Acidic pH and anaerobic conditions, often encountered in the rhizosphere, increase *ipdC* expression in *A. brasilense* Sp245 (43, 61), while in *E. herbicola* 299R, osmotic stress and low water availability induce *ipdC* expression (10). High levels of IAA accumulate in culture media only after entrance into the stationary phase in *A. brasilense* Sp7 and Sp245 and in *E. cloacae* UW5 (14, 44, 61). Consistent with this, the highest expression levels of *ipdC* were observed in stationary phase in *A. brasilense* Sp7 and *E. cloacae* UW5, and it is known that the stationary-phase sigma factor RpoS upregulates *ipdC* expression in *E. cloacae* UW5 and *E. herbicola* 299R (12, 14, 44). IAA and other auxins induced expression of *ipdC* in *A. brasilense* Sp245, and an auxin-responsive element similar to that found in the

* Corresponding author. Mailing address: Biology Department, University of New Brunswick, P.O. Box 4400, Fredericton, New Brunswick E3B 5A3, Canada. Phone: (506) 447-3354. Fax: (506) 453-3583. E-mail: pattenc@unb.

[∇] Published ahead of print on 29 August 2008.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Features ^a	Source or reference
Strains		
<i>E. coli</i>		
S17-1 (λ pir)	Cloning host; λ pir <i>recA thi pro hsdR</i> M ⁺ RP4:2-Tc:Mu:Km Tn7Tp ^r Str ^r	55
JM109	Cloning host; <i>endA1 recA1 gyrA96 thiA hsdR17 relA1 supE44</i> Δ (<i>lac-proAB</i>) [F' <i>traD36 proAB</i> ⁺ <i>laqI</i> ^q <i>lacZ</i> Δ M15]	71
M15	Protein expression host; <i>E. coli</i> K-12 Str ^r F ⁻ Δ lacZ (pREP4)	Qiagen
<i>E. cloacae</i>		
UW5	Wild-type strain	45
J3	UW5 <i>ipdC</i> ::Km ^r	45
J35	UW5 <i>tyrR</i> ::Tc ^r	This study
J51	J35 <i>ipdC</i> :: <i>uidA</i> ; Tc ^r Gm ^r	This study
J55	UW5 <i>ipdC</i> :: <i>uidA</i> ; Gm ^r	This study
Plasmids		
pJP2	Source of Tc ^r cassette	50
pGEM-T Easy	Cloning vector; Amp ^r	Promega
pG16	124-bp 16S rRNA fragment (amplified with 16S 1369F, and 1492R) in pGEM-T Easy; Amp ^r	This study
pGIC	120-bp <i>ipdC</i> fragment (amplified with IPRT1F and -1R) in pGEM-T Easy; Amp ^r	This study
pJQ200SK	Low-copy suicide vector; Gm ^r <i>sacB</i>	52
pJQ200TM	1,177-bp <i>tyrR</i> fragment with 2.1-kb Tc ^r cassette insertion cloned into pJQ200SK; Gm ^r Tc ^r <i>sacB</i>	This study
pJQS	pJQ200SK with <i>sacB</i> deletion; Gm ^r	This study
pJQSIPG	<i>ipdC</i> :: <i>uidA</i> in pJQS; Gm ^r	This study
pREP4	Expresses <i>lac</i> repressor in <i>trans</i> ; Km ^r	Qiagen
pQE30	Protein expression plasmid; N-terminal His ₆ tag for protein purification; Amp ^r	Qiagen
pQEtyrR	1,542-bp <i>tyrR</i> coding region in pQE30; Amp ^r	This study

^a Tc^r, tetracycline resistance; Amp^r, ampicillin resistance; Str^r, streptomycin resistance; Km^r, kanamycin resistance; Tp^r, trimethoprim resistance; Gm^r, gentamicin resistance.

promoters of some auxin-regulated plant genes was found in the *ipdC* promoter region (34, 61).

Production of detectable quantities of IAA usually requires an exogenous source of tryptophan which, in the rhizosphere, is present in host root exudates (13, 14, 27, 43, 44, 45, 64, 72, 73). The addition of tryptophan to culture media induced *ipdC* expression in *E. cloacae* UW5 and in *A. brasilense* strain Sp7 (44, 53, 73), although the regulatory proteins that control tryptophan-mediated *ipdC* expression were not identified in these studies. The upregulation of *ipdC* by an exogenous inducer that is present in root exudates suggests that the host plant may influence production of IAA in PGPR.

In the present study we have identified the regulatory protein that controls *ipdC* expression in response to exogenous tryptophan in *E. cloacae* UW5. The presence of a sequence in the upstream regulatory region of *ipdC* that is highly similar to the recognition site of the transcriptional regulator TyrR that responds to aromatic amino acids and the responsiveness of *ipdC* to tryptophan suggested that TyrR may regulate *ipdC* expression. We confirm here that TyrR is required for activation of *ipdC* expression and for IAA production.

MATERIALS AND METHODS

Bacterial strains, culture conditions and plasmids. Bacterial strains and plasmids used and created in the present study are presented in Table 1. Note that *E. cloacae* UW5 was previously misidentified as *Pseudomonas putida* (44) but has recently been confirmed to be *E. cloacae* by 16S rRNA gene sequence analysis (GenBank accession no. EU136677). All bacteria were routinely grown in Luria-Bertani (LB) medium (Fisher Scientific) and M9 glucose minimal salts medium (Difco) at 30°C for *E. cloacae* or 37°C for *Escherichia coli* in a shaking incubator at 250 rpm. Bacteria grown in the presence of aromatic amino acids were grown in M9 glucose minimal salts medium supplemented with 200 μ g of L-tryptophan,

L-phenylalanine, or L-tyrosine/ml. The antibiotic concentrations routinely used were 100 μ g of ampicillin/ml, 50 μ g of kanamycin/ml, 25 μ g of gentamicin/ml, and 5 μ g of tetracycline/ml.

Sequencing and analysis of *tyrR*. Degenerate primers (T1F and T1R) were designed based on *tyrR* sequences from *E. coli* strains 536, K-12 substrains MG1655 and UT189, *Salmonella enterica* subsp. *enterica* serovar Paratyphi A strain ATCC 9150, *S. enterica* serovar Typhi strain CT18, *S. enterica* serovar Typhimurium LT2, *Shigella boydii* Sb227, *Shigella flexneri* 5 strain 8401, and *Shigella sonnei* Ss046 (GenBank accession nos. CP000247, U00096, CP000243, AL627270, CP000026, AE008774, CP000036, CP000266, and CP000038) and were used to generate a 1,493-bp product using *E. cloacae* UW5 genomic DNA as a template. After this fragment was cloned into pGEM-T Easy (Promega), T7 and SP6 primers were used to acquire a partial sequence, and a primer walking strategy was used to obtain the complete sequence of the fragment. The 3'-terminal end of *tyrR* was not included in the amplicon produced using T1F and T1R primers; therefore, reverse primer tpx1R was designed to a conserved region flanking the 3' end of *tyrR* (within the *tpx* gene), identified using the above-mentioned sequences and *Enterobacter* sp. strain 638 (GenBank accession no. CP000653), to obtain the complete 1,542-bp *tyrR* coding sequence. Sequencing was performed by Robarts Research Institute Sequencing Facility (London, Ontario, Canada) that is equipped with an Applied Biosystems 3730 analyzer. Homology searches were performed by using the Basic Local Alignment Search Tool (BLAST) against the GenBank database (3).

Construction of a *tyrR* insertional mutant. The activity of *tyrR* in *E. cloacae* UW5 was abolished by insertion of a tetracycline resistance cassette into the coding sequence, creating *E. cloacae* J35. The 2.1-kb tetracycline resistance cassette was amplified by using tet-KpnI F and R primers and pJP2 as the template for PCR and then subcloned into pGEM-T Easy. A 1,177-bp *tyrR* fragment, amplified using the primers T1F and T4R, was also subcloned into pGEM-T Easy. The tetracycline resistance cassette was excised from pGEM-T Easy and inserted into a native KpnI site in *tyrR* (708 bp downstream from translation start codon) as a KpnI fragment. The interrupted *tyrR* gene fragment was excised from pGEM-T Easy and cloned into the NotI site in the suicide plasmid pJQ200SK, creating pJQ200TM. pJQ200TM was transformed into calcium chloride-competent *E. coli* S17-1 (λ pir) cells and subsequently introduced into *E. cloacae* UW5 by conjugation. Double recombinants were identified by tetracycline resistance and gentamicin sensitivity. The site of insertion in the

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
16S 1492R	GGWTACCTTGTTACGACTT
16S 1369F	CGGTGAATACGTTTCYCGG
ICRT1F	TCGAACTCAGCAAACAGCAC
ICRT1R	AGGTTTGCAACGTTCTCCAG
IP1F	GCCATGGCAGGAAATCTTC
IP1R-PacI	GCTCGCTTAATTAAGACGGTCCAGCAGGT AATG
IP2F	ACATATTCAGCCCCATACG
IP3	CAGCCTTTTTTGTAAGCATTCTTTCCATG CCCTTCTT
IP3rc	AAGAAGGGCATGGAAAGAATGCTTTACA AAAAAGGCTG
IP3M	CAGCCTTTTTTATAAAGCATTCTTTCTATG CCCTTCTT
IP3Mrc	AAGAAGGGCATAGAAAGAATGCTTTATA AAAAAGGCTG
IP5	GAAAAATCAGTGTATACGTTTACATTTAC ATGAAAAAAA
IP5rc	TTTTTTTTCATGTAAATGTAAACGTATACA CTGATTTTC
SP6	GATTTAGGTGACACTATAG
T1F	ATGCGTYTGGAAGTCTTTTGTG
T1F-BamHI	TGGTACGGATCCATGCGTYTGGAAGTCTT TTGTG
T1R	GCAATCGCGGTRTYGAWAC
T4R	CCTGCTCGTCGGCAAAGC
T5R-PstI	AGTCTACTGCAGTTCGTCACCCTTCTTCT GATT
T7	TAATACGACTCACTATAGGG
tet-KpnI F	GCTAGGTACCTCTTCACGTTCTGCCTTGCG
tet-KpnI R	GTTAGGTACCACGCTAGGGCAGGGCATG
tpx1R	TCACGMMCGARCCGGATTAC
U1F-PacI	GTCGCTTAATTAATAGGTGAGTAGAGGA AACAGCTATGGTCCGTCCTGTAGAAACC CCAAAC ^a
U1R	TCATTGTTTGCCTCCCTG
U2R	TTCCACAGTTTTCGCGATCC

^a Stop codons in all three reading frames and a ribosome-binding site are indicated in boldface for U1F-PacI.

genome was verified by PCR amplification using the primers T1F and T1R (note that the T1R sequence is not present in pJQ200TM). The PCR amplicon generated from the genome of the *tyrR* insertional mutant (*E. cloacae* J35) was 2.1 kb larger than that from the wild-type strain, confirming the replacement of wild-type *tyrR* with the mutant *tyrR* gene fragment.

Quantification of IAA production. *E. cloacae* UW5 (wild-type), *E. cloacae* J35 (*tyrR*), and *E. cloacae* J3 (*ipdC*) were grown overnight in LB broth with appropriate antibiotics, pelleted, and washed twice with saline (0.85% NaCl). Cultures were diluted 1,000-fold into 3 ml of M9 glucose minimal medium with or without 200 µg of tryptophan/ml. After further incubation for 44 h, Salkowski's method (24) was used to quantitate IAA production as follows. Bacterial cells were removed from the culture medium by centrifugation, and then 800 µl of Salkowski's reagent (150 ml of concentrated H₂SO₄, 250 ml of double-distilled H₂O, and 7.5 ml of 0.5 M FeCl₃ · 6H₂O) was mixed with 200 µl of culture supernatant, followed by incubation at room temperature for 20 min. The absorbance at 535 nm was read with a Novaspec II spectrophotometer using M9 glucose minimal medium with or without tryptophan as references. The concentration of IAA in each culture medium was determined by comparison to a standard curve generated from known concentrations of IAA. The absorbance at 600 nm was also recorded at hourly intervals up to 24 h and then at 34 and 44 h to generate growth curves.

RNA extraction and real-time quantitative reverse transcriptase PCR (qRT-PCR). RNA was extracted from *E. cloacae* UW5 and *E. cloacae* J35 cells grown in M9 glucose minimal medium with or without tryptophan as described above. Log-phase cultures (3 ml) were collected at 8 h, and stationary-phase cultures (1.5 ml) were collected at 44 h. Cells were pelleted and treated with 1 ml of RNAProtect (Qiagen) as recommended by the supplier. Pelleted cells were resuspended in 700 µl of homogenization buffer (0.1 M NaCl, 2% sodium

dodecyl sulfate, 50 mM Tris-HCl [pH 9.0], 10 mM EDTA) and incubated at 80°C to lyse the cells (up to 30 min). Nucleic acids were extracted with 700 µl of phenol-chloroform-isoamyl alcohol (124:25:1; pH 4.3). After separation of the organic phase by centrifugation, 500 µl of the aqueous layer was retrieved and subjected to overnight precipitation at -20°C with 1 M LiCl and 62.5% ethanol. After centrifugation for 20 min at 13,000 rpm, the nucleic acid pellet was treated with up to 40 U of DNase I (Invitrogen) for 1.5 h at 37°C in a total volume of 200 µl to remove residual DNA. RNA was extracted with 200 µl of phenol-chloroform-isoamyl alcohol (124:25:1; pH 4.3) and pelleted as mentioned above. The final RNA pellet was resuspended in diethyl pyrocarbonate-treated double-distilled H₂O and quantified spectrophotometrically at 260 nm (using a Beckman Coulter DU series 700 spectrophotometer).

cDNA was generated using 1 µg of RNA; the gene-specific reverse primers ICRT1R and 16S 1492R for *ipdC* and 16S cDNA, respectively; and SuperScript III reverse transcriptase (Invitrogen) according to the supplier's recommendations. Quantitative PCR was performed with Invitrogen's Platinum SYBR green qPCR SuperMix-UDG using cDNA prepared from 100 ng of RNA. The primers ICRT1F and ICRT1R were used to amplify a 120-bp fragment from the *ipdC* cDNA sequence, and the primers 16S 1369F and 1492R were used to amplify a 124-bp fragment from the 16S cDNA sequence for normalization. The cycling conditions were as follows: 2 min at 50°C; 2 min at 95°C; and 45 cycles of 95°C for 15 s, 60°C (*ipdC*) or 50°C (16S) for 30 s, and 72°C for 10 s. Real-time quantification of amplicons was performed by using a Rotor-Gene 6000 thermal cycler (Corbett Life Science, Sydney, Australia). cDNA preparations from each independent triplicate RNA extraction were measured in duplicate, and 100 ng of each DNase I-treated RNA preparation, prior to cDNA preparation, was used as a template for quantitative PCR to verify the lack of DNA contamination in each extraction. Amplification products were subjected to melting-curve analysis to confirm the specificity of the amplicons. Standard curves, prepared in duplicate from known copy numbers (to 10⁹ copies) of plasmids pGIC and pG16 for *ipdC* and 16S rRNA, respectively, were used to extrapolate absolute levels of expression.

Quantification of *ipdC* promoter-driven reporter gene expression. *ipdC* promoter-driven expression was quantified by using β-glucuronidase, encoded by *uidA*. The 1.8-kb *uidA* gene was amplified by PCR using U1F-PacI and U1R primers and pJP2 as a template. A 520-bp fragment containing the *ipdC* promoter sequence was amplified by using the primers IP1F and IP1R-PacI. Both PCR fragments were digested with PacI, purified (PCR purification kit; Qiagen), and ligated together using T4 DNA ligase. To increase the yield of the reporter gene construct, splicing-by-overlapping-extension (SOE) PCR was performed on the ligated product using IP1F and U1R primers. The 2.3-kb *ipdC* promoter-*uidA* SOE PCR products were gel extracted (QIAquick gel extraction kit; Qiagen) and subcloned into pGEM-T Easy. The *ipdC* promoter-*uidA* construct was excised from pGEM-T Easy and inserted into suicide plasmid pJQS as an ApaI and PstI fragment, creating pJQSIPG. Plasmid pJQS was created by sequential double digestion with AclI and BspMI to remove *sacB* from pJQ200SK, filling in the sticky ends produced by the restriction enzymes using Klenow polymerase, and then the plasmid was recircularized by blunt end ligation. pJQSIPG was transformed into calcium chloride-competent *E. coli* S17-1 (*λpir*) and then transferred to *E. cloacae* UW5 and *E. cloacae* J35 by conjugation. Transconjugants were screened for gentamicin resistance, an indication of plasmid integration into the genome. PCR amplification using the primers IP2F (binds to a region upstream of the *ipdC* promoter sequence not present in pJQSIPG) and U2R (binds within *uidA*) yielded a 733-bp fragment that confirmed the site of recombination.

E. cloacae J35 and *E. cloacae* J51, carrying the *ipdC-uidA* fusion in *E. cloacae* UW5 and J35, respectively, were grown in LB broth with antibiotics overnight, pelleted, and washed twice with saline (0.85% NaCl). Cells were diluted 1,000-fold into 3 ml of M9 minimal medium with or without 200 µg of tryptophan, phenylalanine, or tyrosine/ml. β-Glucuronidase activity was assayed in stationary-phase cells after 44 h of growth by using the method reported by Cowie et al. (18). Briefly, 80 µl of reaction buffer (50 mM sodium phosphate [pH 7], 50 mM dithiothreitol, 1 mM EDTA, 0.0125% sodium dodecyl sulfate) containing 0.44 mg of *p*-nitrophenyl β-D-glucuronide (PNPG)/ml was mixed with 20 µl of cell culture in 96-well microtiter plates (CellStar; Greiner Bio One, Frickenhausen, Germany), followed by incubation at room temperature for approximately 1 h. After the addition of 100 µl of 1 M Na₂CO₃ to stop the enzymatic reaction, the absorbance at 405 nm was read in a SpectroMax M5 spectrophotometer (Molecular Devices). The specific activity (in Miller units) was calculated as (1,000 × OD₄₀₅)/(time × OD₆₀₀ × volume of culture in reaction in ml). Wild-type *E. cloacae* UW5 and uninoculated medium were included as controls to assess background levels of absorbance at 405 nm.

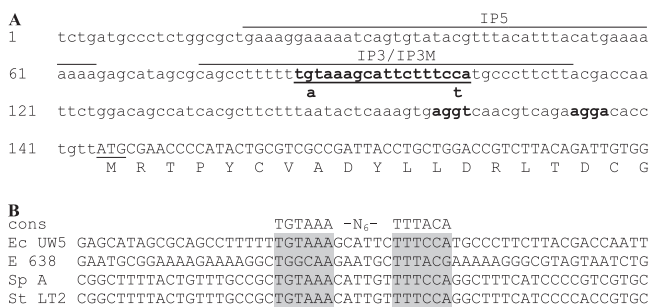


FIG. 1. (A) Nucleotide sequence of the region upstream of *ipdC* in *E. cloacae* UW5 (GenBank accession no. AF285632). The putative TyrR box is indicated in boldface and underlined, the translational start site is underlined, the 5' partial coding sequence is capitalized, and putative ribosome-binding sites are highlighted in boldface and underlined. The location of IP5, IP3, and IP3M used in EMSAs are overlined. Nucleotide substitutions and locations of mutations present in IP3M are shown below the wild-type IP3 sequence. (B) Alignment of *ipdC* promoter sequences from *E. cloacae* UW5 (Ec UW5), *Enterobacter* sp. strain 638 (E 638), *S. enterica* subsp. *enterica* serovar Paratyphi A (Sp A), and serovar Typhimurium LT2 (Sp LT2) (GenBank accession nos. AF285632, CP000653, CP000026, and AE008808, respectively). The consensus sequence for the TyrR binding site in *E. coli* (cons) is shown. Shaded in gray are the conserved putative TyrR boxes in the *ipdC* promoter sequences.

TyrR purification and electrophoretic mobility shift assays (EMSAs). The T1F-BamHI and T5R-PstI primers were used to amplify the entire *tyrR* coding sequence, and the resulting PCR fragment was cloned as a BamHI-PstI fragment into pQE30, which harbors the sequence for an N-terminal His₆ tag (Qiagen). An N-terminal His₆ tag was chosen to avoid impacting the function of TyrR's DNA-binding motif, which is located in the C-terminal domain (26, 67). The resulting plasmid, pQETyrR, was transformed into calcium chloride-competent *E. coli* M15(pREP4). Expression of His₆-TyrR was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the protein was purified by using Ni-NTA (Qiagen). Instructions provided by the supplier were followed for purification, with the exception of a modified wash protocol to eliminate nonspecific protein binding to the Ni-NTA. Wash buffer 1 (WB1) consisted of 50 mM NaH₂PO₄, 300 mM NaCl, and 50 mM imidazole (pH 8.0); WB2 was as described for WB1 but with the addition of 10% glycerol; WB3 was as described for WB2 but with an increase in NaCl to 1 M; and WB4 was as described for WB3 but with 100 mM imidazole. Each wash buffer was applied twice to the column. Elution of His₆-TyrR was performed according to the supplier's protocol. Bradford reagent (Bio-Rad) was used to determine the concentration of the purified protein (31).

The ability of TyrR to bind to the *ipdC* promoter region was tested by using EMSAs. Three oligonucleotides were used, all derived from sequences found in the *ipdC* promoter: IP3, CAGCCTTTTTGTAAAGCATTCTTTCCATGCCCTTCTT; IP3M, CAGCCTTTTTTaTAAAGCATTCTTTcATGCCCTTCTT; and IP5, GAAAAATCAGTGTATACGTTTACATTTACATGAAAAAAA. The locations of these sequences in the *ipdC* promoter region are depicted in Fig. 1A. Underlined in IP3 and IP3M is the putative TyrR box. Bases known to be critical in the interaction between TyrR boxes and TyrR in *E. coli* (26, 39) were substituted in IP3M (lowercase nucleotides in the sequences above and shown below the wild-type sequence in Fig. 1A). IP5 is a sequence that is found upstream of IP3 in the *E. cloacae* UW5 genome that possesses a sequence similar to the TyrR box consensus sequence; however, the length of the sequence is not optimal for TyrR interaction (underlined in the IP5 sequence above) (26, 39, 49). Single-stranded oligonucleotides were synthesized with a digoxigenin (DIG) moiety at the 5' end (Sigma Prolog, Toronto, Ontario, Canada) and annealed to their complementary unlabeled oligonucleotides (IP3rc, IP3Mrc, and IP5rc) (Sigma Genosys, Toronto, Ontario, Canada) to generate double-stranded fragments for the EMSAs.

To assess TyrR binding, purified His₆-TyrR (90 nM), 1 μg of poly(dI-dC), 0.1 mM ATP, and a 0.1 mM concentration of either L-tryptophan, L-phenylalanine, or L-tyrosine were combined with 4 μl of 5× binding buffer (20% glycerol, 250 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 2.5 mM dithiothreitol, 2.5 mM EDTA) to a final volume of 19 μl, followed by incubation for 5 min at room temperature. Then, 1 μl (3 ng) of DIG-labeled oligonucleotide IP3, IP3M, or IP5

was added, followed by incubation for 30 min at room temperature. Next, 5 μl of 5× gel loading buffer (0.25× Tris-borate-EDTA, 60% glycerol, 0.02% bromophenol blue) was added to stop the reaction. A competition assay was also performed to ensure that TyrR binding to the IP3 promoter sequence was sequence specific. The reaction mixtures were similar to those described above except that 1.5, 3, or 6 ng of unlabeled double-stranded IP3 was first added to each reaction, followed by incubation for 5 min at room temperature. Lastly, 1 μl of 5' DIG IP3 was added, followed by incubation for 30 min at room temperature.

Binding reaction mixtures were run on 6% nondenaturing gels (6% acrylamide-bisacrylamide [37.5:1]; 2.5% glycerol; 0.15% ammonium persulfate; 0.1 mM ATP; 0.1 mM L-tryptophan, L-phenylalanine, or L-tyrosine; 0.5× Tris-borate-EDTA; 0.1% TEMED [*N,N,N',N'*-tetramethylethylenediamine]) at 50 V for approximately 2.5 h. A Trans-Blot SD Semi-Dry electrophoretic transfer cell (Bio-Rad) was used to transfer the DIG-labeled oligonucleotides to positively charged nylon membranes (Roche), and these were visualized by using anti-DIG-alkaline phosphatase antibodies and NBT/BCIP according to the manufacturer's instructions (Roche).

RESULTS

The *ipdC* promoter contains a putative TyrR box. The promoter region of *ipdC* in *E. cloacae* UW5 contains a sequence that is highly similar to the TyrR recognition sequence in *E. coli* (TGTA AAA-N₆-TTTACA), known as the TyrR box, shown in Fig. 1A (22, 28, 49). This box is centered 91 bp upstream of the *ipdC* start codon (Fig. 1A). Sequences matching the TyrR box are also found in the region upstream of genes annotated as *ipdC* in the genomic sequences of several closely related bacteria, *Enterobacter* sp. strain 638, *S. enterica* subsp. *enterica* serovar Paratyphi A, and *S. enterica* serovar Typhimurium LT2, although the sequence in this region is otherwise not well conserved (Fig. 1B). Conservation of these nucleotides upstream of the *ipdC* coding region suggests that they may be important for the regulation of *ipdC* expression and that TyrR may be required for *ipdC* transcription.

Sequence analysis of *tyrR*. The *tyrR* coding sequence (GenBank accession no. EU570974) in *E. cloacae* UW5 is 1,542 bp and is predicted to encode a 513-amino-acid protein with a molecular mass of 57.5 kDa. The nucleotide sequence shares identity with *tyrR* of other enteric bacteria, including *Enterobacter* sp. strain 638 (81%), *Citrobacter koseri* (80%), serovar Typhimurium (79%), *S. enterica* subsp. *enterica* serovar Paratyphi A (79%) and B (78%), *Klebsiella pneumoniae* subsp. *pneumoniae* (78%), *Citrobacter braakii* (78%), *E. coli* (78%), *Enterobacter sakazakii* (76%), and the *Shigella* species *S. flexneri*, *S. dysenteriae*, *S. sonnei*, and *S. boydii* (77%). TyrR is a regulatory protein that interacts with aromatic amino acid effector molecules and is both an activator and a repressor of gene expression (19, 22, 47, 49). The structure of TyrR is divided into three domains. The N-terminal domain of TyrR in *E. coli* was shown to be vital for activation of gene expression and is known to interact with the α-subunit of RNA polymerase. This domain is also required for dimerization and contains an ATP-independent binding site for aromatic amino acids (20, 22, 37, 48, 49, 66, 70). An ATP binding site and an ATP-dependent tyrosine binding site are located in the central domain and are required for tyrosine-dependent hexamerization that results in gene repression (22, 33, 67). The C-terminal domain contains a helix-turn-helix motif responsible for DNA binding (26, 67). Analysis of the *E. cloacae* UW5 TyrR amino acid sequence using InterProScan (<http://www.ebi.ac.uk/InterProScan/>; performed May 2008) confirmed the presence of an N-terminal amino

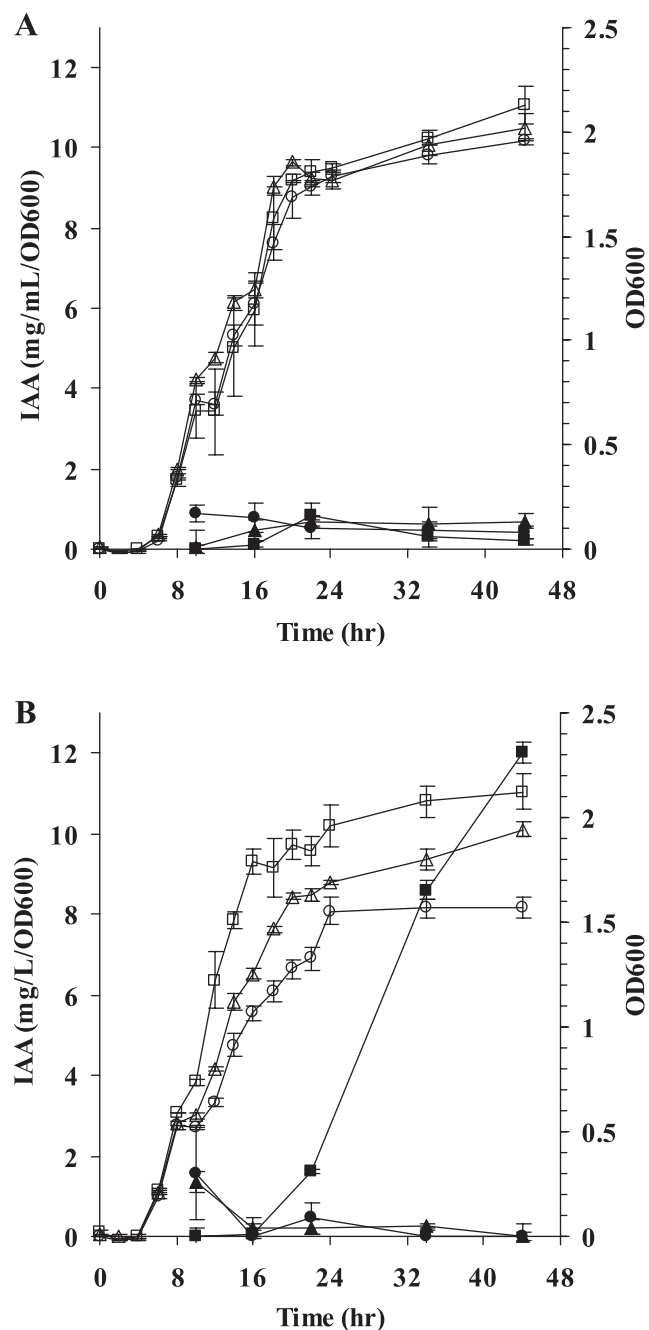


FIG. 2. Growth (open symbols) and IAA produced (closed symbols) by *E. cloacae* UW5 (squares), *E. cloacae* J35 (circles), and *E. cloacae* J3 (triangles) in the absence (A) or presence (B) of 200 μ g of L-tryptophan/ml over 44 h. The data points represent the average from three independent colonies of each strain. Error bars indicate the standard errors of the mean.

acid binding domain, an ATP binding and hydrolyzing AAA-ATPase core in the central domain, and a DNA-binding motif in the C-terminal domain. Interestingly, the central domain also contains a putative RpoN (σ^{54}) domain, although, to date, TyrR has only been shown to interact with RpoD (σ^{70}) (22, 33, 48). The central domain shares homology with RpoN interacting regulatory proteins NtrC (activates expression of nitrogen-

responsive genes) and a TyrR homologue, PhhR (regulates phenol degradation), in pseudomonads (6, 22, 40, 41, 49, 56).

TyrR is required for IAA biosynthesis. To test the hypothesis that TyrR regulates IAA production, a *tyrR* insertional mutant, *E. cloacae* J35, was generated. Accumulation of IAA in the culture medium of wild-type *E. cloacae* UW5 occurred only in the presence of tryptophan after entrance into stationary phase as previously described (Fig. 2) (44). In contrast, neither *tyrR* mutant *E. cloacae* J35 nor *ipdC* mutant *E. cloacae* J3 produced detectable levels of IAA in the presence or absence of tryptophan over a period of 44 h that encompasses both logarithmic and stationary phases of growth (Fig. 2). Although *E. cloacae* strains J35 and J3 had longer generation times than the wild-type strain when grown in M9 glucose minimal medium with 200 μ g of L-tryptophan/ml (434, 315, and 286 min for *E. cloacae* strains J35, J3, and UW5, respectively), all three strains entered stationary phase within 24 h. These results indicate that TyrR and tryptophan are required for IAA production.

TyrR induces *ipdC* expression. The requirement for TyrR for IAA production and the presence of a sequence matching the TyrR box consensus sequence in the *ipdC* promoter region suggested that TyrR may regulate transcription of *ipdC* that encodes a key enzyme in the IAA biosynthetic pathway (44). Quantification of *ipdC* transcript levels by real-time qRT-PCR revealed that *ipdC* transcript levels were highest in wild-type *E. cloacae* UW5 cells grown to stationary phase in media supplemented with tryptophan (Table 3), as was predicted from the high levels of IAA produced under these conditions. In contrast, in the absence of TyrR, in mutant *E. cloacae* J35, *ipdC* was expressed only at low levels (ca. 1 to 1.5 transcripts/million transcripts of 16S rRNA) in both log and stationary phases even in tryptophan-supplemented cultures. Similarly low levels of *ipdC* expression were also seen in log-phase wild-type cultures grown in the absence of tryptophan, conditions under which IAA is not normally produced. Although IAA was not produced by wild-type cultures in log-phase grown with tryptophan or in stationary phase in the absence of exogenous tryptophan, transcription of *ipdC* was apparent under both conditions, albeit at lower levels than the transcript levels produced by stationary-phase cultures supplemented with tryptophan (Table 3). These results confirm that activation of *ipdC* transcription is TyrR dependent. They also suggest that even when conditions are appropriate for IAA production, that is,

TABLE 3. Levels of *ipdC* transcription in wild-type *E. cloacae* UW5 and *tyrR* mutant *E. cloacae* J35 as determined by real-time qRT-PCR analysis

Growth phase ^a	Trp ^b	No. of <i>ipdC</i> transcripts/million transcripts of 16S rRNA \pm SE ^c	
		UW5	J35
Log	-	1.6 \pm 0.5	1.1 \pm 0.1
	+	9.3 \pm 0.6	1.0 \pm 0.1
Stationary	-	7.2 \pm 1.8	1.0 \pm 0.2
	+	23.2 \pm 4.4	1.5 \pm 0.1

^a RNA was extracted from log- and stationary-phase cells at 8 h (average OD₆₀₀ = 0.5) and 44 h (average OD₆₀₀ = 2.0), respectively, postinoculation.

^b Cultures were grown in M9 glucose without (-) or with (+) 200 μ g of L-tryptophan (Trp)/ml.

^c The values shown are the means of at least three independent replicates.

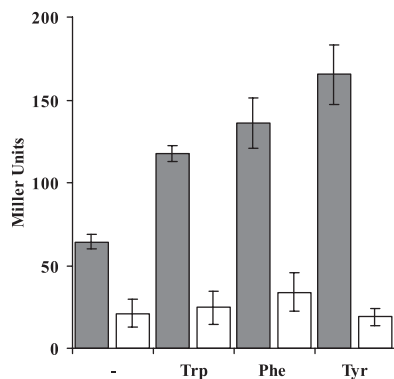


FIG. 3. *ipdC* promoter driven β -glucuronidase activity in *E. cloacae* J55 (■) and *E. cloacae* J51 (□) in stationary phase. Bacteria were grown for 44 h in M9 glucose minimal medium with 200 μ g of L-tryptophan, L-phenylalanine, or L-tyrosine/ml or in the absence of exogenously supplied aromatic amino acids. The data shown are the averages of three independent replicates, and error bars represent the standard errors of the mean.

when exogenous tryptophan is present, TyrR is produced (*tyrR* mRNA levels are relatively constant throughout the *E. cloacae* UW5 growth cycle; data not shown), and *ipdC* is expressed, cells must be in stationary phase before IAA is synthesized. It is possible that regulation of IAA production involves other factors in addition to TyrR.

Expression of *ipdC* is upregulated by aromatic amino acids.

To further investigate TyrR-dependent *ipdC* activation, *ipdC* promoter-driven reporter gene assays were performed. Measurement of β -glucuronidase activity in *ipdC::uidA* transcriptional fusions confirmed the results from real-time qRT-PCR analysis that upregulation of *ipdC* expression in stationary phase is TyrR dependent and that high levels of expression required an exogenous source of tryptophan (Fig. 3). Because TyrR is known to regulate gene expression in response to all three aromatic amino acids in *E. coli* (48, 49), *ipdC* promoter activity was also quantified in *E. cloacae* UW5 in response to phenylalanine and tyrosine. Both amino acids increased TyrR-dependent *ipdC* expression to levels comparable to those expressed in response to tryptophan (Fig. 3). The finding that *ipdC* expression increased in response to all three aromatic amino acids suggests that this gene may be important in aromatic amino acid metabolism in this bacterium.

TyrR binds to the *ipdC* promoter. To differentiate between *ipdC* activation as a consequence of a direct interaction between TyrR and the *ipdC* promoter, or regulation at a point upstream of *ipdC* activation, EMSAs were used to assess the ability of TyrR to bind to the putative TyrR box found in the *ipdC* upstream sequence (see Fig. 1A for the promoter sequences used). Purified His₆-TyrR bound to oligonucleotide fragment IP3, containing a putative TyrR box, in the presence of tryptophan, phenylalanine, or tyrosine (lane 2 in Fig. 4B to D). As was predicted from the nucleotide sequence, the TyrR box in the *ipdC* promoter is a strong box since TyrR also bound to IP3 in the absence of aromatic amino acids (Fig. 4A, lane 2). This corroborates our observations from expression studies that showed that while aromatic amino acids supplied exogenously increased *ipdC* expression, they were not absolutely required for *ipdC* promoter activity. This is consistent with

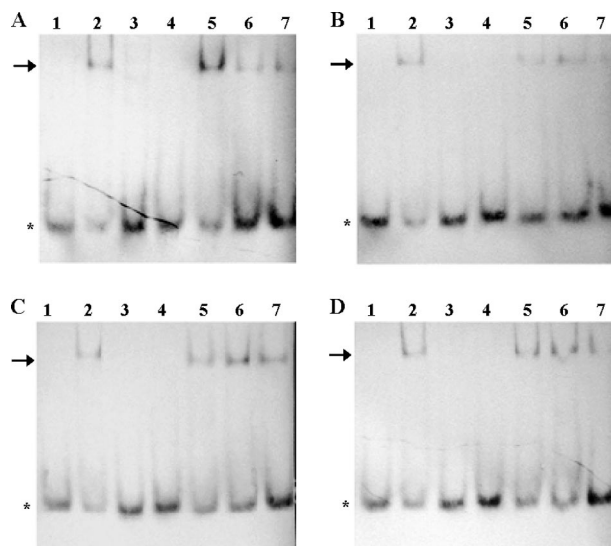


FIG. 4. Binding of purified TyrR to the *ipdC* promoter in the presence of aromatic amino acids as determined by EMSAs. The conditions tested in these assays were as follows: no aromatic amino acids (A), 0.1 mM L-tryptophan (B), 0.1 mM L-phenylalanine (C), and 0.1 mM L-tyrosine (D). Lanes 1 to 4 contained 90 nM His₆-TyrR with 3 ng of 5'-DIG-labeled *ipdC* promoter sequence as follows: lane 1, IP3; lane 2, IP3 plus TyrR; lane 3, IP3M plus TyrR; and lane 4, IP5 plus TyrR. Competition assays were conducted with 90 nM His₆-TyrR and 3 ng of 5'-DIG-labeled IP3 and various amounts of unlabeled IP3 as follows: lane 5, 1.5 ng; lane 6, 3 ng; and lane 7, 6 ng. The asterisks and arrows show the location of free and TyrR-bound 5'-DIG-labeled IP3, respectively.

binding studies in *E. coli* that showed that TyrR can bind to strong boxes, sequences that are highly similar to the consensus sequence (TGTA₃-N₆-TTTACA), without binding aromatic amino acids. Binding of aromatic amino acids to TyrR increases its affinity for DNA binding, especially at weak TyrR boxes (4, 49). Binding of His₆-TyrR to IP3 was shown to be sequence specific since the addition of TyrR did not cause a shift when incubated with fragment IP3M containing the promoter sequence with a mutated TyrR box (Fig. 4, lanes 3). In this fragment, two nucleotides that are essential for TyrR binding in *E. coli*, G near the 5' end of the TyrR box and C near the 3' end (26, 39), were replaced with A and T, respectively (Fig. 1A). The specificity of the interaction was further supported by competition assays performed with 0.5:1, 1:1, and 2:1 molar ratios of unlabeled IP3 to 5'-DIG-labeled IP3; as the proportion of unlabeled IP3 increased, the amount of 5'-DIG-labeled IP3 bound to TyrR decreased (Fig. 4A to D, lanes 5 to 7). A sequence similar to the TyrR box consensus sequence but with a nonoptimal length for TyrR interaction (IP5) was also assessed but did not bind to TyrR (Fig. 4A to D, lane 4). These results confirm that TyrR directly activates expression from the *ipdC* promoter in *E. cloacae* UW5.

DISCUSSION

IAA production is widespread among PGPR and its positive effects on plant growth have been well documented (for reviews, see references 36, 46, 57, and 62). Bacterial IAA has been shown to enhance the development of the host plant root

system (7, 23, 45, 58, 65), which increases the surface area through which soil nutrients are absorbed and helps to establish young seedlings firmly in soil. Thus, IAA-producing PGPR contribute to increased yields of healthy crops and are of interest as a replacement for chemical fertilizers in agricultural applications. Optimal exploitation of the beneficial effects of PGPR can be accomplished only if the factors that induce IAA-producing pathways are fully understood. This is especially important because IAA is also a virulence factor in plant pathogens, and higher levels of IAA production in plants have been proposed to explain the differential effect on plants (46, 57, 65). Several studies have shown that environmental signals present in the rhizosphere increase bacterial IAA production; however, the mechanisms by which these signals are transduced in bacteria are not well understood.

In many PGPR, IAA is synthesized only when tryptophan is supplied in the culture medium (13, 14, 43, 44, 64, 73). This may be due to increased availability of the biosynthetic precursor tryptophan and/or to a requirement for tryptophan for induction of the genes in the IAA biosynthetic pathway. It has been shown that the expression of *ipdC* encoding indole-3-pyruvate decarboxylase, a key enzyme in the IAA biosynthetic pathway of some PGPRs, including *E. cloacae* UW5 and *A. brasilense* Sp7, is upregulated by tryptophan (44, 53, 73). In the rhizosphere, tryptophan is present in root exudates and from dead microorganisms and plant tissue (27, 72), suggesting that a signal for upregulation of the indole-3-pyruvate pathway and, therefore, IAA production, in PGPR is present in the rhizosphere. Indeed, *ipdC*-driven reporter gene expression studies have shown that expression of *ipdC* in *E. herbicola* 299R is plant inducible. Induction of *ipdC* increased by a factor of 32 on bean and tobacco leaves, and 1,000-fold on pear flowers compared to induction in culture medium (10, 12).

We show here that the transcription factor TyrR directly and positively controls *ipdC* expression and IAA production in the PGPR *E. cloacae* UW5 and that TyrR-dependent expression increases in response to exogenous tryptophan. A sequence with only a single base mismatch to the consensus sequence for the TyrR box (TGTA₆-N₆-TTTACA) in *E. coli* (49) was identified in the promoter region of *ipdC* in this bacterium and in other closely related bacteria, even though the promoter sequences were otherwise quite dissimilar. Loss of IAA production and lower levels of *ipdC* expression following disruption of TyrR function through insertional mutagenesis in mutant strain *E. cloacae* J35 confirmed the requirement for TyrR. The high degree of nucleotide sequence identity to the consensus sequence for the TyrR binding site suggests a strong TyrR protein-promoter DNA interaction. This is supported by the ability of purified TyrR to bind to the *ipdC* promoter fragment containing the TyrR box in vitro, and by the induction of *ipdC* expression, in the absence of an effector molecule. The observed transcription of *ipdC* in the absence of tryptophan supplements to the culture medium may be mediated by binding of endogenous aromatic amino acid cofactors to TyrR. Although TyrR can bind to strong boxes in the absence of cofactors, the addition of aromatic amino acids strengthens the interaction between TyrR and its recognition sequence (4, 49). The increased affinity of TyrR for the promoter results in increased transcription, as was observed here by an increase in

ipdC transcript abundance measured by real-time qRT-PCR and by an increase in *ipdC* promoter-driven β -glucuronidase activity after addition of the TyrR cofactor tryptophan. The single base mismatch, at nucleotide position 15 in the TyrR box, is at a noncritical position for TyrR binding, whereas mutations introduced into positions previously determined to be essential (5, 26, 49) abolished TyrR binding to the *ipdC* promoter.

Much of what we know about regulation by TyrR has been determined in *E. coli*; however, *E. cloacae* is closely related to *E. coli* and is also a member of the *Enterobacteriaceae*, and therefore regulation may be similar. TyrR has a rather complex mode of regulation. All three aromatic amino acids can bind to TyrR and act as cofactors for either repression or activation of the TyrR regulon. Often a single promoter can be both induced and repressed by TyrR, and each of the aromatic amino acids can differentially regulate expression as is exemplified by the *tyrP* promoter (69). Genes known to be repressed by TyrR in *E. coli* include *tyrR* itself, *aroF*, *aroL*, *tyrP*, *aroP*, *tyrB*, and *aroG*; all are involved with aromatic amino acid synthesis and transport (22, 37, 48, 49). Of the four *E. coli* genes that are positively regulated by TyrR, three, namely, *aroP*, *mtr*, and *tyrP*, encode aromatic amino acid transporters (49). A fourth positively regulated member of the *E. coli* TyrR regulon, *folA*, catalyzes the reduction of dihydrofolate to tetrahydrofolate an important intermediate required for synthesis of folate from chorismate in *E. coli* (68). Previously, TyrR was thought to regulate only promoters that interact with the housekeeping sigma factor, RpoD (22, 33, 48). The addition of *ipdC*, which is induced by the stationary-phase sigma factor RpoS (12, 44), to the TyrR regulon reveals that TyrR can also regulate RpoS-responsive genes.

A broader function for IPDC in aromatic amino acid transport or metabolism is suggested by the positive TyrR-dependent response of the *ipdC* promoter to all three aromatic amino acids. The induction of *ipdC* by aromatic amino acids has also been shown in *A. brasilense* Sp7 (53). Consistent with this, Spaepen et al. (59) propose that the *A. brasilense* indolepyruvate decarboxylase is a phenylpyruvate decarboxylase based on the higher rate of catalysis with the substrate phenylpyruvate, derived from phenylalanine, compared to indolepyruvate, although Koga et al. (29) had previously determined that phenylpyruvate is not a substrate for indolepyruvate decarboxylase in *E. cloacae*. The increased transcription of *ipdC* in the presence of tyrosine observed here probably reflects the increased strength of promoter binding by TyrR, which binds as a hexamer when bound to tyrosine (49).

The *ipdC* gene encoding indole-3-pyruvate decarboxylase is a newly recognized member of the TyrR regulon. Consistent with other members of this regulon, *ipdC* is involved in the metabolism of tryptophan, and possibly other aromatic compounds. Soil bacteria are attracted to nutrients such as amino acids in root exudates that not only supply carbon, nitrogen, and energy but also influence bacterial gene expression. Exogenous aromatic amino acids induce indolepyruvate decarboxylase expression, via TyrR, that leads to production and secretion of IAA, and perhaps other compounds, that benefit the host plant.

ACKNOWLEDGMENTS

This study was supported by grants to C.L.P. and a scholarship to R.J.R. from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

- Abel, S., and A. Theologis. 1996. Early genes and auxin action. *Plant Physiol.* **111**:9–17.
- Akiyoshi, D. E., R. O. Morris, R. Hinz, B. S. Mischke, T. Kosuge, D. J. Garfinkel, M. P. Gordon, and E. W. Nester. 1983. Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. *Proc. Natl. Acad. Sci. USA* **80**:407–411.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Andrews, A. E., B. Lawley, and A. J. Pittard. 1991. Mutational analysis of repression and activation of the *tyrP* gene in *Escherichia coli*. *J. Bacteriol.* **173**:5068–5078.
- Andrews, A. E., B. Dickson, B. Lawley, C. Cobbett, and A. J. Pittard. 1991. Importance of the position of TyrR boxes for repression and activation of the *tyrP* and *aroF* genes in *Escherichia coli*. *J. Bacteriol.* **173**:5079–5085.
- Austin, S., and R. Dixon. 1992. The prokaryotic enhancer binding protein NTRC has an ATPase activity which is phosphorylation and DNA dependent. *EMBO J.* **11**:2219–2228.
- Barbieri, P., T. Zanelli, E. Galli, and G. Zanetti. 1986. Wheat inoculation with *Azospirillum brasilense* Sp6 and some mutants altered in nitrogen fixation and indole-3-acetic acid production. *FEMS Microbiol. Lett.* **36**:87–90.
- Bartel, B. 1997. Auxin biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**:51–66.
- Benjamins, R., and B. Scheres. 2008. Auxin: the looping star in plant development. *Annu. Rev. Plant Biol.* **59**:443–465.
- Brandl, M. T., and S. E. Lindow. 1997. Environmental signals modulate the expression of an indole-3-acetic acid biosynthetic gene in *Erwinia herbicola*. *Mol. Plant-Microbe Interact.* **10**:499–505.
- Brandl, M. T., and S. E. Lindow. 1996. Cloning and characterization of a locus encoding an indolepyruvate decarboxylase involved in indole-3-acetic acid synthesis in *Erwinia herbicola*. *Appl. Environ. Microbiol.* **62**:4121–4128.
- Brandl, M. T., B. Quinones, and S. E. Lindow. 2001. Heterogeneous transcription of an indoleacetic acid biosynthetic gene in *Erwinia herbicola* on plant surfaces. *Proc. Natl. Acad. Sci. USA* **98**:3454–3459.
- Brandl, M. T., E. M. Clark, and S. E. Lindow. 1996. Characterization of the indole-3-acetic acid (IAA) biosynthetic pathway in an epiphytic strain of *Erwinia herbicola* and IAA production in vitro. *Can. J. Microbiol.* **42**:586–592.
- Carreno-Lopez, R., N. Campos-Reales, C. Elmerich, and B. E. Baca. 2000. Physiological evidence for differently regulated tryptophan-dependent pathways for indole-3-acetic acid synthesis in *Azospirillum brasilense*. *Mol. Gen. Genet.* **264**:521–530.
- Clark, E., S. Manulis, Y. Ophir, I. Barash, and Y. Gafni. 1993. Cloning and characterization of *iaaM* and *iaaH* from *Erwinia herbicola* pathovar *gypsophylae*. *Mol. Plant Pathol.* **83**:234–240.
- Comai, L., and T. Kosuge. 1982. Cloning characterization of *iaaM*, a virulence determinant of *Pseudomonas savastanoi*. *J. Bacteriol.* **149**:40–46.
- Costacurta, A., V. Keijers, and J. Vanderleyden. 1994. Molecular cloning and sequence analysis of an *Azospirillum brasilense* indole-3-pyruvate decarboxylase gene. *Mol. Gen. Genet.* **243**:463–472.
- Cowie, A., J. Cheng, C. D. Sibley, Y. Fong, R. Zaheer, C. L. Patten, R. M. Morton, G. B. Golding, and T. M. Finan. 2006. An integrated approach to functional genomics: construction of a novel reporter gene fusion library for *Sinorhizobium meliloti*. *Appl. Environ. Microbiol.* **72**:7156–7167.
- Cui, J., and R. L. Somerville. 1993. Mutational uncoupling of the transcriptional activation function of the TyrR protein of *Escherichia coli* K-12 from the repression function. *J. Bacteriol.* **175**:303–306.
- Cui, J., L. Ni, and R. L. Somerville. 1993. ATPase activity of TyrR, a transcriptional regulatory protein for sigma 70 RNA polymerase. *J. Biol. Chem.* **268**:13023–13025.
- Delker, C., A. Raschke, and M. Quint. 2008. Auxin dynamics: the dazzling complexity of a small molecule's message. *Planta* **227**:929–941.
- Dixon, M. P., R. N. Pau, G. J. Howlett, D. E. Dunstan, W. H. Sawyer, and B. E. Davidson. 2002. The central domain of *Escherichia coli* TyrR is responsible for hexamerization associated with tyrosine-mediated repression of gene expression. *J. Biol. Chem.* **277**:23186–23192.
- Dobbelaere, S., A. Croonenborghs, A. Thys, A. Vande Broek, and J. Vanderleyden. 1999. Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. *Plant Soil* **212**:155–164.
- Gordon, S. A., and R. P. Weber. 1951. Colorimetric estimation of indoleacetic acid. *Plant Physiol.* **26**:192–195.
- Hagen, G., and T. Guilfoyle. 2002. Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Mol. Biol.* **49**:373–385.
- Hwang, J. S., J. Yang, and A. J. Pittard. 1997. Critical base pairs and amino acid residues for protein-DNA interaction between the TyrR protein and *tyrP* operator of *Escherichia coli*. *J. Bacteriol.* **179**:1051–1058.
- Jaeger, C. H., III, S. E. Lindow, W. Miller, E. Clark, and M. K. Firestone. 1999. Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. *Appl. Environ. Microbiol.* **65**:2685–2690.
- Katayama, T., H. Suzuki, K. Yamamoto, and H. Kumagai. 1999. Transcriptional regulation of tyrosine phenol-lyase gene mediated through TyrR and cAMP receptor protein. *Biosci. Biotechnol. Biochem.* **63**:1823–1827.
- Koga, J., T. Adachi, and H. Hidaka. 1992. Purification and characterization of indolepyruvate decarboxylase. A novel enzyme for indole-3-acetic acid biosynthesis in *Enterobacter cloacae*. *J. Biol. Chem.* **267**:15823–15828.
- Koga, J., T. Adachi, and H. Hidaka. 1991. Molecular cloning of the gene for indolepyruvate decarboxylase from *Enterobacter cloacae*. *Mol. Gen. Genet.* **226**:10–16.
- Kruger, N. J. 1994. The Bradford method for protein quantitation. *Methods Mol. Biol.* **32**:9–15.
- Kuo, T., and T. Kosuge. 1970. Role of amino transferase and indole-3-pyruvic acid in the synthesis of indole-3-acetic acid in *Pseudomonas savastanoi*. *J. Gen. Appl. Microbiol.* **16**:191–204.
- Kwok, T., J. Yang, A. J. Pittard, T. J. Wilson, and B. E. Davidson. 1995. Analysis of an *Escherichia coli* mutant TyrR protein with impaired capacity for tyrosine-mediated repression, but still able to activate at sigma 70 promoters. *Mol. Microbiol.* **17**:471–481.
- Lambrecht, M., A. Vande Broek, F. Dosselaere, and J. Vanderleyden. 1999. The *ipdC* promoter auxin-responsive element of *Azospirillum brasilense*, a prokaryotic ancestral form of the plant AuxRE? *Mol. Microbiol.* **32**:889–891.
- Liu, S. T., K. L. Perry, C. L. Schardl, and C. I. Kado. 1982. *Agrobacterium* Ti plasmid indoleacetic acid gene is required for crown gall oncogenesis. *Proc. Natl. Acad. Sci. USA* **79**:2812–2816.
- Lucy, M., E. Reed, and B. R. Glick. 2004. Applications of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek* **86**:1–25.
- MacPherson, K. H., P. D. Carr, D. Verger, T. Kwok, B. E. Davidson, and D. L. Ollis. 1999. Crystallization of the N-terminal domain of the *Escherichia coli* regulatory protein TyrR. *Acta Crystallogr. D Biol. Crystallogr.* **55**:1923–1924.
- Mazzola, M., and F. F. White. 1994. A mutation in the indole-3-acetic acid biosynthesis pathway of *Pseudomonas syringae* pv. *syringae* affects growth in *Phaseolus vulgaris* and syringomycin production. *J. Bacteriol.* **176**:1374–1382.
- Mironov, A. A., E. V. Koonin, M. A. Roytberg, and M. S. Gelfand. 1999. Computer analysis of transcription regulatory patterns in completely sequenced bacterial genomes. *Nucleic Acids Res.* **27**:2981–2989.
- Morett, E., and L. Segovia. 1993. The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. *J. Bacteriol.* **175**:6067–6074.
- Ng, L. C., C. L. Poh, and V. Shingler. 1995. Aromatic effector activation of the NtrC-like transcriptional regulator PhhR limits the catabolic potential of the (methyl)phenol degradative pathway it controls. *J. Bacteriol.* **177**:1485–1490.
- Offringa, I. A., L. S. Melchers, A. J. Regensburg-Tuinik, P. Costantino, R. A. Schilperoort, and P. J. Hooykaas. 1986. Complementation of *Agrobacterium tumefaciens* tumor-inducing *aux* mutants by genes from the T(R)-region of the Ri plasmid of *Agrobacterium rhizogenes*. *Proc. Natl. Acad. Sci. USA* **83**:6935–6939.
- Ona, O., J. Van Impe, E. Prinsen, and J. Vanderleyden. 2005. Growth and indole-3-acetic acid biosynthesis of *Azospirillum brasilense* Sp245 is environmentally controlled. *FEMS Microbiol. Lett.* **246**:125–132.
- Patten, C. L., and B. R. Glick. 2002. Regulation of indoleacetic acid production in *Pseudomonas putida* GR12-2 by tryptophan and the stationary-phase sigma factor RpoS. *Can. J. Microbiol.* **48**:635–642.
- Patten, C. L., and B. R. Glick. 2002. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Appl. Environ. Microbiol.* **68**:3795–3801.
- Patten, C. L., and B. R. Glick. 1996. Bacterial biosynthesis of indole-3-acetic acid. *Can. J. Microbiol.* **42**:207–220.
- Pittard, A. J., and B. E. Davidson. 1991. TyrR protein of *Escherichia coli* and its role as repressor and activator. *Mol. Microbiol.* **5**:1585–1592.
- Pittard, J. 1996. The various strategies within the TyrR regulation of *Escherichia coli* to modulate gene expression. *Genes Cells* **1**:717–725.
- Pittard, J., H. Camakaris, and J. Yang. 2005. The TyrR regulon. *Mol. Microbiol.* **55**:16–26.
- Prell, J., B. Boesten, P. Poole, and U. B. Priefer. 2002. The *Rhizobium leguminosarum* bv. *viciae* VF39 gamma-aminobutyrate (GABA) aminotransferase gene (*gabT*) is induced by GABA and highly expressed in bacteroids. *Microbiology* **148**:615–623.
- Prinsen, E., A. Costacurta, K. Michiels, J. Vanderleyden, and H. Van Onckelen. 1993. *Azospirillum brasilense* indole-3-acetic acid biosynthesis: evidence for a non-tryptophan-dependent pathway. *Mol. Plant-Microbe Interact.* **6**:609–615.
- Quandt, J., and M. F. Hynes. 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* **127**:15–21.

53. Rothballer, M., M. Schmid, A. Fekete, and A. Hartmann. 2005. Comparative in situ analysis of *ipdC-gfpmut3* promoter fusions of *Azospirillum brasilense* strains Sp7 and Sp245. *Environ. Microbiol.* **7**:1839–1846.
54. Schroder, G., S. Waffenschmidt, E. W. Weiler, and J. Schroder. 1984. The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. *Eur. J. Biochem.* **138**:387–391.
55. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**:784–791.
56. Song, J., and R. A. Jensen. 1996. PhhR, a divergently transcribed activator of the phenylalanine hydroxylase gene cluster of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **22**:497–507.
57. Spaepen, S., J. Vanderleyden, and R. Remans. 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* **31**:425–448.
58. Spaepen, S., S. Dobbelaere, A. Croonenborghs, and J. Vanderleyden. 2008. Effects of *Azospirillum brasilense* indole-3-acetic acid production on inoculated wheat plants. *Plant Soil* doi:10.1007/s11104-008-9560-1.
59. Spaepen, S., W. Versees, D. Gocke, M. Pohl, J. Steyaert, and J. Vanderleyden. 2007. Characterization of phenylpyruvate decarboxylase, involved in auxin production of *Azospirillum brasilense*. *J. Bacteriol.* **189**:7626–7633.
60. Teale, W. D., I. A. Paponov, and K. Palme. 2006. Auxin in action: signalling, transport, and the control of plant growth and development. *Nat. Rev. Mol. Cell. Biol.* **7**:847–859.
61. Vande Broek, A., P. Gysegom, O. Ona, N. Hendrickx, E. Prinsen, J. Van Impe, and J. Vanderleyden. 2005. Transcriptional analysis of the *Azospirillum brasilense* indole-3-pyruvate decarboxylase gene and identification of a *cis*-acting sequence involved in auxin responsive expression. *Mol. Plant-Microbe Interact.* **18**:311–323.
62. Vessey, J. K. 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* **255**:571–586.
63. White, F. F., and S. F. Ziegler. 1991. Cloning of the genes for indoleacetic acid synthesis from *Pseudomonas syringae* pv. *syringae*. *Mol. Plant-Microbe Interact.* **4**:207–210.
64. Xie, B., K. Xu, H. X. Zhao, and S. F. Chen. 2005. Isolation of transposon mutants from *Azospirillum brasilense* Yu62 and characterization of genes involved in indole-3-acetic acid biosynthesis. *FEMS Microbiol. Lett.* **248**:57–63.
65. Xie, H., J. J. Pasternak, and B. R. Glick. 1996. Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 that overproduce indoleacetic acid. *Curr. Microbiol.* **32**:67–71.
66. Yang, J., H. Camakaris, and A. J. Pittard. 1996. Further genetic analysis of the activation function of the TyrR regulatory protein of *Escherichia coli*. *J. Bacteriol.* **178**:1120–1125.
67. Yang, J., S. Ganesan, J. Sarsero, and A. J. Pittard. 1993. A genetic analysis of various functions of the TyrR protein of *Escherichia coli*. *J. Bacteriol.* **175**:1767–1776.
68. Yang, J., Y. Ogawa, H. Camakaris, T. Shimada, A. Ishihama, and A. J. Pittard. 2007. *folA*, a new member of the TyrR regulon in *Escherichia coli* K-12. *J. Bacteriol.* **189**:6080–6084.
69. Yang, J., J. S. Hwang, H. Camakaris, W. Irawaty, A. Ishihama, and A. J. Pittard. 2004. Mode of action of the TyrR protein: repression and activation of the *tyrP* promoter of *Escherichia coli*. *Mol. Microbiol.* **52**:243–256.
70. Yang, J., K. Murakami, H. Camakaris, N. Fujita, A. Ishihama, and A. J. Pittard. 1997. Amino acid residues in the alpha-subunit C-terminal domain of *Escherichia coli* RNA polymerase involved in activation of transcription from the *mtr* promoter. *J. Bacteriol.* **179**:6187–6191.
71. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
72. Yaryura, P. M., M. Leon, O. S. Correa, N. L. Kerber, N. L. Pucheu, and A. F. Garcia. 2008. Assessment of the role of chemotaxis and biofilm formation as requirements for colonization of roots and seeds of soybean plants by *Bacillus amyloliquefaciens* BNM339. *Curr. Microbiol.* **56**:625–632.
73. Zimmer, W., M. Wesche, and L. Timmermans. 1998. Identification and isolation of the indole-3-pyruvate decarboxylase gene from *Azospirillum brasilense* Sp7: sequencing and functional analysis of the gene locus. *Curr. Microbiol.* **36**:327–331.