THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 9, Issue of February 27, pp. 7777–7784, 2004

Printed in U.S.A.

Novel Physiological Modulation of the *Pu* Promoter of TOL Plasmid

NEGATIVE REGULATORY ROLE OF THE TURA PROTEIN OF *PSEUDOMONAS PUTIDA* IN THE RESPONSE TO SUBOPTIMAL GROWTH TEMPERATURES*

Received for publication, September 24, 2003, and in revised form, December 3, 2003
Published, JBC Papers in Press, December 12, 2003, DOI 10.1074/jbc.M310580200

Emanuela Rescalli, Silvia Saini, Cristina Bartocci, Leszek Rychlewski‡, Víctor de Lorenzo§, and Giovanni Bertoni¶

From the Dipartimento di Scienze Biomolecolari e Biotecnologie, Universita' degli Studi di Milano, via Celoria 26, 20133 Milan, Italy, ‡BioInfoBank Institute, Limanowskiego 24A/16, 60-744 Poznan, Poland, and the §Department of Microbial Biotechnology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Cientificas, Campus de Cantoblanco, 28049 Madrid, Spain

From crude protein extracts of *Pseudomonas putida* KT2440, we identified a small protein, TurA, able to bind to DNA fragments bearing the entire *Pu* promoter sequence of the TOL plasmid. The knock-out inactivation of the *turA* gene resulted in enhanced transcription initiation from the *Pu* promoter, initially suggesting a negative regulatory role of TurA on *Pu* expression. Ectopic expression of TurA both in *P. putida* and in *Escherichia coli* reporter strains and transcription *in vitro* of the *Pu* promoter in the presence of purified TurA confirmed the TurA repressor role on *Pu* activity. *turA* gene inactivation did not significantly alter two well characterized physiological regulations of the *Pu* expression in routine conditions of cultivation, exponential silencing, and carbon-mediated repression, respectively. However, the growth at suboptimal temperatures resulted in a TurA-dependent increase of *Pu* repression. These results strongly suggest that a physiological significance of the negative role of TurA on *Pu* activity could be limitation of the expression of the toluene-degrading enzymes at suboptimal growth temperatures. Therefore, the identification of TurA as *Pu*-binding protein revealed a novel physiological modulation of *Pu* promoter that is different from those strictly nutritional described previously.

The number of genes that must be expressed for the aerobic degradation of aromatic compounds is usually high (1), and their well timed silencing increases host bacterium fitness in environmental conditions where the exploitation of the aromatic source is inefficient or unessential for bacterial growth (2, 3). In terms of evolution, to reach such physiological control, specific carbon regulatory devices have supposedly integrated in global networks able to sense the environmental stimuli. One example of this is the degradation of the aromatic hydrocarbons, toluene and *m-/p*-xylene, by the *Pseudomonas putida*, encoded by two large operons, *upper* and *meta*, harbored by the TOL mega-plasmid (for a review, see Ref. 4). Expression from the *upper* operon is driven by the σ^{54}-dependent promoter *Pu* (Fig. 1*C*), activated at a distance in the presence of aromatic substrates of the catabolic pathway by the toluene-responsive regulator XylR (5, 6), belonging to the family of bacterial enhancer-binding transcriptional activators that act upon the σ^{54}-containing RNA polymerase (σ^{54}-RNAP[1]; for reviews, see Refs. 7–10). In addition to the specific response to aromatic substrates, it is conceivable that the TOL system had integrated additional co-regulatory devices to avoid the expression of its energetically expensive pathway, under unfavorable physiochemical conditions (temperature, pH, osmolarity, etc.) and/or in the presence of more appetizing carbon and energy sources than toluenes. The influence of the physiochemical conditions on *Pu* expression has not been extensively characterized. On the contrary, the expression performance of *Pu* appears to be strictly dependent on the energetic status of the cell (2, 11). An abundance of nutrients results in strong repression of the *Pu* promoter during the exponential growth phase, a phenomenon that has been referred to as exponential silencing (12). On the other hand, the presence of carbon sources, such as glucose, gluconate, or α-chetoglutarate, exerts a form of carbon-mediated inhibition on *Pu* activity (13, 14). Furthermore, the small signal molecule (p)ppGpp may participate in the *Pu* nutrient-responsive regulatory network (15). By contrast, the search for *Pu* regulatory protein components, which may be the effector molecules of environmental stimuli, has not been examined in any great depth to date. In this study, we aimed to identify novel protein factors involved in the physiological and/or environmental regulation of *Pu* expression. We envisaged the existence of host protein factor(s) that possibly played a co-regulatory role through direct protein-*Pu* DNA interactions. We decided to screen the *P. putida* proteome for proteins able to interact with *Pu* DNA. Using this approach, we identified a small *Pu*-interacting protein, which we named TurA. It shares *in silico* a degree of structural similarity with H-NS, the major protein constituent of the *Escherichia coli* nucleoid, widespread in Gram-negative bacteria (for reviews, see. Refs 16 and 17). H-NS is involved in the condensation of the bacterial chromosome (18, 19) and pleiotropically modulates the transcription of many environmentally regulated genes (*e.g.* by osmolarity, temperature, anaerobiosis, pH, growth phase, etc), including those related to environment adaptation and/or virulence (16, 20, 21). H-NS inhibits transcription at most of the target promoters, and *hns* gene inactivation generally results in increased expression of the cognate

* This work was supported by QLK3-CT-2000-00170 Contract of the European Union and by "Ministero dell'Istruzione, dell'Università e della Ricerca, Roma" (Fondo per G4 Investimenti della Ricerca di Base Grant RBAU01KHM2). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 39-0250315027; Fax: 39-0250315044; E-mail: giovanni.bertoni@unimi.it.

[1] The abbreviations used are: RNAP, RNA polymerase; DTT, dithiothreitol; IHF, integration host factor; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

gene products. Furthermore, a number of genes negatively affected by H-NS are regulated by specific positive transcription factors. In this study, we show that the knock-out of the *turA* gene leads to increased expression of the *Pu* promoter. Furthermore, we present evidence, both *in vivo* and *in vitro*, demonstrating that TurA strongly represses XylR-mediated transcription of the *Pu* promoter. Inactivation of *turA* gene does not affect either exponential silencing or carbon-mediated inhibition of *Pu* expression. On the contrary, we present evidence showing that TurA-mediated repression of *Pu* activity can strengthen at suboptimal growth temperatures. Therefore, this study revealed a novel physiological modulation of *Pu* promoter that is different from those strictly nutritional described previously. Furthermore, the identification of TurA also represents a novelty in the field of the regulation of aromatic degradation pathways. In fact, to date, only protein regulatory components involved in the specific response to the aromatic inducers have been described.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and General Procedures—The *P. putida* strain MAD1 (*Pu::lacZ*, *xylR*$^+$) is a derivative of *P. putida* KT2442 (rifampicin-resistant derivative of the reference strain *P. putida* KT2440) (22) carrying a chromosomal *Pu-lacZ* fusion along with the *xylR* gene assembled within a tellurite resistance mini-transposon (23). MAD1 *turA*::Km^R, the MAD1 derivative carrying a Km^R cassette insertion in the PP1366 locus of KT2440 genome sequence (*turA* gene), was constructed by allelic exchange with a Km^R-inserted allele as follows. The DNA region containing the PP1366 locus was amplified as an 832-bp BamHI-XbaI fragment from *P. putida* KT2440 genome by PCR utilizing the direct primer 5′-CGGGATCC(BamHI underlined) GGCGCCTATTCTAGCGGTCCGCC-3′ and the reverse primer 5′-CGTCTAGA(XbaI underlined)CGAAAAGGCGCGCCAGCAAGCCT-3′. This 832-bp fragment was cloned into the pCR® 2.1-TOPO® plasmid vector (Invitrogen), giving rise to pTBA1 plasmid. For an efficient selection of the integration event of a Km^R cassette into the *turA* gene, the pTBA1 native Km^R gene plasmid was deleted by digestion with both BglII and NcoI enzymes, treatment with Klenow, and religation, giving rise to pTBA2 plasmid. The plasmid pTBA::Km, carrying a Km^R cassette into the *tur*A gene, was constructed by digestion of pTBA2 DNA with BclI, cutting at the third codon of *turA*, and ligation with a 2.2-kb BamHI fragment containing a mini-Tn5 Km^R cassette (24). The 3.2-kb BamHI-XbaI fragment of pTBA::Km containing the Km^R-inserted *turA* gene was cloned into pKNG101 (25), a mobilizable suicide vector, hosted routinely in the permissive *E. coli* strain CC118λ*pir* (24), bearing the positive counter selection marker *sacB* and conferring resistance to streptomycin. The resulting plasmid pKNG101-*turA*::Km was transferred to *P. putida* MAD1 strain through triparental mating with the helper strain *E. coli* HB101 (RK600) as described previously (24). Homologous recombination events between the wild-type and the Km^R-inserted *turA* alleles generating co-integration of pKNG101-*turA*::Km into the MAD1 chromosome were selected on M9-0.2% citrate plates with kanamycin and streptomycin. To select for second homologous recombination events leading to both co-integrate resolution and allelic exchange, about 100 Km^R Sm^R MAD1 co-integrate colonies were pooled, grown overnight at 30 °C in a non-selective medium, and plated in the presence of 5% sucrose and kanamycin. The resulting Suc^R Km^R colonies were screened for pKNG101 release by streptomycin sensitivity (Sm^S). Finally, one such Suc^R Sm^S Km^R clone was named MAD1 *turA*::Km. The correct replacement of the wild-type gene was assessed by PCR with the oligonucleotide pairs 5′-GGCAGGAAGCTGTGATGGATGTTGATCACC-3′/5′-GCTCAATCAATC ACCGGATCAGGGACATGG-3′ and 5′-GGTATGGCTTCTG CCAACAGCGCCTGAGCG-3′/5′-TTCCGGGACGCCGGCTGGATGGTCCTCCAG 3′, designed to probe the linkage of the ends of the Km^R cassette with the chromosomal regions flanking upstream and downstream of the *turA* coding sequence, respectively.

E. coli CC118 *Pu-lacZ* (5) carries a chromosomal *Pu-lacZ* fusion assembled within a streptomycin resistance mini-transposon. Plasmid pEZ9 (5) contains the entire *Pu* promoter sequence as a 312-bp EcoRI-BamHI insert in pUC18, spanning positions −208 to +93. Plasmid pVLT-*turA* was generated for *turA* expression both in *E. coli* and in *P. putida* by cloning the 844-bp EcoRI-XbaI fragment of pTBA1 containing turA into the broad host range plasmid vector pVLT31 (26) under control of the *lacI*^{q}/P_{tac} pair. To obtain higher expression levels of TurA in *E. coli*, the 832-bp *turA*-containing BamHI-XbaI fragment of pTBA1 was cloned into pUC18, giving rise to pUC-*turA*. pACYC*xylR* (57) is a XylR-expressing plasmid derived by cloning a 2.2-kb BamHI-XbaI fragment containing the *xylR* gene under the control of its native promoter into pACYC184 vector (27). All cloned inserts were verified before use by automated sequencing (MWG Biotech). Recombinant DNA manipulations were carried out according to published protocols (28). Accumulation of β-galactosidase raised by *lacZ* fusions was measured in *P. putida* cells as described previously (29) under the conditions specified in each case. The β-galactosidase accumulation by *E. coli* CC118 *Pu-lacZ* derivatives was qualitatively evaluated through intensity of blue pigmentation upon growth on LB agar plates added with 20 μg/ml X-gal.

Proteins and Protein Techniques—Crude protein extracts of the *P. putida* host strain KT2440 were obtained from cells of 2-liter overnight cultures in LB medium at 30 °C collected by centrifugation at 8000 × *g* for 10 min, resuspended in 20 ml of column buffer (50 mM Tris-HCl, 150 mM NaCl), and disrupted in a French press device. After cell disruption, cell debris was separated from the soluble protein crude extract by centrifugation at 12,000 × *g* for 20 min. Typically, the soluble protein concentration determined by Bradford assay ranged from 5 to 15 mg/ml. For protein fractionation, about 60 mg of soluble protein were loaded onto a 3-ml HiTrap™ heparin-Sepharose™ HP chromatographic column (Amersham Biosciences). Upon loading, the column was washed with 15 ml of column buffer, and bound proteins were eluted through a 30-ml 0.1–1 M NaCl linear gradient and collected in 0.4-ml fractions. The presence of TurA was detected by standard protocols of Western blot on PROTRAN® nitrocellulose membrane (Schleicher & Schuell) with preadsorbed anti-TurA polyclonal rabbit antibodies. The screening of the heparin chromatography fractions for *Pu*-interacting proteins was performed by South-Western blot as follows. The proteins were separated by SDS-PAGE, transferred onto PROTRAN® membrane, and incubated overnight at 4 °C in renaturation buffer (20 mM Tris-HCl, 150 mM NaCl, 2.5 mM DTT, 2.5% Nonidet P-40, 10% glycerol, 5% skimmed milk, pH 7.5). After renaturation, the membrane-bound proteins were incubated for 6 h at 4 °C in binding buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.125% skimmed milk, pH 8) added with 25 ng of a 312-bp EcoRI-BamHI *Pu* fragment from pEZ9, end-labeled by in-filling of the overhanging ends with Klenow and [α-^{32}P]dATP. After incubation with the *Pu* probe, the membrane was washed three times with binding buffer, dried, and autoradiographed.

Purified σ^{54} factor and integration host factor (IHF) were kindly donated by B. Magasanik and S. Goodman, respectively. Native core RNAP and σ^{70}-RNAP were purchased from Epicenter Technology. XylRΔA, the amino-terminal A domain-deleted constitutive variant of XylR able to activate transcription from *Pu* in the absence of any aromatic inducer, was purified as described previously (30). For TurA purification, *E. coli* DH5α strain cells harboring pUC-*turA* were grown overnight at 37 °C in 200 ml of LB medium, collected by centrifugation at 8000 × *g* for 10 min, resuspended in 10 ml of lysis buffer (20 mM sodium phosphate buffer, 4% glycerol, pH 6.8), and disrupted in a French press cell. Cell debris was separated from the soluble protein crude extract by centrifugation at 20,000 × *g* for 30 min at 4 °C. About 600 μg of soluble proteins were separated by 15% polyacrylamide SDS-PAGE and stained with Tangerine Green (Molecular Probes Inc.). Bands corresponding to TurA were cut out of the SDS-PAGE gel and eluted by incubation for 16 h at room temperature in elution buffer (50 mM Tris-HCl, pH 7.9, 100 mM EDTA, 0.1% SDS, 5 mM DDT, and 150 mM NaCl). To remove SDS and allow renaturation, the eluted TurA protein was dialyzed in Spectra-Por Micro-DispoDialyzer tubes at 4 °C for 24 h against renaturation buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM DTT, 0.1% Nonidet P-40, and 5% glycerol). The purity of the TurA preparation was estimated in SDS-PAGE stained with Brilliant Blue G-Colloidal Concentrate (Sigma).

In Vitro Transcription Assays—The linear template for *in vitro* transcription of *Pu* promoter was obtained as a 310-bp SmaI-HincII restriction fragment from the pEZ9. *In vitro* transcription reactions were prepared on ice, in a total volume of 25 μl of transcription buffer containing 35 mM Tris acetate, pH 8.0, 5 mM $MgAc_2$, 70 mM KAc, 20 mM NH_4Ac, 1 mM DTT. The DNA template, added at the buffer to a final concentration of 40 μM, was incubated at 37 °C for 20 min with 4 mM ATP, 50 nM core RNAP, 150 nM σ^{54}, 100 nM IHF, 100 nM XylRΔA, and increasing TurA concentrations of 2, 3.5, and 7 nM, respectively. Transcription reactions were initiated by adding a mixture of ATP, CTP, GTP (400 μM each), UTP (50 μM), and [α-^{32}P]UTP (5 μCi; 3000 Ci/mmol). After incubation for 10 min at 37 °C, the reactions were stopped by the addition of 25 μl of STOP mix containing 0.1 M EDTA and 4 M

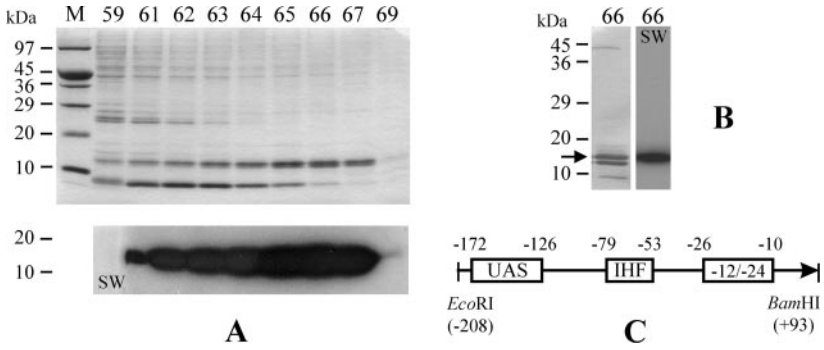

FIG. 1. **Organization of the Pu promoter of TOL plasmid and screening for novel Pu-binding proteins by South-Western blotting.** As shown in *A*, a *P. putida* KT2440 crude protein extract was fractionated through a 3-ml heparin chromatographic column into 75 fractions of 0.4 ml each eluted by a 30-ml linear 0.1–1 M NaCl gradient. Proteins of the fractions were separated by 10% SDS-PAGE (*top*), blotted onto a nitrocellulose membrane, renatured, and incubated in the presence end-labeled *Pu* promoter (*bottom*). The results presented in this figure refer only to 10 such protein fractions eluted at NaCl concentrations ranging from 0.82 to 0.95 M. As shown in *B*, protein fraction 66 was retested for binding to *Pu* DNA by South-Western blotting subjected with more resolutive 15% SDS-PAGE. Two abundant protein species in the range of 15 kDa could be clearly separated. The *arrow* indicates the protein species able to bind to the end-labeled *Pu* DNA. *C*, schematic representation of the distribution of the *Pu* functional elements with respect to the transcription start site (+1) on the 312-bp EcoRI-BamHI fragment of plasmid pEZ9. These include the sequence recognized by σ^{54}-RNAP (−12/−24 motif), the binding site for the IHF (56), and the upstream activating sequences (*UAS*), which are the targets of the activator, XylR.

NH_4Ac. Transcripts were precipitated with 20 μg of glycogen and 100 μl of ethanol, resuspended in denaturing loading buffer (7 M urea, 0.025% bromphenol blue, and 0.025% xylene cyanol in 20 mM Tris, pH 8), electrophoresed on 7% DNA sequencing gel, dried, and autoradiographed. For the RNA I transcription, pEZ9 DNA at 2 μM was incubated with 50 nM σ^{70}-RNAP in the same reaction conditions as above.

RESULTS

Screening for Novel Regulatory Proteins of the Host Strain P. putida KT2440 Able to Bind to the Pu Promoter of the TOL Plasmid—To identify *P. putida* KT2440 proteins involved in *Pu* co-regulation, we aimed to screen for novel *Pu* DNA-protein interactions by protein chromatography coupled with South-Western blotting using a radioactive *Pu* probe. To this end, a crude cell extract derived from an overnight culture of the TOL plasmid host strain *P. putida* KT2440 was fractionated through binding to a heparin affinity chromatography column, extensive washing of unbound proteins, and elution with 0.1–1 M NaCl linear gradient. Samples of the ~70 chromatographic fractions collected along the elution gradient were analyzed by South-Western blotting with end-labeled DNA fragments bearing the entire *Pu* sequence spanning from −208 to +93 with respect to the transcription start site. Fig. 1 shows the results of this screening procedure, conducted on protein fractions eluted from the heparin column by concentrations of NaCl ranging from ~0.82 to 0.95 M. Among the proteins of these fractions, a strong hybridization signal coincided with a protein band present in the fractions 61–67, corresponding to a protein species of about 15 kDa (Fig. 1*A*). To retest the results obtained in this first round of screening, the proteins of fraction 66 were analyzed again by South-Western blotting with end-labeled *Pu* DNA. In this case, as we wanted to obtain better resolution than before in a range of molecular masses lower than 20 kDa, proteins were separated by more resolutive SDS-PAGE for a longer period of time. As shown in Fig. 1*B*, this procedure confirmed that the *Pu* DNA could interact with a protein species of about 15 kDa. However, the 15-kDa band of Fig. 1*A* could be resolved into two protein species of very similar molecular weights, which we initially called P15 and P16, respectively. It is interesting to note that P16 alone could interact efficiently with labeled *Pu* DNA in a South-Western blotting assay (Fig. 1*B*).

Identification and in Silico Analysis of TurA Protein—The amino acid sequence of P16 amino-terminal end was determined, searched through the KT2440 deduced protein data base at The Institute for Genomic Research (TIGR) Comprehensive Microbial Resource (31, 32), and found at the amino-terminal of a small deduced protein of 125 amino acids, accession number PP1366, annotated as sharing a high degree of similarity with the transcriptional regulator MvaT, P16 subunit (33). Standard BLAST search (34) also found in KT2440 genome four open reading frames (loci PP3765, PP0017, PP3693, PP2947, respectively) that code for protein paralogues of P16. Furthermore, P16 orthologue proteins, organized in paralogue pairs, can be also found in *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* genomes. No significant sequence similarity in bacterial species not belonging to the genus *Pseudomonas* was found. To extend our search of similarities from primary to tertiary structure, we adopted predictive software packages able to detect structural similarity between a query protein and experimentally determined structural domains. Firstly, preliminary architecture domain analysis of P16 both by SMART server and by COILS program (35–37) predicted a structure that resembled the *E. coli* nucleoid-associated protein H-NS. Secondly, the combined use of ORFeus, a profile-profile comparison program (38), and PSI-BLAST search (39) validated the structural similarity between the P16 protein and the cluster of orthologous groups (COGs) COG2916 (40), which was annotated as an H-NS-like protein family. Thirdly, this structural similarity was further confirmed by confident transitive similarity searches conducted with ORFeus on protein families extracted from Part B of the Pfam data base (41).

Since the data presented (see below) strongly indicated that P16 protein binds to the *Pu* promoter and represses its activity, we called P16 "TOL upper operon repressor A" (TurA). The amino acid sequence of P15 amino-terminal end was also determined. Interestingly, it corresponded to the amino-terminal of P16 paralogue PP3765 (see above). This could explain the tight similarity of molecular weights and elution profiles in heparin affinity chromatography, respectively, between P15 and P16. Inactivation of the PP3765 gene did not influence *Pu* activity (data not shown). However, by analogy with TurA, we named P15 TurB.

Deficiency of TurA Protein Causes an Increase in Pu Promoter Activity at the End of the Exponential Silencing Phase—To determine the role of TurA protein in *Pu* regulation, we generated, by kanamycin-resistance (Km^R) cassette insertion, the knock-out mutant allele of the *turA* gene in the *P. putida* KT2442 derivative strain MAD1 (KT2442::*Pu-lacZ*, *xylR*$^+$), which harbors both a *Pu-lacZ* transcriptional fusion

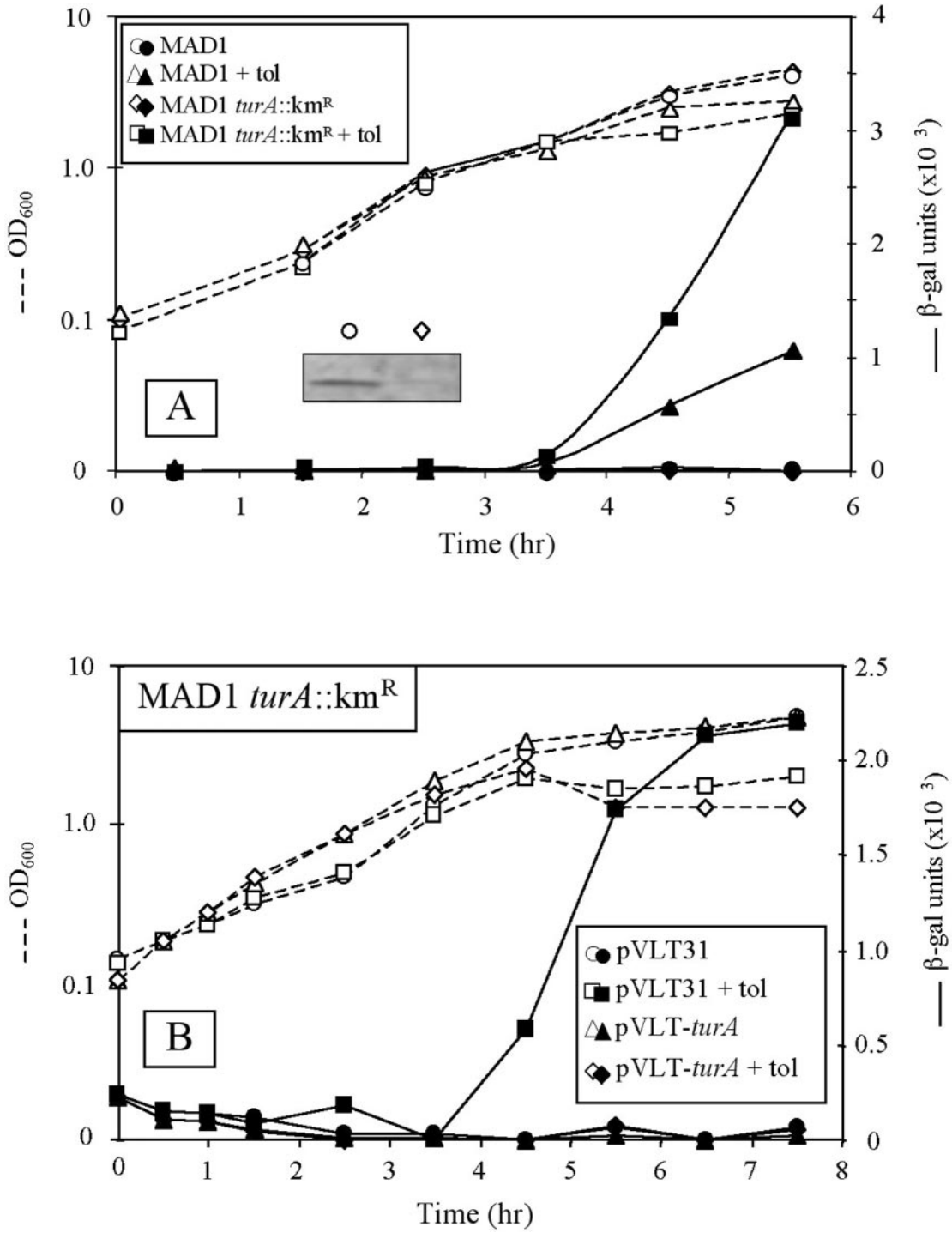

FIG. 2. **Effects of inactivation or overexpression of *turA* gene on Pu activity.** As shown in *A*, cultures of both MAD1 and isogenic MAD1 turA::km^R strains bearing a *Pu-lacZ* transcriptional fusion, stably inserted into the chromosome, were grown in LB medium at 30 °C until A_{600} of 0.1 and then divided into two subcultures. Saturating toluene vapors were supplemented in one of them to induce *Pu* expression. Incubation at 30 °C was then continued for each subculture. Accumulation of β-galactosidase (*β-gal*) activity expressed by the cells of each subculture after A_{600} = 0.1 is shown as an average of samples taken during three independent experiments. A Western blot with anti-TurA antibodies on culture samples taken in the mid-logarithmic phase is shown in the *inset*. As shown in *B*, cultures of MAD1 *turA*::km^R, harboring either pVLT31 or pVLT31-*turA,* were grown in LB medium supplemented with 20 μg/ml tetracycline and then induced with saturating toluene vapors and monitored for β-galactosidase accumulation as in *A*.

and a *xylR* gene stably integrated in the chromosome (23). Fig. 2*A* shows the comparison of β-galactosidase accumulation by MAD1 and MAD1 *turA*::Km^R strains, respectively, when cultured in rich LB medium, with and without toluene induction. Growth in LB medium in the absence of an inducer resulted in no or very low, basal expression of *Pu* (23). In the case of the toluene-supplemented cultures, both induction curves, wild-type and mutant, displayed the typical exponential silencing phenomenon, consisting of very low levels of *Pu* activity during exponential growth followed by rapid induction at the onset of the stationary phase (12). However, in terms of the magnitude of the *Pu* expression after the exponential silencing, MAD1 and MAD1 *turA*::Km^R behaved differently. In fact, after exponential silencing, the expression curves of MAD1 and MAD1

turA::Km^R diverged consistently, with levels of β-galactosidase accumulation three times higher in the mutant strain. Thus, the deficiency of TurA protein revealed unexpected repression on *Pu* that appeared to be different from the exponential silencing phenomenon described previously (12). To test whether TurA might be involved in the regulatory cascade behind the carbon catabolite repression exerted by glucose on *Pu* that was shown to be distinct from *Pu* exponential silencing (11, 14), we examined the *Pu* performance *in vivo* in both MAD1 and MAD1 *turA*::Km^R when cultured in M9 minimal medium, supplemented with 0.2% casamino acids as the main carbon source, in the presence and in the absence of 10 mM glucose. The results of these experiments (not shown) clearly suggested that TurA does not participate in the mechanism of carbon catabolite repression of *Pu* by glucose. In fact, the higher β-galactosidase *Pu* expression levels in the MAD1 *turA*::Km^R derivative continued to be down-regulated by glucose administration to the same extent as in the parental MAD1.

The Ectopic Expression of TurA Silences the Pu Promoter Both in P. putida and in E. coli—To directly assess the negative role of TurA in *Pu* regulation, we set out to monitor the *Pu* activity in the MAD1 *turA*::Km^R strain, transformed with either the *lacI*^{q}/P_{tac}-based broad host range expression vector pVLT31 (26) or its TurA-expressing derivative pVLT-*turA*, with or without toluene administration. As shown in Fig. 2*B*, the amount of TurA expressed from uninduced P_{tac} of pVLT-*turA* could completely silence the toluene-mediated increase of *Pu* activity at the onset of the stationary phase. Furthermore, to obtain stronger evidence of direct TurA involvement in *Pu* regulation, the influence of TurA expression on *Pu* activity was also evaluated in the heterologous genetic background of CC118 *Pu-lacZ*/pACYC*xylR*, a XylR-expressing *E. coli* derivative harboring in the chromosome the same *Pu-lacZ* transcriptional fusion as MAD1, transformed either with pVLT31 or with pVLT-*turA*. To prevent a possible less efficient TurA expression in *E. coli*, we set out to monitor *Pu* activity also in the presence of isopropyl-1-thio-β-D-galactopyranoside. The quantitative evaluation of *Pu* expression along the growth of CC118 *Pu-lacZ*/pACYC*xylR* pVLT-*turA* was hindered by the fact that TurA expression resulted in extremely long growth lags following inoculation of fresh liquid medium. Thus, we assayed *Pu* activity by the amount of blue pigmentation accumulated by cell spots grown on LB plated supplemented with X-gal. As shown in Fig. 3 (*lanes 1*, *3*, *5*, and *7*), CC118 *Pu-lacZ*/pACYC*xylR* cells harboring pVLT31 displayed a comparable intense blue pigmentation both in the absence and in the presence of toluene administration. This can be attributed to a saturating accumulation of blue pigmentation during prolonged growth on agar surface, even in the case of uninduced *Pu* activity, which was at least 10-fold lower when tested by standard Miller's assay on liquid cultures (data not shown). However, both toluene-independent and toluene-stimulated *Pu* activity could be repressed by TurA expression (Fig. 3, *lanes 2*, *4*, *6*, and *8*). Apparently, P_{tac} promoter induction could improve TurA expression from pVLT-*turA* in *E. coli* since isopropyl-1-thio-β-D-galactopyranoside addition seemed to enhance the repression of *Pu* activity, although to a limited extent only. These results represent further evidence that TurA plays a negative regulatory role on *Pu* activity. Furthermore, the fact that TurA repression on *Pu* activity can be reproduced in *E. coli* strongly corroborated the notion that TurA directly modulates the *Pu* activity. Finally, these results also suggested that *Pu* activity modulation by TurA could be achieved by the fine-tuning of TurA abundance *in vivo*.

TurA Protein Represses XylR-mediated Activation of the Pu Promoter in Vitro—The results presented above indicated that

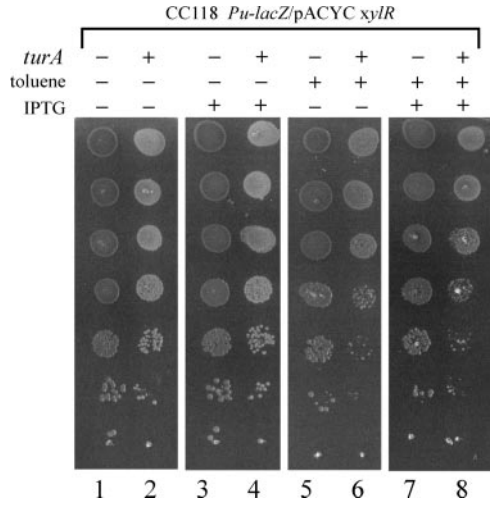

FIG. 3. **TurA represses Pu activity in an *E. coli* reporter strain.** Stationary cultures of the *E. coli* reporter strain CC118 *Pu-lacZ* (pACYC*xylR*), transformed either with pVLT31 or pVLT31-*turA*, were diluted to A_{600} = 1. 2-μl 10-fold serial dilutions were then spotted onto LB agar plates supplemented with 100 μg/ml ampicillin, 20 μg/ml tetracycline, and 20 μg/ml X-gal and incubated at 30 °C for ~18 h in the absence and in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (*IPTG*) and/or saturating vapors of toluene. The β-galactosidase accumulation reflecting *Pu* activity under different conditions is shown as pigmentation intensity of the cell spots.

TurA protein directly represses *Pu* activity. To assess this rigorously, we purified TurA protein (Fig. 4*A*) to evaluate its influence on the transcription of the *Pu* promoter *in vitro*. With this objective, the 310-bp SmaI-HincII DNA fragment, spanning the *Pu* sequence from positions −203 to +93, was transcribed *in vitro* by XylRΔA-activated σ^{54}-RNAP (23, 30), in the presence of increasing concentrations of purified TurA, 2, 3.5, and 7 nM, respectively. As a control, to monitor possible generic effects of purified TurA on transcriptional activity, RNA I (42) was transcribed in the presence of the same gradient of TurA concentrations. As shown in Fig. 4*B*, the addition of TurA at 7 nM could almost completely repress *Pu* transcription. The same TurA concentration could not repress the transcription of RNA I by σ^{70}-RNAP when the pUC18-based plasmid pEZ9 was used as a template. The pattern of the TurA-mediated repression did not have linear correlation with TurA concentration. In fact, at TurA concentrations less than or equal to 3.5 nM, the production of the 93-nucleotide transcript did not decrease significantly (Fig. 4*B*, *lanes 2–4*). On the contrary, the addition of twice the amount of TurA (7 nM) was sufficient to achieve a strong reduction of *Pu* transcription (Fig. 4*B*, *lanes 2* and *5*). Therefore, the behavior of purified TurA protein appeared to be consistent with the notion presented above, that TurA plays a negative regulatory role on *Pu* expression.

TurA Can Modulate Pu Expression in Response to Suboptimal Growth Temperatures—None of the results presented above directly suggested a role for the negative modulation of TurA on *Pu* activity. Indirectly, since *turA* inactivation did not affect either exponential silencing or glucose repression, it indicated that TurA could play a role on *Pu* activity not strictly correlated to availability of more appetizing carbon and energy sources than toluenes. Therefore, we speculated, also on the basis of the putative structural TurA similarity with H-NS (see above), which modulates the transcription of many environmentally regulated genes, that TurA could co-regulate the expression of the toluene-degrading enzymes in response to suboptimal physiochemical conditions. To address this issue, we set out to compare β-galactosidase accumulation by MAD1 and MAD1 *turA*::Km^R strains, respectively, when cultured with

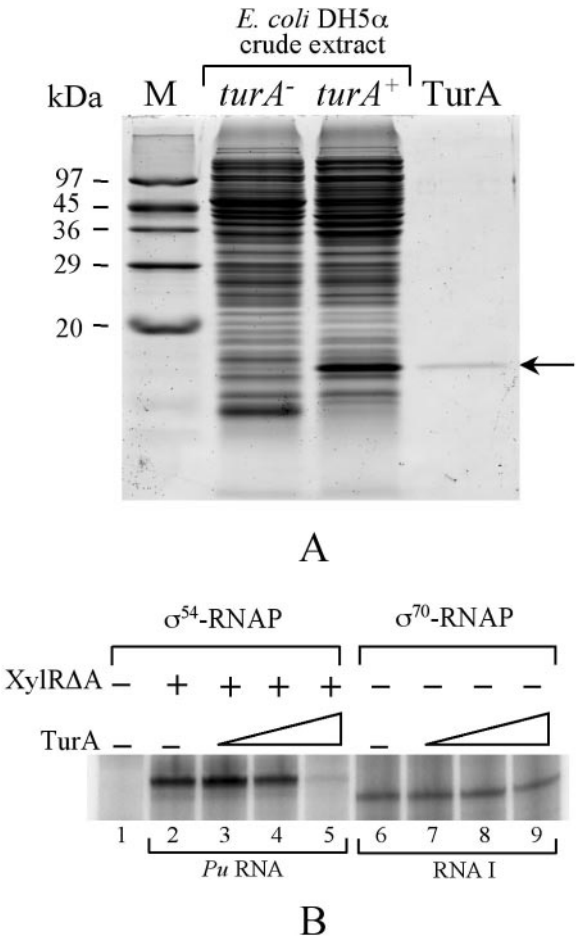

FIG. 4. **Purification of TurA and its repression activity on the transcription in vitro of the Pu promoter.** As shown in *A*, bands corresponding to TurA were cut out of SDS-PAGE gels, and the included TurA was eluted and then renatured through SDS removal. SDS-PAGE of crude protein extracts of *turA*$^-$ and *turA*$^+$ *E. coli* DH5α strains and purified TurA is shown. *M*, marker of molecular masses. As shown in *B*, *left*, a 310-bp SmaI-HincII restriction DNA fragment spanning the *Pu* promoter (final concentration of 40 μM) was transcribed in the presence of increasing TurA concentrations of 2, 3.5, and 7 nM, respectively. The concentrations of XylRΔA, core RNAP, σ^{54}, and IHF were 100, 50, 150, and 25 nM, respectively. As shown on the *right*, the 3-kb pUC18-based plasmid pEZ9 (final concentration of 2 μM) was transcribed by σ^{70}-RNAP in the presence of increasing concentrations of TurA as above. The concentration of σ^{70}-RNAP was 50 nM.

toluene induction in rich LB medium, both at optimal (30 °C) and at suboptimal (16 °C) growth temperatures. As shown in Fig. 5, both for MAD1 and for MAD1 *turA*::Km^R strains, the incubation at 16 °C resulted in approximately two times lower growth rate during the exponential growth phase and no significant change of the highest cellular density ($\sim A_{600}$ = 2.5–3.0) in the stationary phase as compared with incubation at 30 °C. Furthermore, incubation at 16 °C did not change the value of cellular density ($\sim A_{600}$ = 1), at which β-galactosidase accumulation starts to increase significantly, both in MAD1 and in MAD1 *turA*::Km^R strains (see also Fig. 2). Similarly, at both temperatures, the maximum β-galactosidase accumulation in each strain occurred when cellular density settled approximately at A_{600} of 2.5–3.0 in the stationary phase. Therefore, the growth at the suboptimal temperature of 16 °C did not alter the overall profile of *Pu* expression. However, MAD1 expressed at 16 °C a maximum β-galactosidase value reflecting β-galactosidase accumulation/generation that was ~3 times lower than at 30 °C. On the contrary, MAD1 *turA*::Km^R could express at 16 °C maximum β-galactosidase values comparable with and even higher than those at 30 °C. Taken together, these results strongly suggested that the TurA-mediated repression of *Pu* activity can strengthen in response to suboptimal environmental physiochemical conditions, such as, for example, a temperature 14 °C lower than the optimal for growth.

DISCUSSION

The main protein components of the toluene-responsive genetic switch assembled at the *Pu* promoter are the σ^{54}-dependent activator XylR and σ^{54}-RNAP. It is currently believed that, in the regulatory cascade leading from toluene sensing by XylR protein to the escape of σ^{54}-RNAP, there are at least three limiting multistep events able to influence the rate of transcription initiation: (i) the process of intramolecular derepression of XylR on toluene binding that permits the switch from an inactive to an active form capable of establishing productive contacts with σ^{54}-RNAP (6, 43) (for this step in the related DmpR protein, see Refs. 44–47); (ii) the closed complex formation of σ^{54}-RNAP with the *Pu* promoter DNA that involves both σ^{54} and α subunits of σ^{54}-RNAP (48–50); and (iii) the open complex formation that requires contacts between a toluene-activated XyR and the σ^{54} subunit of σ^{54}-RNAP, both prebound at a distance to upstream activating sequences and −12/-24 motif, respectively (51). It was already known that at least two of these three limiting steps, the formation of both closed and open complex, are positively influenced by the promoter geometry imposed by the IHF protein (48–51). Limiting steps of transcription initiation are potential targets for gene regulation (52). However, IHF seems to provide a uniquely structural aid for overcoming these limiting steps and is not a key regulator involved in the physiological control of *Pu* expression (53). Therefore, other factors must contribute to the fine modulation of toluene-responsive *Pu* expression by environmental stimuli. For these reasons, we aimed to identify novel modulator proteins of *Pu* activity. To do this, we envisaged that some of the putative *Pu* modulators may have a regulatory role through direct protein-*Pu* DNA interactions. Therefore, we fractionated the *P. putida*-soluble proteins by heparin affinity chromatography, able to enrich the DNA-binding proteins, and then screened the different protein fractions for their ability to bind to the *Pu* DNA by South-Western blotting. Using this method, we identified a small protein, TurA, that could interact with the *Pu* promoter DNA. Moreover, we presented evidence that strongly supports the notion that TurA-*Pu* DNA interactions participate in direct negative modulation of *Pu* activity. Firstly, *in vivo*, (i) the inactivation of the *turA* gene gave rise to an increase in *Pu* expression (Fig. 2*A*), and (ii) the overexpression of *turA* both in *P. putida* and in *E. coli* resulted in strong decrease of *Pu* activity (Figs. 2*B* and 3). Secondly, *in vitro*, purified TurA is able to efficiently repress *Pu* transcription by XylR-activated σ^{54}-RNAP (Fig. 4).

It is interesting to note that our results suggest that the TurA protein has no role in either exponential silencing or glucose repression of *Pu* activity (11). Therefore, with this study, for the *Pu* physiological tuning, we identified a novel regulatory device that is different from those responding to growth phase and/or nutrient availability. Our results clearly show that, in routine conditions of cultivation, *Pu*-induced expression is higher in the absence of TurA (Fig. 2*A*). This suggests that *Pu*-induced expression might be maintained by TurA at levels below those that can be sustained purely by the currently available cellular quantities of XylR, IHF, and σ^{54}-RNAP. Thus, TurA may have the role of limiting an otherwise unessential *Pu* extra-expression, and it may possibly produce efficient rates of promoter switching-off when toluene runs out. Moreover, we cannot rule out the possibility of a restrictive role of TurA (referred to previously as "restrictor") to either prevent

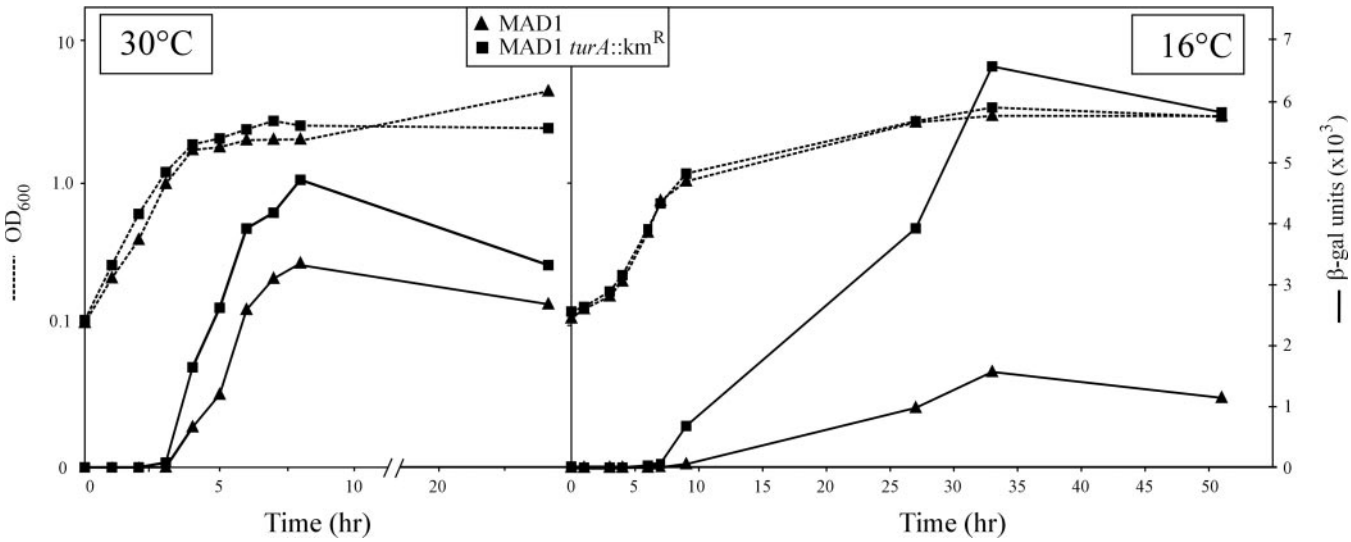

FIG. 5. **Strengthening of TurA-mediated repression of Pu at suboptimal growth temperature.** Overnight cultures in LB medium at 30 °C, of both MAD1 and isogenic MAD1 *turA*::km^R strains bearing a *Pu-lacZ* transcriptional fusion, were diluted in fresh LB medium to A_{600} of 0.1 and grown until saturation at both 30 and 16 °C. Saturating toluene vapors were supplemented in each culture at A_{600} of 0.25 to induce *Pu* expression. Accumulation of β-galactosidase (*β-gal*) activity expressed by the cells of each culture along growth is shown as an average of samples taken during three independent experiments.

or at least minimize both spurious (*i.e.* by other σ^{54}-dependent activators) and gratuitous (*i.e.* not by toluene addition) *Pu* activations (54). Furthermore, we present evidence that TurA-mediated repression of *Pu* activity can strengthen at suboptimal growth temperatures. Therefore, under suboptimal conditions of cultivation, the role of TurA could be limitation of the expression of the toluene-degrading enzymes. At suboptimal temperatures, the physiological significance of such modulation could be the prevention of (i) energy-consuming biosynthesis of a large set of toluene-degradative enzymes under unfavorable conditions, (ii) toxicity associated with the activity of the degradative enzymes, and (iii) enzyme bottlenecks leading to accumulation of toxic intermediates of the toluene degradation pathway. Therefore, our results strongly suggest that TurA can play the role of environmental rather than strictly nutritional modulator of *Pu* activity.

BLAST search analysis indicated that TurA belongs to the MvaT protein family, identified by sequence similarity in the *Pseudomonas* species (55). Remarkably, TurA matches the behavior of the negative transcription factor of MvaT in *P. aeruginosa* (55). However, in *P. aeruginosa*, MvaT appears to modulate the expression of a different class of genes (namely, *quorum sensing*-controlled virulence genes) and participates in a growth phase-dependent control system (55). In this study, we extended the comparative analysis to the structural features of TurA. This analysis indicated that TurA and, possibly, the other members of the MvaT family are structurally related to the nucleoid-associate protein H-NS (16, 17). Surprisingly, no gene product with significant sequence similarity with H-NS can be identified by BLAST search in the currently available *Pseudomonas* genome sequences. Based on these observations, it can be suggested that the members of the MvaT family have roles in the *Pseudomonas* species, analogous to H-NS-like proteins in other Gram-negative species (17). Thus, the functional features of both *P. putida* TurA and *P. aeruginosa* MvaT appear to match the H-NS behavior as a global modulator of gene expression principally through a negative effect on transcription, and acting, in many cases, on environmental signal-responsive promoters that are positively regulated by specific transcription factors (16).

Acknowledgments—We are grateful to I. Cases, M. Valls, E. Galli, F. Vidal, and G. Dehò for inspiring debate, to S. Goodman (University of Southern California, Los Angeles, CA) for the kind donation of IHF protein, and to J. Garmendia for providing the plasmid pACYC*xylR*. We also thank I. Cases for assistance in protein sequencing.

REFERENCES

1. van der Meer, J. R., de Vos, W. M., Harayama, S., and Zehnder, A. J. (1992) *Microbiol. Rev.* **56,** 677–694
2. Cases, I., and de Lorenzo, V. (1998) *Curr. Opin. Microbiol.* **1,** 303–310
3. Cases, I., and de Lorenzo, V. (2001) *EMBO J.* **20,** 1–11
4. Ramos, J. L., Marques, S., and Timmis, K. N. (1997) *Annu. Rev. Microbiol.* **51,** 341–373
5. de Lorenzo, V., Herrero, M., Metzke, M., and Timmis, K. N. (1991) *EMBO J.* **10,** 1159–1167
6. Pérez-Martín, J., and de Lorenzo, V. (1996) *Cell* **86,** 331–339
7. Buck, M., Gallegos, M. T., Studholme, D. J., Guo, Y., and Gralla, J. D. (2000) *J. Bacteriol.* **182,** 4129–4136
8. Xu, H., and Hoover, T. R. (2001) *Curr. Opin. Microbiol.* **4,** 138–144
9. Zhang, X., Chaney, M., Wigneshweraraj, S. R., Schumacher, J., Bordes, P., Cannon, W., and Buck, M. (2002) *Mol. Microbiol.* **45,** 895–903
10. Studholme, D. J., and Dixon, R. (2003) *J. Bacteriol.* **185,** 1757–1767
11. Cases, I., and de Lorenzo, V. (2000) *J. Bacteriol.* **182,** 956–960
12. Cases, I., de Lorenzo, V., and Pérez-Martín, J. (1996) *Mol. Microbiol.* **19,** 7–17
13. Holtel, A., Marques, S., Mohler, I., Jakubzik, U., and Timmis, K. N. (1994) *J. Bacteriol.* **176,** 1773–1776
14. Cases, I., Perez-Martin, J., and de Lorenzo, V. (1999) *J. Biol. Chem.* **274,** 15562–15568
15. Sze, C. C., Bernardo, L. M., and Shingler, V. (2002) *J. Bacteriol.* **184,** 760–770
16. Atlung, T., and Ingmer, H. (1997) *Mol. Microbiol.* **24,** 7–17
17. Bertin, P., Hommais, F., Krin, E., Soutourina, O., Tendeng, C., Derzelle, S., and Danchin, A. (2001) *Biochimie (Paris)* **83,** 235–241
18. Spassky, A., Rimsky, S., Garreau, H., and Buc, H. (1984) *Nucleic Acids Res.* **12,** 5321–5340
19. Spurio, R., Durrenberger, M., Falconi, M., La Teana, A., Pon, C. L., and Gualerzi, C. O. (1992) *Mol. Gen. Genet.* **231,** 201–211
20. Falconi, M., Colonna, B., Prosseda, G., Micheli, G., and Gualerzi, C. O. (1998) *EMBO J.* **17,** 7033–7043
21. Hommais, F., Krin, E., Laurent-Winter, C., Soutourina, O., Malpertuy, A., Le Caer, J. P., Danchin, A., and Bertin, P. (2001) *Mol. Microbiol.* **40,** 20–36
22. Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990) *J. Bacteriol.* **172,** 6557–6567
23. Fernández, S., de Lorenzo, V., and Pérez-Martín, J. (1995) *Mol. Microbiol.* **16,** 205–213
24. de Lorenzo, V., and Timmis, K. N. (1994) *Methods Enzymol.* **235,** 386–405
25. Kaniga, K., Delor, I., and Cornelis, G. R. (1991) *Gene* (*Amst.*) **109,** 137–141
26. de Lorenzo, V., Eltis, L., Kessler, B., and Timmis, K. N. (1993) *Gene* (*Amst.*) **123,** 17–24
27. Chang, A. C., and Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156
28. Sambrook, J., and Russel, D. (2000) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Pérez-Martín, J., and de Lorenzo, V. (1996) *J. Mol. Biol.* **258,** 575–587
31. Peterson, J. D., Umayam, L. A., Dickinson, T., Hickey, E. K., and White, O. (2001) *Nucleic Acids Res.* **29,** 123–125
32. Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins Dos Santos, V. A., Fouts, D. E., Gill, S. R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R. T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris, L. P., Holtzapple,

E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K. N., Dusterhoft, A., Tummler, B., and Fraser, C. M. (2002) *Environ. Microbiol.* **4,** 799–808
33. Rosenthal, R. S., and Rodwell, V. W. (1998) *Protein Sci.* **7,** 178–184
34. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
35. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5857–5864
36. Letunic, I., Goodstadt, L., Dickens, N. J., Doerks, T., Schultz, J., Mott, R., Ciccarelli, F., Copley, R. R., Ponting, C. P., and Bork, P. (2002) *Nucleic Acids Res.* **30,** 242–244
37. Lupas, A., Van Dyke, M., and Stock, J. (1991) *Science* **252,** 1162–1164
38. Ginalski, K., Pas, J., Wyrwicz, L. S., von Grotthuss, M., Bujnicki, J. M., and Rychlewski, L. (2003) *Nucleic Acids Res.* **31,** 3804–3807
39. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
40. Tatusov, R. L., Koonin, E. V., and Lipman, D. J. (1997) *Science* **278,** 631–637
41. Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., Eddy, S. R., Griffiths-Jones, S., Howe, K. L., Marshall, M., and Sonnhammer, E. L. (2002) *Nucleic Acids Res.* **30,** 276–280
42. Morita, M., and Oka, A. (1979) *Eur. J. Biochem.* **97,** 435–443
43. Garmendia, J., and de Lorenzo, V. (2000) *Mol. Microbiol.* **38,** 401–410
44. O'Neill, E., Ng, L. C., Sze, C. C., and Shingler, V. (1998) *Mol. Microbiol.* **28,** 131–141
45. O'Neill, E., Sze, C. C., and Shingler, V. (1999) *J. Biol. Chem.* **274,** 32425–32432
46. Wikstrom, P., O'Neill, E., Ng, L. C., and Shingler, V. (2001) *J. Mol. Biol.* **314,** 971–984
47. O'Neill, E., Wikstrom, P., and Shingler, V. (2001) *EMBO J.* **20,** 819–827
48. Bertoni, G., Fujita, N., Ishihama, A., and de Lorenzo, V. (1998) *EMBO J.* **17,** 5120–5128
49. Carmona, M., de Lorenzo, V., and Bertoni, G. (1999) *J. Biol. Chem.* **274,** 33790–33794
50. Macchi, R., Montesissa, L., Murakami, K., Ishihama, A., de Lorenzo, V., and Bertoni, G. (2003) *J. Biol. Chem.* **278,** 27695–27702
51. Pérez-Martín, J., Timmis, K. N., and de Lorenzo, V. (1994) *J. Biol. Chem.* **269,** 22657–22662
52. Geiselmann, J. (1997) *Biol. Chem.* **378,** 599–607
53. Valls, M., Buckle, M., and de Lorenzo, V. (2002) *J. Biol. Chem.* **277,** 2169–2175
54. Pérez-Martín, J., and de Lorenzo, V. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7277–7281
55. Diggle, S. P., Winzer, K., Lazdunski, A., Williams, P., and Camara, M. (2002) *J. Bacteriol.* **184,** 2576–2586
56. Rice, P. A., Yang, S., Mizuuchi, K., and Nash, H. A. (1996) *Cell* **87,** 1295–1306
57. Garmendia, J. (2001) *The Transcriptional Regulator XylR of Pseudomonas putida: Response to Aromatic Inducers and Biotechnological Applications.* Ph.D thesis, Universidad Autonoma de Madrid, Madrid

Novel Physiological Modulation of the *Pu* Promoter of TOL Plasmid: NEGATIVE REGULATORY ROLE OF THE TURA PROTEIN OF PSEUDOMONAS PUTIDA IN THE RESPONSE TO SUBOPTIMAL GROWTH TEMPERATURES

Emanuela Rescalli, Silvia Saini, Cristina Bartocci, Leszek Rychlewski, Víctor de Lorenzo and Giovanni Bertoni

J. Biol. Chem. 2004, 279:7777-7784.
doi: 10.1074/jbc.M310580200 originally published online December 12, 2003
