A Two-Component Regulatory System Integrates Redox State and Population Density Sensing in *Pseudomonas putida*[†]

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A two-component system formed by a sensor histidine kinase and a response regulator has been identified as an element participating in cell density signal transduction in *Pseudomonas putida* KT2440. It is a homolog of the *Pseudomonas aeruginosa* RoxS/RoxR system, which in turn belongs to the RegA/RegB family, described in photosynthetic bacteria as a key regulatory element. In KT2440, the two components are encoded by PP_0887 (*roxS*) and PP_0888 (*roxR*), which are transcribed in a single unit. Characterization of this two-component system has revealed its implication in redox signaling and cytochrome oxidase activity, as well as in expression of the cell density-dependent gene *ddcA*, involved in bacterial colonization of plant surfaces. Whole-genome transcriptional analysis has been performed to define the *P. putida* RoxS/RoxR regulon. It includes genes involved in sugar and amino acid metabolism and the sulfur starvation response and elements of the respiratory chain (a *cbh3* cytochrome oxidase, Fe-S clusters, and cytochrome *c*-related proteins) or genes participating in the maintenance of the redox balance. A putative RoxR recognition element containing a conserved hexamer (TGCCAG) has also been identified in promoters of genes regulated by this two-component system.

Bacteria have a variety of systems that allow them to perceive alterations in the environment and adjust their physiology to the new situation. In many cases, the functions required to detect and respond to environmental changes rest upon a sensor histidine kinase and a response regulator, respectively, which couple to form the so-called two-component regulatory systems. Transduction of the environmental signal is carried out through the transfer of a phosphate group from a phosphorylated histidine in the sensor protein to an aspartic acid in the regulator, which thus becomes active, resulting in transcription of its target genes. Two-component systems have been described to regulate a wide variety of cell functions and are perhaps the chief machinery for signal transduction in bacteria. Well-studied examples of this type of regulatory element include the redox RegB/RegA system of *Rhodobacter* (9), the *Pseudomonas putida* toluene-responding TodS/TodT system (18), and the envelope stress-responding Cpx system (26) or PhoP/PhoQ, which in different gram-negative bacteria responds to limiting Mg²⁺ concentrations, activating Mg²⁺ transport and modulating virulence and lipopolysaccharide modifications that lead to increased antibiotic resistance (13).

Cells may sense and respond not only to environmental cues but also to chemical signals produced by the bacterial population or by eukaryotic hosts. Many microbial species are known to produce small diffusible molecules, termed autoinducers, which mediate cell-cell communication in a pop-

ulation density-dependent manner, inducing changes in gene expression when a certain concentration of the signal molecule is reached. These signaling mechanisms, known as quorum sensing, regulate a variety of cellular processes (3) ranging from virulence mechanisms to swarming motility or biofilm architecture (2, 6) and may also participate in bacteria-host signaling in both pathogenic and mutualistic interactions (14). Different molecules have been described to function as quorum-sensing signals, the most extended in gram-negative bacteria being acyl-homoserine lactones (AHL) of various chain lengths. Other signals include the so-called universal autoinducer (furanosyl borate diester) or the *Pseudomonas* quinolone signal. In gram-positive bacteria, modified peptides have been described as quorum-sensing molecules (20). Whereas AHL signals are often recognized intracellularly by a transcriptional regulator, peptide signals are transduced via two-component regulatory systems.

In a previous work we had characterized a gene, *ddcA*, involved in colonization of plant seeds by *P. putida* KT2440, which shows a cell density-dependent expression profile (11). Although this microorganism is not known to produce any of the usual quorum-sensing signals and appears not to have the genetic potential to synthesize AHL or *Pseudomonas* quinolone signal, *ddcA* expression was shown to respond to a pH-sensitive signal accumulated in the supernatant of KT2440 cultures during growth (11). In this paper we report the identification and characterization of a two-component regulatory system that modulates expression of *ddcA* and appears to be a key sensor/regulator of the redox state of *P. putida* cells.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *P. putida* KT2440 is a plasmid-free derivative of strain mt-2 (24, 27). Transposon mutants were generated by random mutagenesis with mini-Tn5Km1 (7) as previously described (12). *Escherichia coli*

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DH5 α was used as the host for cloning experiments. The *P. putida* KT2440 cosmid library used in this work has been described before (28). Plasmid pME510 carries a transcriptional fusion of *ddcA* to the reporter *lacZ* gene (11). Plasmid pRF1 was constructed by cloning an NsiI/BglII fragment containing *roxS* and *roxR* into plasmid pBBR1MCS-5 (16), as detailed in the Results section below. *P. putida* cultures were grown at 30°C in LB medium or M9 minimal medium (29) supplemented with 1 mM MgSO₄, 50 μ M FeCl₃ or iron citrate, and trace metals, with glucose (20 mM) or citrate (15 mM) as a carbon source. *E. coli* cultures were grown in LB medium at 37°C. When appropriate, antibiotics were added at the following concentrations (μ g/ml): kanamycin (Km), 25; tetracycline, 15; ampicillin, 50; streptomycin (Sm), 100; and gentamicin, 10 (for *E. coli*) or 100 (for *P. putida*).

General molecular biology techniques. Plasmid and chromosomal DNA preparations and agarose gel electrophoresis were done following standard procedures (1, 29). Restriction enzymes, DNA ligase, and shrimp alkaline phosphatase (Roche and New England Biolabs) were used following the manufacturers' instructions. A DIG-DNA Labeling and Detection Kit (Roche) was used for Southern hybridization. PCRs were done with *Taq* DNA polymerase (Eppendorf) using an Eppendorf Mastercycler personal thermocycler. Arbitrary PCR followed by sequencing was used to define transposon insertion sites, as described previously (12).

***P. putida* microarrays.** The genome-wide *P. putida* KT2440 chip used in this work consists of 5,539 oligonucleotides (50-mers) spotted in duplicate onto γ -aminosilane-treated microscope slides and linked with UV light and heat. The oligonucleotides represent 5,350 of the 5,516 genes annotated in the KT2440 genome (<http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gpp>), along with 140 of the 148 open reading frames predicted for the TOL plasmid pWW0 and several commonly used reporter genes and antibiotic resistance markers. The chips also include homogeneity controls corresponding to the *rpoD* and *rpoN* genes, as well as duplicate negative controls at 203 positions. A detailed description of the array characteristics has been reported elsewhere (38).

RNA isolation and preparation of labeled cDNA. Cultures were grown in LB medium at 30°C to an optical density at 660 nm (OD₆₆₀) of 2.8. Cells from 13-ml culture samples were collected by centrifugation at 4°C in tubes precooled in liquid nitrogen. Pellets were immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using Tri-Reagent (Ambion) and subjected to DNase treatment followed by purification with RNeasy columns (Qiagen).

To obtain fluorescent probes for hybridization, cDNA generated from RNA samples was labeled by indirect incorporation of Cy3 and Cy5 dyes. For each reaction, 25 μ g of RNA was primed with 7.5 μ g of random hexamers (Amersham) at 70°C for 10 min, followed by 10 min at 25°C, and then chilled on ice for 2 min. A mix containing 1 $\times$ reverse transcription buffer; 0.5 mM (each) dATP, dCTP, and dGTP; 0.25 mM (each) dTTP and aminoallyl-dUTP; 10 mM dithiothreitol; 40 U of RNaseOUT (Invitrogen), and 400 U of SuperScript II reverse transcriptase (Invitrogen) was added, and the mixture was incubated at 42°C for 120 min. After the reaction was stopped with EDTA, the RNA template was hydrolyzed by adding 10 μ l of 1 N NaOH, followed by incubation at 65°C for 15 min. Samples were then neutralized with 25 μ l of 1 M HEPES (pH 7.5), and the hydrolyzed RNA and unincorporated nucleotides were removed through Qia-Quick PCR purification columns (Qiagen) using phosphate wash and elution buffers. Samples were completely dried, resuspended in 9 μ l of 0.1 M sodium carbonate buffer (pH 9), mixed with either Cy3 (KT2440) or Cy5 (EU58) fluorescent dyes (Amersham Biosciences), and allowed to couple for 2 h at room temperature in the dark. After coupling, the reaction was quenched with 4 to 5 μ l of 4 M hydroxylamine for 15 min. Finally, labeled cDNA probes were again purified, and labeling efficiency was assessed using a Nanodrop ND1000 spectrophotometer (Nanodrop Technologies).

Microarray hybridization and data analysis. Before hybridization, the microarray slides were blocked by immersion into 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin for 1 h at 42°C. After two successive immersions in distilled, deionized water, followed by a final wash with isopropanol, slides were spin-dried by centrifugation at 1,500 $\times$ g for 5 min. Equal amounts of purified Cy3- and Cy5-labeled cDNA probes, corresponding to KT2440 and EU58, respectively, were mixed, dried, and reconstituted in 35 μ l of hybridization buffer (5 $\times$ SSC, 25% formamide, 0.5% SDS, 5 $\times$ Denhardt's solution, 5% dextran sulfate) preheated to 42°C. The labeled probe was denatured at 98°C for 3 min, applied directly onto the microarray slide, and covered with a glass coverslip. After hybridization for 20 h at 42°C in a dark, humidified chamber (AHC ArrayIt Hybridization Cassettes; Telechem International, Inc.), the slides were washed by gentle agitation in 2 $\times$ SSC-0.1% SDS at 42°C for 5 min, followed by two 5-min washes at room temperature in 1 $\times$ SSC, two 5-min washes in 0.2 $\times$ SSC, and a final 5-min wash in 0.1 $\times$ SSC.

Residual salts were removed by centrifugation before the slides were scanned on a GenePix 4100A scanner (Axon Instruments, Inc.). Separate images were acquired for Cy3 and Cy5 at 10- μ m resolution, and the background-subtracted median spot intensities were determined using GenePix Pro, version 5.1, image analysis software (Axon Instruments, Inc.). To filter out unreliable data, spots with anomalies and those where intensities of at least 70% of the pixels were not 2 standard deviations (SD) above the background in the two channels were discarded. Intensities were normalized by applying the Lowess intensity-dependent normalization method (37) and were statistically analyzed using the Almazan System Software (Alma Bioinformatics S.L.). Three independent biological replicates were performed for each experiment, and a Student's *t* test algorithm based on the differences between the log 2 ratio for each biological replicate was used. Genes were considered differentially expressed when the relative change was at least 1.95-fold and the *P* value was below 0.05.

RT-PCR. Reverse transcription-PCR (RT-PCR) analyses were performed by using a Titan One-Tube RT-PCR Kit (Roche) in accordance with the manufacturer's recommendations. For each reaction, 0.5 μ g of total RNA was used. To rule out DNA contamination, a negative control was included whereby the mixture was heated for 5 min at 94°C to inactivate reverse transcriptase before the PCR amplification step. As positive controls, primers corresponding to the *hlpBA* operon (23) or to the *rpoB* gene were used. A list of the oligonucleotides used is included as Table S1 in the supplemental material.

Nadi and β -galactosidase activity assays. The Nadi test was used for visual assessment of cytochrome *c* oxidase activity. Reactions were carried out as described by Marrs and Gest (21) by the addition of a freshly prepared 1:1 mixture of 1% α -naphthol in 95% ethanol and 1% *N,N*-dimethyl-*p*-phenylenediamine monohydrochloride (DMPD) to colonies grown on LB agar plates such that all colonies were covered, and then excess reagent was removed. Formation of indophenol blue was timed as an indicator of cytochrome *c* oxidase activity.

β -Galactosidase activity was assayed during growth in LB or minimal medium as described previously (22). Data are given in Miller units. Unless otherwise specified, overnight cultures were inoculated (1:100 dilution) in fresh medium and grown for 1.5 h, diluted 1:1 every 30 min, before the collection of samples was initiated. This was done to ensure proper dilution of β -galactosidase that might have accumulated after overnight growth. Experiments were done at least three times.

Biofilm formation, seed adhesion, and rhizosphere colonization assays. Biofilm formation assays were done in rotating glass tubes. Adhesion to the solid surface was followed over time during growth in LB medium by staining with crystal violet, as previously described (12). Bacterial attachment was quantified after solubilization of the dye with ethanol and spectrophotometric measurement of absorbance at 550 nm. Attachment to corn seeds was tested as previously described (11, 12). For rhizosphere colonization experiments, surface-sterilized seeds were allowed to germinate for 48 h before being inoculated with a 1:1 mixture of the mutant strain and a KT2440 derivative carrying an Sm resistance gene in single copy in the chromosome (KT2440-Sm), generated by site-specific insertion of mini-Tn7 Ω Sm1 (15) at an extragenic location. Plants were maintained in a controlled chamber at 24°C and 55 to 65% humidity with a daily light period of 16 h. After 1 week, plants were collected, and the roots were cut and placed in tubes with 20 ml of M9 basal medium and 4 g of glass beads. Tubes were vortexed for 2 min, and dilutions were plated on selective medium (LB medium with Km or Sm, respectively).

Microarray data accession number. Data have been deposited in the Array-Express repository (<http://www.ebi.ac.uk/microarray-as/aer/>) under accession number E-MEXP-1684.

RESULTS

Identification of functions modulating expression of the cell density-dependent gene *ddcA*. Random transposon mutagenesis was done on *P. putida* KT2440 using mini-Tn5Km1 to identify genes affecting expression of the *ddcA::lacZ* fusion carried on plasmid pME510. Km^r clones were selected on minimal medium with citrate, pooled, and transformed with pME510 by electroporation. After samples were plated on LB medium with tetracycline and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), eight white colonies were isolated from approximately 5,000 clones. These were checked to confirm the presence of the plasmid, and β -galactosidase activity was assayed during growth in LB medium. All clones showed reduced

FIG. 1. Expression of the *ddcA::lacZ* transcriptional fusion in the wild type and two representative mutants harboring pME510. Recovery of expression of the *ddcA::lacZ* fusion in EU58 by plasmid pRF1 is also shown. β -Galactosidase activity was measured during growth in LB medium at 30°C. Open squares, KT2440(pME510); filled circles, EU58(pME510); filled triangles, EU59(pME510); open circles, EU58 harboring both pME510 and pRF1. Results are averages and SD of two experiments with two replicas.

levels of expression of the *ddcA::lacZ* fusion (Fig. 1) although to different extents. Residual activity was observed in four clones (represented in Fig. 1 by EU58), with a slight increase in late exponential phase, while the rest (EU55, EU56, EU57, and EU59) showed no β -galactosidase activity throughout the growth curve.

Arbitrary PCR followed by sequencing was performed to determine the insertion site of the transposon in each mutant, except EU57, for which identification was unsuccessful. Mutants EU55, EU56, and EU59 were affected in PP_0108, PP_0031, and PP_4947, respectively. PP_0031 and PP_0108 correspond to hypothetical proteins of unknown function; while the latter is conserved in different microorganisms, the former seems to be unique to *P. putida*. No homologs could be found in the databases, and this protein showed only limited sequence similarity with a region of a methyl-accepting chemotaxis transducer from *Magnetococcus*. PP_4947 corresponds to *putA*, the gene encoding proline dehydrogenase, a multifunctional protein responsible for the flavin adenine dinucleotide-dependent catabolism of proline to glutamate, which also regulates its own expression, as well as of that of the adjacent *putP* gene, the main proline transporter under nonstress conditions (34, 35).

The remaining four mutants (EU51, EU52, EU53, and EU58), where β -galactosidase activity was not completely abolished, were determined to be siblings with the mini-Tn5 inserted in locus PP_0887. Sequence analysis revealed that the protein encoded by this open reading frame is a putative sensor histidine kinase, which would be part of a two-component regulatory system, along with a response regulator encoded by the downstream locus PP_0888. These two proteins show significant similarity (71% and 89% identical residues, respectively) with the RoxS/RoxR two-component regulatory system of *Pseudomonas aeruginosa* (5), and we have therefore named the genes accordingly (PP_0887, *roxS*; PP_0888, *roxR*). It is worth noting that the actual start codon of *roxS* seems to correspond to an ATG located 96 bp downstream of the GTG codon annotated as the start site in the TIGR database entry of PP_0887, for which no Shine-Dalgarno sequence can be iden-

tified. Upstream of the ATG, a putative Shine-Dalgarno sequence (AGGAGA) can be found, and the size of the resulting polypeptide (417 amino acids) is comparable to that of RoxS of *P. aeruginosa* (422 amino acids) and related proteins found in the databases.

One of the four *roxS* mutants (EU58) was cured of the pME510 plasmid by successive passage through nonselective medium and was chosen for further analysis.

***roxS* and *roxR* form an operon.** Given the 1-base overlap existing between the stop codon of *roxS* (underlined in TGAA) and the start codon of *roxR* (ATG), it seemed likely that these two genes formed a single transcriptional unit. RT-PCR was performed using primers designed to determine if that was the case. The downstream gene (PP_0889, with its start codon at a distance of 108 bp from the stop codon of *roxR*) coding for a putative β -lactamase of the AmpC family was also included in the analysis since it is transcribed in the same direction (Fig. 2). Total RNA was extracted from KT2440 cultures grown in LB medium to two different densities (OD_{660} s of 0.8 and 1.5) and used as the template in RT-PCRs. After electrophoresis of the reaction products, a band of the expected size was observed, regardless of the growth state of the culture, confirming the existence of a single transcript corresponding to *roxSR* (Fig. 2). No amplification was obtained with two different pairs of primers designed to test if PP_0889 was part of the same transcriptional unit, indicating that the reduced *ddcA::lacZ* expression observed in EU58 is not due to a polar effect of the transposon on PP_0889.

RoxS/RoxR participate in cell density signal transduction. Complementation studies were done to confirm the involvement of the RoxS/RoxR two-component system in cell density-dependent expression of *ddcA*. For this purpose, a cosmid library of KT2440 was screened by colony hybridization to identify cosmids harboring both genes. A ~ 4.4 -kb NsiI/BglIII fragment containing the *roxSR* operon was subcloned from one of the identified cosmids into plasmid pBBR1MCS-5. The resulting plasmid, pRF1, was electroporated into EU58(pME510), and β -galactosidase activity was assayed. As shown in Fig. 1, expression of the *ddcA::lacZ* fusion was restored, reaching levels higher than those of the wild-type strain.

We have previously shown that *ddcA* expression responds to an as yet undefined signal accumulated in the supernatant of cells grown to early stationary phase (11). Given the predicted roles of RoxR and RoxS, it seemed likely that this two-component system would participate in transduction of such a cell density signal. To test this hypothesis, cultures of KT2440 and EU58 were grown to an OD_{600} of ≈ 3 and centrifuged, and the supernatants were collected and added to mid-exponential cultures of either strain harboring pME510. After 1 h of incubation, β -galactosidase activity was measured and compared to that of cultures without the addition of conditioned medium. A fourfold increase in activity was observed in KT2440(pME510) incubated with either KT2440 or EU58 supernatant compared to the control, whereas no significant increase in activity could be detected in EU58(pME510) regardless of the treatment (Fig. 3). This result suggests that EU58 is impaired in its ability to respond to the signal rather than in its synthesis.

Pleiotropic effects of the mutation in *roxSR*. In *P. aeruginosa*, the function of RoxS/RoxR has been linked with the redox state of the cells, via modulation of the activity of a cyanide-

FIG. 2. (A) Map of the chromosomal region where *roxS* and *roxR* are located. The transposon insertion is indicated, as well as the primers and expected sizes of RT-PCR products. (B) Electrophoresis of RT-PCR products obtained using RNA from KT2440 as a template and primer pairs a and b, designed to verify the existence of a single mRNA molecule corresponding to *roxS* and *roxR* (lanes 2 to 4) and/or to *roxR*-PP0889 (lanes 5 to 7). Lane 1, molecular weight marker; lanes 2 and 5, template RNA obtained from cultures at an OD₆₆₀ of 0.8; lanes 3 and 6, template RNA obtained from cultures at an OD₆₆₀ of 1.5; lanes 4 and 7, controls without reverse transcriptase to ensure that amplification was not due to residual DNA. Results with primer pair c were also negative (not shown).

insensitive cytochrome oxidase (5). In order to determine if this two-component system has a similar role in *P. putida*, KT2440 and EU58 were grown in liquid medium in the presence of increasing concentrations of sodium azide. As shown in Fig. 4, EU58 was more sensitive to sodium azide than the parental strain, indicating that the activity of an azide-insensitive cytochrome oxidase was altered in the mutant. Tolerance was restored to the levels of the wild type in EU58 harboring plasmid pRF1, confirming an analogous implication of *P. putida* RoxS/RoxR in cytochrome oxidase activity as that observed in *P. aeruginosa*.

Similar results were observed in the presence of other agents with a potential to alter the redox state, such as hydrogen peroxide. In this case, tolerance was tested on plates where bacterial lawns had been sown and a paper disk containing 35% H₂O₂ was placed in the center. The diameter of inhibition

halos was measured after overnight incubation. Growth of EU58 was reduced to scattered colonies near the edges of the plate, whereas the inhibition halo was smaller in the complemented mutant than in the wild type (Fig. 4).

FIG. 3. Participation of RoxS/RoxR in cell density signal transduction. Exponentially growing cultures (OD₆₆₀ of 0.3) of KT2440 (open bars) and EU58 (filled bars) harboring pME510 were mixed with cell-free supernatants (sup) of cultures of the same strains without plasmid and grown to an OD₆₆₀ of 3. Expression of *ddcA::lacZ* was measured after 1 h of incubation. As a control, cultures without the addition were used. Results are averages and SD of three experiments. wt, wild type.

FIG. 4. (A) Role of RoxS/RoxR in the activity of azide-insensitive cytochrome oxidase. Strains KT2440 (circles), EU58 (squares), and EU58 harboring pRF1 (triangles) were grown overnight in LB medium with increasing concentrations of sodium azide, and turbidity (OD₆₆₀) was measured. Results are the averages and SD of three experiments. (B) Sensitivity of KT2440, EU58, and EU58(pRF1) to 35% H₂O₂ (see text for details).

FIG. 5. (A) Adhesion of KT2440 and EU58 to corn seeds. Seed attachment was analyzed as described previously (11), and the percentage of cells attached per seed after 1 h of incubation was determined. Results are the average and SD of six assays. (B) Biofilm formation on glass tubes during growth in LB medium under orbital rotation, evaluated at different times by crystal violet staining, followed by solubilization and measurement of A_{550} . White bars, KT2440; black bars, EU58; gray bars, EU58(pRF1). Results are averages and SD of three experiments.

Since *ddcA* was initially identified as a gene involved in bacterial colonization of corn (*Zea mays*) seeds, it might be expected that a mutant affected in a positive regulator of *ddcA* showed defects in its ability to establish on plant surfaces. Seed adhesion assays were performed comparing the mutant and the wild-type strains. As shown in Fig. 5, the proportion of EU58 cells that attached to seeds after 1 h of incubation was significantly lower than that of KT2440 and slightly lower than that observed previously for a *ddcA* mutant (12). Competitive colonization assays were also performed to analyze the fitness of this mutant in the rhizosphere of corn plants in competition with the wild type. Seeds were inoculated with a 1:1 proportion of KT2440-Sm and EU58 and sown in sterile sand. After 1 week, the rhizosphere population of the wild type was twice as high as that of EU58 (1.4×10^7 CFU/g of root versus 6.2×10^6), a difference that was statistically significant ($P = 0.05$). Biofilm formation on abiotic surfaces, however, was not significantly different between KT2440 and EU58, whereas the complemented mutant formed a more compact biofilm at the air-

liquid interface, suggesting that overexpression of *roxSR* may influence biofilm development (Fig. 5).

Genome-wide analysis of the *roxSR* regulatory network. To further define the role of the RoxSR two-component system in *P. putida*, whole-genome transcriptional profiling of the wild-type and mutant strains was performed using DNA microarrays, as described in Materials and Methods. The comparative analysis revealed that a significant number of genes are influenced directly or indirectly by the RoxSR two-component system. In fact, we found that 173 genes showed reduced expression in EU58 in comparison to the wild type, whereas 84 genes were upregulated in the mutant (see Tables S2 and S3 in the supplemental material). It is worth noting that in many cases genes were physically organized into operons (some known and others deduced from sequence data) so that the number of transcriptional units whose expression is reduced in the *roxSR* background is 127, and 73 are upregulated in the mutant. The fact that several or all of the genes in the operons appear with the same expression pattern is indicative of the consistency of the results.

Table 1 shows representative genes having increased expression in the wild type with respect to the mutant, grouped by functional categories. One significant group corresponds to genes encoding respiratory chain proteins, including the cluster PP_0312 to PP_0316; the operon coding for a *cbh3*-type cytochrome oxidase; *ubiG*, involved in ubiquinone biosynthesis; a SenC family protein, presumably involved in cytochrome oxidase assembly (31); and a cytochrome *c*-type protein (PP_3332), whose expression is reduced 10-fold in the EU58 mutant with respect to the wild type. These data indicated that the electron transport system of EU58 mutant cells is generally impaired, despite the fact that the *cioAB* operon was not among the genes differentially expressed according to the microarray data, as would be expected from the azide sensitivity assays. The role of RoxS/RoxR in controlling the level of cytochrome *c* oxidase was further confirmed by means of the Nadi assay (21), a method based on the rapid formation of indophenol blue from colorless α -naphthol catalyzed by cytochrome *c* oxidase, using DMPD as an exogenous electron donor. Strains containing a nonfunctional cytochrome *c* oxidase will not catalyze this reaction, resulting in a substantial delay in spontaneous indophenol formation. Colonies of KT2440, EU58, and EU58(pRF1) grown in LB medium were incubated in the presence of α -naphthol and DMPD. Formation of indophenol blue was observed in a few seconds in the wild type and the complemented mutant, developing maximum coloration within 2 min, whereas in EU58 color appearance was delayed (~ 10 min), indicating reduced cytochrome *c* oxidase activity.

The other large set of genes influenced by RoxS/RoxR correspond to functions involved in amino acid metabolism, specifically, serine, threonine, and glycine, as reflected by L-threonine aldolase (PP_0321), serine hydroxymethyltransferase (PP_0322), and the *sox* operon (PP_0323-PP_0326), which encodes sarcosine oxidase. Arginine and histidine utilization genes also showed differential expression. However, when growth of KT2440 and EU58 strains was tested in liquid medium with arginine or histidine as the sole C and N sources, no significant differences were detected, and only a slight decrease in the growth rate of the mutant (~ 96 min, versus 88 min for KT2440) was observed with serine as the sole C and N sources.

TABLE 1. Selected genes with increased expression in KT440 with respect to the *roxSR* mutant^a

Gene function and locus (name)	Gene product	Fold change
Transport		
PP_4756	Amino acid permease	5.0
PP_0112	ABC transporter, periplasmic binding protein	2.3
PP_0113	ABC transporter, permease	2.1
PP_0114	ABC transporter, ATP binding protein	2.0
PP_0793 (<i>fruB</i>)	Fructose-specific phosphotransferase system	3.7
PP_1015	Sugar ABC transporter, periplasmic sugar-binding protein	6.8
PP_1017	Sugar ABC transporter, permease	2.3
PP_1018	Sugar ABC transporter, ATP-binding subunit	3.4
PP_1019 (<i>oprB</i>)	Porin B	3.1
Sugar metabolism		
PP_0794 (<i>fruK</i>)	1-Phosphofructokinase	2.8
PP_1010 (<i>edd</i>)	6-Phosphogluconate dehydratase	2.6
PP_1023 (<i>pgl</i>)	6-Phosphogluconolactonase	2.2
Amino acid metabolism		
PP_0310	Oxidoreductase FMN-binding (glycine-betaine metabolism)	3.5
PP_0311	Iron-sulfur cluster-binding (glycine-betaine metabolism)	2.9
PP_0321	Low-specificity L-threonine aldolase	3.0
PP_0322 (<i>glyA</i>)	Serine hydroxymethyltransferase	2.4
PP_0323 (<i>soxB</i>)	Sarcosine oxidase, beta subunit	4.8
PP_0324 (<i>soxD</i>)	Sarcosine oxidase, delta subunit	3.2
PP_0325 (<i>soxA</i>)	Sarcosine oxidase, alpha subunit	3.1
PP_1088 (<i>argG</i>)	Argininosuccinate synthase	2.1
PP_1110	Serine O-acetyltransferase	4.5
PP_1111	Aminoacyl-tRNA synthetase, putative	4.6
PP_1112	Conserved hypothetical protein	2.2
PP_1113	Pyridoxal-phosphate-dependent enzyme family protein	2.5
PP_1654 (<i>cysM</i>)	Cysteine synthase B	3.1
PP_5029 (<i>hutG</i>)	Formiminoglutamase (histidine utilization operon)	4.1
PP_5030 (<i>hutI</i>)	Imidazolonepropionase	2.6
PP_5032 (<i>hutH</i>)	Histidine ammonia-lyase	2.6
PP_5033 (<i>hutU</i>)	Urocanate hydratase	3.3
Electron transport and respiratory chain		
PP_0312	Electron transfer flavoprotein, alpha subunit	3.3
PP_0313	Electron transfer flavoprotein, beta subunit	2.7
PP_0315	Rieske 2Fe-2S family protein	3.9
PP_0316	Flavin adenine dinucleotide-binding oxidoreductase	2.1
PP_1765 (<i>ubiG</i>)	3-Demethylubiquinone-9 3-methyltransferase	2.2
PP_3332	Cytochrome <i>c</i> -type protein	10.9
PP_3823	Cytochrome <i>c</i> -type protein	2.0
PP_4250 (<i>ccoN-1</i>)	Cytochrome <i>c</i> oxidase <i>ccb3</i>-type, subunit I	2.1
PP_4251 (<i>ccoO-1</i>)	Cytochrome <i>c</i> oxidase <i>ccb3</i>-type, subunit II	2.9
PP_4253 (<i>ccoP-1</i>)	Cytochrome <i>c</i> oxidase <i>ccb3</i>-type, subunit III	2.0
PP_2379	Sco1/SenC family protein (cytochrome oxidase assembly)	4.6
Protein fate		
PP_0308	Putative dipeptidase	5.3
PP_0403 (<i>surA</i>)	Chaperonin, survival protein SurA	2.3
PP_1360 (<i>groES</i>)	Chaperonin	2.3
PP_2300 (<i>clpP</i>)	ATP-dependent Clp protease, proteolytic subunit	3.2
Other functions		
PP_0328 (<i>fdhA</i>)	Formaldehyde dehydrogenase, glutathione-independent	7.3
PP_0362 (<i>bioB</i>)	Biotin synthetase	3.2
PP_0493 (<i>selA</i>)	L-Seryl-tRNA selenium transferase	2.1
PP_0494 (<i>selB</i>)	Selenocysteine-specific translation elongation factor	2.0
PP_1133, PP_3381, PP_3586	ISPpu9 transposase	2.1, 2.7, 2.3
PP_3821 (<i>galU</i>)	UTP-glucose-1-phosphate uridylyltransferase	2.3
PP_1280 (<i>algI</i>)	Alginate O-acetylation protein AlgI	2.0
PP_1281 (<i>algL</i>)	Alginate lyase	2.2
PP_1014	Conserved hypothetical protein	7.8
PP_1366	Transcriptional regulator	3.8
PP_0894	Conserved hypothetical protein	4.7

^a Putative operons are in boldface.

FIG. 6. Validation of microarray results by RT-PCR using RNA from KT2440 (left) and EU58 (right) as templates and primers corresponding to the indicated genes.

Other genes with a relevant decrease in expression in the mutant were *surA*, encoding a periplasmic chaperone involved in stationary phase survival (30) and other chaperones; *fdhA* (encoding glutathione-independent formaldehyde dehydrogenase); a putative dipeptidase; functions required for the incorporation of the alternative amino acid selenocysteine into proteins; and genes belonging to the alginate biosynthesis operon. Although alginate production has so far been observed in KT2440 in biofilms only under matrix stress conditions (4), we tested KT2440, EU58, and EU58(pRF1) growing on plates in the presence of D-cycloserine, an inhibitor of peptidoglycan synthesis that induces the production of alginate in other bacteria (36). No differences were observed among the three strains.

The highest upregulation in EU58 with respect to the wild type (see Table S3 in the supplemental material) corresponds to a gene encoding ornithine cyclodeaminase (PP_3533), the enzyme responsible for the interconversion of ornithine and proline. This, and the fact that mutant EU58 is affected in proline catabolism, could indicate that *ddcA* expression was inhibited by proline accumulation. However, addition of exogenous proline did not have a negative effect on *ddcA::lacZ* expression; rather, an increase in β -galactosidase activity was observed, associated to increased growth of the proline-supplemented cultures (data not shown).

Other functions with increased expression in the mutant include a number of stress proteins, functions related to nucleic acid metabolism or modification, type I pili, genes involved in coenzyme A metabolism, and a significant number of hypothetical proteins. Interestingly, a gene coding for one of the components of an *aa3*-type cytochrome oxidase is also upregulated in EU58.

Several genes were chosen for validation of the microarray data by RT-PCR. The *cioA* gene was also included in this analysis. As expected, the RT-PCR products obtained for PP_3332, *fdhA*, *cysD*, *hutG*, and PP_0110 were more abundant in the wild type than in EU58, whereas the opposite was true for PP_3533 (Fig. 6). Despite the lack of significant changes observed in the microarrays, the RT-PCR product for *cioA* was more abundant in the wild type (Fig. 6), as was anticipated from the azide sensitivity assays.

DISCUSSION

In this work we have characterized the RoxS/RoxR two-component system of *P. putida* encoded by PP_0887 and PP_0888. This element is a homolog of the *P. aeruginosa* RoxS/RoxR system (5), which in turn belongs to the family of RegA/RegB and PrrA/PrrB, described in *Rhodobacter* as redox-re-

sponding two-component regulatory systems with a key role in modulating respiration, photosynthesis, and carbon and nitrogen fixation, among other processes (9). It has been proposed that changes in the redox state are sensed by the histidine kinase, RegB, via the *cbb3* cytochrome oxidase and direct interaction with ubiquinone through a ubiquinone-binding site (GGXXNPF), present in the sequence of RegB (32). This site can also be found in RoxS of *P. putida* (GGSTNPF), pointing to a similar signal transduction pathway. However, our results show that expression of the ubiquinone biosynthesis gene *ubiG* and at least one of the two *cbb3*-type cytochrome oxidases present in *P. putida* is regulated by RoxS/RoxR, which suggests the existence of a positive feedback mechanism. Both the transcriptional data and the physiology of mutant EU58 indicate that this two-component system has a central role in the balance of cytochromes and terminal oxidases in the respiratory chain of *P. putida*. Changes in the interplay between the different *P. putida* terminal oxidases depending on the environmental conditions have been previously reported, with the global regulator ANR participating in the coordination of these responses (33). Our results add a new element to this regulatory complexity.

The RoxS/RoxR system also controls a broad set of functions that have an influence on energy metabolism, such as formaldehyde and formate dehydrogenases, and metabolic routes for sugars and amino acids. Some of the genes under the control of RoxS/RoxR also point toward a role in the sulfur starvation response; replacement of sulfur-containing cysteine by the alternative amino acid selenocysteine, revealed by the activation of *selA* and *selB*, is suggestive of this response. Further indication is provided by the reduced expression in EU58 of the PP_1110-PP_1113 operon as well as the *cysD* and *cysNC* genes. These two genes have been shown to be induced by sulfur depletion and also by oxidative stress in *Mycobacterium tuberculosis* (25). All of these data and the fact that a *roxSR* mutant loses its ability to sense the population density signal that modulates *ddcA* expression indicate that this two-component system is a key element in a complex regulatory network. Intriguingly, although *ddcA* (PP_4615) showed increased expression in the wild type with respect to EU58, the relative change in expression (1.54-fold) was below the cutoff (1.95-fold), in contrast with the significant differences observed with the transcriptional *lacZ* fusion. It is possible that the low stability of the native *ddcA* transcript can explain this unexpected result. Similarly, the *cioAB* operon showed a relative change in expression below the cutoff (1.7-fold), but RT-PCR results indicate that its expression is reduced in the mutant.

The large number of genes with differential expression in KT2440 and EU58 and the fact that several of them are in turn regulators suggest that not all are under the direct control of this two-component system. In an attempt to define common elements that could constitute a recognition sequence for the response regulator, the upstream regions of 24 of the genes with reduced expression in the *roxSR* mutant were aligned. The sequence CGTGCCAGCC was identified as a potential RoxR box, with the underlined hexamer highly conserved. This sequence differs from sequences proposed in *Bradyrhizobium japonicum* and *Rhodobacter sphaeroides*, where a degenerate hexamer followed by a pentamer located at a variable distance has been described as a binding site for the RoxR orthologs

TABLE 2. Overlap between RoxR-dependent genes and genes induced in an *rpoT* mutant

Locus (gene name)	Gene product	Fold induction	
		<i>rpoT</i> vs WT ^a	WT vs <i>roxSR</i>
PP0887	Sensor histidine kinase	2.4	1.9
PP0888	Response regulator	2.3	2.1
PP1110	Serine <i>O</i> -acetyltransferase, putative	3.5	4.5
PP1112	Conserved hypothetical protein	2.1	2.2
PP1747	Conserved hypothetical protein	2.2	3.5
PP2379	Sco1/SenC family protein	2.9	4.6
PP3286 (<i>phaN</i>)	Phenylacetic acid degradation transcriptional repressor	2	2.0
PP4250 (<i>ccoN-1</i>)	Cytochrome <i>c</i> oxidase <i>cbb3</i> -type, subunit I	7	2.1
PP4252 (<i>ccoQ-1</i>)	Cytochrome <i>c</i> oxidase <i>cbb3</i> -type CcoQ, subunit	4	1.9
PP4253 (<i>ccoP-1</i>)	Cytochrome <i>c</i> oxidase <i>cbb3</i> -type, subunit III	3.6	2.0
PP5030 (<i>hutI</i>)	Imidazolonepropionase	3.7	2.6
PP5033 (<i>hutU</i>)	Urocanate hydratase	3.7	3.3
PP4278 (<i>xdhA</i>)	Xanthine dehydrogenase, XdhA subunit	2	−2.8
PP4487 (<i>acsA</i>)	Acetyl-coenzyme A synthetase	2	−2.5

^a Relative induction data are from Duque et al. (8). WT, wild type.

RegR and PrrA (10, 19). Sequences resembling the consensus can be found both in the *cioA* and the *ddcA* promoters (CCC TGCGATCG and CGTGCCATGCC, respectively; nucleotides identical to the consensus sequence are underlined).

While comparing the transcriptional profiling data obtained here with data from previous studies, we noticed that the *roxSR* operon was among the genes that showed increased expression in an *rpoT* mutant of *P. putida* (8). Consistently, a subset of genes with reduced expression in EU58 is upregulated in the *rpoT* background, as shown in Table 2. RpoT belongs to the family of extracytoplasmic sigma factors and plays a role in tolerance to toluene, mainly through increased expression of the *tigGHI* efflux pump genes (8). The significance of this observation in the physiology and stress responses of *P. putida* deserves further investigation.

With respect to the other genes identified in this work that affect expression of the *ddcA::lacZ* fusion, the role of PP_0031 is difficult to postulate since no homologs are found in any other organism except *P. putida*. PP_0108 is located between the genes coding for the *aa3*-type cytochrome oxidase (PP_0103–PP_0106) and those presumed to participate in its assembly (PP_0109–PP_0111), but whether its function is related to them is not known. In the case of PutA (PP_4947), this protein possesses the two enzymatic activities required for the conversion of proline into glutamic acid, as well as a regulatory role. Although so far PutA has been reported to repress expression of only *putA* and *putP* in the absence of proline (34), regulation of other genes cannot be discarded. In fact, we have noticed similarities between the promoter regions of *ddcA* and *putP* (data not shown). Proline can accumulate as an osmoprotectant and has also been shown to influence the redox state of bacterial cells (17), which may explain the increased activity of the *ddcA* promoter in the presence of proline, thereby con-

necting this result with the role of RoxS/RoxR. Future research will be aimed at deciphering the relationships between all these elements and the precise nature of the signal(s) transduced by this two-component system.

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