

The *davDT* Operon of *Pseudomonas putida*, Involved in Lysine Catabolism, Is Induced in Response to the Pathway Intermediate δ -Aminovaleric Acid

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Pseudomonas putida KT2440 is a soil microorganism that attaches to seeds and efficiently colonizes the plant's rhizosphere. Lysine is one of the major compounds in root exudates, and *P. putida* KT2440 uses this amino acid as a source of carbon, nitrogen, and energy. Lysine is channeled to δ -aminovaleric acid and then further degraded to glutaric acid via the action of the *davDT* gene products. We show that the *davDT* genes form an operon transcribed from a single σ^{70} -dependent promoter. The relatively high level of basal expression from the *davD* promoter increased about fourfold in response to the addition of exogenous lysine to the culture medium. However, the true inducer of this operon seems to be δ -aminovaleric acid because in a mutant unable to metabolize lysine to δ -aminovaleric acid, this compound, but not lysine, acted as an effector. Effective induction of the *P. putida* *P*_{*davD*} promoter by exogenously added lysine requires efficient uptake of this amino acid, which seems to proceed by at least two uptake systems for basic amino acids that belong to the superfamily of ABC transporters. Mutants in these ABC uptake systems retained basal expression from the *davD* promoter but exhibited lower induction levels in response to exogenous lysine than the wild-type strain.

Fluorescent pseudomonads are good colonizers of the rhizosphere of most crop plants, and it has been established that root exudates play a central role in plant-*Pseudomonas* sp. interactions (8, 11, 24, 36). The main organic compounds of root exudates are sugars, various organic acids, flavonoids, and a number of amino acids (3, 7). Although sugars account for most of the organic matter in exudates (18), they do not seem to play a major role in plant-bacterium interactions, as deduced from the finding that they do not contribute to tomato root colonization by a well-studied *Pseudomonas* biocontrol strain (25). Bacterial genes involved in the metabolism of amino acids are differentially expressed in the rhizosphere, or in response to root exudates, and mutants deficient in amino acid utilization are less competitive in rhizosphere colonization than the wild-type strain (4, 5, 12, 44–46). A similar observation was made with mutants of a *Rhizobium* sp. unable to use proline as the sole carbon and nitrogen source (19).

By means of a transposon carrying a promoterless *lux* operon to generate transcriptional fusions by insertional mutagenesis, Espinosa-Urgel and Ramos (12) identified the *Pseudomonas putida* *davT* gene, whose expression increased in bacteria colonizing the rhizosphere. The *davT* gene encodes the enzyme δ -aminovalerate aminotransferase, which participates in a key step in lysine catabolism. Expression of the *davT::lux* fusion was enhanced in response to the addition of lysine in the culture medium. This is consistent with the relatively high concentration of lysine (0.42 mM) estimated to be present in corn root exudates (3, 7, 12, 29, 31). The *davT*

mutant was unable to use lysine or δ -aminovalerate as a carbon source, in consonance with the latter chemical being a point of convergence in the degradation of lysine through two different branches (Fig. 1). Although the *davT* mutant of *P. putida* showed a chemoattractive response to root exudates, it was less competitive in root colonization assays than its parental strain (11). Root exudates contain small amounts of different compounds. The ability of rhizobacteria to use different carbon and energy sources simultaneously could be important for the establishment in the rhizosphere.

In the present study we characterized in detail the *davT* chromosomal region, and we show that this gene forms an operon with *davD*, the gene encoding glutaric semialdehyde dehydrogenase. We have identified the promoter of the *davDT* operon and found that its maximal expression requires efficient transport of lysine into the cell and transformation of this amino acid into δ -aminovaleric acid, which appears to be the true effector for the expression of the *davDT* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *P. putida* KT2440, a derivative of the *P. putida* soil isolate mt-2, was used (13, 32). *P. putida* rei2 is a KT2440 derivative that carries the *luxCDABE* genes fused in frame to the *davT* gene (12). *Escherichia coli* strain DH5 α was used for cloning experiments, and *E. coli* CC118 λ pir was used as a host for pUT plasmids (9). These strains were grown at 37°C in Luria-Bertani medium (40). *P. putida* strains were grown at 30°C either in Luria-Bertani medium or in minimal medium, which was basal M9 medium (40) supplemented with Fe-citrate, MgSO₄, and trace metals, as described previously (1), and with benzoate (15 mM), glucose (0.4% [wt/vol]), or sodium citrate (10 mM) as a carbon source. When indicated lysine (10 mM), δ -aminovaleric acid (5 mM) or glutaric acid (10 mM) were added to the culture medium. When appropriate, antibiotics were added at the following concentrations: chloramphenicol, 30 μ g/ml; gentamicin (Gm), 30 μ g/ml; kanamycin (Km), 50 μ g/ml; and tetracycline, 15 μ g/ml.

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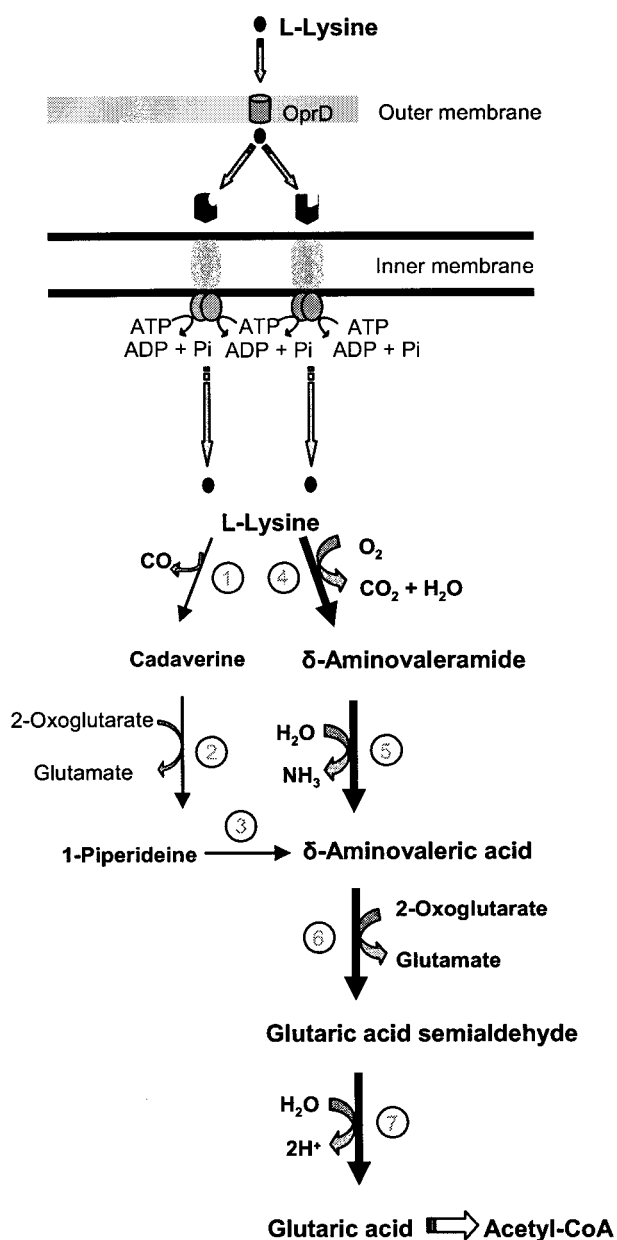


FIG. 1. L-Lysine catabolism by *P. putida*. L-Lysine has been proposed to enter into the periplasmic space through the OprD porin (43). Subsequently, it is transported to the cytoplasm (the present study) and is metabolized through two branches that converge at δ -aminovaleric acid (12, 35). Further metabolism of δ -aminovaleric acid yields glutaric acid that is channeled to Krebs cycle intermediates. The thick arrows indicate the main catabolic pathway (Revelles, unpublished). The enzymatic steps are indicated as follows: 1, L-lysine decarboxylase; 2, cadaverine aminotransferase; 3, 1-piperidine dehydrogenase; 4, L-lysine monooxygenase; 5, 5-aminovaleramide amidase; 6, 5-aminovalerate aminotransferase; 7, glutarate semialdehyde dehydrogenase.

DNA techniques. Preparation of plasmid and chromosomal DNA, digestion with restriction enzymes, ligation, electrophoresis, and Southern blotting were done by using standard methods (40). *P. putida* cells were electroporated as described previously (23). Hybridizations were done by using the DIG DNA labeling and detection kit (Roche Laboratories, Mannheim, Germany) according to the manufacturer's instructions. Plasmid sequencing was done with universal or reverse pUC19/M13 oligonucleotides as primers. Sequencing was done on an

ABI Prism 310 automated sequencer. Sequences were analyzed with Omega 2.0 software (Oxford Molecular) and ORF FINDER (available at the National Center for Biotechnology Information [NCBI]) and compared to data from the *P. putida* KT2440 genome, obtained from NCBI, and with the GenBank database by using basic local alignment search tool (BLAST; www.ncbi.nlm.nih.gov) programs (2).

Preparation of RNA, primer extension analysis, and RT-PCR. *P. putida* strains KT2440 and *rei2* were grown overnight in M9 minimal medium with benzoate. Cells were then diluted 100-fold in fresh medium, and different aliquots were incubated in duplicate in the absence or in the presence of 5 mM lysine and 5 mM δ -aminovaleric acid until the culture reached a turbidity of ca. 1.0 at 660 nm. Cells (30 ml for primer extension and 1.5 ml for reverse transcription-PCR [RT-PCR]) were harvested by centrifugation ($5,000 \times g$ for 10 min) and processed for RNA isolation according to the method of Marqués et al. (27). Extracts were treated with RNase-free DNase I (50 U) in the presence of 3 U of an RNase inhibitor cocktail (Roche Laboratories). For primer extension analysis, we used as a specific primer an oligonucleotide complementary to the *davD* mRNA (5'-GGTCACCTTGATGGTCTGGCC-3'). The primer was labeled at its 5' end with [γ - ^{32}P]ATP by using T4 polynucleotide kinase as described previously (27). About 10^5 cpm of the labeled primer was hybridized to 20 μg of total RNA, and extension was carried out by using avian myeloblastosis virus reverse transcriptase as described previously (27). Electrophoresis of cDNA products was done in a urea-polyacrylamide sequencing gel to separate the reaction products.

RT-PCR was done with 1 μg of RNA in a final volume of 20 μl by using the Titan OneTube RT-PCR system according to the manufacturer's instructions (Roche Laboratories). The annealing temperature used for RT-PCR was 60°C, and the cycling conditions were as follows: 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min. Positive and negative controls were included in all assays. The primers used to test contiguity in the mRNA of the *davD* and *davT* genes are available upon request.

Construction of a *P. putida* *orf4* mutant strain. A mutant strain bearing an inactivated chromosomal *orf4* was constructed as follows: plasmid pCHESI Ω Km is a pUC18 derivative containing the origin of transfer *oriT* of RP4 and the Ω -Km interposon of plasmid pHP45 Ω Km cloned as a HindIII fragment (23). To generate the *orf4* mutation, a 593-bp fragment of *P. putida* *orf4* was amplified by PCR with primers provided with EcoRI and BamHI sites and subsequently cloned between the EcoRI and BamHI sites of pCHESI Ω Km in the same transcriptional direction as the P_{lac} promoter. The resulting plasmid, pCHESI-ORF4, was mobilized from *E. coli* CC118 λ *pir* into *P. putida* KT2440 by triparental mating with the *E. coli* HB101(pRK600) helper strain (15). *P. putida* transconjugants bearing a cointegrate of the plasmid in the host chromosome were selected on M9 minimal medium with benzoic acid (10 mM) as the sole carbon source and Km. A few Km-resistant clones were chosen to confirm that pCHESI-ORF4 integrated into and disrupted the *orf4* gene. All clones analyzed contained an inactivated *orf4* gene, and a single random clone was chosen and called *P. putida* ORF4:: Ω Km.

Mini-Tn5 mutagenesis of *rei2* mutant and selection of mutants with decreased light emission. *P. putida* *rei2* (*lux*⁺, Km^r) was mated with *E. coli* CC118 λ *pir*(pUT-Gm) (S. Molin, unpublished data) and the helper strain *E. coli* HB101(pRK600) as described above. Transconjugants of *P. putida* *rei2* were selected on M9 minimal medium with citrate as the C source and with Km and Gm. Approximately 5,000 independent transconjugants were selected, and light emission was examined in the presence of 5 mM lysine added to M9 minimal medium plates with citrate as a C source. We used a charge-coupled device camera to monitor light emission (38) and selected six mutants that did not emit light or emitted significantly lower levels of light than the parental strain. To exclude those that may have resulted from insertion of mini-Tn5 in the *lux* genes, total DNA of the mutants was hybridized against the entire *lux* operon labeled with digoxigenin. Four clones were discarded because of inactivation of the *lux* genes, and the other two were retained for further characterization (see below).

β -Galactosidase assays. The low-copy-number promoter probe pMP220 (42) is a useful vector for gene expression analysis because it carries a promoterless '*lacZ*' gene with the appropriate Shine-Dalgarno sequence upstream from the first ATG. We amplified the intergenic regions between *davD* and *davT* and between *orf3* and *davD* and cloned them in front of '*lacZ*' in pMP220 to yield pOR1 and pOR2, respectively. The intergenic region between *orf3* and *davD*, and between *davD* and *davT* (Fig. 2) was amplified by PCR with primers incorporating restriction sites, an EcoRI site in the primer designed to meet the 5' end, and a KpnI site in the primer designed to meet the 3' end. Upon amplification, DNA was digested with EcoRI and KpnI and ligated to EcoRI-KpnI-digested pMP220 to create the appropriate fusion to '*lacZ*' to yield pOR1 and pOR2. These plasmids were sequenced to make sure that no mutations were

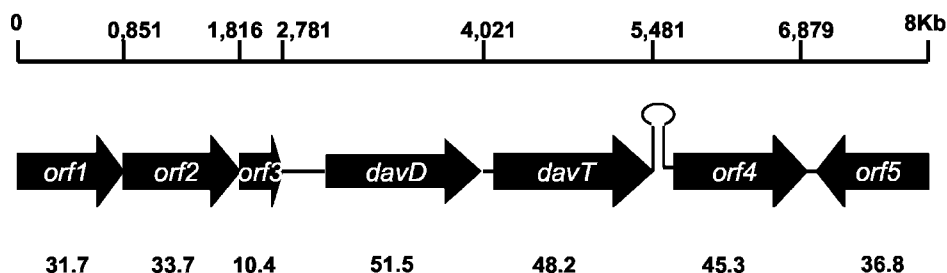


FIG. 2. Structure of an 8-kb fragment of the chromosome of *P. putida* KT2440 bearing the *davD* and *davT* genes. The *davDT* genes were sequenced as described in Materials and Methods, and our results agreed with the *P. putida* genome sequence available at the NCBI (33). Sequence analysis was carried out as described in Materials and Methods. The size of the product of each ORF is given below the corresponding gene in kilodaltons.

introduced in the amplified fragments. The plasmids were electroporated into *P. putida* KT2440, and the corresponding transformants were grown overnight on M9 minimal medium with 15 mM benzoate and tetracycline. Then cultures were diluted 100-fold in the same medium. After 1.5 h of incubation at 30°C with shaking, the cultures were supplemented or not with inducer compounds, and 4 h later β -galactosidase activity was assayed in permeabilized whole cells according to the method of Miller (28).

¹⁴C-labeled lysine uptake assays. *P. putida* KT2440 and its mutant derivatives were grown on M9 minimal medium with benzoate until they reached mid-logarithmic phase (0.7 to 0.8 turbidity units at 660 nm). Cells were harvested by centrifugation ($3,500 \times g$ for 15 min), washed with M9 medium and benzoate, and incubated with 0.5 μ M lysine (7.8 μ Ci of ¹⁴C per assay) for different periods of time. The cells were then filtered and washed with 2 volumes of medium, and the filter was transferred to a scintillation vial. Nonspecific lysine-binding assays were done as described above except that the cells were preincubated for 30 min at 4°C and, after the addition of [¹⁴C]lysine, were kept at 4°C.

RESULTS

The *davD-davT* genes form an operon that is expressed from the P_{davD} promoter. Espinosa-Urgel and Ramos (12) reported that the *davD* and *davT* genes were closely linked in the chromosome of *P. putida* KT2440 and oriented in the same transcriptional direction (Fig. 2), but it was not determined whether the genes formed an operon. Previous work indicated that *davT* encodes δ -aminovalerate aminotransferase, and the sequence similarity led us to postulate that the preceding gene, *davD*, encodes the next enzyme in the lysine catabolic route, glutaric acid semialdehyde dehydrogenase (12). No further analysis of the chromosomal region was done in the original study by Espinosa-Urgel and Ramos (12). We have now identified three open reading frames (ORFs) upstream of *davD* (*orf1*, *orf2*, and *orf3*) whose translated products correspond to PP0209, PP0210, and PP0211, respectively (33), and one gene (*orf4* PP0215) downstream from *davT* (Fig. 2). All of the genes are predicted to be transcribed in the same direction. Further downstream from *orf4* there is another ORF that is transcribed divergently with respect to *orf4* (Fig. 2).

orf1 is 852 bp long and its translated gene product exhibits a high degree of similarity (~75%) to a number of ATP-binding components of ABC transporter systems, such as the nitrate ABC transporter system of *Methanococcus janaschii* and *Synechocystis* sp., as well as the sulfonate transporter system of *Methanosarcina acetivorans* (6, 14, 20). The stop codon of *orf1* overlaps with the start codon of *orf2*, which is 966 bp long and whose translated protein exhibits in some stretches a low degree of similarity to fibroproteins. However, the function of this protein is unknown. The putative *orf3* presumably encodes

a hypothetical protein of unknown function. At 487 bp downstream from the stop codon of *orf3* we identified the putative first ATG of *davD* gene. The intergenic space between *davD* and the downstream *davT* gene is 293 bp, whereas the distance between the stop codon of *davT* and the start codon of *orf4* is only 138 bp. The translated product of *orf4* exhibits similarity to DNA-binding regulators belonging to the two-component response regulator family.

We decided to test whether all six genes with the same orientation could be transcribed as a single mRNA. To test the potential operon structure of these genes, we grew *P. putida* KT2440 cells in M9 minimal medium with benzoate as the carbon source and with 5 mM lysine and isolated mRNA from cells in the late-log growth phase. Then we carried out RT-PCRs with primers based on the 3' end of each of the ORFs and the 5' end of the next ORFs. We found cDNA products of the expected sizes with the primers corresponding to *orf1* and *orf2* and *davD* and *davT* (not shown) but not with primers corresponding to *orf3* and *davD* or *davT* and *orf4*. It should be mentioned that we have not been able to detect mRNA corresponding to *orf3* under any growth condition tested in the present study. We therefore considered that the six ORFs could be organized in three transcriptional units, with *davD* and *davT* forming a unit that seems to be transcribed independently from *orf4* and *orf1/orf2*.

The similarity of *orf4* to genes encoding response regulators led us to test whether the *orf4* gene product is involved in the regulation of the *davDT* operon. To this end, we generated a mutant in *orf4* as described in Materials and Methods. The mutant grew as fast as the wild type with lysine as the sole carbon and energy source, and the gene product was therefore considered not to be involved in the regulation of lysine metabolism.

***davD* promoter region.** Plasmids pOR1 (intergenic *davD-davT* region':':*lacZ*) and pOR2 (P_{davD} ':':*lacZ*) were electroporated in *P. putida* KT2440, and cells were grown as described in Materials and Methods in the absence or in the presence of 5 mM lysine. We found no β -galactosidase activity with pOR1 regardless of the growth conditions. In contrast, with pOR2 we detected basal expression in the absence of lysine, which increased when cells were grown in its presence (Fig. 3). This suggested that a functional promoter could only be located in front of the *davD* gene. These findings, along with the RT-PCR

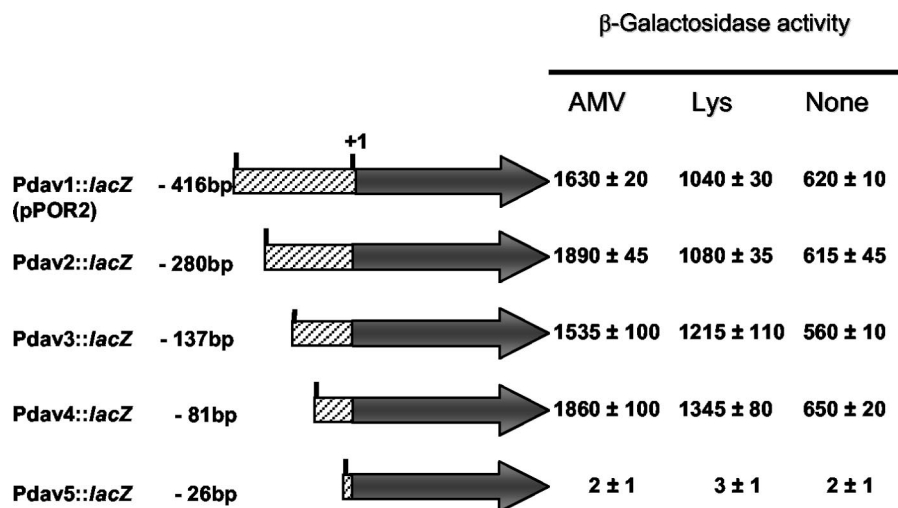


FIG. 3. Identification of the minimal region of the *davD* promoter that allowed regulated expression. Intergenic regions of different length between *davD* and *orf3* were amplified by PCR with the same primer to meet the 3' end (provided with a *Kpn* site) and different primers at the 5' end (provided with an *EcoRI* site). The PCR products, once digested with these restriction enzymes, yielded fragments of 416, 280, 137, 81, and 26 bp upstream from the +1 transcription start point. *P. putida* KT2440 cells bearing the indicated fusion were grown on M9 minimal medium with benzoate alone or with 5 mM lysine or 5 mM δ -aminovaleric acid. The β -galactosidase activity was determined as described in Materials and Methods, and values are the averages of three independent assays done in duplicate.

results, suggest that transcription of *davDT* genes is driven exclusively from a promoter upstream of *davD*.

To identify the transcription initiation point of the *davDT* operon and define the minimum region required for induction to take place, we carried out the following assays. First, we prepared mRNA from KT2440 grown with 5 mM lysine and used a primer located 50 bp within the *davD* gene for primer extension. Our results revealed a major transcription initiation point (Fig. 4) located 66 bp upstream with respect to the A of the first ATG in *davD*. Analysis of the sequence upstream revealed a -10 region (5'-TAGCAT-3') very similar to 5'-TATCAT-3', which is best recognized by the *P. putida* sigma-70 factor (10). The promoter also exhibited a relatively well-conserved -35 region (5'-TTGTCC-3') with 17 bases between the two boxes.

To determine the region upstream from the +1 position in *davD* necessary for the regulated expression of the operon, we amplified different portions of the region by PCR using in all cases the same primer at the 3' end and different primers at the 5' end, so that we generated PCR-amplified fragments of different sizes (Fig. 3). These fragments were cloned in pMP220 and β -galactosidase activity was measured. We found high β -galactosidase levels ($>1,500$ Miller units) when the primer allowed amplification of at least 81 bp upstream from the +1 point.

Sequence analysis of the region between positions -81 and $+1$ revealed, in addition to the $-10/-35$ boxes described above, a T-rich region between -48 and -62 that could function as a UP element or a curvature element, and an imperfect inverted repeat that extended from -76 to -58 and that could be the target for a regulator responsible for the induction of this promoter (Fig. 4).

Efficient lysine transport is required for full expression of the *davDT* operon. Mutant *rei2* carries an in-frame fusion of *davT* to the *luxABCDE* genes, and the cells show increased

light emission upon exposure to lysine. We mutagenized the *rei2* mutant with a mini-Tn5-Gm transposon as described in Materials and Methods and searched for mutants with reduced luminescence. These mutants were expected to result from the limited uptake of lysine or from inactivation of a putative regulator. Two mutants called *rei2-6* and *rei2-7* emitted a basal level of light and only a small increase in light emission was found in response to lysine (Table 1). Southern blotting assays revealed a single insertion of the mini-Tn5-Gm in each of the mutants, and we sequenced the insertion site. In *rei2-6* and *rei2-7* mutants, the mini-transposon had interrupted ORFs of 753 and 885 bp, respectively (corresponding to PP0283 and PP4486). BLAST analyses against different data banks were carried out with the translated sequence of each ORF. We found that in the *rei2-6* and *rei2-7* mutants the inactivated genes were related to amino acid uptake systems belonging to the ABC superfamily (17, 30, 39, 41). The gene inactivated in mutant *rei2-6* (PP0283) exhibited high similarity to the ATP-binding component of the arginine/ornithine transport system of *Pseudomonas aeruginosa* (94% similarity), the ATP-binding protein of the histidine ABC transporter of *P. syringae* pv. tomato strain DC3000 (92% similarity) and *E. coli* (79% similarity), and the ATP-binding component of the ornithine transport of *Salmonella enterica* serovar Typhimurium LT2 (79% identity). The insertion in mutant *rei2-7* had inactivated a periplasmic binding protein (PP4486) of a transport system for basic amino acids (16, 34). This periplasmic binding protein is a member of the LAO (lysine/arginine/ornithine) group (21). We hypothesized that the lower induction from P_{davD} in these mutants probably resulted from their limited lysine uptake. To test this hypothesis, we carried out assays with [14 C]lysine in cells grown on minimal medium with benzoate as a C source and NH_4^+ as an N source. We found that uptake of [14 C]lysine in the *rei2*, *rei2-6*, and *rei2-7* mutants was linear in time for about 3 min. [14 C]lysine was incorporated by *rei2* cells at a rate

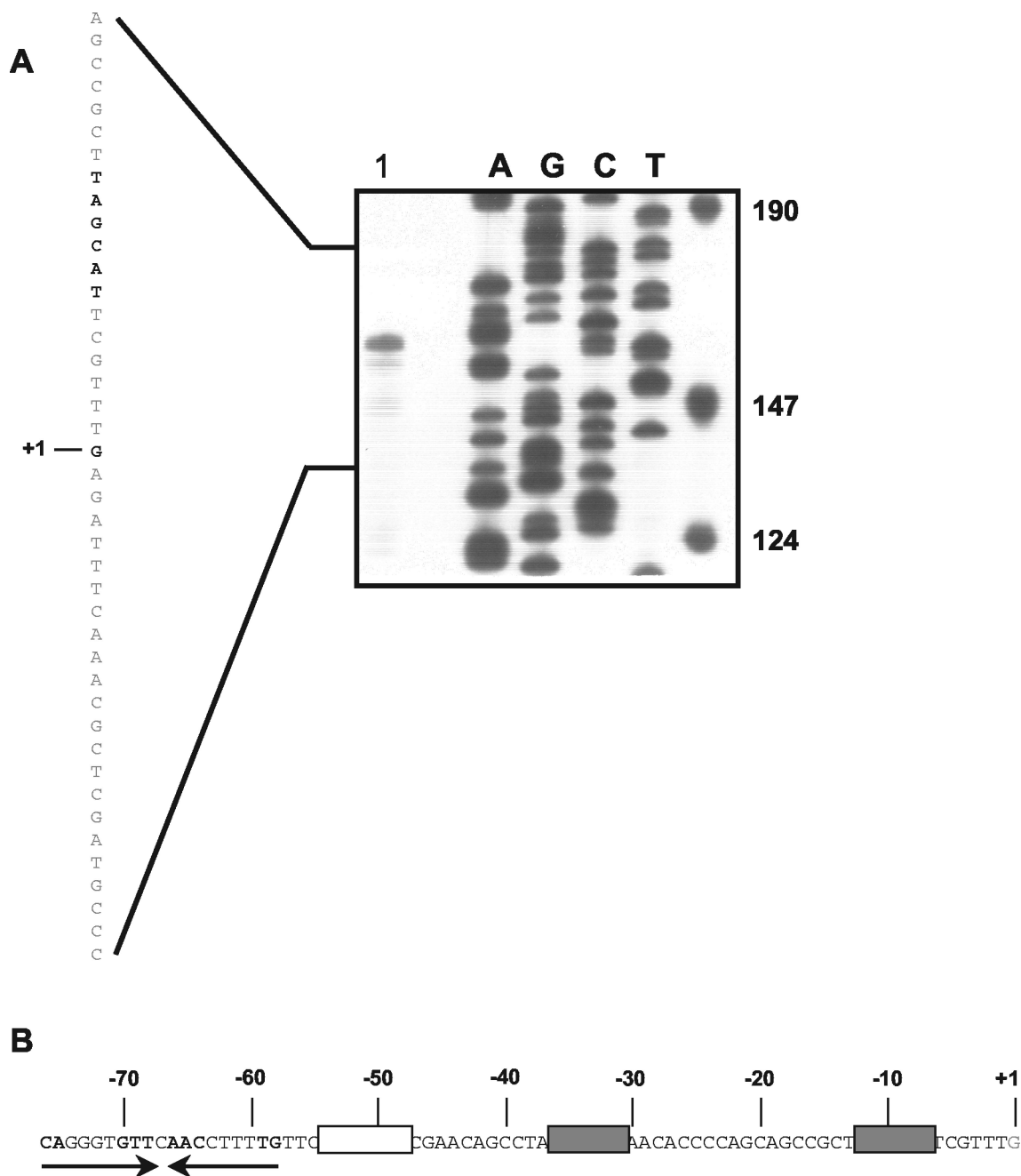


FIG. 4. Transcription initiation point of the *davD* promoter and key sequence features in the proximal 5' region. (A) Transcription initiation mapping of the *davD* gene. Lane 1, RNA isolated from cells grown on M9 minimal medium with 5 mM lysine. The other lanes correspond to a sequencing ladder. The DNA sequence corresponding to the *davD* promoter region is shown on the left. The transcription start point is in boldface and is marked +1. The proposed -10 box is in boldface. (B) Organization of the *davD* promoter region. The -10 and -35 regions are indicated in boldface and by gray boxes. A T-rich region is shown in an open box. Convergent arrows indicate a potential inverted repeat.

of 2.5 nmol/mg of protein per min, whereas in the mutants the rate of [¹⁴C]lysine incorporation was ca. 30% of that determined for the *rei2* strain.

δ-Aminovaleric acid enhances expression of the *davDT* operon. To identify the signal molecule(s) that induces expression from the *davD* promoter, we cultured *P. putida* KT2440 bearing pOR2 on M9 minimal medium with 5 mM lysine (the first substrate in the pathway), 5 mM δ-aminovaleric acid (the

product in which the two potential lysine degradation pathways converge [Fig. 1]), 2.5 mM cadaverine (a potential intermediate in one of the lysine branches that can yield δ-aminovaleric acid), and 5 mM glutaric acid (the product resulting from δ-aminovaleric acid metabolism). Our results revealed that, whereas glutaric acid was not an inducer (Table 2), the other three compounds induced expression from the *P_{davD}* promoter. Whether lysine and cadaverine are true inducers or whether

TABLE 1. Relative level of light emission by *rei2* and its mutant derivatives^a

| Strain | Relative light units \pm SD | | Increase (fold) |
|---------------|-------------------------------|---------------|-----------------|
| | -Lys | +Lys | |
| <i>rei2</i> | 5 \pm 0.1 | 20 \pm 0.2 | 4 |
| <i>rei2-6</i> | 5 \pm 0.1 | 6 \pm 0.1 | 1.2 |
| <i>rei2-7</i> | 5 \pm 0.1 | 7.5 \pm 0.1 | 1.5 |

^a Bacterial cells were grown on M9 minimal medium with citrate as the carbon source without (-) and with (+) 5 mM lysine (Lys) for 3 h at 30°C. Light emission was then determined in a luminometer as described in Materials and Methods.

δ -aminovaleric acid resulting from the metabolism of lysine and cadaverine is the true inducer could not be discerned from these results.

To further elucidate the nature of the inducer we repeated the above assays in *P. putida* KT2440 mut-1, a mutant unable to convert δ -aminovaleramide into δ -aminovaleric acid (O. Revelles, unpublished data). In this mutant, δ -aminovaleric acid induced expression from P_{davD} at a level similar to that found in the wild type (\sim 3-fold over the basal level), whereas induction by lysine was null, and induction with cadaverine was low (<2-fold). This suggests that δ -aminovaleric acid resulting from the metabolism of lysine or cadaverine may be the true inducer of the *davDT* operon (Table 2).

Köhler et al. (22) reported that an *rpoN* mutant that lacked σ^{54} was unable to grow on lysine. Although the *davDT* operon promoter presented σ^{70} -dependent characteristics, its expression could be influenced by σ^{54} if the operon were part of a regulatory cascade with a master regulator involving σ^{54} , as is the case in 3-methylbenzoate metabolism by the TOL plasmid of *P. putida* when cells are grown on *m*-xylene (37). To test this hypothesis, we transformed pOR2 in *P. putida* strain TK19, an *rpoN* mutant, and determined the β -galactosidase activity in the presence or in the absence of lysine cadaverine and δ -aminovaleric acid. We found an induction ratio of about 2.5, a level similar to the one obtained in the wild type. Therefore, σ^{54} is not directly or indirectly involved in expression of the *davD* gene, and the inability of the RpoN mutant to grow on lysine should therefore be ascribed to the need to express other catabolic segments of the lysine catabolic pathway.

DISCUSSION

Espinosa-Urgel and Ramos (12) showed before that mutagenesis of *P. putida* KT2440 with a mini-Tn5-*lux* transposon was useful for identifying genes whose expression increased in cells that colonize plant roots or are exposed to plant root

exudates. The inability of the *rei2* mutant to use lysine identified a gene, called *davT*, involved in the use of this amino acid as a C source.

DNA sequencing and the series of RT-PCR assays carried out in the present study have now shown that *davT* forms an operon with the *davD* gene. Our *in vivo* assays revealed light emission by cells of the *rei2* strain growing in the absence of lysine, which increased \sim 3-fold in response to the exogenous addition of this amino acid.

It has been proposed that in *P. putida*, the initial metabolism of lysine occurs through two branches that converge at δ -aminovaleric acid (Fig. 1). *P. putida* mut-1 is blocked at δ -aminovaleramide aminotransferase—a key step in one of the loops (Fig. 1)—and grows slowly on lysine (Revelles, unpublished). This suggests that the δ -aminovaleramide branch is the major lysine assimilation pathway in this strain. In agreement with this hypothesis is the finding that in the mut-1 strain, lysine did not induce the expression of the *davDT* operon promoter, and cadaverine only induced low levels of expression of the *davDT* genes. In contrast, δ -aminovaleric acid behaved as the strongest effector. We are therefore tempted to propose that δ -aminovaleric acid is the true inducer of the *davDT* operon.

The transcription start point of the *davD* promoter was identified, and deletion analysis of the promoter region revealed the need for at least 80 bp upstream from this point for expression from the *davD* promoter. Sequence analysis of the promoter region revealed good -10 and -35 boxes for σ^{70} promoters. Because expression from P_{davD} can be induced by exogenous lysine, we hypothesized the participation of an as-yet-unidentified regulator, which might possibly bind to an inverted repeat in the -58/-76 region. Although this motif is relatively far from the RNA polymerase binding site, the presence of a putative UP element in the -50/-60 region might facilitate contacts between RNA polymerase and the putative regulator. This type of organization is seen in some *E. coli* CRP regulated promoters (7, 26) and, to our knowledge, this is the first such case proposed for a pseudomonad.

Downstream of *davT* we identified *orf4*, whose translated product showed homology to regulators belonging to the two-component family. An *orf4::* Ω Km mutant exhibited a pattern of *davDT* expression similar to that of the wild type, which ruled out this regulator as responsible for the regulated expression of this operon. An alternative approach to search for a potential regulator was designed. We reasoned that the intensity of light emitted by the mutants in the putative regulator of the *davDT* operon would be diminished. After Tn5 mutagenesis of the *rei2* strain we searched for such mutants among 5,000 transconjugants; only two of them exhibited low

TABLE 2. Induction of the *davD* promoter in the wild-type *P. putida* KT2440 and its isogenic mutant KT2440 mut-1 and RpoN^a

| Strain | Activity (mean Miller units \pm SD) on substrate | | | | |
|--------|--|-----------------|-----------------|-----------------|--------------|
| | None | Lysine | Cadaverine | Aminovalerate | Glutarate |
| KT2440 | 620 \pm 10 | 1,280 \pm 100 | 930 \pm 60 | 1,730 \pm 20 | 600 \pm 10 |
| mut-1 | 470 \pm 10 | 530 \pm 30 | 840 \pm 10 | 1,700 \pm 120 | 580 \pm 20 |
| RpoN | 620 \pm 40 | 1,450 \pm 30 | 1,370 \pm 100 | 1,800 \pm 50 | 550 \pm 10 |

^a Wild-type, mut-1, and RpoN strains bearing pOR2 were grown in M9 minimal medium with benzoate as the C source. Where indicated, cultures were supplemented with 5 mM lysine, 2.5 cadaverine, 5 mM δ -aminovalerate, and 5 μ M glutarate. The β -galactosidase activity was determined 7 h later in cells growing exponentially. The activity is given in Miller units, and values are averages of three to six independent determinations.

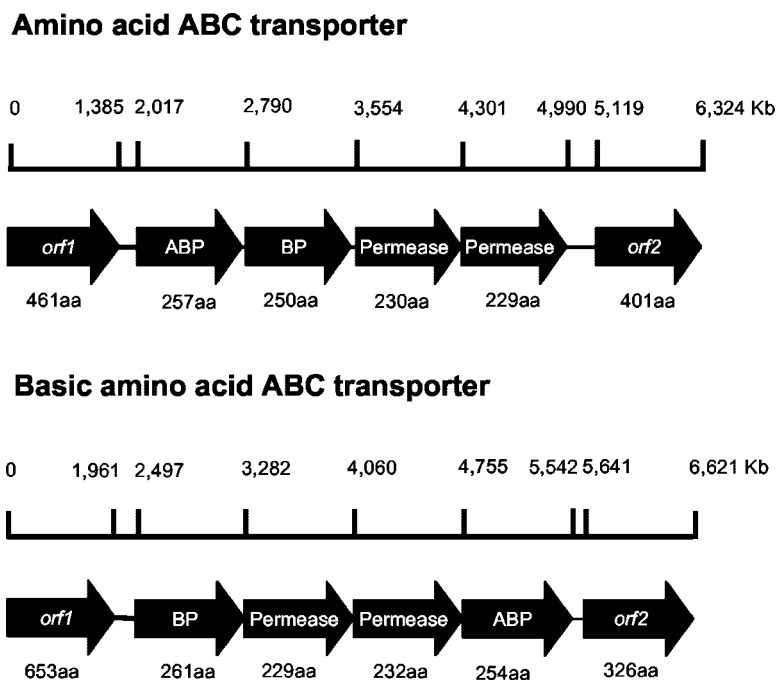


FIG. 5. Genetic organization of the two ABC transporter systems for L-lysine uptake in *P. putida* KT2440. (Top) Organization of the L-lysine transport system inactivated in mutant rei2-6; (bottom) organization of the L-lysine transport system inactivated in rei2-7. Genome organization is shown according to the findings of Nelson et al. (33). The number of amino acids of each translated product is given below the gene. BP stands for periplasmic binding protein, and ABC stands for ATP-binding protein. The function of the products encoded by adjacent ORFs in both loci is unknown.

induction levels. Analysis of the knockout genes suggested that the mutants might exhibit defects in lysine uptake. The results with mutants rei2-6 and rei2-7 showed that efficient induction of the *davDT* operon by exogenous lysine requires effective uptake systems.

Lysine in *P. putida* KT2440 seems to be taken up through at least two ABC transporters that appear to consist of four proteins. Figure 5 shows the genomic organization of the two systems. In mutant rei2-6 the gene that encodes the ATP-binding protein was followed by the gene that encodes the solute-binding protein and two integral membrane permeases. In mutant rei2-7 the genes encoding the two permeases lay between the genes that encode the solute-binding protein and the ATP-binding protein. Inactivation of either of the two uptake systems resulted in a significant decrease in the rate of lysine uptake and resulted in reduced induction from the *davD* promoter.

Köhler et al. (22) reported that an RpoN mutant of *P. putida* did not grow with lysine. In the RpoN mutant strain both lysine and δ -aminovaleric acid induced expression from P_{davD} , and therefore the inability to grow could not be ascribed to the prevention of expression of the branches that converge in δ -aminovaleric acid or of the *davDT* operon itself. Instead, the RpoN mutant seems to be unable to metabolize glutaric acid (Revelles, unpublished). It follows that lysine catabolism in *P. putida* involves the assemblage of several pathway stretches that are independently regulated: (i) the two loops that convert lysine into δ -aminovaleric acid, (ii) the *davD* operon for metabolism of δ -aminovaleric acid to glutaric acid, and (iii) a segment that converts glutaric acid into Krebs cycle interme-

diates. This modular organization endows the strain with high metabolic versatility to respond to different carbon or nitrogen sources supplied exogenously or generated internally during cell metabolism.

ACKNOWLEDGMENTS

This study was supported by EU project QLRT-02884 and national grant BIO2003-00515 from the CICYT.

We thank K. Shashok and C. Lorente for checking the English in the manuscript.

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