8

TOWARDS A GENOME-WIDE MUTANT LIBRARY OF *PSEUDOMONAS PUTIDA* STRAIN KT2440

Estrella Duque, Antonio J. Molina-Henares, Jesús de la Torre, María A. Molina-Henares, Teresa del Castillo, Jennifer Lam, and Juan L. Ramos*

Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, C/ Prof. Albareda, 1, E-18008 Granada, Spain
*Corresponding author: jlramos@eez.csic.es

Key Words: Mutant bank, mini-transposons, functional genomics, auxotrophs, gene regulation, DNA sequencing

1. INTRODUCTION

Microbiology is experiencing exciting times thanks to the current explosion of knowledge. About 25 years after Watson and Crick resolved the structure of DNA,[104] Sanger's and Maxam's laboratories[56,86] developed easy ways to determine the nucleotide sequence of a segment of DNA. This in turn led to the development of new technologies that now make it possible not only to decipher the complete genome sequence of an organism, but also to analyze the global patterns of expression of genes based on genomic microarrays or the results of proteomic assays. Nonetheless, although transcriptional arrays and proteomic techniques can identify large numbers of genes expressed under particular conditions, the biological meaning of these correlations is generally unclear without further analysis.

Juan-Luis Ramos and Alain Filloux (eds.), Pseudomonas, 227–251.

The availability of the *Pseudomonas putida* KT2440 genome sequence has made transcriptomic[11,21,22,39,81,82,93,107] of this microorganism possible. These approaches focus on the monitoring of RNA, protein, and metabolite levels. The creation of large libraries of mutants defined by site-directed or random mutagenesis meant another step forward in our functional understanding of the *P. putida* genome. Such mutant libraries can be used to study each mutant's phenotype under defined conditions.[42]

With the use of transposons for mutagenesis, a link can be established between the insertion site and the phenotype, which can help to elucidate the biochemistry and the physiology of processes under study.[19,27,33,40,47,48,81,84,90] However, the generation of mutations that inactivate candidate genes is often time-consuming and commonly limits the number of genes that can be examined. Moreover, all of us who have worked in the laboratory have expressed countless times how much we would like to have a particular mutant to proceed with the analysis of our favorite organism. Indeed, this has been the case for us and for others working in our laboratory, and was one of the reasons that lead us to construct a large mini-Tn*5* transposon mutant library of *P. putida* KT2440, a saprophytic bacterium used as a model system to study biodegradation and interactions of a nonsymbiotic microorganism with plants. We determined the site of insertion of the mini-transposon by sequencing the DNA adjacent to the transposon border. The *P. putida* KT2440 mutant collection includes multiple insertions in a significant fraction of nonessential genes of the bacterium, and can be used systematically to examine, with other approaches, the phenotypes of inactivated candidate genes that have been associated with a biological process of interest. Alternatively, the mutant collection in itself can be used to screen for specific properties, that is, mutants unable to use a carbon or nitrogen source, identification of auxotrophs, identification of mutants tolerant or sensitive to a particular stress, and basically whatever phenotypic marker one might be interested in.

P. putida KT2440 is a plasmid-free derivative of a 3-methylbenzoate-degrading bacterium isolated from garden soils in Japan, which was originally designated *Pseudomonas arvilla* strain mt-2[68] and subsequently reclassified as *P. putida* mt-2.[65,80,105] This strain grows rapidly in minimal salt medium as well as in complex media such as Luria–Bertani (LB). Prof. Kenneth N. Timmis defined this strain as a nutritional opportunist *par excellence* and a paradigm of metabolically versatile microorganisms that recycle organic wastes in aerobic and microaerophilic compartments of the environment.[96] *P. putida* KT2440 is probably the best-characterized saprophytic laboratory pseudomonad. This strain was the first Gram-negative soil bacterium to be certified by the Recombinant DNA Advisory Committee of the United States Institute of Health as the host strain of a host-vector biosafety system for gene cloning in Gram-negative soil bacteria.[30] This, in turn, has facilitated work with recombinant derivatives in the

laboratory and in field assays – work which revealed that the strain retains its ability to survive and function in the environment when reintroduced in soils and waters.[5,25,57,58,60,61,62,69,73,74,76,77,80,83,85,103]

An extensive spectrum of versatile genetic tools developed in the last three decades has been particularly important for these analyses.[7,19,20,40,59,85,106] Indeed, the early genetic characterization of *P. putida* KT2440 and analyses of the pattern of gene expression of a good number of genes were based on the development of wide-host-range vectors for gene cloning, and promoter probe vectors for gene expression analysis.[6,59,75] Later, mini-transposons were developed for (i) mutagenic analysis, (ii) introduction of useful markers for environmental monitoring, and (iii) analysis of the heterologous expression of cloned genes from other soil organisms.[7,19,20,40,85]

Sequencing the KT2440 genome, analysis of mutants and BLAST comparisons of the genomes of KT2440 with those of other *Pseudomonas*, for example, *Pseudomonas aeruginosa* strain PAO1,[94] *Pseudomonas fluorescens* Pf5,[71] several *Pseudomonas syringae* strains,[32] and *Pseudomonas entomophila*,[102] revealed that the *Pseudomonas* sp. core genome consists of about 2,100 genes. These analyses also provided significant new insights into the biology of KT2440 and the underlying genomic basis of its biosafety features, which have further increased the usefulness of this model laboratory organism and its potential biotechnological applications.

2. BASIC CHARACTERISTICS OF THE GENOME OF *P. PUTIDA* KT2440

The global features of the genome of KT2440 were described in the original report by Nelson *et al.*[66] and its subsequent reanalysis in this series of books by Martin dos Santos *et al.*[53,55] In this chapter, we review some of the most relevant characteristics that are useful to consider for the construction of a collection of mutants.

The genome of strain KT2440 consists of a single circular chromosomes of 6,181,863 basepairs (bp), and has a mean G + C value of 61.6%. The initial annotation suggested 5,420 open reading frames (ORFs) ranging in size from 90 bp (the arbitrary size cut-off the authors applied to the algorithm to find genes) to almost 30,000 bp (see below). This set of genes specifies the proteome of KT2440. In addition, there are seven ribosomal RNA operons,[66,78] one of which occurs as a tandem (171,000–182,000) with a spacer of just a few hundred bp. Also present are 74 tRNA genes and two structural RNAs. The intergenic sequences were estimated to comprise 12.5% of the genome.[66]

Nelson *et al.*[66] proposed that *P. putida* strain KT2440 has independently evolved its own repertoire of transcription factors, indicating that an

important factor in the adaptation of an organism to a new environment is the emergence of a distinct set of these proteins. This is similar to what Madan Babu *et al*.[52] proposed for *Escherichia coli*. These authors observed that organisms with similar lifestyles have similar regulatory networks and have incorporated orthologous genes with similar patterns of network interconnection. At the global level, analysis of the dataset revealed that conservation of transcription factors is independent of the number of their target genes, and depends on the lifestyle of the organism rather than phylogenetic relatedness.

One characteristic we reported in the intergenic space of KT2440 is that the genome contains more than 800 copies of a species-specific 35-bp Repetitive Extragenic Palindromic (REP) element.[3] The REP sequence consists of a central palindromic motif and conserved nucleotides that define the head and the tail of the REP sequence. The consensus sequence is 5′-ccggcctcTTC*GCGGG*t*aa*a*CCCGC*tcctacaggg-3′ (small letters: 50–89% conserved residue; capital letter: 90–100% conserved residue). In contrast to *P. aeruginosa* and *E. coli*, in which the REP elements are typically organized in complex "bacterial interspersed mosaic elements," most REP elements in *P. putida* occur as single units or pairs: 225 REP sequences occur singly, 372 are located in tandem arrangements on opposite strands, and clusters of three, four, and five REP sequences are found in 36, 12 and 1 case, respectively. The role of these repeats in *P. putida* is still unknown, but they do not seem to be involved in gene regulation. Recently, Ramos-González *et al*.[79] reported that the REP sequence is the target of the so-called ISPpu*10* insertion sequence.

As noted above, based on BLAST comparisons of the genomes from five representative species of *Pseudomonas*, Vodovar *et al*.[102] identified a set of almost 2,100 genes that constitute the core genome of *Pseudomonas*. This indicates that the number of noncore genes of KT2440 is larger than the number of core genes. In this context it should be noted that the genome of KT2440 contains more than 100 regions of atypical oligonucleotide composition, including a number of gene islands.[66] Nine of these islands are larger than 20 kb, and apart from mobilization functions, they also encode functions such as amino acid and opine uptake and metabolism, arsenate resistance, resistance to heavy metals (e.g., copper and cadmium), oxidative stress response (peroxidase), biosynthesis of secondary metabolites, and a set of proteins that exhibit typical motifs of enzymes of a restriction-modification system.[35] These noncore functions are considered to contribute to the fitness and versatility of *P. putida* in its natural habitat. Therefore, the analysis of mutant libraries of a given pseudomonad can provide clues on their lifestyle in certain ecological niches and reveal specific properties of each microbe. In addition, analysis

of core mutants can reveal information of general interest for the genus *Pseudomonas*.

Table 1 lists the assignment of the identified coding sequences in the TIGR annotation of metabolic categories and the number of ORFs in each group that have been knocked out (July, 2006).

Among the 5,420 genes predicted according to Table 1 to code for proteins, around 2,143 have no functional annotation and were annotated as genes encoding "hypothetical proteins" (600 ORFs), which indicated that in most cases, no significant similarity to any other gene could be found. Other genes have been annotated as conserved hypothetical proteins (1,039 ORFs) and proteins of unknown function (504 ORFs); as of this writing no clues are available on the function of the proteins in these two groups.

Of the remaining proteins, some were annotated erroneously since the annotation was based mainly on BLAST analysis without functional tests. Some of these annotations have been corrected on the basis of the isolation of mutants and/or enzyme characterization (Table 2). For instance, PP3591 was originally annotated as a potential malate synthase; subsequent genetic and biochemical analyses revealed that it was in fact the second enzyme in the catabolism of D-lysine in the so-called AMA pathway.[64,82] The current version of TIGR (July, 2006) incorporates the

Table 1. Metabolic categories of annotated genes in *P. putida* KT2440.

Metabolic function	Number of genes	Number of knockout genes
Amino acid biosynthesis	126	36
Biosynthesis of cofactors, prosthetic groups, and carriers	149	27
Fatty acid and phospholipid metabolism	112	30
Central intermediary metabolism	79	15
Energy metabolism	459	124
Purines, pyrimidines, nucleosides, and nucleotides	65	6
DNA metabolism	118	29
Transcription	66	19
Protein synthesis	132	14
Protein fate	180	47
Cellular processes	361	97
Regulatory functions	535	143
Signal transduction	140	50
Transport and binding proteins	656	190
Cell envelope	327	74
Related to mobile elements	183	31
Conserved hypothetical proteins, hypothetical proteins, and proteins of unknown function	2,143	321

Table 2. Re-annotation of some open reading frames in *P. putida* based on functional analyses.

PP number	Gene symbol	Current annotation	Previous annotation	Reference
0166	*lapC*	Membrane fusion protein, ABC transporter. Secretion of LapA	HlyD family secretion protein	[29]
0167	*lapB*	ATPase, ABC transporter. Secretion of LapA	Secretion ATP-binding protein	[29]
0168	*lapA*	Large adhesion, surface associated. Biofilm formation	Surface adhesion protein	[29]
0213	*davD*	Glutaric semialdehyde dehydrogenase	Succinate-semialdehyde dehydrogenase	[82]
0214	*davT*	δ-Aminovalerate aminotransferase	4-Aminobutyrate aminotransferase	[82]
0382	*davA*	δ-Aminovaleramide aminohydrolase	Carbon-nitrogen hydrolase family protein	[82]
0383	*davB*	Lysine monooxygenase	Tryptophan 2-monooxygenase, putative	[82]
0691	*proB*	Glutamate-β-semialdehyde dehydrogenase	Glutamate 5-kinase	[78]
0806	*lapF*	Adhesion protein. Involved in seed colonization	Surface adhesion protein, putative	[29]
1002	*lysP*	Lysine permease	Arginine/ornithine antiporter	Revelles *et al.*, unpublished
1206	*acrD*	Arginine/ornithine, lysine/ ornithine antiporter	Porin D	Revelles *et al.*, unpublished
1449	*hlpA*	Hemolysin-like protein. Seed colonization and iron acquisition	Surface colonization protein, putative	Molina-Henares *et al.*, unpublished
1450	*hlpB*	HplB transporter/activator protein	Activation/secretion protein, TPS family, putative	Molina-Henares *et al.*, unpublished
1530	*dapD*	Tetrahydrodipicolinate succinylase	2,3,4,5-tetrahydropyridine-2-carboxylate *N*-succinyltransferase, putative	Molina-Henares *et al.*, unpublished
1588	*dapC*	*N*-succinyl diaminopimelate aminotransferase	Aminotransferase, class I	Molina-Henares *et al.*, unpublished
3590	*amaC*	D-Lysine 6-aminotransferase	Amino transferase	Revelles *et al.*, unpublished

Table 2. Re-annotation of some open reading frames in *P. putida* based on functional analyses—cont'd.

PP number	Gene symbol	Current annotation	Previous annotation	Reference
3591	*dkpA*	Piperidine-2-carboxylate dehydrogenase	Malate dehydrogenase, putative	64, 82
3596	*amaD*	D-Lysine dehydrogenase	D-Amino acid dehydrogenase	Revelles *et al.*, unpublished
4140	*cadA*	Lysine decarboxylase	Decarboxylase, Orn /Lys/Arg family	[82]
4473	*lysC*	Aspartate kinase	Aspartate kinase, monofunctional class	Molina-Henares *et al.*, unpublished
4486	*ltpA*	Basic amino acid ABC transporter, periplasmic protein	Basic amino acid ABC transporter, periplasmic basic amino acid-binding protein	Duque *et al.*, unpublished
4519	*lapE*	Outer membrane protein, ABC transporter. Secretion of LapA	Agglutination protein	29
4615	*ddcA*	Membrane protein involved in seed colonization	Conserved hypothetical protein	29
4695	*cbrA*	Two-component system, sensory box histidine kinase; amino acid utilization regulator	Sensory box histidine kinase	Vílchez, S., and Ramos, J. L., unpublished
4696	*cbrB*	Two-component system, response regulator	Nitrogen regulation protein NR(1)	Vílchez, S., and Ramos, J. L., unpublished
5257	*amaB*	L-Pipecolate oxidase	Oxidoreductase, FAD-binding	Revelles *et al.*, unpublished
5258	*amaA*	Δ^1-Piperidine-6-carboxylate dehydrogenase	Aldehyde dehydrogenase family protein	Revelles *et al.*, unpublished

corrected information. However, we feel that in contrast with the *P. aeruginosa* community, no real community efforts have been devoted to updating the annotation of *P. putida* KT2440. We wish to call our colleagues' attention to this issue since continuous updating of the annotations will make the mutant collection more useful to researchers and will help disseminate better information about KT2440.

3. CONSTRUCTION OF THE TRANSPOSON MUTANT COLLECTION

A mini-Tn*5* derivative carrying a Km^R gene was used to generate the mutant library (Figure 1).[3,12] The mini-Tn*5* for mutagenesis was chosen because it was previously shown to integrate at relatively random positions in the chromosome of KT2440.[92]

Transposon insertions were generated in *P. putida* by triparental mating of the strain with *E. coli* donor CC118λ*pir* bearing pUT-Km and *E. coli* HB101 bearing the helper plasmid pRK600, which carries conjugation-proficient functions.[19,40] The mutagenized cultures were plated on large bioassay-scale M9 minimal medium agar plates containing kanamycin (to select for transposon insertions), rifampicin (the *P. putida* strain used is a Rif^R strain), and benzoate or citrate as the carbon source (to select against donor cells). We found that the best way to obtain randomness in the insertion was by using different cultures of KT2440 and by carrying out separate mutagenesis assays for short periods of time, that is, 6 h. In the early steps of construction of the library, when we began sequencing we found a higher rate of siblings than expected because the conditions included a single mutagenesis mating and a long (24 h) incubation period.

In addition to these steps to ensure random selection of mutants, at certain stages clones were used to screen directly for a number of phenotypes. Since mini-Tn*5* transconjugants were selected on M9 minimal medium with citrate, a search was set up to identify clones deficient in the use of different carbon sources, for example, glucose, L-lysine, vanillate, quinate, etc. In this series of assays we kept specific clones deficient in the use of a given carbon source. In another series of assays we set up a specific program to identify the genes necessary for the biosynthesis of several amino acids, or genes necessary to use inorganic nitrogen sources (M. A. Molina-Henares, unpublished data).

We performed several statistical tests to ensure that the distribution of transposon insertions was random and that the mini-Tn*5* transposons had no hot spots in the *P. putida* genome. An important parameter that attests to the randomness of transposon insertions is the number of genes that carry a transposon insertion. A low number of genes hit by the transposon indicates bias in the pattern of transposition. To determine the theoretical number of genes that should be hit by at least one transposon, we used the neutral-base-pair model.[45] This model makes it possible to estimate the number of gene hits based on genome length, the number of transposon insertions, and the gene sizes. After applying this model to the library of 2,200 *P. putida* transposon mutants sequenced so far, we predicted that 2,000 genes would be mutated. The actual number of genes that were hit by at least one transposon was 1,680 (84% of the predicted

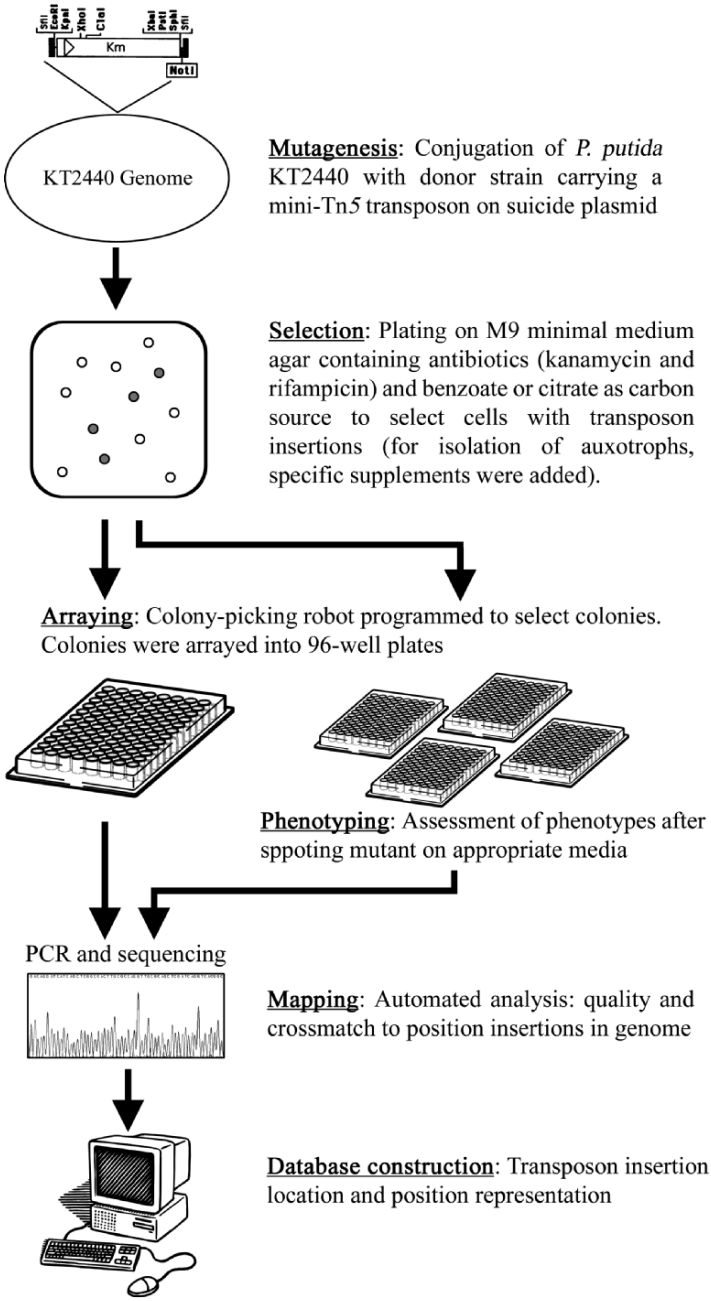

Figure 1. Generation analysis and maintenance of the *P. putida* KT2440 mutant library. (Details are given in the text.)

number in *P. putida*), which was consistent with expectations based on the neutral-base-pair model.

We also performed a genome-wide analysis of all transposon insertion sites in relation to G + C (A + T) content. Using a 100-bp window centered on the transposon insertion position, we calculated the mean G + C content. The differences between the mean G + C and A + T contents in all windows and the mean G + C and A + T contents of the whole genome were 0.4% (G + C) and 0.3% (A + T).

Once randomness had been ensured, we determined the best way to screen the mutant collection. We found that to avoid cross-contamination, 96-well plates were best, and in addition offered a format that allowed for efficient storage of all strains. The mutant collection consists of plates (96 strains per plate) that are stored frozen at –80°C. In our experience *P. putida* mutant strains stored in glycerol at –80°C are stable for several years, although certain clones lose viability rapidly. This is the case for mutants in a set of outer membrane proteins such as *oprL*, *tolB*, and *tolC*.[47,48] To avoid loss of viability, we set up a program for the long-term storage of specific mutant strains through lyophilization.[63] To prevent accidental losses, several copies of the collection are kept in different locations.

The methods used for sequence mapping and storage of mutants were originally developed for sequencing genomes in which no long reads are necessary. To identify the insertion site we have found it useful to be able to read the mini-Tn*5* border sequence because it unequivocally guarantees that the rest of the sequence is adjacent to it. DNA fragments which included transposon insertion junctions were amplified and sequenced using a semi-degenerate PCR scheme (Figure 1). The procedure consists of two PCRs. In the first, a series of mixed random primers are used together with a primer based on the sequence of the mini-transposon. After the first amplification, a second amplification is carried out with an internal primer based on the mini-Tn*5* sequence. This guarantees that DNA amplifications are based on previously amplified DNA.

An automatic search program written in HPP and available upon request was used to crossmatch the junction sequences against the *P. putida* genome, and then to determine the position of the mini-Tn*5* insertion relative to annotated ORFs. Data from the collection can be accessed at www.eez.csic.es/mutants.

3.1. Composition of the Mutant Collection

The collection is made up of more than 25,000 independent clones, and as of this writing we have identified the transposon insertion site in nearly 2,200 strains (see Table 1). Since the kanamycin resistant marker functions very well in counter selection, 100% of the Km^R

clones had an insertion at a location in the *P. putida* genome. Approximately 90% of the insertions were within ORFs, corresponding well to the fraction of the genome predicted to have coding functions.[15] Some of these insertions are in the same genes, but we kept only those that were located at different sites within the ORF. The ORFs that we hit most often were PP0168 and PP0806, which are the two longest ORFs in the genome and are the target of 9 and 11 independent insertions, respectively. The ORF encoding PP5076 (large subunit of glutamate synthase) was selected five times in our searches for specific types of mutants deficient in the use of inorganic nitrogen sources (nitrate or ammonia), or unable to use proline as a nitrogen source. Curiously, genes encoding peptidases, that is, PP0098, PP0435, and PP5320, were hit three times. Mutants in *catB*, *vanA* were found four times due to selective pressure. Only about 60 ORFs were the target of the mini-Tn*5* in two cases. In all, based on the limited number of clones analyzed to date, we can report that in almost 95% of the cases the mini-Tn*5* is located in a different gene. This is in accordance with the randomness of insertions by mini-Tn*5*.

3.2. Mutant Distribution

The assembly of the *P. putida* mutant collection, limited as it is for the time being, provides a source of mutants for researchers worldwide. Researchers can identify genes of interest in *P. putida* using bioinformatic searches, microarray or proteomic data. Once a list of interesting genes is generated, it can be compared with the mutant collection and the corresponding mutant strains can be ordered, saving time and resources that would otherwise be required to construct the mutants. To facilitate the distribution of mutant strains, we created a publicly accessible website at http://www.artemisa.eez.csic.es.

Researchers may search for strains by ORF number, gene name or gene abbreviation. If the requested mutant strain has been lyophilized we send an ampoule; if not we prepare mutants for distribution by streaking the appropriate strain onto an LB agar plate with kanamycin, and preparing a stab once the strain has grown. We recommend that immediately after receipt, each strain should be streaked out and a representative sample frozen and stored. We also strongly recommend that the identity of all strains be confirmed prior to use. Several tests can be run to confirm the nature of the mutation. One is to use individual colonies, PCR and appropriate primers based on the intact gene corresponding to the insertion, to verify that the target gene has been inactivated. Depending on the PCR conditions, either no fragment or a fragment

corresponding to a very large product will result from a correctly assigned mutant strain. To corroborate the exact position of a transposon insertion, the easiest approach is to carry out a PCR with a transposon-specific primer (5′-CGACCTGCAGGCATGCAAGCTTCGGC-3′) and a primer based on the sequence of the target gene. Sequencing of the PCR product identifies the precise insertion site.

4. METABOLIC FUNCTIONS

4.1. Brief Analysis of Transposon Mutants in Basic Carbon and Nitrogen Utilization Sources

The availability of the whole genomic sequence of *P. putida* KT2440 makes it possible to combine bioinformatics and experimental data on the metabolism of different nutrients[46,51,66,80,82,89,90,105] and allows us to reconstruct catabolic and anabolic pathways. Velázquez *et al*.[97] reconstructed the metabolic pathways available for the use of glucose, fructose, and gluconate in *P. putida* KT2440. Every protein involved in each of the steps was assigned a PP number as specified in the TIGR database (www.tigr.org). Figure 2 summarizes the steps in these pathways. It should be mentioned that the network of transformations that results from projecting genomic data is consistent with all observations made in various laboratories since the early 1970s on glucose, fructose, and gluconate metabolism in this bacterium.[46,89,98,99] Since *P. putida* KT2440 lacks fructose-6-phosphate kinase, glucose, and gluconate appear to be metabolized exclusively by the Entner–Doudoroff pathway,[28] while fructose is channeled through the Embden–Meyerhof (EM) route. An analysis of glucose metabolism in KT2440 provided a novel set of data (del Castillo *et al*., unpublished results) indicating that glucokinase and glucose dehydrogenase function simultaneously in the assimilation of glucose in strain KT2440, which contrasts with findings in other pseudomonads. The prediction that mutants in *edd* (PP1010 or phosphogluconate dehydratase) and in *eda* (PP1024 or keto-deoxy-phosphogluconate aldolase) would fail to grow on glucose was confirmed by del Castillo *et al*. (unpublished results), since these mutants appeared in screening assays intended to find mutants unable to use glucose (our unpublished results). Mutants in each of the steps confirm the proposed pathway based on bioinformatics analyses.

Regarding the use of inorganic nitrogen sources, mutants unable to use nitrate, nitrite, and ammonium were isolated as well as mutants that cannot derive nitrogen from certain sources of organic nitrogen (amino acids such as glycine, leucine, glutamate, phenylalanine, lysine, and proline). Mutants in assimilatory nitrate reductase (PP1703) and nitrite reductase

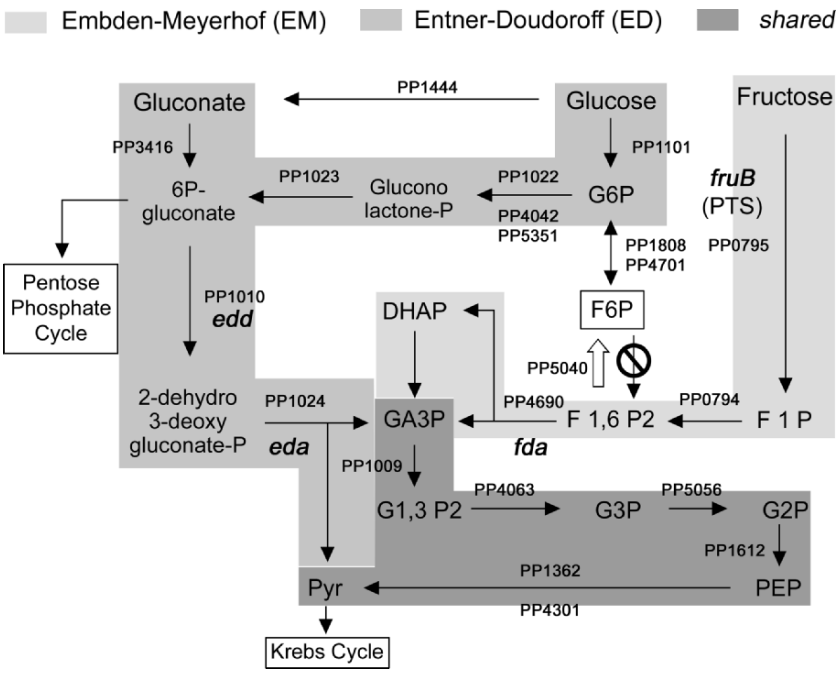

Figure 2. The metabolic network for glucose, gluconate, and fructose metabolism in *P. putida* KT2440. The Figure is based on the bioinformatics and experimental work of Velázquez *et al*. (2004), reproduced in part with permission from the authors and from the American Society for Microbiology, and on the work by del Castillo *et al*. (in preparation).

(PP1705) are available. We also identified clones unable to use NH_4^+ as a nitrogen source. A number of these mutants exhibited a knockout in one of the subunits of glutamate synthase,[9] which suggests that the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway is preferred for the assimilation of ammonium in spite of the fact that the strain encodes a glutamate dehydrogenase. An oddity of KT2440 is that it exhibits three GSs, and although mutants in each of them are available, each mutant grew well on inorganic and organic nitrogen sources, indicating that the three copies of GS can replace each other in functional terms.

Very few auxotrophs have been described for *P. putida* in the literature,[36,72,87] so we set up a specific program to isolate auxotrophs for Trp, Leu, and Ser. These mutants were selected to confirm the biosynthetic pathways for these essential metabolites (see Figure 3 for leucine and threonine biosynthesis). Alaminos and Ramos[1] showed that like other eukaryotic and prokaryotic microorganisms, *P. putida* KT2440 uses homoserine as the starting point for the biosynthesis of methionine. This first step

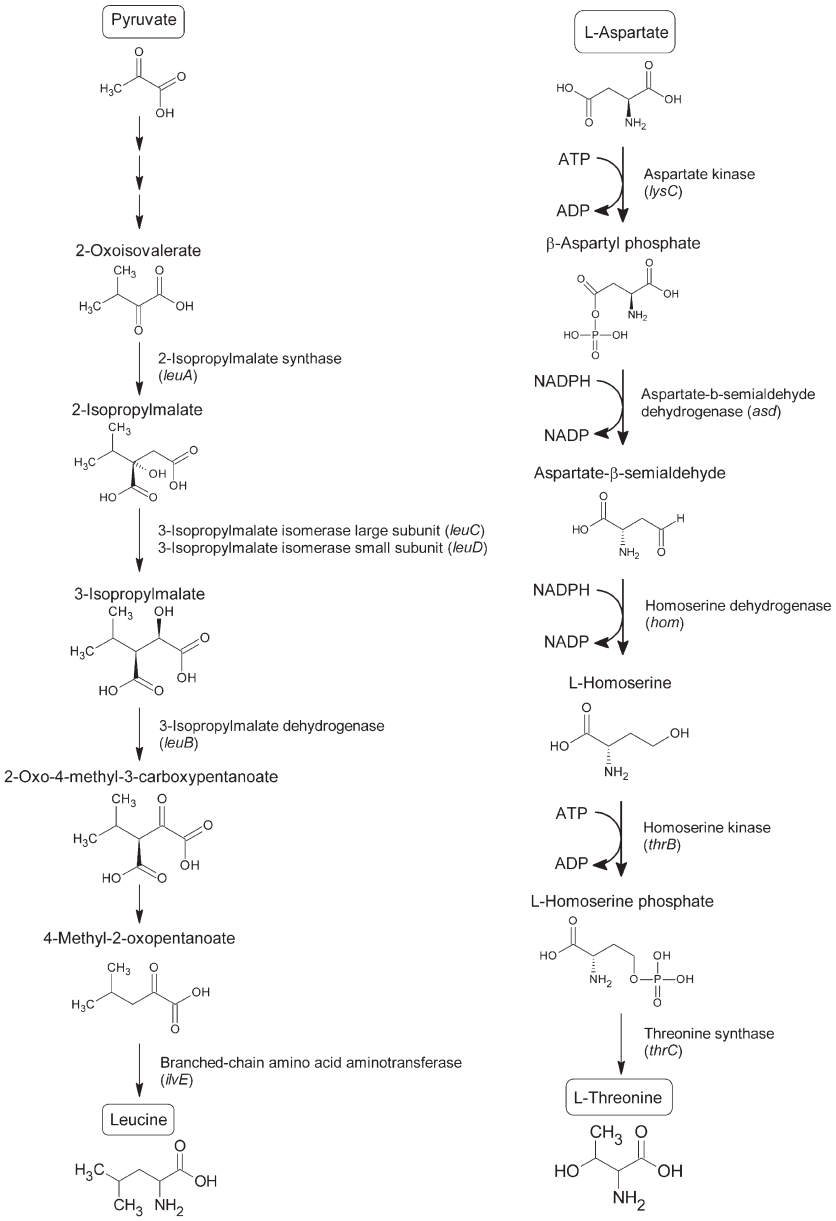

Figure 3. Biosynthesis of threonine and leucine in *P. putida*.

consisted on the acylation of homoserine to yield *O*-acetyl-homoserine in a reaction catalyzed by the *metW*, *metX* gene products, as is also the case in *P. syringae*.[2] The second step, as in Gram-positive bacteria and certain fungi, consisted on the direct sulfhydrylation of *O*-acetyl-homoserine into homocysteine, a reaction catalyzed by MetZ. This reaction also occurs in *P. aeruginosa*.[31] Eventually, homocysteine was converted into methionine in a reaction catalyzed by one of the two methionine synthase enzymes.

The use of rich LB medium facilitated the isolation of mutants in which metabolic steps related with the biosynthesis of an extensive range of amino acids and cofactors were affected, for example, amino acid auxotrophs or mutants deficient in the biosynthesis of biotin, folic acid, ubiquinone, pantothenate, coenzyme A, and thiamine.

A general strategy exploited by pseudomonads to degrade diverse aromatic compounds is to oxidize them to catechols, which can be funneled into a limited number of central pathways.[17,38] In KT2440, for example, Jiménez *et al*.[43] proposed that the initial steps in ferulate, *p*-coumarate, and *p*-hydroxybenzoate metabolism are mediated by different enzymes (upper pathways), but all routes ultimately converge via protocatechuate (ferulate and *p*-coumarate) or catechol (benzoate) on the 3-oxoadipate/β-ketoadipate pathway. Interestingly, this pathway is found almost exclusively in soil- and plant-associated microorganisms,[37,70,77,100] and presumably evolved in response to the large number of phenolic compounds synthesized by plants. Other relevant central pathways are the phenylacetate and homogentisate pathways. Some mutants unable to use these chemicals have been isolated, although despite several attempts, mutants unable to use quinate were not obtained, probably because several different pathways are available for the initial metabolism of this compound.

4.2. Nutrient Uptake Systems

P. putida KT2440 has very broad transport capabilities, with approximately 370 cytoplasmic membrane transport systems – 15% more than *P. aeruginosa* – that constitute about 12% of the whole genome. The largest family corresponds to the ATP-Binding Cassette (ABC) transporter (94 paralog members), a significant proportion of which is predicted to be devoted to amino acid uptake (Duque *et al*., unpublished). This is consistent with the ability of KT2440 to colonize plant roots, since root exudates are rich in amino acids, and reflects the physiological emphasis on the metabolism of amino acids and their derivatives to successfully compete in the rhizosphere.[44,49,50] In addition, *in vivo* expression technology (IVET) studies confirmed the induction of this type of transporter in *P. putida* when colonizing plant roots.[79] In the case of amino acids, multiple uptake systems can exist for a single compound. For

instance, we have reported that at least two complex uptake systems in KT2440 are involved in the uptake of L-lysine, and recently a third uptake system was identified as part of a cluster in which D-lysine dehydrogenase is present.[82]

P. putida KT2440 encodes various uptake systems for osmoprotectants such as glycine betaine (PP0871-PP0868) and proline (PP0294-PP0296 and PP2774-PP2775). Mutants in these systems are available and can shed light on the process. It has been proposed that up to five transporters are available for gamma-aminobutyric acid (GABA) uptake, namely PP4106, PP2911, PP4756, PP2543, and PP0284; however, no functional proof is available. We have found that one of these proteins, PP4106, is involved in the uptake of tyrosine, since PP4106 was isolated specifically as a mutant that failed to use this amino acid.

Consistent with its exceptional metabolic versatility, the KT2440 genome encodes more putative transporters for aromatic substrates than any currently sequenced microbial genome, including multiple homologs of the *Acinetobacter calcoaceticus* benzoate transporter BenK (PP3165), and of the *P. putida* 4-hydroxybenzoate transporter PcaK (PP1376).[67] In addition, in the KT2440 mutant collection we have identified porins such as PP3168 specifically involved in benzoate uptake. The genome of KT2440 also encodes the determinants for the import of some sugars; so far we have identified mutants in the fructose uptake system (PP0792-PP0793) as well as in gluconate uptake (PP3417).[95]

Martínez-Bueno *et al*.[54] reported that *P. putida* KT2440 exhibits up to 13 iron-siderophore uptake systems in spite the fact that the strain produces only one siderophore, pyoverdine, whose genes are clustered in three groups (PP4243-PP4246, PP4319-PP4327, and PP4219-PP4223) organized in a manner similar to that found in other pseudomonads.[14,34,101] Although the siderophore receptor for pyoverdine in each strain is highly specific for the siderophore the strain produces, fluorescent pseudomonads have been shown to use siderophores produced by other strains, uptake being mediated by other ferric-siderophore receptors. In the current collection, 11 mutants in different uptake systems are available, and their importance in niche colonization and biocontrol can now be tested.

Strain KT2440 has a large number of determinants for active efflux systems for metals such as arsenite (PP1929, PP2717), copper (PP5378-PP5379 and PP2204-PP2205), cadmium (PP0041-PP0045, PP2408-PP2411, PP5139), chromate (PP2556) and other toxic chemicals such as cyanate (PP0970, PP3751). Other efflux systems identified to date extrude paraquat (two paralogous sets: PP2576-PP2577 and PP0598-PP0599) and, interestingly, fusaric acid (PP1266-PP1263), a common fungal toxin produced by phytopathogens such as *Fusarium oxysporum*.[91] Mutants in the efflux systems that extrude arsenate and cadmium are available.

4.3. Regulation and Signal Transduction

As in other soil-dwellers, the genome of *P. putida* is large, and this is thought to reflect the fact that soil is relatively nutrient-poor, such that nutrients are varied and patchily distributed. To survive in soils, microorganisms need to use a variety of scarce nutrients, and therefore need several sets of metabolic genes and gene regulators.

Almost 10% of the genes in the KT2440 genome encode products involved in signal transduction and gene regulation, which reflects the evolutionary emphasis in this bacterium on monitoring and responding to a large number of environmental signals. Common transcription factors include the sigma factors RpoD (sigma-70), RpoN (sigma-54), RpoS (sigma-38), RpoH (sigma-32), FliA (sigma-27) and AlgT (sigma-22, homologous to RpoE in *E. coli*). The genome also contains a large number (19) of genes for extracytoplasmic function (ECF) sigma-70 factors, many of which are clustered with sensing or transport genes[54] (see Chapters 11 and 12 of Volume II in this series). The function of these ECFs is unknown. Site-directed mutagenesis mutants have been obtained for ECF-PP12, but only in *P. putida* DOT-T1E, a solvent-tolerant strain. A reduced number of genes making up less than 1% of the genome have been shown to be under the direct or indirect influence of this ECF.[26] Very few phenotypes have been associated to this mutant, the clearest example of it being increased solvent sensitivity.

4.4. Swarming and Chemotaxis

P. putida has previously been shown to attach as single cells to solid matrices, and can form biofilms on various surfaces. Intriguingly, proteomic analysis revealed that proteins belonging to the *P. putida* motility complex, including the flagellins, the basal body proteins and the chemotactic proteins, are expressed at higher levels in biofilm-grown cells than in planktonic cells.[4,13,88] Additionally, in liquid culture an aflagellate mutant was unable to form a pellicle at the air–water interface, and insertional inactivation of flagellar filament genes delayed pellicle formation, indicating that the motility complex is important for cell–cell interactions in mature biofilms.[10,41]

Bacteria respond to chemical stimuli by moving towards attractants or retreating from repellents. This phenomenon, called chemotaxis, enables bacteria to position themselves in their environments. The signals (stimuli) are perceived by membrane-spanning methyl-accepting chemotaxis proteins (MCP), which monitor environmental composition and transduce the signal via a number of receptor and transducer proteins to the flagellar apparatus, and thus influence the mode of rotation and the swimming

direction of the cell. *P. putida* KT2440 encodes 27 MCPs, suggesting that it can respond to a large variety of environmental signals. Adaptation is mediated by the level of methylation of MCP, which is controlled by methyltransferase CheR and methylesterase CheB. The activity of transducer CheY is regulated by the phosphatase CheZ.

It has been proposed that an MCP is involved in inorganic phosphate sensing (PP0562), that three MCPs (PP0320, PP1371, PP2249) are involved in aerotaxis, and that PP2257, PP2111, and PP4521 are able to sense amino acids. Mutants in PP1371 and PP4521 are available, and their role in energy sensing and movement towards oxygen can be tested. It is interesting to note that the uncharacterized chemotaxis cluster (PP1494-PP1488) is shared with *P. aeruginosa and P. syringae*, and a mutant is currently available for analysis.

Recently, Matilla *et al*. (unpublished) have shown that *P. putida* can swarm on solid plates. The process is dependent on lipopolysaccharides (LPS) and the presence of type IV pili, which are involved in the efficient colonization of the plant root.[18,23,24] The availability of the mutant collection has been of great importance in analyses of swarming in KT2440 (Matilla *et al*., unpublished results).

4.5. Adherence to Surfaces

The genome of *P. putida* encodes two unusually large proteins, the 6,310-amino acid PP0806 and the 8,628-amino acid PP0168, the second largest bacterial protein described so far. On the basis of work by Espinosa-Urgel *et al*.,[29] who found that transposon insertion mutants of both proteins fail to adhere to the surface of plant seeds, both proteins, as well as another large protein (PP1449, 1,508 amino acids), have been annotated as surface adhesion proteins. All three proteins exhibit a complex repetitive structure and atypical amino acid composition, and are threonine-rich and free of cysteine.[16,53] These clusters have often been knocked out through mini-Tn*5* insertions due to their large size rather than through sequence composition, since insertions occurred in different sites within the long ORFs. The availability of mutants in ORFs adjacent to these genes has been instrumental to elucidate their role in adhesion to surfaces.

Protein PP0168 contains three types of peptide sequence repeats: nine highly conserved N-terminal repeats of 100 amino acids, 29 highly conserved repeats consisting of 219 amino acids that form two subgroups (1–7, 8–29), and five hemolysin-type calcium-binding repeats at the C-terminus. Moreover, a C-terminal domain shows similarity to a von Willebrand domain involved in adhesion and signal transduction.[8] The second largest protein, PP0806, consists of about 65 repeats with an

average size of 85 amino acids. The first 35 repeats (with the exception of repeats 1 and 2) form seven clusters of five repeats. Towards the C-terminal end, the repeats exhibit an increasing degree of sequence variation. In-depth characterization at the molecular level of these proteins will help us to understand how *P. putida* KT2440 interacts with surfaces playing a role in the social behavior of this microorganism in different habitats.

ACKNOWLEDGMENTS

This work was supported by grants BIO2003-0515 and BIO2006-05668 from the CICYT. We thank Fernando Rojo and Maria-Isabel Ramos-González for reading the manuscript and K. Shashok for improving the use of English in the manuscript.

REFERENCES

1. Alaminos, M., and Ramos, J.L., 2001, The methionine biosynthetic pathway from homoserine in *Pseudomonas putida* involves the *metW*, *metX*, *metZ*, *metH* and *metE* gene products. *Arch. Microbiol*., 176:151–154.
2. Andersen, G.L., Beattie, G.A., and Lindow, S.E., 1998, Molecular characterization and sequence of a methionine biosynthetic locus from *Pseudomonas syringae. J. Bacteriol*., 180:4497–4507.
3. Aranda-Olmedo, I., Tobes, R., Manzanera, M., Ramos, J.L., and Marques, S., 2002, Species-specific repetitive palindromic (REP) sequences in *Pseudomonas putida. Nucleic Acids Res*., 30:1826–1833.
4. Auerbach, I.D., Sorensen, C., Hansma, H.G., and Holden, P.A., 2000, Physical morphology and surface properties of unsaturated *Pseudomonas putida* biofilms. *J. Bacteriol*., 182:3809–3815.
5. Bagdasarian, M., Lurz, R., Rueckert, B., Franklin, F.C.H., Bagdasarian, M.M., Frey, J., and Timmis, K.N., 1981, Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas. Gene*, 16:237–247.
6. Bagdasarian, M., and Timmis, K.N., 1982, Host: vector systems for gene cloning in *Pseudomonas. Curr. Top Microbiol. Immunol*., 96:47–67.
7. Bailey, J., and Manoil, C., 2002, Genome-wide internal tagging of bacterial exported proteins. *Nature Biotechnol*., 20:839–841.
8. Bork, P., and Rohde, K., 1991, More von Willebrand factor type A domains? Sequence similarities with malaria thrombospondin-related anonymous protein, dihydropyridine-sensitive calcium channel and inter-alpha-trypsin inhibitor. *Biochem. J*., 279:908–910.
9. Caballero, A., and Ramos, J.L., 2006, A double mutant of *Pseudomonas putida* JLR11 deficient in the synthesis of the nitroreductase PnrA and assimilatory nitrite reductase NasB is impaired from growth on TNT. *Environ. Microbiol*., 8:1305–1310.
10. Caiazza, N.C., and O'Toole, G.A., 2004, SadB is required for the transition from reversible to irreversible attachment during biofilms formation by *Pseudomonas aeruginosa* PA14. *J. Bacteriol*., 186:4476–4485.
11. Cánovas, D., Cases, I., and de Lorenzo, V., 2003, Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. *Environ. Microbiol*., 5:1242–1256.

12. Cao, H., Baldini, R.L., and Rahme, L.G., 2001, Common mechanisms for pathogens of plants and animals. *Annu. Rev. Phytopathol*., 39:259–84.
13. Chang, W.-S., and Halverson, L.J., 2003, Reduced water availability influences the dynamics, development, and ultrastructural properties of *Pseudomonas putida* biofilms. *J. Bacteriol*., 185:6199–6204.
14. Cornelis, P., and Matthijs, S., 2002, Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. *Environ. Microbiol*., 4:787–798.
15. Cowles, C.E., Nichols, N.N., and Harwood, C.S., 2000, BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida. J. Bacteriol*., 182:6339–6346.
16. Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penades, J.R., 2001, Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J. Bacteriol*., 183:2888–2896.
17. Dagley, S., 1971, Catabolism of aromatic compounds by micro-organisms. *Adv. Microb. Physiol*., 6:1–46.
18. de Groot, A., Heijnen, I., de Cock, H., Filloux, A., and Tommassen, J., 1994, Characterization of type IV pilus genes in plant growth-promoting *Pseudomonas putida* WCS358. *J. Bacteriol*., 176:642–650.
19. de Lorenzo, V., Herrero M., Jakubzik, U., and Timmis K.N., 1990, Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol*., 172:6568–6572.
20. de Lorenzo, V., and Timmis, K.N., 1994, Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived mini-transposons. *Meth. Enzymol*., 235:386–405.
21. Djordjevic, M.A., Chen, H.C., Natera, S., Van Noorden, G., Menzel, C., Taylor, S., Renard, C., Geiger, O., and Weiller, G.F., 2003, A global analysis of protein expression profiles in *Sinorhizobium meliloti*: discovery of new genes for nodule occupancy and stress adaptation. *Mol. Plant–Microbe Interact*., 16:508–524.
22. Domínguez-Cuevas, P., González-Pastor, J.E., Marqués, S., Ramos, J.L., and de Lorenzo, V., 2006, Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. *J. Biol. Chem*., 281:11981–11991.
23. Dorr, J., Hurek, T., and Reinhold-Hurek, B., 1998, Type IV pili are involved in plant-microbe and fungus-microbe interactions. *Mol. Microbiol*., 30:7–17.
24. Drake, S.L., Sandstedt, S.A., and Koomey, M., 1997, PilP, a pilus biogenesis lipoprotein in *Neisseria gonorrhoeae*, affects expression of PilQ as a high-molecular-mass multimer. *Mol. Microbiol.*, 23:657–668.
25. Duque, E., Ramos-González, M.I., Delgado, A., Contreras, A., Molin, S., and Ramos, J.L., 1992, Genetically engineered *Pseudomonas* strains for mineralization of aromatics: survival, performance, gene transfer, and biological containment, pp. 429–437. In Gaily, E., Silver, S., Withold, B. (eds.), *Pseudomonas-91. Molecular Biology and Biotechnology*, Chapter 46. American Society for Microbiology, Washington DC.
26. Duque, E., Rodríguez-Herva, J.J., de la Torre, J., Domínguez-Cuevas, P., Muñoz-Rojas, J., and Ramos, J.L., 2007. The RpoT regulon of *Pseudomonas putida* and its role in endurance against solvents. *J. Bacteriol*., 189:207–219.
27. Erb, R.W., Eichner, C.A., Wagner-Dobler, I., and Timmis, K.N., 1997, Bioprotection of microbial communities from toxic phenol mixtures by a genetically designed pseudomonad. *Nature Biotechnol*., 15:378–382.
28. Entner, N., and Doudoroff, M., 1952, Glucose and gluconic acid oxidation of *Pseudomonas saccharophila. J. Biol. Chem*., 196:853–862.

29. Espinosa-Urgel, M., Salido, A., and Ramos, J.L., 2000, Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *J. Bacteriol.*, 182:2363–2369.
30. Federal Register, 1982, Appendix E, Certified host-vector systems. 47:17197.
31. Foglino, M., Borne, F., Bally, M., Ball, G., and Patte, J.C., 1995, A direct sulfhydrylation pathway is used for methionine biosynthesis in *Pseudomonas aeruginosa. Microbiology*, 141:431–439.
32. Fouts, D.E., Abramovitch, R.B., Alfano, J.R., Baldo, A.M., Buell, C.R., Cartinhour, S., et al., 2002, Genome wide identification of *Pseudomonas syringae* pv. *tomato* DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc. Natl Acad. Sci. U.S.A.*, 99:2275–2280.
33. Galán, B., Díaz, E., and García, J.L., 2000, Enhancing desulphurization by engineering a flavin reductase-encoding gene cassette in recombinant biocatalysts. *Environ. Microbiol.*, 2:687–694.
34. Godoy, P., Ramos-Gonzalez, M.I., and Ramos, J.L., 2001, Involvement of the TonB system in tolerance to solvents and drugs in *Pseudomonas putida* DOT-T1E. *J. Bacteriol.*, 183:5285–5292.
35. Hallsworth, J.E., Heim S., and Timmis, K.N., 2003, Chaotropic solutes cause water stress in *Pseudomonas putida. Environ. Microbiol.*, 5:1270–1280.
36. Harder, W., and Quayle, J.R., 1971, Aspects of glycine and serine biosynthesis during growth of Pseudomonas AM1 on C compounds. *Biochem. J.*, 121:763–769.
37. Harwood, C.S., and Parales, R.E., 1996, The β-ketoadipate pathway and the biology of self-identity. *Annu. Rev. Microbiol.*, 50:553–590.
38. Harayama, S., and Timmis, K.N., 1989, Catabolism of aromatic hydrocarbons by *Pseudomonas*, pp. 151–174. In D.A. Hopwood and K.F. Chater (eds.), *Genetics of Bacterial Diversity*, Academic Press, London.
39. Heim, S., Ferrer, M., Heuer, H., Regenhardt, D., Nimtz, M., and Timmis, K.N., 2003, Proteome reference map of *Pseudomonas putida* strain KT2440 for genome expression profiling: distinct responses of KT2440 and *Pseudomonas aeruginosa* strain PAO1 to iron deprivation and a new form of superoxide dismutase. *Environ. Microbiol.*, 5:1257–1269.
40. Herrero, M., de Lorenzo V., and Timmis K.N., 1990, Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.*, 172:6557–6567.
41. Itoh, Y., Wang, X., Hinnebusch, B.J., Preston III, J.F., and Romeo, T., 2005, Depolymerization of β-1,6-*N*-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. *J. Bacteriol.*, 187:382–387.
42. Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenthner, D., Bovee, D., Olson, M.V., and Manoil, C., 2003, Comprehensive transposon mutant library of *Pseudomonas aeruginosa. Proc. Natl Acad. Sci. U.S.A.*, 100:14339–14344.
43. Jiménez, J.I., Miñambres, B., García, J.L., and Díaz, E., 2002, Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ. Microbiol.*, 4:824–841.
44. Kamilova, F., Validov, S., Azarova, T., Mulders, I., and Lugtenberg, B., 2005, Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. *Environ. Microbiol.*, 7:1809–1817.
45. Lehoux, D.E., Sanschagrin, F., and Levesque, R.C., 1999, Defined oligonucleotide tag pools and PCR screening in signature-tagged mutagenesis of essential genes from bacteria. *BioTechniques*, 26:473–480.
46. Lessie, T.G., and Phibbs, P.V. Jr., 1984, Alternative pathways of carbohydrate utilization in pseudomonads. *Annu. Rev. Microbiol.*, 38:359–388.

47. Llamas, M.A., Ramos, J.L., and Rodríguez-Herva, J.J., 2003, Transcriptional organization of the *Pseudomonas putida tol-oprL* Genes. *J. Bacteriol*., 185:184–195.
48. Llamas, M.A., Rodríguez-Herva, J.J., Hancock, R.E.W., Bitter, W., Tommasen, J., and Ramos, J.L., 2003, Role of *Pseudomonas putida tol-oprL* gene products in uptake of solutes through the cytoplasmic membrane. *J. Bacteriol*., 185:4707–4716.
49. Lugtenberg, B.J.J., and Dekkers, L.C., 1999, What makes *Pseudomonas* bacteria rhizosphere competent? *Environ. Microbiol*., 1:9–13.
50. Lugtenberg, B.J.J., Kravchenko, L.V., and Simon, M., 1999, Tomato seed and root exudates sugars: composition, utilization by *Pseudomonas* biocontrol strains and role in rhizosphere colonization. *Environ. Microbiol*., 1:439–446.
51. Ma, J.-F., Hager, P.W., Howell, M.L., Phibbs, P.V., and Hassett, D.J., 1998, Cloning and characterization of the *Pseudomonas aeruginosa zwf* gene encoding glucose-6-phosphate dehydrogenase, an enzyme important in resistance to methyl viologen (paraquat). *J. Bacteriol*., 180:1741–1749.
52. Madan Babu, M.M., Teichmann, S.A., and Aravind, L., 2006, Evolutionary dynamics of prokaryotic transcriptional regulatory networks. *J. Mol. Biol*., 358:614–633.
53. Martin dos Santos, V., Timmis, K., Tümmler, B., and Weinel, C., 2004, Genomic features of *Pseudomonas* Strain KT2440, pp. 77–112. In J.L. Ramos (ed.), *Pseudomonas*, Vol. 1. Kluwer Academic/Plenum Publishers, New York.
54. Martínez-Bueno, M.A., Tobes, R., Rey, M., and Ramos, J.L., 2002, Detection of multiple extracytoplasmic function (ECF) sigma factors in the genome of *Pseudomonas putida* KT2440 and their counterparts in *Pseudomonas aeruginosa* PA01. *Environ. Microbiol*., 4:842–855.
55. Martin dos Santos, V.A.P., Heim, S., Moore, E.R.B., Strätz, M., and Timmis, K.N., 2004, Insights into the genomic basis of niche specificity of *Pseudomonas putida* KT2440. *Environ. Microbiol*., 6:1264–1286.
56. Maxam, A.M., and Gilbert, W., 1977, A new method for sequencing DNA. *Proc. Natl Acad. Sci. U.S.A*., 74:560–564.
57. McClure, N.C., Fry, J.C., and Weightman, A.J., 1991, Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory-scale activated-sludge unit. *Appl. Environ. Microbiol*., 57:366–373.
58. McClure, N.C., Weightman, A.J., and Fry, J.C., 1989, Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated-sludge unit. *Appl. Environ. Microbiol*., 55:2627–2634.
59. Mermod, N., Lehrbach, P.R., Reineke, W., and Timmis, K.N., 1984, Transcription of the TOL plasmid toluate catabolic pathway operon of *Pseudomonas putida* is determined by a pair of co-ordinately and positively regulated overlapping promoters. *EMBO J*., 3:2461–2466.
60. Molina, L., Ramos, C., Ronchel, M.C., Molin, S., and Ramos, J.L., 1998, Construction of an efficient biologically contained *Pseudomonas putida* strain and its survival in outdoor assays. *Appl. Environ. Microbiol*., 64:2072–2078.
61. Molina, L., Ramos, C., Duque, E., Ronchel, M.C., García, J.M., Wyke, L., and Ramos, J.L., 2000, Survival of *Pseudomonas putida* KT2440 in soil and in the rhizosphere of plants under greenhouse and environmental conditions. *Soil Biol. Biochem*., 32:315–321.
62. Molina, L., Ramos, C., Ronchel, M.C., Molin, S., and Ramos, J.L., 2000, Construction of an efficient biologically contained *Pseudomonas putida* strain and its survival in outdoor assays. pp. 170–177. In: Biotechnology of soil: Monitoring, conservation and remediation, EC-COST, Brussels.
63. Muñoz-Rojas, J., Bernal, P., Duque, E., Godoy, P., Segura, A., and Ramos, J.L., 2006, Involvement of Cyclopropane fatty acids in the response of *Pseudomonas putida* KT2440 to freeze-drying. *Appl. Environ. Microbiol*., 72:472–477.

64. Muramatsu, H., Mihara, H., Kakutani, R., Yasuda, M., Ueda, M., Kurihara, T., and Esaci, N., 2005, The putative malate/lactate dehydrogenase from *Pseudomonas putida* is an NADPH-dependent Δ^1-piperideine-2-carboxylate/Δ^1-pyrroline-2-carboxylate reductase involved in the catabolism of D-lysine and D-Proline. *J. Biol. Chem*., 280:5329–5335.
65. Nakazawa, T., 2002, Travels of a *Pseudomonas*, from Japan around the world. *Environ. Microbiol*., 4:782–786.
66. Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martin 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 Lee, 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.A., Timmis, K.N., Dusterhoft, A., Tummler, B., and Fraser, C.M., 2002, Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol*., 4:799–808.
67. Nichols, N.N., and Harwood, C.S., 1997, PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida. J. Bacteriol*., 179:5056–5061.
68. Nozaki, M., Kagamiyama, H., and Hayaishi, O., 1963, Metapyrocatechase. I. Purification, crystallization and some properties. *Biochem Z*., 338:582–590.
69. Nüsslein, K., Maris, D., Timmis, K.N., and Dwyer, D.F., 1992, Expression and transfer of engineered catabolic pathways harbored by *Pseudomonas* spp. introduced into activated sludge microcosms. *Environ. Microbiol*., 58:3380–3386.
70. Olivera, E.R., Minambres, B., Garcia, B., Muniz, C., Moreno, M.A., Ferrández, A., Díaz, E., García, J.L., and Luengo, J.M., 1998, Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: the phenylacetyl-CoA catabolon. *Proc. Natl Acad. Sci. U.S.A*., 95:6419–6424.
71. Paulsen, I.T., Press, C.M., Ravel, J., Kobayashi, D.Y., Myers, G.S.A., Mavrodi, D.V., DeBoy, R.T., Seshadri, R., Ren, Q., Madupu, R., Dodson, R.J., Durkin, A.S., Brinkac, L.M., Daugherty, S.C., Sullivan, S.A., Rosovitz, M.J., Gwinn, M.L., Zhou, L., Schneider, D.J., Cartinhour, S.W., Nelson, W.C., Weidman, J., Watkins, K., Tran, K., Khouri, H., Pierson, E.A., Pierson III, L.S., Thomashow, L.S., and Loper, J.E., 2005, Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nature Biotechnol*., 23:873–878.
72. Phillips, A.T., 1986, Biosynthetic and catabolic features of amino acid metabolism in *Pseudomonas*. In J.R. Sokatch (ed.), *The Bacteria*, Vol. X. Academic Press, New York.
73. Pipke, R., Wagner-Döbler, I., Timmis, K.N., and Dwyer, D.F., 1992, Survival and function of a genetically engineered Pseudomonad in aquatic sediment microcosms. *Appl. Environ. Microbiol*., 58:1259–1265.
74. Ramos, J.L., Díaz, E., Dowling, D., de Lorenzo, V., Molin, S., O'Gara, F., Ramos, C., and Timmis, K.N., 1994, The behavior of bacteria designed for biodegradation. *Bio/Technol*., 12:1349–1356.
75. Ramos, J.L., Duque, E., Godoy, P., and Segura, A., 1998, Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. *J. Bacteriol*., 180:3323–3329.
76. Ramos, J.L., Duque, E., and Ramos-González, M.I., 1991, Survival in soils of an herbicide-resistant *Pseudomonas putida* bearing a recombinant TOL plasmid. *Appl. Environ. Microbiol*., 57:260–266.
77. Ramos, J.L., Wasserfallen, A., Rose, K., and Timmis, K.N., 1987, Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. *Science*, 235:593–596.
78. Ramos-Díaz, M.A., and Ramos, J.L., 1998, Combined physical and genetic map of the *Pseudomonas putida* KT2440 Chromosome. *J. Bacteriol*., 180: 6352–6363.

79. Ramos-González, M.I., Campos, M.J., Ramos, J.L., and Espinosa-Urgel, M., 2006, Characterization of the *Pseudomonas putida* mobile genetic element ISPpu*10*: an occupant of repetitive extragenic palindromic sequences. *J. Bacteriol*., 188:37–44.
80. Regenhardt, D., Heuer, H., Heim, S., Fernandez, U.D., Strömpl, C., Moore, E.R.B., and Timmis, K.N., 2002, Pedigree and taxonomic credentials of *Pseudomonas putida* strain KT2440. *Environ. Microbiol*., 4:912–915.
81. Reva, O.N., Weinel, C., Weinel, M., Böhm, K., Stjepandic, D., Hoheisel, J.D., and Tümmler, B., 2006, Functional genomics of stress response in *Pseudomonas putida* KT2440. *J. Bacteriol*., 188:4079–4092.
82. Revelles, O., Espinosa-Urgel, M., Fuhrer, T., Sauer, U., and Ramos, J.L., 2005, Multiple and interconnected pathways for L-Lysine catabolism in *Pseudomonas putida* KT2440. *J. Bacteriol*., 187:7500–7510.
83. Ronchel, M.C., Ramos, C., Jensen, L.B., Molin, S., and Ramos, J.L., 1995, Construction and behavior of biologically contained bacteria for environmental applications in bioremediation. *Appl. Environ. Microbiol*., 61:2990–2994.
84. Rodríguez-Herva, J.J., Ramos-González, M.I., and Ramos, J.L., 1996, The *Pseudomonas putida* peptidoglycan-associated outer membrane lipoprotein is involved in maintenance of the integrity of the cell envelope. *J. Bacteriol*., 178:1699–1706.
85. Sánchez-Romero, J.M., Diaz-Orejas, R., and de Lorenzo, V., 1998, Resistance to tellurite as a selection marker for genetic manipulations of *Pseudomonas* strains. *Appl. Environ. Microbiol*., 64:4040–4046.
86. Sanger, F., Nicklen, S., and Coulson, A.R., 1977, DNA Sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. U.S.A*., 74:5463–5467.
87. Santiago, M.F., and West, T.P., 2002, Control of pyrimidine formation in *Pseudomonas putida* ATCC 17536. *Can. J. Microbiol*., 48:1076–1081.
88. Sauer, K., and Camper, A.K., 2001, Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J. Bacteriol*., 183:6579–6589.
89. Sawyer, M.H., Baumann, L., Berman, S.M., Canovas, J.L., and Berman, R.H., 1977, Pathways of D-fructose catabolism in species of *Pseudomonas. Arch. Microbiol*., 112:49–55.
90. Schleissner, C., Reglero, A., and Luengo, J.M., 1997, Catabolism of D-glucose by *Pseudomonas putida* U occurs via extracellular transformation into D-gluconic acid and induction of a specific gluconate transport system. *Microbiology*, 143:1595–1603.
91. Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D., and Keel, C., 2000, Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J. Bacteriol*., 182:1215–1225.
92. Schweizer, H., and de Lorenzo, V., 2004, Molecular tools for genetic analysis of Pseudomonads, pp. 317–350. In J.L. Ramos (ed.), *Pseudomonas*, Vol. I. Kluwer Academic/ Plenum Publishers, New York.
93. Segura, A., Godoy, P., van Dillewijn, P., Hurtado, A., Arroyo, N., Santacruz, S., and Ramos, J.L., 2005, Proteomic analysis reveals the participation of energy- and stress-related proteins in the response of *Pseudomonas putida* DOT-T1E to toluene. *J. Bacteriol*., 187:5937–5945.
94. Stover, C.K., et al., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature*, 406:959–964.
95. Swanson, B.L., Hager, P., Phibbs, P. Jr., Ochsner, U., Vasil, M.L., and Hamood, A.N., 2000, Characterization of the 2-ketogluconate utilization operon in *Pseudomonas aeruginosa* PAO1. *Mol. Microbiol*., 37:561–573.

96. Timmis, K.N., 2002, *Pseudomonas putida*: a cosmopolitan opportunist par excellence. *Environ. Microbiol*., 4:779–781.
97. Velázquez, F., di Bartolo, I., and de Lorenzo, V., 2004, Genetic evidence that catabolites of the Entner-Doudoroff pathway signal C source repression of the sigma-54 Pu promoter of *Pseudomonas putida. J. Bacteriol*., 186:8267–8275.
98. Vicente, M., and Canovas, J.L., 1973, Glucolysis in *Pseudomonas putida*: physiological role of alternative routes from the analysis of defective mutants. *J. Bacteriol*., 116:908–914.
99. Vicente, M., and Canovas, J.L., 1973, Regulation of the glucolytic enzymes in *Pseudomonas putida. Arch. Mikrobiol*., 93:53–64.
100. Venturi, V., Zennaro, F., Degrassi, G., Okeke, B.C., and Bruschi, C.V., 1998, Genetics of ferulic acid bioconversion to protocatechuic acid in plant-growth-promoting *Pseudomonas putida* WCS358. *Microbiology*, 144:965–73.
101. Visca, P., Leoni L., Wilson, M.J., and Lamont, I.L., 2002, Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas. Mol. Microbiol*., 45:1177–90.
102. Vodovar, N., Vallenet, D., Cruveiller, S., Rouy, Z., Barbe, V., Acosta, C., Cattolico, L., Jubin, C., Lajus, A., Segurens, B., Vacherie, B., Wincker, P., Weissenbach, J., Lemaitre, B., Medigue, C., and Boccard, F., 2006, Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila. Nature Biotechnol*., 24:673–679.
103. Wagner-Döbler, I., Pipke, R., Timmis, K.N., and Dwyer, D.F., 1992, Evaluation of aquatic sediment microcosms and their use in assessing possible effects of introduced microorganisms on ecosystem parameters. *Appl. Environ. Microbiol*., 58:1249–1258.
104. Watson, J.D., and Crick, F.H.C., 1953, Genetical implications of the structure of deoxyribonucleic acid. *Nature*, 171:964–967.
105. Weinel, C., Nelson, K.E., and Tummler, B., 2002, Global features of the *Pseudomonas putida* KT2440 genome sequence. *Environ. Microbiol*., 4:809–818.
106. Wubbolts, M.G., and Timmis, K.N., 1990, Biotransformation of substituted benzoates to the corresponding *cis*-diols by an engineered strain of *Pseudomonas oleovorans* producing the TOL plasmid-specified enzyme toluate-1,2-dioxygenase. *Appl. Environ. Microbiol*., 56:569–71.
107. Yuste, L., Hervás, A.B., Canosa, I., Tobes, R., Jiménez, J.I., Nogales, J., Pérez-Pérez, M.M., Santero, E., Díaz, E., Ramos, J.-L., de Lorenzo, V., and Rojo, F., 2006, Growth phase-dependent expression of the *Pseudomonas putida* KT2440 transcriptional machinery analysed with a genome-wide DNA microarray. *Environ. Microbiol*., 8:165–177.