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Molecular Characterization of Chloranilic Acid Degradation in *Pseudomonas putida* TQ07

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Pentachlorophenol is the most toxic and recalcitrant chlorophenol because both aspects are directly proportional to the halogenation degree. Biological and abiotic pentachlorophenol degradation generates *p*-chloranil, which in neutral to lightly alkaline environmental conditions is hydrolyzed to chloranilic acid that present a violet-reddish coloration in aqueous solution. Several genes of the degradation pathway, *cadR-cadCDX*, as well as other uncharacterized genes (ORF5 and 6), were isolated from a chloranilic acid degrading bacterium, *Pseudomonas putida* strain TQ07. The disruption by random mutagenesis of the *cadR* and *cadC genes* in TQ07 resulted in a growth deficiency in the presence of chloranilic acid, indicating that these genes are essential for TQ07 growth with chloranilic acid as the sole carbon source. Complementation assays demonstrated that a transposon insertion in mutant CAD82 (*cadC*) had a polar effect on other genes contained in cosmid pLG3562. These results suggest that at least one of these genes, *cadD* and *cadX,* also takes part in chloranilic acid degradation. Based on molecular modeling and function prediction, we strongly suggest that CadC is a pyrone dicarboxylic acid hydrolase and CadD is an aldolase enzyme like dihydrodipicolinate synthase. The results of this study allowed us to propose a novel pathway that offers hypotheses on chloranilic acid degradation (an abiotic by-product of pentachlorophenol) by means of a very clear phenotype that is narrowly related to the capability of *Pseudomonas putida* strain TQ07 to degrade this benzoquinone.

Keywords: pentachlorophenol, chloranilic acid, biodegradation, *Pseudomonas putida,* pyrone dicarboxylic acid hydrolase, LysR regulator

Highly halogenated aromatic compounds, tetra- and penta-halogenated phenols, are currently used for a wide variety of purposes because of their biocide properties. Their toxicity and recalcitrance is directly proportional to their halogenation degree (Zhao *et al.,* 1995), for this reason, Pentachlorophenol (PCP) is the most important chlorophenol in both aspects. Since 1992 in the USA and 2000 in the European Union, PCP production was prohibited. However, it is still manufactured in countries like China and Mexico. PCP is used even nowadays, in several countries, as the best synthetic wood preservative. In fact, the PCP production volume in 2009 was 7,257 tons, which were commercialized in the United States, Canada, China and Mexico (Zheng *et al.,* 2011). PCP toxicological and environmental impact has been assessed before (Ahlborg *et al.,* 1980; Jensen, 1996; Proudfoot, 2003), PCP is toxic to all life forms because it disables oxidative phosphorylation (Yang *et al.,* 2006). In addition, PCP is a weak mutagen capable of forming DNA adducts; therefore, it is considered a potential carcinogen and teratogen (Dai *et al.,* 2005).

The PCP aerobic degradation pathway in some bacteria contains five catalytic enzymes responsible for its mineralization. The first enzyme of this pathway is PCP-4-monooxygenase, which catalyzes the oxygenolytic elimination of the first PCP chlorine, in order to generate tetrachloro-*p*-benzoquinone (*p*-chloranil) (Chen and Yang, 2008). *p*-chloranil is also the main PCP by-product generated by enzymatic *in vitro* oxidation (Mileski *et al.,* 1988; Longoria *et al.,* 2009), fungal metabolism (Ruckdeschel and Renner, 1986) and photolysis (Czaplicka, 2006).

In neutral to lightly alkaline environmental conditions, *p*-chloranil is hydrolyzed to chloranilic acid (CA), that presenting a violet-reddish coloration in aqueous solution (Sarr *et al.,* 1995). CA has been identified too as a by-product in kraft pulp mill bleachery effluents, which is produced in similar concentrations as chloroguaiacols and chlorocatechols, the most abundant compounds found on this kind of waste (Remberger *et al.,* 1991). In a previous work we reported the isolation of a *Pseudomonas putida* strain TQ07 capable of using CA as its sole source of carbon; as well as the identification of the *cadA* gene, which codes for a FAD-monooxygenase involved in degradation (Treviño-Quintanilla *et al.,* 2002). In the present work we report the characterization of three new genes from the same strain involved in CA mineralization. In addition, we propose a possible pathway for the catabolism of this compound. The coloration of CA and possibly other benzoquinones facilitates the isolation, characterization, and manipulation (at molecular level) of microorganisms, able to degrade them, by the same method that we used in this report.

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Materials and Methods

Bacterial strains, plasmids, and growth media

Escherichia coli strains HB101, DH5α, and S17.1λpir were grown in Luria-Bertani (LB) medium or nutrient broth (NB) at 37°C. For cultures of *E. coli* cells carrying antibiotic resistance markers, the media were supplemented with ampicillin, 200 μg/ml; kanamycin, 50 μg/ml and tetracycline, 20 μg/ml. *Pseudomonas putida* TQ07 was grown in NB or minimal salts medium (MM): deionized water 1000 ml; K_2HPO_4 1.0 g; KH_2PO_4 0.5 g; NH_4NO_3 1.0 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; NaCl 0.1 g; and $FeCl_3$ 0.02 g (final pH 6.8). Antibiotics were incorporated at the following concentrations for *P. putida* TQ07: chloramphenicol, 50 μg/ml; nalidixic acid, 50 μg/ml; kanamycin, 50 μg/ml and tetracycline, 20 μg/ml.

Biodegradation assays

Biodegradation assays were performed in 250 ml Erlenmeyer flasks, each containing 50 ml of MM, supplemented with chloranilic acid (Aldrich Chemical Company, USA) that was dissolved in filter-sterilized water and added to the medium at a final concentration of 400 mg/L. Then these cultures were inoculated with 2.5 ml of a 48 h MM culture of *P. putida* TQ07 that presented an optical density at (600 nm) of 0.2, the cells were then incubated at 30°C for 48 h while being shaken at 250 rpm. Cell growth was monitored by measuring optical density at 600 nm.

Analytical methods

Chloranilic acid degradation was quantified by UV spectrophotometry, measuring absorbance reduction at 243 nm and determining the concentration by comparison with different chloranilic acid solutions of known concentration.

Random mutagenesis

Random TQ07 transposon mutagenesis was carried out with a pUT derivative containing the mini-Tn5Km*lacZ*2 transposon (de Lorenzo *et al.,* 1990). Transformants were selected on NB plates supplemented with chloramphenicol and kanamycin. CAD mutants were identified by their defective halo formation on NB plates supplemented with 400 mg/L of chloranilic acid, after 48 h of incubation at 30°C. The CAD mutants were then grown in MM supplemented with 400mg/L of pyruvate or succinate to establish that the transposon in CAD mutants did not affect enzymes of the tricarboxylic acid cycle (TCA cycle).

Bacterial matings

A *P. putida* TQ07 library was mobilized into chloranilic acid mutants by triparental mating from *E. coli* HB101 using the helper plasmid pRK2013 (Figurski and Helinki, 1979). Transformants were selected on NB plates supplemented with chloramphenicol and tetracycline.

DNA manipulations

Standard procedures for restriction endonuclease digestion, agarose gel electrophoresis, DNA purification from agarose, DNA ligation and Southern blot hybridization were carried out as described previously (Sambrook *et al.,* 1989).

Sequence analysis

Sequence analysis was performed using the GeneWorks program (Intelligenetics Inc., USA). Function prediction and homology modeling were performed using the programs BLAST available at the National Center for Biotechnology Information (Altschul *et al.,* 1990) and I-TASSER available at http://zhanglab.ccmb.med.umich.edu/I-TASSER (Roy *et al.,* 2010). The relationship of BLAST and I-TASSER results with CAD mutants were realized under the criterion of comparing genes with experimental results to corroborate the predicted functions of *cad* genes.

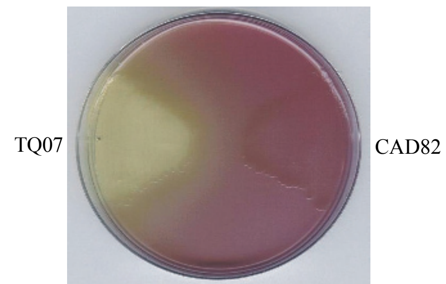


Fig. 1. Phenotype of *Pseudomonas putida* TQ07 and a mutant affected in its CA degradation pathway, growing up in a NB plate supplemented with 600 mg/L of CA.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been deposited in the GenBank nucleotide sequence databases under accession number JN819206.

Results

Mutagenesis and selection of CAD mutants

Based on the phenotype of uncolored halos around the colonies when *P. putida TQ07* grown on NB plates supplemented with chloranilic acid, 16 mutants of *P. putida* TQ07 with mini-Tn5Km*lacZ*2 insertions were isolated (Fig. 1). These 16 CAD mutants were able to grow using pyruvate or succinate as substrates, suggesting that they were specifically affected in the chloranilic acid degradation pathway. Afterwards, we selected mutants CAD50 and CAD82 containing a *Pst*I 10 kb fragment that included the 5 kb mini-Tn5Km*lacZ*2 transposon in order to characterize them fully. Next, we created a DNA genomic library of TQ07 in pCP13, a broad-host-range cosmid vector. This library was screened by colony hybridization, using as a probe the *Pst*I 10 kb fragment of CAD mutants isolated the cosmid pLG3562, which contains the genes affected in both mutants. The *Pst*I 10 kb fragments of both CAD mutants were cloned into pBluescript II KS(+) vector in order to generate pTQ50 and pTQ82. These fragments as well as the *Pst*I 5 kb fragment of the pLG3562 cosmid were sequenced. In this last *Pst*I 5 kb fragment, six Opening Reading frames (ORFs) were identified using the Clone Manager 2.5 ORF Finder software (Fig. 2).

Complementation of mutants affected in the chloranilic acid degradation pathway

The cosmid clone pLG3562 that hybridized with the *Pst*I 10 kb fragment of the CAD mutants was later shown to completely complement the CAD50 (ORF1) and CAD82 (ORF2) mutants, restoring halo formation when grown in nutrient agar supplemented with chloranilic acid as well as 100% of

the growth on MM with this compound as sole carbon source. In order to localize in more detail the genes that complemented mutants CAD50 and CAD82, a series of deletion plasmids were constructed with several restriction endonucleases. First, we subcloned the 5 kb *Pst*I fragment in the expression vector pMP220 (Spaink *et al.,* 1987) producing the pLG05PP plasmid. As expected, the two CAD mutants were fully complemented by this plasmid, because pLG05PP contains all the ORFs previously described (Fig. 2). To establish if the insertion of the mutant CAD82 was polar over other genes of the *Pst*I 5 kb fragment, we constructed the pLG03PB plasmid by subcloning a 2946 bp *Pst*I-*Bam*HI fragment that contained only ORF1 and ORF2. This last plasmid fully complemented mutant CAD50, but not mutant CAD82. This assay demonstrated that the insertion of the mini-Tn5Km*lacZ*2 transposon in the CAD82 mutant was polar on other genes contained in the *Pst*I 5 kb fragment. These results suggest that at last one of these genes (ORF3 and 4) also take part in CA degradation. Additionally, *E. coli* HB101 containing cosmid pLG3562 did not make degradation halos when grown on nutrient agar supplemented with chloranilic acid and it was unable to grow with this compound as sole source of carbon. This result suggests that not all genes encoding proteins involved in chloranilic acid degradation are clustered in the TQ07 region cloned in cosmid pLG3562.

Based on the results of complementation assays and amino acid sequence similarity analysis, we propose that four ORFs (1 to 4) are probably involved in chloranilic acid degradation. Two of them, ORF1 and ORF2, have transposon insertion in mutants CAD50 and CAD82, respectively. The two last ORFs (ORF5 and 6) enclosed in this fragment are encoded divergently with respect to the others, and do not seem to take part in chloranilic acid degradation process. To take advantage of these results we renamed the ORF1 to 4 as *cadR, cadC, cadD* and *cadX*, respectively (Fig. 2).

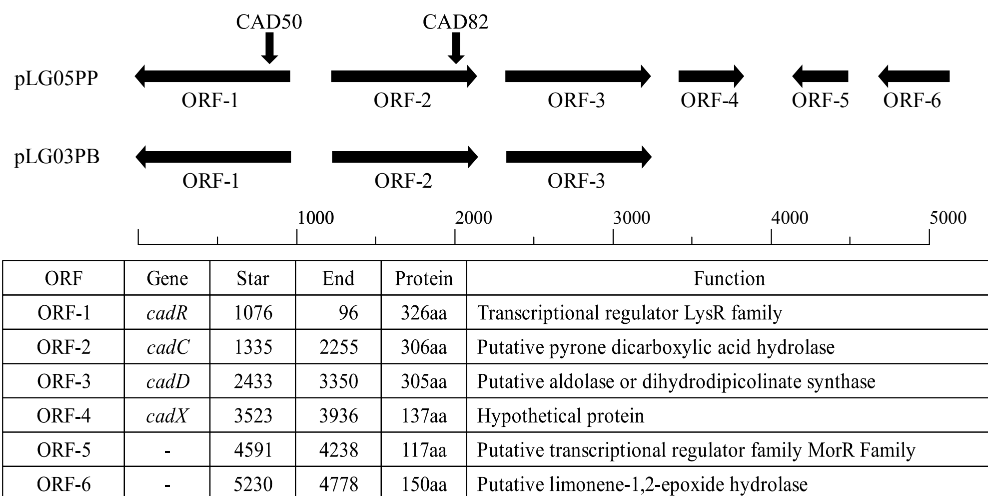
cadR encodes a LysR family transcriptional regulator

The *cadR* gene is encoded divergently with regard to other three genes (*cadC, D* and *X*) contained in the 5 kb *Pst*I fragment of cosmid pLG3562 (Fig. 2). The *cadR* gene encodes a 326 amino acid-residue polypeptide that is similar to members of the LysR family of transcriptional regulators (Henikoff *et al.,* 1988).

CadR presents the highest identity (34%) against a regulator of the plasmid IncP R751 (Thorsted *et al.,* 1998). Regarding genes involved in degradation, the highest identity of this regulator was of 27% in relation to the *fldY* gene involved in the regulation of fluorene mineralization across the 4,5-protocatechuate pathway of *Sphingomonas* sp. LB126 (Wattiau *et al.,* 2001). Jointly with other members of this family, this polypeptide contains a putative helix-turn-helix (HTH, amino acids 37 to 57) DNA binding motif. In the case of the CAD50 mutant, which presents an altered CA degradation pathway, it is precisely in this DNA binding motif where the mini-TnKm5*lacZ*2 insertion is located (amino acid 55). This suggests that the product of this gene takes part in the transcriptional regulation of other genes that encode enzymes involved in CA degradation. The presence of intergenic regulatory sequences between *cadR* and *cadC,* which are consistent with the organization proposed previously for divergent LysR regulators (Schell, 1993) (data not shown), reinforces the possibility that the product of this gene takes part in the regulation of one or more of the divergently encoded genes.

cadC encodes a putative pyrone dicarboxylic acid hydrolase

The *cadC* gene encodes a 306 amino acid-residue protein (Fig. 2), which shows 32% identity with genes that encode for the 2-pyrona-4,6-dicarboxylic acid hydrolase enzyme. This enzyme takes part in the degradation of aromatic compounds such as vanillic acid, syringic acid, phthalate, naphthalene, phenanthrene, anthracene and fluorene throughout protocatechuate synthesis. The latter is a general intermediary in these pathways, which is metabolized to 2-pyrone-4,6-dicarboxylic acid (PDC) by a 4,5-dioxygenase. PDC is then hydrolyzed by the 2-pyrone-4,6-dicarboxylic acid hydrolase enzyme



ORF	Gene	Star	End	Protein	Function
ORF-1	*cadR*	1076	96	326aa	Transcriptional regulator LysR family
ORF-2	*cadC*	1335	2255	306aa	Putative pyrone dicarboxylic acid hydrolase
ORF-3	*cadD*	2433	3350	305aa	Putative aldolase or dihydrodipicolinate synthase
ORF-4	*cadX*	3523	3936	137aa	Hypothetical protein
ORF-5	-	4591	4238	117aa	Putative transcriptional regulator family MorR Family
ORF-6	-	5230	4778	150aa	Putative limonene-1,2-epoxide hydrolase

Fig. 2. Genes map of complementation plasmids. The transcription direction of each gene is indicated by the arrows, the location of mutants insertions is indicated and the possible function of each gene is specified.

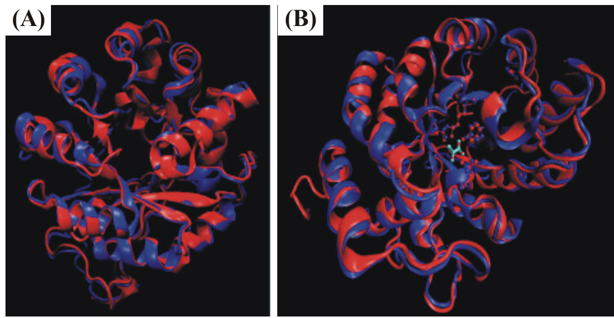


Fig. 3. Schematic representations of the superimposition of Cα traces. (A) CadC molecular modeling (red) and 2-pyrone-4,6-dicarboxylic acid hydrolase from *Sphingomonas paucimobilis* 2QAH (blue). (B) CadD molecular modeling (red) and dihydrodipicolinate synthase of *E. coli* 3I7S (blue), ball and sticks show implicated residues in the catalysis (blue and red) and pyruvate (light blue).

to 4-oxalomesaconic acid. This enzyme was characterized at molecular and biochemical level for the first time in *Sphingomonas paucimobilis* SYK-6, which degrades protocatechuate through the previously mentioned pathway (Masai *et al.,* 1999). The insertion of the mini-Tn5Km*lacZ2* transposon in the CAD82 mutant, which presents an altered CA degradation catabolism, took place 15 amino acid-residues before the carboxylic end of this protein (amino acid 291). To verify the possible function of *cadC* we obtained a *de novo* protein modeling, it was performed by using an iterative implementation of (multi) threading/assembly/refinement approach (I-TASSER). The structure was predicted as *(α/β)* 8-barrel (TIM-barrel), being the 2-pyrone-4,6-dicarboxylic acid hydrolase from *Sphingomonas paucimobilis* SYK-6, the structurally closest protein (pdb entry 2QAH). The TM-score obtained by this homology modeling was 0.8 with an *R.M.S.D.* of 4.8 Å, where a TM-score >0.5 indicates a model of correct topology. This prediction suggests that both enzymes present the same fold (Fig. 3A).

cadD encodes a putative aldolase

Sequence analysis of *cadD* showed that this gene encodes a 305 amino acid-residue protein. Based on amino acid sequence similarity, this protein presents 33% identity with dihydrodipicolinate synthase (DHDPS), which is encoded by the *dapA* gene in *E. coli* (Pisabarro *et al.,* 1993). *cadD* showed a 28% identity with 2-keto-3-deoxygluconate aldolase from *Sulfolobus solfataricus*, which is encoded by the *kdg*A gene, this enzyme catalyzes a reversible aldol cleavage C6 to C3 producing pyruvate and glyceraldehyde (Buchanan *et al.,* 1999).

To verify the possible function of *cadD*, we predicted a 3D structure of CadD using the I-TASSER online server. The homology modeling obtained present a (α/*β*) 8-barrel fold, with a TM-score of 0.9 and a R.M.S.D. of 3.6 Å in cadC y cadD, indicating that this model have a correct topology. The proteins in PDB which are structurally closest to the CadD are dihydrodipicolinate synthase (DHDPS) from *Bartonella henselae* (PDB entry 3SI9A), *Neisseria meningitidis* (PDB entry 3FLUA), and *Salmonella typhimurium* (PDB entry 3G0S). The key catalytic residues T47, Y140, K170, and I211 (equivalent to T44, Y133, K161 and I203 of DHDPS from *N. meningitides* and *E. coli*) are all conserved in CadD and are situated within the active site in the centre of the TIM-barrel (Devenish *et al.,* 2009; Soares da Costa *et al.,* 2010). This approach strongly

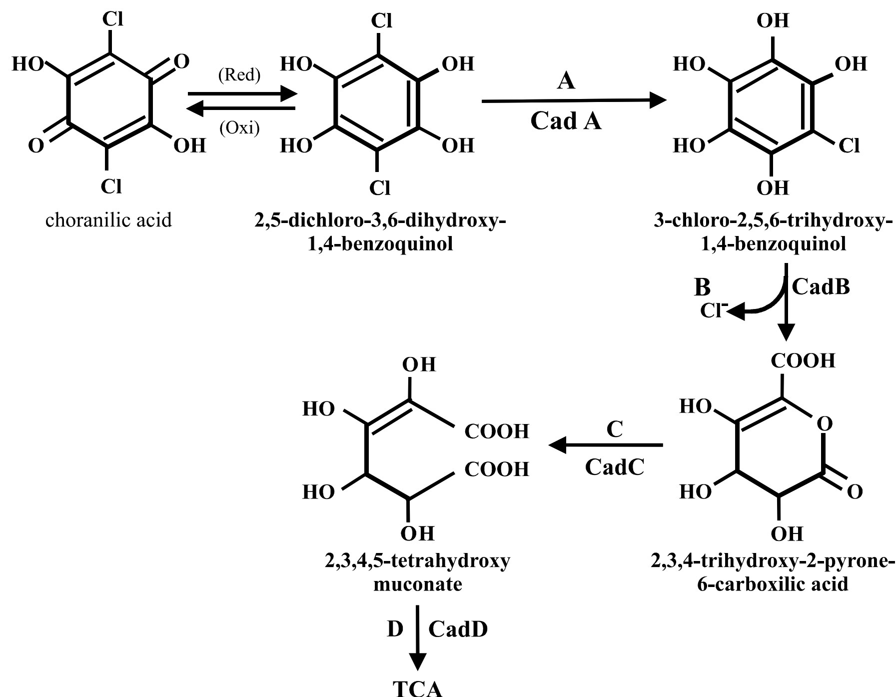


Fig. 4. Hypothetical pathway of chloranilic acid degradation in *Pseudomonas putida* TQ07.

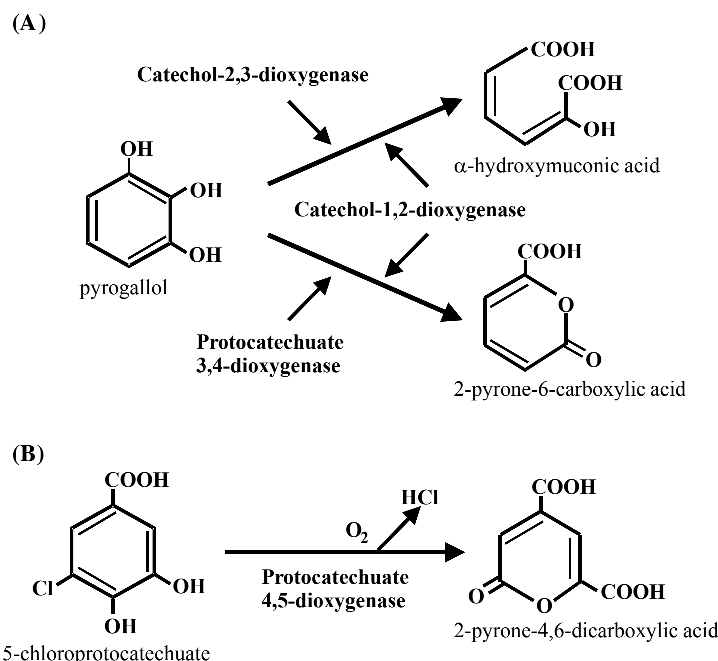


Fig. 5. (A) Oxidative cleavage of pyrogallol by three different dioxygenases, catechol-1,2-dioxygenase, catechol-2,3-dioxygenase and protocatechuate-3,4-dioxygenase. (B) Oxidative cleavage of 5-chloroprotocatechuate by a protocatechuate-4,5-dioxygenase.

suggests that this protein is an enzyme with a similar aldolase function like dihydrodipicolinate synthase (Fig. 3B).

cadX encodes a hypothetical protein

The *cadX* gene encodes a 137 amino acid-residue protein, which during the amino acid sequence similarity analysis showed a 41% identity with several gene products annotated as unknown function. However all these genes are included in operons with genes involved in the catechol *meta*-cleavage degradation pathway, which includes *cmp*X of *Sphingomonas* sp. HV3 (Yrjälä *et al.,* 1997), *cbz*X of *Pseudomonas putida* GJ31 (Mars *et al.,* 1999) and the protein encoded by ORF1126 of the pNL1 plasmid of *Sphingomonas aromaticivorans* F199 (Romine *et al.,* 1999).

Discussion

Based on the obtained results, the amino acids sequence homology and 3D structure prediction analyses, we propose a choranilic acid hypothetical degradation pathway in *Pseudomonas putida* TQ07. The first enzyme involved in CA degradation (CadA) was characterized previously as a FAD-monooxygenase of aromatic compounds (Treviño-Quintanilla *et al.*, 2002). The general mechanism of these enzymes is the introduction of a hydroxyl group to the aromatic ring generating a phenolic compound. During the introduction of this hydroxyl, another group is removed from the ring, (*e.g.* hydrogen, nitro, amino, carboxyl, and halogen). There are monooxygenases that remove chlorine groups that are in *ortho* position regarding a hydroxyl group. For example, chlorophenol-4-monooxygenase of *Burkholderia cepacia* AC1100 (Garrec *et al.,* 2001) and 2,6-dichloro-*p*-hydroquinone chlorohydrolase of *Flavobacterium* sp. ATCC 39723 (Lee and Xun, 1997), which transform 2,5-dichlorohydroquinone and 2,6-dichlorohydroquinone into 5-chlorohydroxyquinone and 6-chlorohydroxyhydroquinone respectively, the previous facts suggest that CadA function is to remove a chlorine group of 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinol generating 5-chloro-2,3,6-trihydroxy-1,4-benzoquinol (Fig. 4A).

The following step in the degradation pathway would involve the participation of a dioxygenase (still not identified), which participates in the cleavage of this compound (Fig. 4B). In analogy to the mechanism proposed by Kersten and collaborators in 1985 for the degradation of 5-chloroprotocatechuate (Kersten *et al.,* 1985) (Fig. 5B), a compound similar to 2-pyrone-6-carboxylic acid would be obtained from chloranilic acid (reduced and hydroxylated) from the action of a protocatechuate 4,5-dioxygenase. This compound is formed also when protocatechuate 3,4-dioxygenase and catechol 1,2-dioxygenase, which are intradiol aromatic-ring-cleavage dioxygenases, cleave and open the aromatic ring of 1,2,3-trihydroxybenzene "pyrogallol" (Fig. 5A) (Saeki *et al.,* 1980). The removal of a chlorine group from reduced chloranilic acid by CadA would allow that a wider variety of dioxygenases to cleave this compound, generating 2,3,4-trihidroxy-2-pyrone-6-carboxylic acid from 3-chloro-2,5,6-trihydroxy-1,4-benzoquinol, which in turn would be the substrate for the putative pyrone dicarboxylic acid hydrolase (encoded by *cadC*). This would generate 2,3,4,5-tetrahydroxymuconate, a compound similar to 2-hydroxymuconate, which is an intermediary in protocatechuate degradation (Fig. 4C).

Finally, in order to generate from 2,3,4,5-tetrahydroxymuconate intermediaries of the tricarboxylic acid cycle, the enzyme codified by *cad*D might be at work. This enzyme presents

a 33% of average-residue identity and a 3D structure prediction modeling with enzymes involved in the synthesis or degradation of similar compounds to 2,3,4,5-tetrahydroxymuconate. In consequence, due to the fact that the insertion in mutant CAD82 was polar on this gene, it is possible that *cad*D encodes an enzyme involved in the degradation of chloranilic acid which would produce intermediaries for the tricarboxylic acid cycle (Fig. 4D).

The present work allowed us to characterize three genes involved in CA degradation, whose homologs in other microorganisms have not been associated with the degradation of chloroaromatic compounds. In addition, we report the use of a chlorinated benzoquinone (chloranilic acid) for the isolation and characterization of genes involved in the aerobic metabolism of an abiotic by-product of pentachlorophenol, by means of a discolored-halo forming phenotype, which is narrowly related to the capacity of *Pseudomonas putida* strain TQ07 to degrade this benzoquinone. In general, our results allowed us to propose a putative novel pathway which offers new hypotheses on the chloranilic acid degradation (Fig. 4).

Acknowledgments

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