Title: *Ralstonia pickettii* in environmental biotechnology: Potential and Applications

Running Title: *Ralstonia pickettii* and Environmental Biotechnology

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1. Summary

Xenobiotic pollutants such as toluene and trichloroethylene are released into the environment by various industrial processes. *Ralstonia pickettii* possess significant biotechnological potential in the field of bioremediation and has the ability to breakdown many of these toxic conditions. Here we provide a description of the major compounds that various strains of *R. pickettii* are capable of degrading and a brief review of their breakdown pathways and an argument for its use in bioremediation.

2. Introduction

Ralstonia is a newly designated genus that includes former members of *Burkholderia* species (*Burkholderia pickettii* and *Burkholderia solanacearum*). These organisms have been renamed as *Ralstonia pickettii* and *Ralstonia solanacearum* respectively (Yabuuchi *et al*. 1995). *R. pickettii* is an aerobic Gram-negative, oxidase-positive, nonfermentative rod and is a ubiquitous micro-organism that is found in water and soil (Gilligan *et al.* 2003). *R. pickettii* has been identified in biofilm formation in plastic water piping (Anderson *et al.* 1990). The bacterium has been identified in ultrapure water in industrial systems (Kulakov *et al*. 2002), in the Space Shuttle water system (Koenig and Pierson 1997) and in laboratory based purified water systems (Adley *et al*. 2005). The organism has the ability to survive and thrive in low nutrient (oligotrophic) conditions (McAlister *et al.* 2002), it is theorised that in ultrapure water systems the bacteria may be able to scavenge from the polymers in plastic piping. In addition *R. pickettii* has been shown to have biodegradative abilities, demonstrating its large metabolic diversity (Table 1). Many different species of bacteria are being investigated for bioremediation capabilities; three of best characterised are *Burkholderia vietnamiensis*, *Pseudomonas putida* and *Pseudomonas fluorescens*. However these species have drawbacks that may limit their use. *Burkholderia vietnamiensis* strain G4 (O'Sullivan and Mahenthiralingam 2005) is part of the

Burkholderia cepacia complex (Bcc) (Genomovar V) which in Cystic Fibrosis (CF) patients leads to a deterioration of prognosis and an increased risk of death (Corey and Farewell 1996; Isles *et al.* 1984; Tablan *et al*. 1985). In a study carried out by LiPuma *et al*. 2001, *B. vietnamiensis* accounted for 5.1% of all Bcc cases found in 606 CF patients. *B. vietnamiensis* can also cause a phenomenon called plant tissue water soaking, which can cause disease and tissue damage in onions. As a result of the clinical relevance of Bcc species and their close interspecies relatedness, the biotechnological applications of all Bcc species have been severely restricted by the U.S. EPA (Anon 2003). *P. fluorescens* has been demonstrated to cause fin rot in fish (Sakai *et al*. 1989) and *P. putida* has also been shown to cause disease in fish (Wakabayashi *et al.* 1996; Nakatsugawa and Iida 1996). The use of these bacteria as bioremediators is therefore not advised as environmental release could lead to environmental damage such as disease in plants, the depletion of fish stocks and the potential to cause disease in humans. The use of these organisms could also cause public concern. *R. pickettii* demonstrates many advantages over these bacteria. Though the bacterium has been shown to be involved in a number of clinical situations in unusual circumstances (Ryan *et al.* 2006), it has never been detected as a phytopathogen or as an animal pathogen (Gilligan *et al.* 2003). This may allow more widespread environmental release. The organism's ability to survive in low nutrient environments like water and soil would also help it to survive on environmental release. These factors illustrate why *R. pickettii* is a good candidate organism for the study of its bioremediation capabilities and potential applications. A complete list of the known compounds that can be degraded by various strains of *R. pickettii* are included in Table 1.

3. Xenobiotic Compounds

3.1. Aromatic hydrocarbons

Aromatic hydrocarbons are volatile organic compounds (VOC's), which include phenol (C_6H_5OH), cresol (C_7H_8O), benzene (C_6H_6) and toluene (C_7H_8). They are found in a variety of household and

industrial products such as germicides, antiseptics, and several different household cleaners. Many of the aromatic hydrocarbons are toxic, carcinogenic, and otherwise hazardous compounds that are frequently found as contaminants of soil and ground water (Siegrist 1992; Anon 2002). Strains of *R. pickettii* are capable of degrading many of these aromatic hydrocarbons and using them as both a carbon and an energy sources (Kukor and Olsen 1990, 1992). This is achieved by means of multi-enzyme pathways such as the *Tbu* pathway of *R. pickettii* PKO1, a soil isolate, which can convert aromatic hydrocarbons into catechols (Kukor and Olsen 1990, 1991, Olsen *et al.* 1994). The *tbu* pathway of *R. pickettii* PKO1 has been cloned as a 26.5-kbp DNA fragment designated pRO1957 and expressed in *Pseudomonas aeruginosa* (Olsen *et al*. 1994). The genes encoding enzymes for this catabolic pathway have been shown to be organized into three operons. The first is the *tbuA1UBVA2C* and *tbuT* (Genbank accession numbers: AY541701 and U04052) (Genbank accession number U72645) operon encoding the initial toluene- 3-monooxygenase and the transcriptional activator *TbuT* (Byrne *et al.* 1996) The second is *tbuD* operon encoding phenol/cresol hydroxylase (Genbank accession number M98806) (Kukor and Olsen 1990, 1992), and the third is the *tbuWEFGKIHJ* operon encoding enzymes of the *meta*cleavage pathway for conversion of catechol and methylcatechols to tricarboxylic acid cycle intermediates (Kukor and Olsen 1991). The first step in the degradation pathway is the hydroxylation of toluene or benzene into the intermediates cresol or phenol by a monooxygenase enzyme (Fig. 1) (Kukor and Olsen 1990, 1992; Olsen *et al*. 1994). Toluene- 3-monooxygenase (T3MO) was first reported to hydroxylate toluene at the *meta* position, producing primarily *m*-cresol (Olsen *et al*. 1994). It has subsequently been discovered that T3MO actually hydroxylates mono-substituted benzenes predominantly at the *para* position (Fishman *et al.* 2004). Therefore the enzyme was renamed from T3MO to toluene *para*-monooxygenase (TpMO). TpMO is regulated by the *tbuT* locus and is induced by other compounds such as ethylbenzene (C_8H_{10}) xylene (C_8H_{10}) and trichloroethylene (C_2HCl_2) (Byrne and Olsen 1996). The phenol or *p*-cresol is then hydroxylated into catechol by a phenol/cresol hydroxylase (Fig. 1). The *tbuD* operon encodes phenol hydroxylase (EC

Number 1.14.13.7) and is regulated by the *tbuR* gene. This phenol hydroxylase is a flavoprotein that is capable of degrading a wide assortment of phenols. It exists in the form of a dimer and uses NADPH as a co-substrate (Kukor and Olsen 1992). The final component of the *tbu* pathway consists of the *tbuWEFGKIHJ* operon that encodes seven enzymes responsible for the *meta* cleavage of catechol (Fig 1). These are catechol 2, 3-dioxygenase (Genbank accession number U20258, EC Number 1.13.11.2), 2-hydroxymuconate semialdehyde hydrolase (EC Number 3.7.1.9), 2-hydroxymuconate semialdehyde dehydrogenase (EC Number 1.2.1.45), 4-hydroxy-2-oxovalerate aldolase (EC Number 4.1.3.39), 4-oxalocrotonate decarboxylase (EC Number 4.1.1.77), 4-oxalocrotonate isomerase, and 2-hydroxypent-2, 4-dienoate hydratase, respectively (Kukor and Olsen 1991). The steps of this pathway can be seen in Fig. 1. The *tbuWEFGKIHJ* operon is controlled by *tbuS*, which represses the operon in the absence of phenol and activates it when the effector molecules are present by forming a transcription activator complex. *R. pickettii* also has the ability to metabolise toluene and other aromatic hydrocarbons under hypoxic conditions. *R. pickettii* PKO1 can metabolise toluene when oxygen levels are 25% of air-saturated water which distinguishes it from other toluene metabolising bacteria. PKO1 can degrade toluene at oxygen levels as low as two mg of dissolved O_2 per Litre (Kukor and Olsen 1996), this can be attributed to kinetic and binding differences within the enzyme catechol 2, 3-dioxygenase. The *R. pickettii* enzyme has a higher turnover rate and a twofold greater affinity for the substrate than non-hypoxic strains (Kukor and Olsen 1996, Kukor *et al*. 1993). PKO1 adapts to hypoxic environments by its ability to use nitrate as an alternative electron acceptor to oxygen for the catabolism of aromatic hydrocarbons (Kukor and Olsen 1996). The bioremediation of polluted groundwater and toxic waste sites requires that bacteria come into close physical contact with pollutants. This can be accomplished by chemotaxis, where *R. pickettii* PKO1 is attracted to toluene, where the response is dependent on induction by growth with toluene (Parales *et al*. 2000). *R. pickettii* PKO1 was shown to degrade 99.9% of benzene, toluene, ethylbenzene, ortho-, meta-, and para-xylene, and styrene and 75.9% of 1, 2, 4-trimethylbenzene at a concentration of 0.2 mg l^{-1} after 48 hours in a hydrocarbon degradation assay

indicating that this organism has a wide range and high activity against many potential substrates (Leahy *et al.* 2003).

3.2. Trichloroethylene

R. pickettii strain PK01 is one of the most extensively studied degraders of trichloroethylene (TCE) (C_2HCl_2), which is a suspected carcinogen (Anon 1976) and U.S. EPA priority pollutant (Anon 1980). It is one of the most commonly detected VOC's in groundwater (Rajagopal 1986). The key enzyme in the breakdown of TCE in *R. pickettii* PKO1 is Toluene *para*-monooxygenase. This then follows the same pathways as the aromatic hydrocarbons. The biodegradation of toluene and TCE by the organism is induced by TCE at high concentrations via the *tbuT* locus (Byrne and Olsen 1996). *R. pickettii* itself demonstrates a tolerance towards TCE toxicity through two different systems (Park *et al*. 2002). The first system is responsible for the tolerance to solvent stress (e.g. disturbance of bacterial membrane by hydrophobic compounds), and the other system allows the bacteria to tolerate toxic intermediate stress (i.e. the ability of the organisms to survive toxic intermediates produced from the oxidation of TCE). PKO1 also has the ability to degrade TCE under hypoxic conditions using nitrate as an alternative electron acceptor to oxygen as with the aromatic hydrocarbons (Leahy *et al.* 1996). In recent trials, *R. pickettii* PKO1 achieved 83.9% removal of TCE at a concentration of 0.2 mg l^{-1} after 48 hours in a hydrocarbon degradation assay (Leahy *et al.* 2003).

3.3. 1, 4-Dioxane

1, 4-Dioxane ($C_4H_8O_2$) is a likely human carcinogen (DeRosa *et al.* 1996), and a significant emerging water contaminant. It is extensively used as a stabilizer for chlorinated solvents such as 1, 1, 1-trichloroethane (TCA). It is used in the manufacture of several organic chemicals including polystyrene manufacture, wood stains and varnishes and SBR latex production (Zenker *et al.* 2003)

and as a wetting agent in the paper and textile processing industries. It has been detected as a contaminant in both surface waters and groundwaters (Johns *et al.* 1998; Jackson *et al.* 1999; Abe 1999). *R. pickettii* PKO1 has been shown to degrade 50 mg/L of 1, 4-Dioxane in the presence of a hydrocarbon inducer at a rate of 0.31 ± 0.007-mg/hr/ mg protein (Mahendra and Alvarez-Cohen 2006).

3.4. Chlorinated Phenolic Compounds

Chlorophenol compounds are used widely as pesticides and biocides and some, in particular monochlorophenols, can be formed during the chlorination of wastewaters and by the breakdown of chlorinated aromatic compounds (Pritchard *et al*. 1987). Chlorinated compounds have a high degree of environmental persistence as well as a high solubility in water (Smith and Novak 1987). Finding ways to remove chlorophenol compounds presents an important challenge. *R. pickettii* strain LD1 (purified from a bacterial biofilm) can utilise monochlorophenols as carbon sources and can catabolize these compounds without the use of supplemental nutrients or cofactors (Fava *et al*. 1995). LD1 can utilise 2-chlorophenol (1.51 mmol l^{-1}), 3-chlorophenol (0.57 mmol l^{-1}), and 4-chlorophenol (0.75 mmol l^{-1}) (C_6H_5ClO). The chlorophenol compounds are metabolized into chlorocatechols. The mechanism of degradation however is still unclear (Fava *et al*. 1995). The LD1 strains ability to mineralise monochlorophenols can be enhanced by the addition of vitamins to the culture medium. The addition to the culture medium of a vitamin solution (containing biotin, folic acid, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, niacin, pantothenic acid, cyanocobalamin, p-aminobenzoic acid, and thioctic acid [total final concentration: < or = 600 ppb]) resulted in a 7% - 16% increase in the amount of target compounds degraded over the incubation period required for the concentration of the compound in the cultures to drop to approximately zero (Kafkewitz *et al*. 1996).

2, 4, 6-Trichlorophenol (C_6HCl_2O) (2, 4, 6-TCP) is used widely as a biocide, as a wood preservative, as an antiseptic, a glue preservation, as an anti-mildew treatment and in the manufacture of other

chemicals (Anon 1990). The US EPA has classified 2, 4, 6-TCP as a Group B2, probable human carcinogen. These compounds are toxic and accumulate in the environment, as they are highly resistant to degradation. The ability of *R. pickettii* and other organisms e.g. *Rhodococcus chlophenolicus* and *Streptomyces rochei* to degrade this CP compound have been examined (Apajalahti and Salkinoja-Salonen 1987, Zabolina *et al*. 1995). Three known strains of *R. pickettii* (DTP0309, DTP0405, DTP0602) all isolated from soil are capable of using 2, 4, 6-TCP as a carbon source with Strain DTP0602 being the most rapid degrader (Kiyohara *et al*. 1992). These soil isolates were able to use 0.5 mM 2, 4, 6-TCP as a growth substrate and resting cell suspensions degraded up to 2.5 mM 2, 4, 6-TCD. These bacteria consume the CP compound and leave residual chloride ions in the culture media. They can degrade other isomers of TCP as well as pentachlorophenol (PCP) (Kiyohara *et al*. 1992). In *R. pickettii* the *had* locus encodes the genes responsible for degradation of 2, 4, 6-TCP. 2, 4, 6-trichlorophenol-4-dechlorinase (hydroxylase) (Genbank accession number: D86544) isolated from strain DTP0602 is responsible for the first step in the degradation of 2, 4, 6-TCP (Takizawa *et al*. 1995). The pathway is shown in Fig. 2. A hydroxyquinol 1, 2-dioxygenase enzyme (Genbank accession number: D86544, EC Number 1.13.11.37) then degrades the intermediate products created as can be seen in Fig. 2 (Hatta *et al*. 1999). Homology between the 2, 4, 6- trichlorophenol-4-dechlorinase in *R. pickettii* and the 2, 4, 6-Trichlorophenol-4- Monooxygenase in *Azotobacter* sp. GP1 has been found, particularly in the NH2-terminal amino region (Wieser *et al*. 1997). The genes responsible for the degradation of 2, 4, 6-Trichlorophenol from *R. pickettii* have been cloned in both *Escherichia coli* and *P. putida* using a transposon tagging strategy (Takizawa *et al*. 1995).

3.5. Lantadenes

The lantana plant has encroached on many forests, orchards and pastureland in many parts of the world (Sharma *et al*. 1988; Pass *et al*. 1991). Lantadenes are pentacyclic triterpenoid compounds derived

from the lantana plant. If consumed by grazing animals they cause cholestasis, hepatotoxicity and photosentisation (Sharma *et al*. 1988). These cyclic triterpenoids are difficult compounds to degrade (Krasnobajew 1988). They have shown promise as pharmacological compounds as they inhibit the Epstein Barr virus (Inada *et al*. 1995), demonstrate anti-HIV, anti-tumour, anti-bacterial and anti-inflammatory activities (Li *et al*. 1993; Fujioka *et al*. 1994; Pengsuparp *et al*. 1994). A strain of *R. pickettii* isolated from soil sampled proximal to lantana plants have demonstrated the ability to use lantadene A as a carbon source, however, this utilization was inhibited when other carbon-containing compounds were added to the growth medium (Sharma *et al*. 1997). This may lead to the exploitation of this bacterium in combating toxicity in grazing animals and in biotransforming lantadene into useful bioactive compounds.

3.6. 3, 4-Dichloropropionanilide

3, 4-Dichloropropionanilide ($C_9H_9Cl_2NO$) is the chemical name for a family of herbicides that are known as amides. It is used extensively in rice production for control of grasses, especially barnyard grass and broadleaf weeds in rice (Barnes *et al.* 1987). It has been shown to have neurotoxic and immunotoxic effects in mice (Cuff *et al.* 1996). Approximately 70% of the U.S. rice crop is treated with propanil; accounting for more than 95% of the 2.7–3.6 million kg of this active ingredient (AI) used annually (Aspelin and Grube 1999, Gianessi and Anderson 1995a, 1995b). A soil isolate of *R. pickettii* was found that demonstrated resistance to 3, 4-Dichloropropionanilide, due to the enzyme propanil hydrolase (Hirase and Matsunaka 1991). Research is being carried out on the mechanism of *R. pickettii* resistance to these herbicides with the aim of using the genes responsible to produce herbicide resistant transgenic rice plants (Piruzian *et al.* 1998).

3.7. 2, 4-Dichlorophenoxyacetic acid

2, 4-Dichlorophenoxyacetic acid ($C_8H_6Cl_2O_3$) is a systemic chlorophenoxy herbicide used widely in Canada (more than four million kilograms annually) in the control of broadleaf weeds in cereal cropland and on industrial property, lawns, turf, pastures, non-cropland and aquatic weeds (Anon 1986). Commercial 2, 4- dichlorophenoxyacetic acid products are marketed as alkali salts, amine salts and ester formulations. Many microorganisms including members of the *Pseudomonas* family can degrade 2, 4- dichlorophenoxyacetic acid (Ka and Tiedji 1994; Ka *et al*. 1994 a, b, c). The structural gene *tfd*A from plasmid pJP4 encoding the first of the functional enzymes necessary for the transformation of chlorocatechols into 3-oxoadipate (Don and Pemberton 1981); has been used as a probe to detect bacterial populations with 2, 4- dichlorophenoxyacetic acid degrading capability. In *R. pickettii* homologous *tfd* genes have been shown to be plasmid borne (Ka *et al*. 1994a). One *R. pickettii* isolate (designated 712) contains a 40.9-kb plasmid that hybridises to a 2, 4- dichlorophenoxyacetic acid *tfdA* gene probe and shares features in common with pKA2, from *Variovorax paradoxus* (formally *Alcaligenes paradoxus* (Willems *et al.* 1991). pKA4 is self-transmissible, strongly hybridizes to pKA2 and has a similar restriction pattern. It is thought that these microorganisms may have exchanged plasmids by intergeneric transfer (Ka and Tiedji 1994). The 2, 4- dichlorophenoxyacetic acid degrading genes from the pKA2 plasmid may undergo recombination between the chromosome and the plasmid. This is well documented for aromatic hydrocarbon-degradative determinates on the *Pseudomonas* TOL plasmid that integrate into the host chromosome (Jeenes and Williams 1982; Sinclair *et al.* 1986; Ka and Tiedji 1994) and was demonstrated with pKA2 which was transferred to *Burkholderia cepacia* and found to integrate into the chromosome of that organism.

3.8. Nitroaromatics

While nitrobenzene ($C_6H_5NO_2$) is primarily used in the production of aniline and aniline derivatives, such as methyl diphenyl diisocyanate (MDI), it also finds use in the manufacture of rubber chemicals,

pesticides, dyes, and pharmaceuticals (Anon 1991). Nitrobenzene is also used in shoe and floor polishes, leather dressings, paint solvents, and other materials to mask unpleasant odors. Substitution reactions with nitrobenzene are used to form m-derivatives (Anon 1991; Sittig 1991). Redistilled, as oil of mirbane, nitrobenzene has been used as an inexpensive perfume for soaps. A significant market for nitrobenzene is its use in the production of the analgesic acetaminophen (Anon 1991). In 1992, releases of nitrobenzene to the environment reported to the Toxic Chemical Release Inventory by certain types of U.S. industries totaled about 917,000 pounds (Anon 1994). Because of its toxicity, nitrobenzene has been listed as a priority pollutant by the US EPA as far back as 1979 (Keith and Telliard 1979) and has been added to the list of compounds regulated under the Resource Conservation and Recovery Act (Hanson 1990). Two different pathways of degradation of nitroaromatics have been found in two different strains of *R. pickettii*. In *R. pickettii* Strain YH105 (sludge isolate) degradation of nitroaromatics occurs via a two-step enzymatic process that uses two chromosomally encoded genes: *p*-nitrobenzoate reductase and p-hydroxylaminobenzoate lyase as outlined in Fig. 3 (Genbank accession number AF187879). YH105 is able to degrade up to 15 mM *p*-nitrobenzoate to protocatechuate via *p*-hydroxylaminobenzoate; protocatechuate then enters the TCA cycle (Yabannavar and Zylstra 1995). The genes responsible have been cloned and expressed in *E. coli*. Similar nitroreductases have been found in *Comamonas acidovorans* NBA-10 (Groenewegen *et al.* 1992) and *P. pseudoalcaligenes* JS45 (Somerville *et al.* 1995), the enzyme hydroxylaminolyase however has only been purified from *C. acidovorans* NBA-10 (Groenewegen and DeBont 1992). *R. pickettii* PKO1 degrades nitrobenzene, using the TpMO, to 3- and 4-nitrocatechol via 3- and 4-nitrophenol and these nitrocatechols are then slowly degraded to unidentified metabolites. Haigler and Spain had not identified the enzymes responsible for addition of the second hydroxyl group to the nitrophenols to form nitrocatechols; however it has subsequently been shown that this reaction proceeds down the *tbu* pathway (Parales *et al.* 1997; Fishman *et al.* 2004).

3.9. Quinoline

Quinoline (C_9H_7N) and its derivatives occur widely in coal tar, bone oil, oil shale and plant alkaloids and serve as intermediates and solvents in the chemical industry. Quinoline and some of its derivatives are reported to be toxic, carcinogenic and mutagenic (Sideropoulos and Secht, 1984; Minako *et al.* 1997). The widespread use of quinoline and its derivatives entails that these compounds, together with many other environmental chemicals are distributed in the environment thus polluting soil and water (Miethling *et al*. 1993; Sutton *et al*. 1996). Degradation of quinoline by microbial processes has attracted much interest in recent years. In 2002 a pure strain identified as *Burkholderia pickettii* was isolated from the activated sludge of a coke-oven wastewater treatment plant through enrichment using quinoline as sole source of carbon and nitrogen. The isolate was then used to test quinoline biodegradation by free cells and this was shown to remove 500 mg/L of quinoline after nine hours (Jianlong *et al.* 2002).

3.10. N-nitrosodimethylamine

N-nitrosodimethylamine (NDMA) ($C_2H_6N_2O$) is considered a probable human carcinogen (Anon, 1987). This compound is regulated in USA waters with an US EPA cleanup level of 0.7 ng/L (Anon 2001). Its presence in the environment has been linked to aerospace facilities through the decomposition of hydrazine-based rocket fuels (MacDonald, 2002) and more generally to the discharge of water and wastewater disinfected with chlorine (Mitch *et al*. 2003; Njam and Trussell 2001). In the latter case, it appears that secondary amines react with chloramine to form a hydrazine intermediate that is in turn oxidized to NDMA (Mitch and Sedlak 2002). Its persistence in groundwater aquifers has been responsible for the closure of municipal drinking water wells and it's listing as a priority pollutant (Mitch *et al*. 2003). *R. pickettii* PKO1 has been found to degrade this

compound in the presence of toluene at a rate of 1 ng/mg/min. (Sharp *et al*. 2005). It was not stated in the paper whether NDMA was completely degraded or just transformed.

4. Conclusions

R. pickettii with its ability to survive and prosper in oligotrophic environment has the ability to use a variety of compounds as energy and carbon sources. The organism already has demonstrated this capacity being able to degrade a number of toxic substances (Table 1) making it an excellent candidate for bioremediation. It has several advantages over other candidate strains being studied such as *B. vietnamiensis* G4 (which is currently undergoing intense study [O'Sullivan and Mahenthiralingam 2005]) or *P. putida*, in that it is only weakly pathogenic, with no phytopathogenic or animal pathogenic incidents being reported. Several areas of application have the potential to use *R. pickettii,* these include; treatment of contaminated ground water and of municipal and industrial waste and sewage. Examples include the removal of toluene from groundwater of which a successful test was carried out in Hanahan, South Carolina. The use of bioremediators prevented the contamination of residential from a massive fuel leak from a nearby military installation (Vroblesky *et al*. 1997). Natural microbial communities in the area were stimulated with nutrients to increase the biodegradation of toluene. Through the use of nutrient addition (Kafkewitz *et al*. 1996) bioremediation of compounds such as chlorophenols and pesticides found in sewage effluent and groundwater could be increased. The degradation of toxic compounds by microorganisms that are part of the microflora of wastewater treatment plants could be augmented if plasmids and genes responsible for these properties were transferred from *R. pickettii.* When genome sequence data of *R. pickettii* is available, analysis of degradative processes will potentially allow optimisation of the physiological state of *R. pickettii* strains during bioremediation applications, and could potentially lead to the construction of novel or more proficient pathways for degradation. *R. pickettii* strain PKO1 could have the potential to be a

super biodegrader with the introduction of plasmids bearing other degradative enzymes e.g. pKA4 and integrating other genes from different bacteria into the chromosome to assist in the breakdown of toxic compounds. An example of the potential of genome analysis can be seen in the alteration of TpMO so that the enzyme hydroxylates all three positions of toluene as well as both positions of naphthalene. The mutation in the enzyme produced a toluene *para*-monooxygenase variant that formed 75% *m*-cresol from toluene and 100% *m*-nitrophenol from nitrobenzene. This was the first time a true *meta*-hydroxylating toluene monooxygenase was created (Fishman *et al.* 2005). The demand for a safe organism makes *R. pickettii* a natural choice for bioremediation applications.

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Table 1: Toxic compounds degraded by *Ralstonia pickettii*

Substrate	Genes Responsible	Reference(s)
Benzene	*tbu* pathway	Kukor JJ and Olsen RH 1992; Massol-Deya A, *et al*. 1997
Meta-cresol	*tbu* pathway	Kukor JJ and Olsen RH 1992; McClay K, *et al*. 1996
Ortho-cresol	*tbu* pathway	McClay K, *et al*. 1996
Para-cresol	*tbu* pathway	McClay K, *et al*. 1996
1, 4-Dioxane	*tbu* pathway	(Mahendra and Alvarez-Cohen 2006).
2,4-dichlorophenoxy acetic acid	*tdf* genes in pKA4	Ka *et al*. 1994 a, b, c
3,4-Dichloropropionanilide	Unknown	Hirase and Matsunaka, 1991
2, 3 and 4-Monochlorophenol	Unknown	Fava F, *et al*. 1995
N-nitrosodimethylamine	*tbu* pathway	Sharp *et al*. 2005
Nitrobenzene	*pnb* locus	Yabannavar AV and Zylstra JG 1995
	tbu pathway	Haigler BE and Spain JC 1995
Pentacyclic triterpenoids	Unknown	Sharma *et al*. 1997
Phenol	*tbu* pathway	Kukor JJ and Olsen RH 1992
Quinoline	Unknown	Jianlong *et al.* 2002
Toluene	*tbu* pathway	Kahng HY, *et al*. 2000; Kukor JJ and Olsen RH 1992; Massol-Deya A, *et al*. 1997; McClay K, *et al*. 1996
Trichloroethylene	*tbu* pathway	Leahy JG, *et al*. 1996
2, 4, 6-Trichlorophenol	*had* locus	Kiyohara *et al*. 1992; Takizawa *et al*. 1995
Pentachlorophenol	Unknown	Kiyohara *et al*. 1992

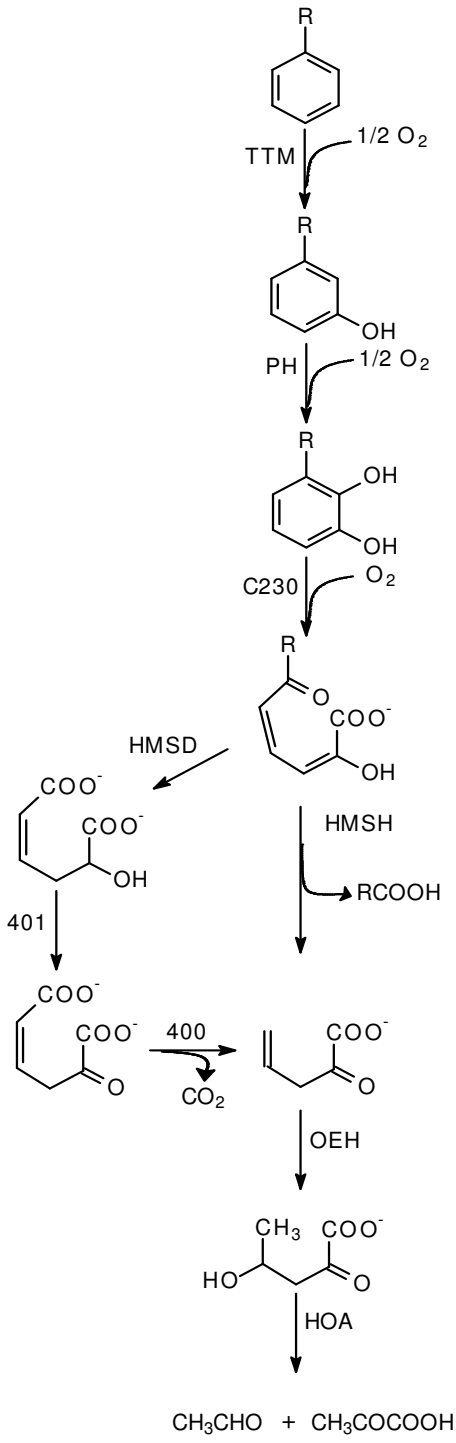

Fig.1: Pathway for degradation of benzene and related aromatic hydrocarbons by *R. pickettii* PKO1. Abbreviations: TTM, toluene-p-monooxygenase; PH, phenol hydroxylase; C230, catechol-2, 3-dioxygenase; HMSH, 2-hydroxymuconate semialdehyde hydrolase; HMSD, 2-hydroxymuconate semialdehyde dehydrogenase; 4O1, 4-oxalocrotonate isomerase; 4OD, 4-oxalocrotonate decarboxylase; OEH, 2-hydroxypent-2, 4-dienoate hydratase; HOA, 4-hydroxy-2- oxovalerate aldolase.

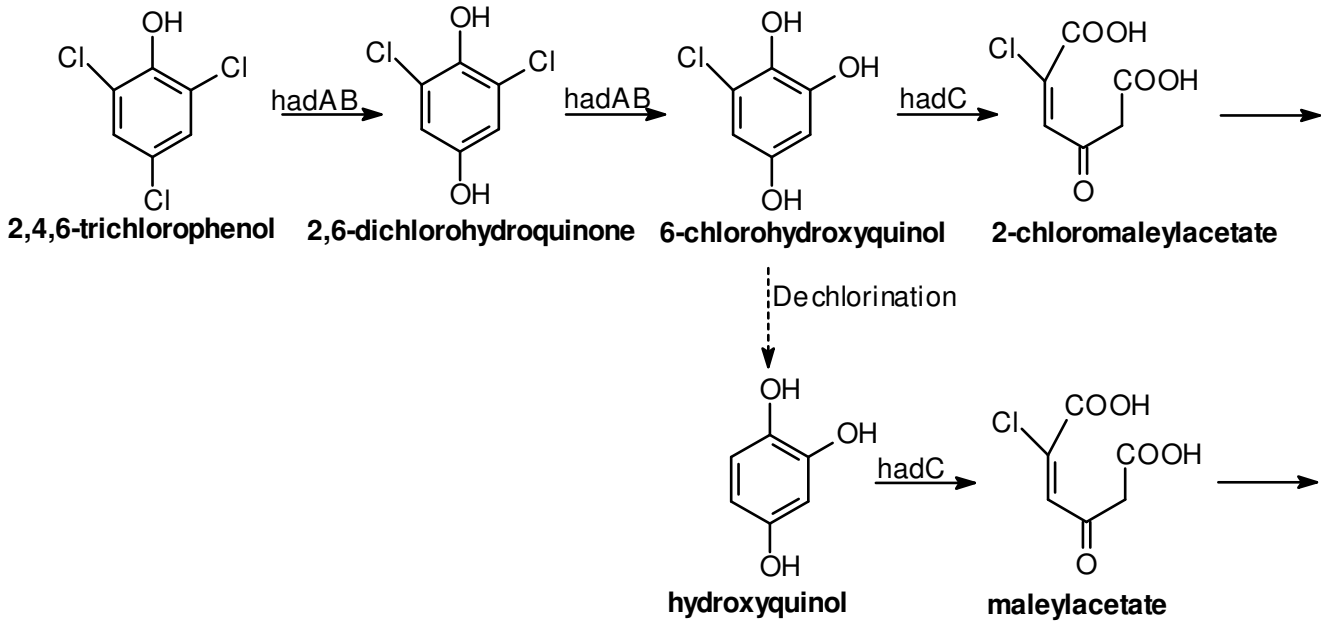

Fig 2: Pathway for the degradation of 2,4, 6-TCP in *R. pickettii* DTP0602. *hadAB* encodes chlorophenol p-hydroxylase and *hadC* encodes hydroxyquinol 1, 2-dioxygenase.

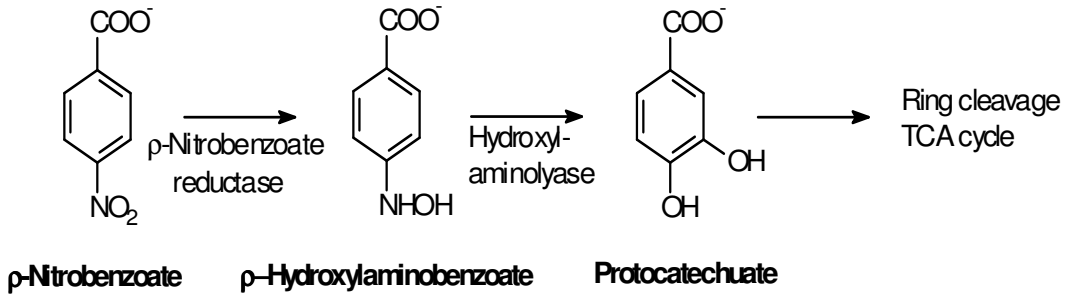

Fig 3: Pathway for the degradation of *p*-nitrobenzoate. TCA, tricarboxylic acid