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Brief report

Microbial responses to xenobiotic compounds. Identification of genes that allow *Pseudomonas putida* KT2440 to cope with 2,4,6-trinitrotoluene

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Summary

***Pseudomonas putida* KT2440 grows in M9 minimal medium with glucose in the presence of 2,4,6-trinitrotoluene (TNT) at a similar rate than in the absence of TNT, although global transcriptional analysis using DNA microarrays revealed that TNT exerts some stress. Response to TNT stress is regulated at the transcriptional level, as significant changes in the level of expression of 65 genes were observed. Of these genes, 39 appeared upregulated, and 26 were downregulated. The identity of upregulated genes suggests that *P. putida* uses two kinds of strategies to overcome TNT toxicity: (i) induction of genes encoding nitroreductases and detoxification-related enzymes (*pnrA*, *xenD, acpD*) and (ii) induction of multidrug efflux pump genes (*mexEF*/*oprN*) to reduce intracellular TNT concentrations. Mutants of 13 up- and 7 downregulated genes were analysed with regards to TNT toxicity revealing the role of the MexE/MexF/OprN pump and a putative isoquinoline 1-oxidoreductase in tolerance to TNT. The ORF PP1232 whose transcriptional level did not change in response to TNT affected growth in the presence of nitroaromatic compounds and it was found in a screening of 4000 randomly generated mutants.**

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Introduction

2,4,6-Trinitrotoluene (TNT) is the most widely used explosive in the world and its production, use and storage have led to the contamination of a great number of sites with TNT or other nitroaromatic compounds (Esteve-Núñez *et al.*, 2001; Lewis *et al.*, 2004; Claus *et al.*, 2007). At severely TNT-contaminated sites, TNT levels of up to 10 g kg^{-1} in soils and up to 100 mg l^{-1} in water have been described (Claus *et al.*, 2007). 2,4,6-Trinitrotoluene recalcitrance has been attributed to the symmetric localization of the nitro groups of the aromatic ring, a disposition that limits attack by classic dioxygenases involved in the microbial metabolism of mono- and dinitrated aromatic compounds. The persistent presence of TNT in ecosystems exerts nonspecific toxic effects, affecting prokaryotic and eukaryotic organisms (Smets *et al.*, 2007). 2,4,6-Trinitrotoluene toxicity has been often associated with the enzymatic reduction of nitro groups to toxic hydroxylamine derivates and to the generation of reactive oxygen species in the cell (George *et al.*, 2008).

A number of microbial enzymes have been described to attack TNT. The most broadly distributed group of such enzymes is that of the oxygen-insensitive nitroreductases, which perform sequential two-electron reductions of nitro groups. These enzymes generally contain FMN as a cofactor and use $NAD(P)H^+$ as an electron donor, and include nitroreductases such as NfsA and NfsB from *Escherichia coli* (Zenno *et al.*, 1996a,b), PnrA and PnrB from *Pseudomonas putida* (Park and Kim, 2000; Caballero *et al.*, 2005), and NitA and NitB from *Clostridium acetobutylicum* (Kutty and Bennett, 2005). Enzymes of this family have also been described in many other bacteria, for example, in *Salmonella enterica* serovar Typhimurium (Nokhbeh *et al.*, 2002), *Enterobacter cloacae* (Bryant and DeLuca, 1991), *Helicobacter pylori* (Goodwin *et al.*, 2002) and *Vibrio fischeri* (Zenno *et al.*, 1994; Riefler and Smets, 2002).

Another family of enzymes that attack TNT is the old yellow enzyme (OYE) family of flavoproteins, which is broadly distributed among bacteria, yeast, plants and

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nematodes (Williams and Bruce, 2002). The physiological function of these enzymes is not well established yet; however, they have often been associated with the reduction of nitroaromatic compounds (Fitzpatrick *et al.*, 2003; Williams *et al.*, 2004; González-Pérez *et al.*, 2007; van Dillewijn *et al.*, 2008a,b). Two types of OYE enzymes have been described. Type I hydride transferases, which like oxygen-sensitive nitroreductases reduce the nitro substituents to hydroxylamine derivatives, and type II hydride transferases, which catalyse a nucleophilic attack on the aromatic ring of TNT (Pak *et al.*, 2000; van Dillewijn *et al.*, 2008a,b). Of bacterial OYE family members, those that are well characterized are XenA through to XenF of *P. putida* KT2440 (van Dillewijn *et al.*, 2008a), XenB from *P. fluorescens* (Pak *et al.*, 2000), PETN reductase (Williams *et al.*, 2004), NemA reductase from *E. coli* (Miura *et al.*, 1997) and YqjM from *Bacillus subtilis* (Fitzpatrick *et al.*, 2003). However, in spite of the thorough biochemical characterization of many TNT-degrading enzymes and the biochemical mechanisms (Wittich *et al.*, 2008), little is known regarding the transcriptional response of bacteria exposed to TNT (Garmendia *et al.*, 2008).

Pseudomonas putida KT2440, for which the genome has been completely sequenced (Nelson *et al.*, 2002), is a model microorganism for soil bioremediation; it has a versatile and broad catabolic metabolism (Jiménez *et al.*, 2002; Timmis, 2002; Reva *et al.*, 2006) and the capacity to colonize bulk and rhizosphere soil (Molina *et al.*, 2000). In this study we report a comparative transcriptomic analysis of *P. putida* KT2440 grown in the presence and absence of TNT, as well as the phenotypical characterization of a number of mutants in up- and downregulated genes. The cellular response to this nitroaromatic compound results in the induction of nitroaromatic detoxifying enzymes and extrusion of TNT via the MexEF/OprN efflux pump.

Results and discussion

Growth of P. putida *KT2440 in the presence of TNT*

The growth of *P. putida* KT2440 in liquid M9 minimal medium with glucose as a carbon source under aerobic conditions was tested in the presence of increasing concentrations of TNT (0.14, 0.29, 0.43 and 0.58 mM). Results revealed that KT2440 grew with a doubling time of about 60 min even in M9 TNT-saturated medium (0.58 mM). The growth yield in the absence of TNT and in the presence of up to 0.43 mM TNT was 1.1 ± 0.1 mg dry weight ml^{-1}; however, the growth yields of cultures (Fig. 1) grown in the presence of 0.58 mM were consistently 10–15% lower than in the absence of the nitroaromatic or in the presence of lower concentrations. The resilient ability of *P. putida* to thrive in the presence of high TNT concentrations is illustrated by comparison with *Stenotrophomonas* sp. OK-5, an isolate from TNT-contaminated soils, which cannot sustain TNT concentrations above 0.4 mM (Ho *et al.*, 2004), and the unicellular green algae *Chlamydomonas reinhardtii*, which does not survive at TNT concentrations as low as 0.01 mM (Patel *et al.*, 2004).

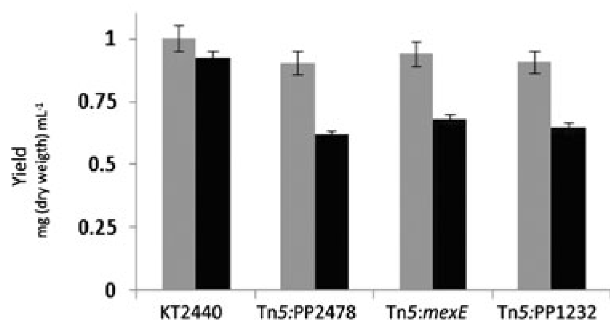

Fig. 1. Growth yield of *P. putida* KT2440 and several isogenic mutants in TNT-free and TNT-saturated medium. Cultures were inoculated at ~0.05 OD_{660} and incubated at 30°C for 24 h with shaking (150 r.p.m.) in a Kühner incubator. Grey bars represent growth in TNT-free M9 minimal medium. Black bars represent growth in TNT-saturated glucose-supplemented M9 minimal medium.

Transcriptional analysis of P. putida *KT2440 growing in the presence of TNT*

To shed light on the mechanisms that *P. putida* KT2440 uses to tolerate high concentrations of TNT, we carried out global transcriptomic DNA microarrays by comparing the expression pattern of cells of the exponential growth phase, growing in the absence and in the presence of an initially saturated solution of TNT (0.58 mM). In cells grown in the presence of TNT, expression of 65 genes appeared to be significantly changed (fold change ≥ 2 or ≤ 2 with P value < 0.05). Of these genes, 39 were upregulated and 26 were downregulated (Tables 1 and 2). Genes were arranged according to the general function of their corresponding gene products. In the set of upregulated genes, group 1 includes those involved in cellular detoxification or metabolism of aromatic compounds. These are genes that encode proteins linked with TNT biotransformation such as *pnrA* (PP_2490) and *xenD* (PP_2489). PnrA is an oxygen-insensitive nitroreductase that is NADPH-dependent (Caballero *et al.*, 2005). On the other hand, XenD is a xenobiotic reductase belonging to the OYE family that *in vitro* was devoid of activity due to the loss of flavin cofactors, although its potential role *in vivo* is unknown (van Dillewijn *et al.*, 2008a). Group 1 also includes genes encoding enzymes that may be involved in TNT biotransformation, for example, the *azo* gene that encodes the azoreductase PP_2866 and a putative nitrobenzoate reductase (PP_3657). Azoreductases are

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Table 1. Upregulated genesin *P. putida* KT2440 grown in TNT-saturated M9 medium.

TIGR identifier	Gene/description	Fold change	Mutant
	1. Detoxification		
PP2474	Glutathione *S*-transferase family protein	2.6	
PP2478	Isoquinoline 1-oxidoreductase beta subunit putative	2.0	M
PP2482	Molybdenum cofactor biosynthesis protein A putative	2.4	
PP2489	*xen D* xenobiotic reductase, putative	2.0	M
PP2490	*pnrA* nitroreductase family protein	2.0	M
PP2866	*acpD* azoreductase	2.1	
PP3657	Nitrobenzoate reductase, putative	2.6	
	2. Resistance efflux pumps		
PP2065	Multidrug efflux RND transporter	2.0	M
PP3427	*oprN* multidrug efflux RND outer membrane protein OprN	3.8	M
	3. Transcriptional regulators		
PP2078	Transcriptional regulator LysR family	2.1	
PP4482	Transcriptional regulator AraC family	2.1	
	4. Metabolism		
PP2650	*gbd* 4-hydroxybutyrate dehydrogenase	2.3	
PP3633	*argC* N-acetyl-gamma-glutamyl-phosphate reductase	2.1	
PP3715	*catB* muconate cycloisomerase	2.4	M
PP4250	*ccoN* 1-cytochrome c oxidase cbb3-type subunit I	2.0	M
	5. Unknown/uncharacterized proteins		
	5.A. Membrane proteins		
PP1976	HlyD family secretion protein	2.0	
PP2647	Major facilitator family transporter	2.1	M
PP3176	Major facilitator family transporter	2.0	
PP3330	Outer membrane ferric siderophore receptor putative	2.1	M
PP3521	Membrane protein putative	2.8	
PP3814	Polyamine ABC transporter periplasmic polyamine-bind	2.8	
	5.B. Uncharacterized oxidoreductases		
PP2507	Oxidoreductase short-chain dehydrogenase/reductase	2.2	M
PP2827	Alcohol dehydrogenase zinc-containing	2.1	
PP3308	Oxidoreductase small subunit putative	2.1	
	5.C. Other unknown proteins		
PP0153	Conserved hypothetical protein	2.6	
PP2307	Conserved hypothetical protein	2.3	
PP2505	GAF domain/GGDEF domain protein	2.2	
PP2506	Hypothetical protein	2.1	
PP2646	Conserved hypothetical protein	2.1	M
PP3428	TPR domain protein	2.8	
PP3522	Conserved hypothetical protein	3.2	
PP3770	Conserved hypothetical protein	3.5	
PP3917	Hypothetical protein	2.0	
PP4021	Esterase	2.0	
PP4081	Conserved hypothetical protein	2.6	M
PP4421	Aminotransferase class III	2.0	
PP4447	Hypothetical protein	2.4	
PP4448	Conserved hypothetical protein	2.2	M
PP4858	Conserved hypothetical protein	2.1	

Experiments were run in triplicate. Data are average of the three assays (done in duplicate) with standard deviation below 15% of the given values. Cells were collected at the beginning of the exponential phase (0.5 of OD_{660}) and total RNA extraction and cDNA synthesis were performed according to Roca and colleagues (2008). *Pseudomonas putida* arrays were produced by Progenika and were employed as described by Yuste and colleagues (2006). M, mutant available from the *Pseudomonas* Reference Culture Collection (Duque *et al*., 2007a).

usually involved in the bond cleavage of aromatic azo compounds, but they have been recently linked with TNT metabolism in *Rhodobacter sphaeroides* AS1.1737 (Liu *et al*., 2007). Induction of enzymes involved in TNT metabolism has been reported in other microorganisms in response to this nitroaromatic chemical, such as clostridial nitroreductases that are encoded by *nitA* and *nitB* (Kutty and Bennett, 2005), and *yqjM* from *B. subtilis* (Fitzpatrick *et al*., 2003).

Other group 1 genes that may be involved in detoxification of TNT or its metabolites include glutathione-*S*-transferase (GST) (PP_2474) and a putative iso-

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Table 2. Downregulated genes in *P. putida* KT2440 grown in TNT-saturated M9 medium.

TIGR identifier	Gene/description	Fold change	Mutant
	3. Transcriptional regulators		
PP1366	Transcriptional regulator MvaT P16 subunit	–2.3	
PP3761	Sensor histidine kinase/response regulator	–2.2	M
	4. Metabolism		
PP1081	Glutaredoxin-related protein	–2.1	
PP3376	*kguD* 2-ketogluconate 6-phosphate reductase	–2.7	M
PP3781	Oxygen-independent Coproporphyrinogen III oxidase fam	–4.7	
	5. Unknown /uncharacterized proteins		
	5.A. Membrane proteins		
PP2322	*oprI* outer membrane lipoprotein OprI	–2.2	
PP2595	ABC transporter permease/ATP-binding protein putative	–2.1	
	5.C. Other unknown proteins		
PP0318	Conserved hypothetical protein	–2.2	
PP0319	Conserved hypothetical protein	–3.1	
PP1225	Radical activating enzyme	–2.4	M
PP1249	Lipoprotein, putative	–3.5	
PP2323	Conserved domain protein	–2.4	
PP3397	Hypothetical protein	–2.4	M
PP3642	Hypothetical protein	–3.2	
PP3679	Hypothetical protein	–2.2	
PP3704	Hypothetical protein	–2.2	
PP3782	Hypothetical protein	–2.7	
PP3783	Conserved hypothetical protein	–2	M
PP3786	Aminotransferase	–3.0	M
PP3921	Hypothetical protein	–2.8	
	6. Transposases		
PP2570	ISPpu9 transposase	–2.3	
PP3381	ISPpu9 transposase	–2.7	
	7. Protein synthesis enzymes		
PP0389	*rpsU* Ribosomal protein S21	–3.8	
PP0721	Ribosomal 5S rRNA E-loop binding protein Ctc/L25/TL5	–2.1	M
PP4007	*infA* Translation initiation factor IF-1	–2.6	
PP5087	*rpmE* Ribosomal protein L31	–2.3	

Conditions as for Table 1.

quinoline oxidoreductase (PP_2478 through PP_2482). Glutathione-*S*-transferase belongs to a set of versatile enzymes that catalyses a wide range of reactions involving the conjugation of glutathione to a variety of electrophilic substrates. Glutathione-*S*-transferases participate in catabolic pathways involved in the aerobic degradation of polychlorinared biphenyls (Vuilleumier and Pagni, 2002). It was suggested that GST was essential for growth of *Burkholderia xenovorans* in the presence of polychlorinated biphenyls (Fortin *et al.*, 2006) and for *Sphingobium chlorophenolicum* with tetrachlorohydroquinone (Kiefer and Copley, 2002). The GST has also been suggested to improve aerobic growth of *Rhodococcus* AD45 with chloroethenes (Rui *et al.*, 2004). The role of isoquinoline reductase in tolerance to toxic chemicals has not been reported previously. In *P. putida* KT2440, a single operon encodes the heterodimeric molybdo isoquinoline oxidoreductase system (ORF PP_2478 through to PP_2482).

In the genome of *P. putida* KT2440 at least 30 extrusion pumps have been found (Nelson *et al.*, 2002). It is of interest to note that two extrusion efflux pumps (group 2) were induced by KT2440 in response to the presence of TNT. This suggests the importance of active extrusion systems in maintaining low intracellular TNT concentration. One of these efflux pumps is the broad-substrate multidrug-resistant MexEF/OprN pump; considered to be a xenobiotic compound transporter (Maseda *et al.*, 2002). *mexEF/oprN* forms an operon in which *oprN* exhibited the highest fold-change in the presence of TNT (3.8-fold, Table 1), whereas genes encoding the other proteins increased their expression level by 1.8- (*mexE*) and 1.6-fold (*mexF*) (not shown). MexEF/OprN is a multidrug efflux pump that has been previously reported to be upregulated in response to formaldehyde and phenylalanine, and has been shown to extrude formaldehyde (Roca *et al.*, 2008), phenylalanine (M.C. Herrera and J.L. Ramos, unpublished), organic solvents (Li *et al.*, 1998)

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and antibiotics (Köhler *et al.*, 1997). The function of the other efflux pump (PP_2065) in KT2440, found to be induced in response to TNT, remains unknown.

The expression of two transcriptional regulators belonging to the LysR (PP2078) and AraC family (PP4482) showed increased expression in response to TNT. It is uncertain if they interact directly with TNT or rely on another inducing signal, and their specific targets are also unknown.

Of the other upregulated genes, four encode enzymes involved in energy and general metabolism (group 4). Two of these genes *ccoN* and *argC* encode proteins related to energy generation under growth-limiting conditions, which agrees with the lower growth yields of *P. putida* that were observed in the presence of TNT.

The remaining ORFs that exhibited induced expression (group 5) corresponded to proteins of unknown function. Within this group we established three subgroups. The first subgroup (5.A) contains several genes encoding membrane proteins, the second (5.B) contains three uncharacterized oxidoreductases and the third (5.C) consists of the genes that encode hypothetical proteins. Therefore, we cannot rule out the contribution of still unknown mechanisms in TNT detoxification.

Microarray data analysis revealed 26 downregulated genes, which have also been arranged according to the putative function of each gene product, and have been included in Table 2. No gene belonging to groups 1 or 2 (genes encoding detoxification enzymes and extrusion pumps respectively) was found downregulated. The expression of two regulators PP1366 and PP3761 was found downregulated, which may in turn influence expression of some of the up- and downregulated genes. We found that some ribosomal proteins were downregulated (Table 2). Downregulation of some ribosomal genes often occurs when cells are under chemical or oxidative stress (Bore *et al.*, 2007; Reid *et al.*, 2008), as shown by Fraga-Muller and colleagues (2007) for *Pseudomonas aeruginosa* growing in the presence of pentachlorophenol. Nonetheless, the transcriptional profiles of *P. putida* with TNT did not reveal changes in other genes encoding proteins typically involved in the general stress responses. This is in contrast with reports in TNT-sensitive microorganisms, such as a *Stenotrophomonas* strain (Ho *et al.*, 2004) and *C. reinhardtii*, where a number of upregulated heat-shock proteins were influenced by TNT (Patel *et al.*, 2004).

Phenotypic analysis of knockout mutants of genes up- or downregulated in response to TNT

A bank of mini-Tn*5* mutants of *P. putida* KT2440 is available at the *Pseudomonas* Reference Culture Collection. The collection consists of independent mutants in almost 30% of all the ORFs (Duque *et al.*, 2007a). For the up- or downregulated genes for which mutants were available (marked with M in Tables 1 and 2), we tested growth in the absence and in the presence of 0.58 mM TNT. All but two mutant strains showed similar growth rates and growth yield to those of the parental KT2440 strain (data not shown). The two mutants that exhibited deficient growth in the presence of TNT were knockouts in the isoquinoline reductase gene cluster and in the *mexEF*/*OprN* efflux pump.

This is the first report linking isoquinoline 1-oxidoreductase and MexEF/OprN with TNT tolerance. For the mutant deficient in isoquinoline l-oxidoreductase, the presence of TNT resulted in slower growth rate in exponential phase (doubling time = 108 $\pm$ 5 min) and lower growth yields (0.65 $\pm$ 0.05 mg l^{-1}) than the parental strain (see Fig. 1). The *mexE*/*mexF*/*oprN* operon mutant showed decreased growth rate (96 min doubling time) and 30–40% decrease in growth yield in TNT-containing medium (Fig. 1). This datum confirms the importance of extrusion mechanisms in bacterial TNT tolerance as discussed above.

It is known that in transcriptomic approaches not all up- or downregulated genes have a demonstrable role in the studied process, as the expression profile by itself does not define critical genes for a response (Segura *et al.*, 2005; Duque *et al.*, 2007b; Roca *et al.*, 2008). An explanation for the apparent lack of effect of many of the mutations could be the existence of paralogous genes that encode proteins with similar (if not identical) roles in the cell; for example, in the case of *xenD*, up to five paralogues are known in *P. putida* KT2440 (van Dillewijn *et al.*, 2008a,b).

Search for potential gene products involved in TNT tolerance but whose gene expression remains unchanged

The existence of genes, whose products are important to particular processes, but whose expression level do not exhibit changes in response to a specific cue remain unrevealed by DNA microarray assays, has been described (Roca *et al.*, 2008). To overcome this technical limitation we constructed a bank of 4000 independent Tn*5* single-insertion mutants obtained by pTn*5*cat (Marsch-Moreno *et al.*, 1998). Each clone was replicated in TNT-saturated solid medium in order to select for mutants that exhibit growth defects in this medium. Only one clone with scarce growth was isolated. Sequencing of the DNA adjacent to the plasposon insertion site revealed that the inactivated gene corresponded to PP_1232 (insertion site was at 138 codon). This gene encodes a conserved periplasmic protein belonging to the M48 peptidase family, a group of zinc-containing metalloproteases (Siddiqui *et al.*,

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2007) that may be involved in maintenance of membrane protein integrity (Sakoh *et al.*, 2005). These proteases have been previously suggested to be involved in linking cellular stress responses with exposure to toxic metals (Bouskill *et al.*, 2007) or heat shock (Kornitzer *et al.*, 1991).

In summary, the resistance of microorganisms to xenobiotics is important for the successful bioremediation of such compounds. *Pseudomonas putida* KT2440 shows a very high level of resistance to TNT. Transcriptional profiles and mutant analyses have revealed that in KT2440 the cellular resistance to this nitroaromatic compound is achieved through the cooperation of at least two mechanisms, involving both multidrug extrusion pumps and detoxifying enzymes. The existence of uncharacterized proteins that are induced in response to TNT does not allow us to exclude the existence of other yet unknown mechanisms involved in the microbial response to TNT.

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