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Physiological responses of *Pseudomonas putida* to formaldehyde during detoxification

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**Summary**

*Pseudomonas putida* KT2440 exhibits two formaldehyde dehydrogenases and two formate dehydrogenase complexes that allow the strain to stoichiometrically convert formaldehyde into CO₂. The strain tolerated up to 1.5 mM formaldehyde and died in the presence of 10 mM. In the presence of 0.5 mM formaldehyde, a sublethal concentration of this chemical, the growth rate decreased by about 40% with respect to growth in the absence of the toxicant. Transcriptomic analysis revealed that in response to low formaldehyde concentrations, a limited number of genes (52) were upregulated. Based on the function of these genes it seems that sublethal concentrations of HCOH trigger responses to overcome DNA and protein damage, extrude this toxic compound, and detoxify it by converting the chemical to CO₂. In strains bearing mutations of the upregulated genes we analysed growth inhibition by 1.5 mM HCOH and killing rates by 10 mM HCOH. Mutants in the MexEF/OprN efflux pump and in the DNA repair genes *recA* and *uvrB* were hypersensitive to 10 mM HCOH, the killing rate being three to four orders of magnitude higher than those in the wild-type strain. Mutants in other upregulated genes died at slightly higher or at similar rates to the parental strain. Regarding growth inhibition, we found that mutants in glutathione biosynthesis, stress response mediated by 2-hydroxy acid dehydrogenases and two efflux pumps of the MSF family were unable to grow in the presence of 1.5 mM HCOH. In an independent screening test we searched for mutants which were hypersensitive to formaldehyde, but whose expression did not change in response to this chemical. Two mutants with insertions in *recD* and *fhdA* were found which were unable to grow in the presence of 1.5 mM HCOH. The *recD* mutant was hypersensitive to 10 mM HCOH and died at a higher rate than the parental strain.

**Introduction**

Formaldehyde is an important building block in the manufacture of polymers and is produced at the rate of almost 30 million metric tons per year. However, formaldehyde is also a ubiquitous pollutant, probably because its highly volatile nature facilitates its dispersion through air to soils and waters. Early studies by Neely (1963) revealed that formaldehyde has bacteriostatic activity at sublethal concentrations due to its ability to disrupt growth and interfere with methionine biosynthesis. At higher concentrations formaldehyde is a potent biocidal agent and has thus been used to selectively inactivate *Bacillus anthracis* and *Bacillus subtilis* spores (Manchee et al., 1994; Sagripanti and Bonifacino, 1996) in soil and liquid suspensions. Formaldehyde has also been found to be efficient as a disinfectant in holding-tank sewages, particularly at alkaline pHs (Sobsey et al., 1974), and has been used to sterilize laboratory rooms and working surfaces (Taylor et al., 1969).

In addition to its artificial production by chemical synthesis, formaldehyde is a subproduct in the metabolism of histidine, choline and of a number of plant-derived methoxylated aromatic chemicals such as vanillate, veratrate and caffeate (Mitsui et al., 2000; Jiménez et al., 2004) by certain microorganisms. Formaldehyde has also been found as a subproduct of the metabolism of certain xenobiotic compounds such as the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Fournier et al., 2004), glyphosate (N-phosphonomethylglycine) (Shinabarger and Braymer, 1986), atropine (Long et al., 1997), and methenamine, a compound used to treat urinary tract infections (Musher and Griffith, 1974).

Because formaldehyde is produced naturally, microorganisms have evolved different mechanisms to counteract its toxicity. These mechanisms involve fixation through a number of metabolic pathways, and detoxification via oxidation to formate and CO₂. In a number of microorganisms formaldehyde has been shown to be assimilated via the serine pathway (Myers and Paretsky, 1961; Nester...
and Spizien, 1961) or the allulose pathway (ribulose phosphate cycle) (Goldberg and Matelis, 1975). *Burkholderia cepacia* growing on vanillate as a carbon source was shown to induce 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuliosomerase (Mitsui et al., 2000) to fix the formaldehyde produced. This pathway has also been shown to operate in the Gram-positive facultative methylo trophs *Mycobacterium gastri* MB19 (Mitsui et al., 2000), *Bacillus methanolicus* (Jakobsen et al., 2006) and in the archaean *Thermococcus koda*. In addition, *Burkholderia fungorum* LB400 exhibits multiple pathways for formaldehyde detoxification (Marx et al., 2004).

Kummerle and colleagues (1996) showed that *Escherichia coli* strain U3695 bears a plasmid encoding a glutathione- and an NAD+-dependent formaldehyde dehydrogenase that conferred a formaldehyde-resistant character to the strain. Similarly, an fghA mutant of *Parococcus denitrificans* defective in glutathione-dependent formaldehyde dehydrogenase was found to be unable to grow on methanol because of the accumulation of formaldehyde. In the *Methyllobacterium extorquens* facultative methylo troph, inactivation of the H4MPT pathway led to increased sensitivity to formaldehyde (Vorholt et al., 2002; Marx et al., 2003).

Our laboratory is interested in applying microbiological and molecular biological findings to the design of a treatment plant to degrade toxic products derived from the work done in molecular biology laboratories. One of the toxic products identified thus far is formaldehyde, which is often used as a disinfectant or as a cross-linker in the study of protein interactions, in sample fixation for microscopy and in other assays such as in northern blotting. *Pseudomonas putida* KT2440 is a soil microorganism (Table 1 and Ramos-González et al., 1991; Molina et al., 2000; Nakazawa, 2002) able to assimilate histidine and a number of methoxylated lignin-derived products (Jiménez et al., 2004; Duque et al., 2007a). Formaldehyde is produced in the metabolism of these chemicals, and this led us to explore whether *P. putida* KT2440 could metabolize formaldehyde, and to test its tolerance to this toxic compound. We found that strain KT2440 is highly tolerant to formaldehyde and that at the transcriptomic level this microorganism responds to sublethal concentrations of HCOH by upregulating 52 genes that fall within the categories of DNA repair, protein folding, efflux pumps, stress response, general metabolism and proteins of unknown function. We also found that the recD gene, whose transcriptional level did not change in response to HCOH, acts as a safeguard in formaldehyde tolerance, and that its inactivation has dramatic effects on the survival of *P. putida* KT2440 in the presence of this toxic compound.

**Results**

**Pseudomonas putida KT2440 detoxifies formaldehyde**

The annotation of the *P. putida* KT2440 genome showed three potential open reading frames (ORFs) that could

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**Table 1. Strains used in this study.**

| Strain                  | Relevant characteristics                                      | Reference                        |
|------------------------|---------------------------------------------------------------|----------------------------------|
| *P. putida* KT2440     | Parental strain                                              | Espinosa-Urgel and Ramos (2004)  |
| *P. putida* KT2440R    | Rifampicin resistant, derivative of KT2440                    | Duque et al. (2007a)             |
| Mutant (locus)         |                                                                |                                  |
| PRCC gshA (PP0243)     | Glutamate-cysteine ligase                                     | Duque et al. (2007a)             |
| PRCC fdhD (PP0257)     | Formate dehydrogenase accessory protein FdhD                 | Duque et al. (2007a)             |
| PRCC (PP0506)          | Translation initiation factor SU1                             | Duque et al. (2007a)             |
| PRCC xenB (PP0920)     | Xenobiopic reductase B                                        | Duque et al. (2007a)             |
| PRCC argl (PP1000)     | Ornithine carbamoyl transferase catabolic                     | Duque et al. (2007a)             |
| PRCC (PP1616)          | l-isomer specific 2-hydroxy acid dehydrogenase family protein | Duque et al. (2007a)             |
| PRCC (PP1617)          | Putative esterase                                             | Duque et al. (2007a)             |
| PRCC recA (PP1629)     | RecA protein                                                  | Duque et al. (2007a)             |
| PRCC (PP1974)          | Excinuclease ABC, B subunit                                    | Duque et al. (2007a)             |
| PRCC (PP3426)          | l-isomer specific 2-hydroxy acid dehydrogenase                | Duque et al. (2007a)             |
| PRCC (PP2546)          | Conserved hypothetical protein                                 | Duque et al. (2007a)             |
| PRCC (PP2647)          | Major facilitator family transporter                           | Duque et al. (2007a)             |
| PRCC (PP2695)          | Transcriptional regulator LysR family                         | Duque et al. (2007a)             |
| PRCC mexE (PP3425)     | Multidrug efflux RND membrane fusion protein MexE             | Duque et al. (2007a)             |
| PRCC (PP3658)          | Aromatic compound MFS transporter, putative                   | Duque et al. (2007a)             |
| PRCC vanA (PP3736)     | Vanillate demethylase A                                        | Duque et al. (2007a)             |
| PRCC (PP5107)          | Formyldehydratase dehydrogenase, putative                     | Duque et al. (2007a)             |
| PRCC (PP4098)          | Excinuclease ABC, C subunit                                    | Duque et al. (2007a)             |
| PRCC (PP4180)          | Conserved hypothetical protein                                 | Duque et al. (2007a)             |
| PRCC (PP4672)          | Exodeoxyribonuclease V, alpha subunit                         | Duque et al. (2007a)             |
| PRCC (PP5174)          | Monofunctional biosynthetic peptidoglycan transglycosylase    | Duque et al. (2007a)             |
| PRCC (PP5184)          | Putative glutamine synthetase                                 | Duque et al. (2007a)             |

All mutant strains are mini-Tn5 derivatives of KT2440 and are therefore kanamycin resistant.

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encode proteins with formaldehyde dehydrogenase activity, and two clusters of genes that could encode potential formate dehydrogenase complexes. Of the three ORFs encoding formaldehyde dehydrogenase, PP0328 and PP3970 were predicted to encode functional proteins, whereas PP1939 was predicted to encode a truncated protein (Nelson et al., 2002). The detoxification pathway from formaldehyde to CO₂ provides two relevant pieces of physiological data. (i) Pseudomonas putida KT2440 cannot use formaldehyde (1 mM) or formate (10 mM) as a carbon source, which is in agreement with the lack of metabolic enzymes for C1 metabolism in the genome of P. putida KT2440. (ii) Incubation of P. putida KT2440 in LB medium with ¹⁴C-formaldehyde (0.5 mM) led to the complete conversion of the labelled aldehyde into ¹⁴CO₂.

In this assay cells grown overnight on LB medium were diluted to an initial turbidity of 0.02 at 660 nm. After 1 h of incubation we added a mixture of H¹⁴COH and HCOH so that the number of counts per minute (CPM) was about 2.2 ± 0.1 x 10⁶ in the culture medium. Growth and CO₂ production were monitored. Figure 1 shows that ¹⁴CO₂ production and growth were parallel and that more than 95% of the original ¹⁴C-CPM was recovered as ¹⁴CO₂-CPM. No ¹⁴C was incorporated in the cell biomass.

To analyse the response of P. putida KT2440 to formaldehyde we first established the strain’s level of tolerance to this toxic compound. To this end we tested the effect of increasing concentrations of formaldehyde on growth in LB culture medium, and found that the parental strain grew with a doubling time of about 1.3 ± 0.1 h.

**Fig. 1.** Growth of P. putida KT2440 in the presence of low H¹⁴COH concentrations and production of ¹⁴CO₂. An overnight culture of KT2440 was diluted 100-fold and supplemented with 0.5 mM H¹⁴COH 1 h later so that the total ¹⁴C count added was around 2.2 ± 0.1 x 10⁶ CPM. ¹⁴CO₂ was collected on a 2 N NaOH solution placed into a tube sealed at the bottom of the culture flask. At the indicated times growth (■) and ¹⁴CO₂ (□) were monitored.

Formaldehyde concentrations ≥ 1.6 mM completely inhibited growth (not shown), although near 100% of the initial cells survived for more than 24 h. Lower concentrations of formaldehyde had less acute effects, i.e. in the presence of 0.5 mM formaldehyde cells grew at a rate of about 2.1 ± 0.1 h⁻¹, which is about 40% slower than in the absence of the toxic chemical.

We also tested the response of KT2440 cells in the exponential phase to sudden shocks of biocidal concentrations of HCOH (10 mM). We found that the addition of this concentration of HCOH resulted in a steady decrease in viable cells from about 2 x 10⁹ to about 10⁶ in 60 min (Fig. 2). The addition of 0.5–1 mM HCOH had no influence on the level of survival (Fig. 2). The above series of results indicated that P. putida can withstand up to 1.5 mM HCOH in the culture medium, and that concentrations as low as 0.5 mM do not affect survival but slightly retard growth, indicating that the strain ‘senses’ this sublethal concentration of HCOH.

**Fig. 2.** Killing kinetics of P. putida KT2440 upon exposure to different HCOH concentrations. The parental KT2440 strain was grown to reach the exponential phase (turbidity of 0.85 ± 0.05 at 660 nm), and at t = 0 the culture was divided in four aliquots to which we added nothing (■), 0.5 mM (□), 1 mM (△) or 10 mM (○) formaldehyde. At the indicated times the number of viable cells was estimated by spreading appropriate dilutions on LB plates.

Global response of P. putida KT2440 to sublethal formaldehyde concentrations

Efficient protection systems involving specific enzymes have emerged throughout evolution as stress responses to the exposure of microorganisms to unfavourable conditions (Mortersz et al., 2004; Palma et al., 2004; Maeda et al., 2008). Based on the physiological data reported above, we decided to establish the global transcriptional response of P. putida KT2440 to a sublethal HCOH con-
centration, i.e. 0.5 mM (see Experimental procedures). Upon formaldehyde treatment, *P. putida* KT2440 upregulated 52 genes and downregulated 14 (see Table 2 for upregulated genes and Table S1 for downregulated genes). Figure 3 shows the upregulated genes arranged according to general functions, and Table 2 shows the set of specific genes induced, their fold change and their role category. We were able to distinguish seven specific groups of genes (Fig. 3): DNA repair (Group 1), protein fate (Group 2), stress responses (Group 3), general metabolism (Group 4), membrane proteins (Group 5), regulators (Group 6) and the set of hypothetical proteins plus proteins of unknown function (Group 7). Group 1 includes a set of three enzymes involved in DNA repair.

Table 2. Genes upregulated in *P. putida* KT2440 (pWW0) 20 min after the addition of HCOH.

| TIGR identifier | Gene product | Gene name | Change (fold) | Role categorya | Mutantb |
|-----------------|--------------|-----------|---------------|----------------|---------|
| PP_0088         | Conserved hypothetical protein |             | 2.57          | 7              |         |
| PP_0243         | Glutamate-cysteine ligase gshA |           | 2.29          | 3              | Y       |
| PP_0257         | Formate dehydrogenase accessory protein FdhD fdhD | | 4.64          | 4              | Y       |
| PP_0266         | Conserved hypothetical protein |             | 6.85          | 7              |         |
| PP_0565         | IPP isomerase type 1 family protein | | 2.11          | 7              |         |
| PP_0566         | Translation initiation factor SU1 | | 2.41          | 2              | Y       |
| PP_0625         | ATP-dependent Clp protease, ATP-binding subunit ClpB clpB | | 1.87          | 2              |         |
| PP_0840         | Serine O-acetyltransferase cysE | | 2.10          | 5              |         |
| PP_0864         | Ornithine decarboxylase, putative | | 2.28          | 2              |         |
| PP_0920         | Xenobiotic reductase B xenB | | 1.89          | 3              | Y       |
| PP_1000         | Ornithine carbamoyltransferase argl | | 1.84          | 4              | Y       |
| PP_1360         | Chaperonin, 10 kDa groES | | 3.30          | 2              |         |
| PP_1361         | Chaperonin, 60 kDa groEL | | 2.40          | 2              |         |
| PP_1383         | BenF-like porin | | 2.22          | 5              |         |
| PP_1410         | Conserved hypothetical protein | | 1.93          | 7              |         |
| PP_1474         | Hypothetical protein | | 2.13          | 7              |         |
| PP_1615         | Transcriptional regulator, LysR family | | 1.95          | 6              |         |
| PP_1616         | D-isomer specific 2-hydroxy acid dehydrogenase family protein | | 9.21          | 3              | Y       |
| PP_1617         | Esterase, putative recA | | 2.59          | 7              | Y       |
| PP_1629         | RecA protein | | 2.02          | 1              | Y       |
| PP_1639         | SprT protein, putative | | 4.91          | 3              |         |
| PP_1640         | Conserved hypothetical protein | | 3.23          | 7              |         |
| PP_1974         | Excinuclease ABC, B subunit uvrB | | 2.17          | 1              | Y       |
| PP_1982         | Heat-shock protein IbpA ibpA | | 8.25          | 2              |         |
| PP_2302         | ATP-dependent protease La lon-2 | | 2.12          | 2              |         |
| PP_2426         | D-isomer specific 2-hydroxy acid dehydrogenase family protein | | 6.11          | 3              | Y       |
| PP_2568         | Ring-cleaving dioxygenase | | 2.06          | 4              |         |
| PP_2646         | Conserved hypothetical protein | | 2.17          | 7              | Y       |
| PP_2647         | Major facilitator family transporter | | 1.85          | 5              | Y       |
| PP_2695         | Transcriptional regulator, LysR family | | 2.12          | 6              | Y       |
| PP_3183         | SCO1/SenC family protein/cytochrome c | | 3.13          | 5              |         |
| PP_3254         | Nucleosidase, putative uvrC | | 2.07          | 1              |         |
| PP_3269         | Conserved hypothetical protein | | 2.64          | 7              |         |
| PP_3314         | Heat shock protein, HSP20 family | | 1.91          | 2              |         |
| PP_3321         | Conserved hypothetical protein | | 1.89          | 7              |         |
| PP_3349         | Major facilitator family transporter | | 2.11          | 5              |         |
| PP_3425         | Multidrug efflux RND transporter, membrane fusion protein MexE mexE | | 3.47          | 5              | Y       |
| PP_3427         | Multidrug efflux RND outer membrane protein OprN oprN | | 3.28          | 5              |         |
| PP_3658         | Aromatic compound MFS transporter, putative | | 3.51          | 5              | Y       |
| PP_3737         | Vanillate O-demethylase oxidoreductase vanB | | 2.40          | 4              | Y       |
| PP_3773         | Hypothetical protein | | 5.10          | 7              |         |
| PP_3901         | Conserved hypothetical protein | | 1.99          | 7              |         |
| PP_4178         | Dienelactone hydrolase family protein | | 2.90          | 4              |         |
| PP_4180         | Conserved hypothetical protein | | 3.54          | 7              | Y       |
| PP_4725         | Dihydricopicinolate reductase dapB | | 2.11          | 4              |         |
| PP_4727         | DnaK protein dnaK | | 2.33          | 2              |         |
| PP_4728         | Heat shock protein GspE gspE | | 3.40          | 2              |         |
| PP_4770         | Conserved hypothetical protein | | 2.23          | 7              |         |
| PP_5000         | Heat shock protein HslV hslV | | 2.40          | 2              |         |
| PP_5001         | Heat shock protein HslVU, ATPase subunit HslU hslU | | 2.83          | 2              |         |
| PP_5119         | Transcriptional regulator TetR family | | 2.33          | 6              |         |
| PP_5362         | Conserved hypothetical protein | | 2.37          | 7              |         |

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a. Role categories: 1, DNA repair; 2, protein fate; 3, stress; 4, metabolism; 5, membrane proteins; 6, regulatory functions; 7, hypothetical proteins and proteins of unknown function.

b. Mutants used in this study (available at the PRCC) are indicated by Y (yes).
Formaldehyde is known to damage DNA (Englesberg, 1952; Liu et al., 2006), and it is therefore not surprising for this kind of enzymes to be induced in response to formaldehyde. Repair and recombination systems, including uvrB (PP1974), uvrC (PP3254) and recA (PP1629), were induced more than twofold in response to sublethal HCHO concentrations (Table 2).

One of the groups best represented by upregulated genes was the protein fate group (Group 2), which includes a number of chaperones and is related with the well-established fact that aldehydes are highly reactive molecules that inactivate proteins (Liu et al., 2006). Activated genes include dnaK (PP4727), groEL (PP1361), and a variety of chaperones and proteases (PP0625, PP1360, PP1982, PP2302, PP4728, PP5000 and PP5001) (Table 2). GroEL was shown to be a general stress response protein that is rapidly produced in activated sludge exposed to stress (Duncan et al., 2002). In E. coli this chaperone, together with its cofactor GroES, promoted folding and could interact with as many as 250 different proteins (Kerner et al., 2005). Proteolysis is another stress mechanism used by bacteria (Henderson et al., 2006). Transcripts for three ATP-dependent proteases (ClpB, Lon-2, HslUV) were present at significantly higher levels in formaldehyde-treated cells than in control cells (Table 2). The Lon protease can degrade misfolded proteins and ClpB can promote ATP-dependent regulation of unfolding or disassembling proteins.

Group 3 was represented by a set of genes that encode proteins that protect against stress induced by toxic chemicals. The cytotoxic effects of formaldehyde may be increased under conditions in which free radicals are produced (Saito et al., 2005). In our assays two 2-hydroxy acid dehydrogenase family proteins (PP1616 and PP2426) were induced more than sixfold. Other genes whose expression levels increased more than twofold were the gshA gene (PP0243), whose gene product is involved in the biosynthesis of glutathione; the gene-encoding PP1639, a stress protein homologue to the E. coli SprT protein; and the xenobiotic reductase, XenB (PP0920). These proteins are involved in responses to oxidative stress (Segura et al., 2005), and some of them work in conjunction to protect biological membranes from degradation through GSH-dependent reduction of peroxi-dized phospholipids (Ferguson et al., 2006).

Among members of the metabolism group (Group 4), formaldehyde induced expression of one of the formate dehydrogenase genes, PP0257, which was induced more than 4.5-fold (Table 2). This confirms the expected response of KT2440 to HCHO. In certain microorganisms exposure to toxic chemicals leads to the general down-regulation of biosynthetic functions such as general metabolism and nucleotide and nucleic acid metabolism. This is the case in Nitrosomonas europaea when exposed to chloroform (Gvakharia et al., 2007) and in P. putida exposed to toluene (Dominguez-Cuevas et al., 2006). In contrast to these observations, we found that exposure of P. putida to formaldehyde induced a number of genes related to amino acid metabolism and breakdown of vanilllic acid. The latter is particularly surprising because formaldehyde is a by-product in the catabolism of vanillin acid, and one would not expect a pathway to be induced by an end-product.

In Group 5 (membrane proteins) three drug transport systems involved in the extrusion of toxic chemicals were induced (Poole, 2004). Pseudomonas putida KT2440 has almost 30 extrusion pumps potentially involved in detoxification (Ramos et al., 2001; 2002; Nelson et al., 2002). The mexEF pump genes together with the oprN gene were induced more than threefold in P. putida KT2440 in response to formaldehyde, which indicates that the MexEF/OprN efflux pump (PP3425, PP3427) could indeed be involved in the extrusion of this chemical. Two other potential efflux pumps belong to the major facilitator family and correspond to PP3349, which was induced 2.11-fold, and PP3658, which was induced 3.51-fold.

We found that three regulatory proteins, namely PP1615, PP2695 and PP5119, were upregulated. This set of proteins were members of Group 6, and at present the specific targets controlled by these regulators are unknown, but they may be involved in the control of some of the other upregulated genes. The last group (Group 7) included 15 proteins of unknown function or hypothetical proteins.

Response to formaldehyde of mutants in genes upregulated in response to formaldehyde

The Pseudomonas Reference Culture Collection (PRCC) has constructed a bank of mini-Tn5 mutants of P. putida KT2440 (Duque et al., 2007a), in which strains with muta-
tions in around 30% of ORFs have been mapped by sequencing the gene with the mini-Tn5 insertion (Duque et al., 2007a). We requested from this collection all available mutants in genes that are upregulated in response to formaldehyde (marked Y in Table 2). This allowed us to test mutants in all seven groups, including recA and uvrB mutants in the DNA repair group, and mutants in PP0566 in the protein fate group. In the group of stress functions we requested a gshA mutant encoding PP0243, annotated as involved in glutathione biosynthesis, and a mutant in the xenB gene encoding PP0920, which is involved in the response to xenobiotics (Segura et al., 2005). In this group we obtained mutants deficient in the synthesis of PP2426 or PP1616, which encodes putative D-isomer-specific 2-hydroxy acid dehydrogenase enzymes, i.e. two of the groups with the highest induction level (the change was 9.2-fold for PP1616 and 6.11 for PP2426). Within the group of membrane proteins, a mutant with a mini-Tn5 insertion in the mexE gene was available; this gene encodes the efflux element of a drug extrusion RND pump. We also requested an argI mutant; this gene encodes PP1000, annotated as involved in ammonium detoxification through the arginine pathway. We also included in the series of assays a mutant in PP2695, one of the regulatory proteins. In the group of hypothetical proteins we used mutants deficient in PP1617 and PP2646. In the general metabolism group, a mutant in the vanB gene was available.

The assays were designed to determine growth rates in the presence of 1.5 mM HCOH and killing kinetics after exposure to 10 mM HCOH. The findings for growth rates disclosed three types of mutants in upregulated genes. Type 1 included four mutants (PP0243, PP1000, PP1616 and PP4180) (Table 3) that failed to grow at all in the presence of 1.5 mM HCOH. The second type consisted of mutants whose growth rates were at least twofold lower than that of the wild type (PP1617, PP2426, PP2647, PP2648, PP3425, PP3658, PP3736, PP4098 and PP4672), and the third type comprised five mutants that grew at a rate similar to the parental strain (PP0566 PP0920, PP1629, PP1974, PP2695).

The results of the killing kinetics assays distinguished two groups: one group in which the killing rate was similar to that of the wild type, and represented in Fig. 4 by mutants in the gshA and xenB genes, and another in which the mutants died at a faster rate than the parental strain. This group includes three mutants, namely, mutants deficient in the DNA repair genes recA (PP1629) and uvrB (PP1974) (see killing kinetics in Fig. 4) and in the MexE efflux pump. All of these mutants were particularly sensitive to HCOH shocks. These results suggest that many upregulated genes indeed play a role in tolerance to formaldehyde.

### Mutant response to formaldehyde in downregulated genes

Two mutants deficient in PP5107 and PP5184 that encode mtgA, and one of the three glutamine synthetases, respectively, were available among the set of downregulated genes. These mutants grew in the presence of 1.5 mM HCOH at the same rate as the parental

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Table 3. Doubling time of *P. putida* and its isogenic mutants in the presence of formaldehyde.

| UP mutant (TIGR identifier) | Gene product | Doubling time (h)a |
|-----------------------------|--------------|--------------------|
| KT2440R                     |              | 2.1                |
| PP_0243 Glutamate-cysteine ligase | No growth    | 15.3              |
| PP_0257 Formate dehydrogenase FdhD | 2.4          | 2.4                |
| PP_0566 Translation initiation factor SUI1 | No growth | 2.4                |
| PP_0920 Xenobiotic reductase B | 2.4          | 2.4                |
| PP_1000 Ornithine carbamoyltransferase | No growth | 2.4                |
| PP_1616 D-isomer specific 2-hydroxy acid dehydrogenase | No growth | 2.4                |
| PP_1617 Esterase, putative | 8.7          | 8.7                |
| PP_1629 RecA protein | 2.8          | 2.8                |
| PP_1974 Uvr B | 2.2          | 2.2                |
| PP_2426 D-isomer specific 2-hydroxy acid dehydrogenase | 9.5          | 9.5                |
| PP_2647 Major facilitator family transporter | 8.8 | 8.8                |
| PP_2648 Universal stress protein family | 8.2 | 8.2                |
| PP_2695 Transcriptional regulator LysR family | 2.5 | 2.5                |
| PP_3425 Multidrug efflux RND membrane fusion protein MexE | 4.7 | 4.7                |
| PP_3658 Aromatic compound MFS transporter, putative | 14.0 | 14.0                |
| PP_3736 Vanillate demethylase A | 17.9 | 17.9                |
| PP_3970 Formaldehyde dehydrogenase | 14.4 | 14.4                |
| PP_4098 Excinuclease ABC, C subunit | 10.7 | 10.7                |
| PP_4180 Conserved hypothetical protein | No growth | 17.1                |
| PP_4672 RecD protein | 17.1 | 17.1                |

a. The parental strain or its mutant derivatives were incubated on LB medium with 1.5 mM formaldehyde to an initial turbidity of about 0.05 units at 660 nm. Growth was monitored for at least 24 h and doubling times were estimated at the exponential phase.
strain, and their killing kinetics were almost identical to that of the parental strain (not shown). This suggests that these gene products play no role in tolerance to formaldehyde.

Identification of other potential gene products involved in HCOH tolerance whose transcriptional level did not change in response to this toxic compound

It is known from studies on innate tolerance to drugs that certain genes that encode stress response functions are not regulated. Therefore, these genes cannot be identified with microarray assays, as they will be masked in the set of genes whose variations are considered non-significant. The approach we took consisted on identifying mutants unable to grow in the presence of 0.5 mM HCOH. To this end a bank of 5000 independent mini-Tn5 mutants was constructed, as described in Experimental procedures, and tested to find mutants in genes other than those that were upregulated. This allowed us to identify six mutants. Sequencing of the mini-Tn5 insertion site revealed knockouts in only two new genes. The insertions were in the recD gene encoding RecD (PP4672), which is involved in DNA repair and in the fhdA gene encoding a glutathione-dependent formaldehyde dehydrogenase (PP3970). These mutants were also exposed to 0.5 mM HCOH (test for growth inhibition) and 10 mM HCOH (to establish their killing kinetics). Figure 4 shows that the killing rate of the recD mutant in the presence of 10 mM HCOH was two orders of magnitude faster than in the parental strain. Growth of the recD mutant was deficient in the presence of 0.5 mM HCOH, with a doubling time that was almost eightfold higher than that of the parental strain (Table 3).

As mentioned above, we found a mutant (PP3970) in the formaldehyde to CO2 pathway was found, and we tested the effect of deficiency in HCOH metabolism in tolerance to this toxic compound. The rate of killing in the presence of 10 mM HCOH was similar to that of the parental strain (Nelson et al., 2002); however, growth was strongly inhibited by 1.5 mM HCOH. The doubling time was above 14.4 ± 0.2 h, which is about sevenfold higher than that of the parental strain.

Discussion

We have shown that P. putida exhibits an innate level of tolerance to formaldehyde that allows it to grow in the presence of up to 1.5 mM of this compound. This level of resistance is similar to that tolerated by E. coli (Kummerle et al., 1996), Burkholderia (Marx et al., 2004), Mycobacterium (Mitsui et al., 2000), Bacillus or Methylobacterium (Trujillo and Lindell, 1973; Jakobsen et al., 2006; Orita et al., 2006), and as it is a non-pathogenic strain with high potential for biodegradation (Abril et al., 1989; Jiménez et al., 2004) we decided to study in detail its response to this toxic compound. We found that concentrations of formaldehyde 6.6-fold higher than those that inhibit growth lead to cell death, which indicates that once the safeguard system against this compound is circumvented, cells are killed.

In this study we identified genes involved in the response of P. putida KT2440 to sublethal concentrations of formaldehyde, which has helped us to understand the initial response of the strain to this toxic compound. We have also analysed a set of mutants in upregulated genes, and studied their ability to grow in the presence of sublethal concentrations of HCOH and survive at high HCOH concentrations. Correlations between gene induction, growth inhibition and increased sensitivity to HCOH allowed us to establish a network of genes that encode proteins involved in response to this toxic chemical.

Several pathways for the metabolism of formaldehyde into formate and their simultaneous functioning have been shown to exist in Burkholderia (Marx et al., 2003; 2004). Mutations in any of these pathways led to increased formaldehyde sensitivity when Burkholderia cells were grown on methanol. Formaldehyde in P. putida KT2440 can be detoxified through two oxidation pathways that allow toxic aldehyde to be converted to the less toxic formate, which is subsequently converted into CO2. 14C assays allowed us to record mass balances, which revealed the complete lack of incorporation of 14C-formaldehyde into cell carbon skeletons. This contrasts with studies in other Pseudomonas sp. strains that have been reported to grow on formaldehyde through the use of the ribulose monophosphate
pathway (Goldberg and Matelis, 1975). One of our mutant strains with a defect in one of the formaldehyde dehydrogenases (PP3970) grew more slowly than the parental strain in the presence of 1.5 mM HCOH. This indicates that HCOH conversion to CO₂ is also a relevant detoxification pathway in *P. putida*.

Based on the analysis of the growth rates in the presence of sublethal concentrations of HCOH and the killing rates of the mutants, we suggest that in addition to the detoxification system, other systems were efficiently operating to protect KT2440 from formaldehyde. A potential mechanism for formaldehyde tolerance is the active extrusion of HCOH by the cells. In this connection it is worth noting that the MexEF/OprN extrusion pump was specifically induced in response to formaldehyde. To provide support for the role of the MexEF efflux pump in tolerance to formaldehyde, we tested HCOH tolerance in a *mexE*-deficient background. We found that the strain died more quickly than the wild type at high HCOH concentrations, and that growth on LB supplemented with 1.5 mM HCOH was significantly more slowly than that of the parental strain (Table 3). This suggests that HCOH extrusion is relevant at any concentration of this toxic compound. To our knowledge this is the first case in which an RND efflux pump is identified as able to remove a small molecule such as HCOH. RND efflux pumps can remove substrates from the periplasmic space, the cell membrane and the bacterial cytoplasm (Yu *et al.*, 2005; Takatsuka and Nikaido, 2006). Given the nature of HCOH, this chemical probably enters the vestibule of the pump from the cytoplasm.

It should also be noted that two other efflux pumps belonging to the MFS family (PP2647 and PP3658) were also induced. These mutants grew at least four times more slowly than the parental strain at low concentrations; however, their behaviour in killing assays was similar to that of the parental strain. This indicates that the two MFS efflux pumps play a role in tolerance to low HCOH concentrations.

In agreement with earlier studies in other microorganisms showing that HCOH damages both DNA and proteins (Englesberg, 1952) is our finding that a set of DNA-repairing genes was induced together with chaperones with protease activity. Mutants deficient in DNA repair *recA*, *recD* and *uvrB* genes were extremely sensitive to high concentrations of formaldehyde with killing rates around 1000-fold faster than the parental strain, indicating that DNA repair is of utmost importance in tolerance to damage at the genetic level. The *uvrB* and *recA* mutants are interesting as at sublethal HCOH concentrations they can grow at rates similar to the parental strain; however, they were hypersensitive at high HCOH concentrations. This contrasts with the *recD* mutant, which grew very slowly at low HCOH concentrations, suggesting that constitutive levels of RecD are *sine qua non* for survival. No chaperone-deficient mutants are available at present in *P. putida*; however, because of the large number of induced chaperones and their overlapping activities, we assumed that single mutants would not be as sensitive as mutants in the DNA repair system.

Formaldehyde can also lead to a kind of oxidative stress through alterations in the functioning of the respiratory chain (Nachin *et al.*, 2005). In agreement with this hypothesis, some oxidative stress systems, such as hydroxy acid dehydrogenases and glutathione biosynthesis, were found to be induced by formaldehyde. Mutants deficient in the synthesis of glutathione or in PP1616 and PP2426, which encode the two α-isomer-specific 2-hydroxy acid dehydrogenases, failed to grow or grew deficiently in the presence of sublethal concentrations of formaldehyde. We propose that under adverse conditions, *P. putida* KT2440 sets up a process intended to survive rather than to grow.

Although transcriptomic approaches helped us to identify genes that were induced or repressed in response to formaldehyde as an environmental cue, not all induced genes play a role in these responses, and gratuitous induction has been observed (Ramos *et al.*, 2001). For instance, in *P. putida* DOT-T1E and *P. putida* S12, exposure to toluene led to an increase in the expression of fructose-1,6-bisphosphate aldolase and phosphoenolpyruvate carboxykinase, but mutants in these genes exhibited parental behaviour (Segura *et al.*, 2005). In this connection we found that mutants in XenB (PP0920) and the translation initiation factor SUI1 (PP0566) showed parental behaviour. This could be due to the presence of multiple equivalent genes, as it is known that up to six *xenB*-like genes exist in *P. putida* (P. van Dillewijn, R. Wittich and J.L. Ramos, unpubl. data), and several translational initiation factors are encoded in the chromosome of the strain. If they have overlapping activities, the loss of one of them may not significantly affect growth.

We also hypothesized that the expression of genes that encode proteins essential for formaldehyde tolerance may not be induced in response to this chemical. To test whether this was the case in HCOH resistance, we generated a bank of 5000 mini-Tn5 independent mutants and searched for HCOH-sensitive ones. In this screening we found two more mutants, one in the *recD* gene and another in the *thdA* gene; the level of expression of these genes did not change significantly in response to formaldehyde. As the RecD protein is involved in DNA repair, our results further support that DNA repair is critical for survival when cells are exposed to low concentrations of HCOH. As mentioned in *Introduction*, *P. putida* metabolizes a number of methoxylated aromatic compounds that yield formaldehyde as a by-product (Jiménez *et al.*, 2004). The constitutively expressed RecD protein may

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represent the first barrier acting as an innate defence against internally produced formaldehyde. In this regard the constitutively expressed PP3970 formaldehyde dehydrogenase participates in the detoxification of HCOH via its conversion to innocuous formate, which is subsequently converted to CO₂.

In summary, our results show that *P. putida* is highly resistant to formaldehyde, and that resistance is multifactorial. According to our observations, resistance can involve detoxification via conversion of the toxic chemical to CO₂, extrusion of this molecule by the MexEFOprN efflux pump, induction of a large series of enzymes that protect DNA and protein from direct HCOH damage, and from lateral effects such as oxidative damage. Mutants in any of the systems lead to increased sensitivity to the toxic compound. However, the intimate mechanisms leading to the activation of genes and the potential cascade of regulation have not yet been elucidated.

**Experimental procedures**

**Bacterial strains and growth conditions**

The strains and plasmids used in this study are shown in Table 1. Bacterial cells were grown on LB medium at 30 °C or on modified M9 minimal medium with glucose [0.5% (w/v)] or citrate (16 mM) as a carbon source (Abril et al., 1989; Duque et al., 2007b).

**Mineralization of ¹⁴C-formaldehyde**

In experiments with ¹⁴C-formaldehyde, erlenmeyer flasks with a small tube sealed at the bottom of the flask were used. In the small tube 5 ml of a 2 N NaOH solution was placed to collect evolved ¹⁴CO₂.

**Mini-Tn5 mutagenesis**

For mini-Tn5 mutagenesis we followed the protocol described by De Lorenzo and colleagues (1990). *Pseudomonas putida* KT2440 was mated for 4 h with *E. coli* CC118λpir (pUT-Km, pRK600). Cells were then suspended and around 20 000 transconjugants were selected on M9 minimal medium with citrate and 25 μg ml⁻¹ kanamycin. Transconjugants were screened for formaldehyde sensitivity using a Pick Up robot. Clones were spotted onto 96-well ELISA gants and without 0.5 mM formaldehyde. Clones were spotted onto 96-well ELISA plates with and without 0.5 mM formaldehyde. Clones that did not grow with formaldehyde were considered formaldehyde-sensitive and were kept for further assays.

**Formaldehyde shock assays in liquid culture medium**

Cells were grown overnight in 30 ml of LB medium. On the following day the cultures were diluted 1:100 in the same medium and grown under the same conditions. When the cultures reached the mid-exponential growth phase (turbidity of 0.85 ± 0.05 at 660 nm) they were divided in two halves. We added 10 mM HCOH to one of the aliquots, and the other one was kept as a control. The number of viable cells was determined as cfu ml⁻¹ before the stressor agent was added and 10, 30 and 60 min later. These assays were run in duplicate and repeated at least three times. The values reported here are the average of at least four determinations.

**DNA techniques**

Plasmid DNA was isolated according to the alkaline lysis method with the QiAprep Spin Plasmid Minipreps Kit. Total DNA was isolated as described by Ramos-González and colleagues (1991). DNA digestions with restriction enzymes, ligations and transformations were performed with standard procedures. For PCRs the standard mixture (25 μl) contained 10 ng of DNA, 200 μM of each deoxynucleoside triphosphate, 50 pmol of each primer, 2 μl of dimethyl sulfoxide and 0.25 U of Taq polymerase. The PCR conditions were as follows: 4 min at 95°C and then 35 cycles at 60°C for 45 s, 72°C for 30–180 s and 94°C for 4 s, followed by a final 5 min step at 72°C.

**RNA isolation and preparation of labelled cDNA**

*Pseudomonas putida* microarrays

*Pseudomonas putida* arrays (Progenika, Spain) consisting of 5539 gene-specific oligonucleotides (50-mer) spotted in duplicate onto γ-amino silane-treated 25 × 75 microscope slides and bonded to the slide with UV light and heat (Yuste et al., 2006; Duque et al., 2007b). The oligonucleotides represented 5350 of the 5421 predicted ORFs annotated in the *P. putida* KT2440 genome (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org_search=pse&org=pp). In addition, 140 of the 148 putative ORFs predicted for the TOL plasmid pWW0 were also represented. The chips were also endowed with homogeneity controls consisting of oligonucleotides for the *rpoD* and *rpoN* genes spotted at 20 different positions, as well as duplicate negative controls at 203 predefined positions. Further details of the array characteristics were reported elsewhere by Yuste and colleagues (2006) and Duque and colleagues (2007b). Assays under each set of conditions were replicated at least three times.

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owed by purification with RNeasy columns (Qiagen). Then the concentration of RNA was determined spectrophotometrically and its integrity was assessed by agarose gel electrophoresis. Preparation of fluorescently labelled cDNA, hybridization and data analysis were carried out as described by Duque and colleagues (2007b). Images were obtained at 10 μm resolution and the background-subtracted median spot intensities were determined using GenePix Pro 5.1 image analysis software (Axon Instruments). Signal intensities were normalized by applying the lowest intensity-dependent normalization method (Yang et al., 2002). Data were analysed in the ‘log ratio’ mode with Almazen System software (Alma Bioinformatics S.L.). The results obtained from experiments run in triplicate were normalized and filtered to identify genes with statistically significant increases or decreases and significant P-values. A particular ORF was considered differentially expressed if the change was at least 1.8-fold and if the P-value was $\leq 0.05$.

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**Supplementary material**

The following supplementary material is available for this article online:

**Table S1.** Gene downregulated in *P. putida* KT2440 (pWW0) 20 min after the addition of HCOH.

This material is available as part of the online article from http://www.blackwell-synergy.com