Growth of *Pseudomonas putida* F1 on styrene requires increased catechol-2,3-dioxygenase activity, not a new hydrolase

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Pseudomonas putida F1 cannot grow on styrene despite being able to degrade it through the toluene degradation (*tod*) pathway. Previous work had suggested that this was because TodF, the *meta*-fission product (MFP) hydrolase, was unable to metabolize the styrene MFP 2-hydroxy-6-vinylhexa-2,4-dienoate. Here we demonstrate via kinetic and growth analyses that the substrate specificity of TodF is not the limiting factor preventing F1 from growing on styrene. Rather, we found that the metabolite 3-vinylcatechol accumulated during styrene metabolism and that micromolar concentrations of this intermediate inactivated TodE, the catechol-2,3-dioxygenase (C23O) responsible for its cleavage. Analysis of cells growing on styrene suggested that inactivation of TodE and the subsequent accumulation of 3-vinylcatechol resulted in toxicity and cell death. We found that simply overexpressing TodE on a plasmid (pTodE) was all that was necessary to allow F1 to grow on styrene. Similar results were also obtained by expressing a related C23O, DmpB from *Pseudomonas* sp. CF600, in tandem with its plant-like ferredoxin, DmpQ (pDmpQB). Further analysis revealed that the ability of F1 (pDmpQB) and F1 (pTodE) to grow on styrene correlated with increased C23O activity as well as resistance of the enzyme to 3-vinylcatechol-mediated inactivation. Although TodE inactivation by 3-halocatechols has been studied before, to our knowledge, this is the first published report demonstrating inactivation by a 3-vinylcatechol. Given the ubiquity of catechol intermediates in aromatic hydrocarbon metabolism, our results further demonstrate the importance of C23O inactivation as a determinant of growth substrate specificity.

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INTRODUCTION

Much is known about the mechanisms by which bacteria degrade aromatic compounds (reviewed by Díaz, 2004). However, even in model systems we still have much to learn about the determinants of growth substrate specificity. The toluene degradation (*tod*) pathway in *Pseudomonas putida* F1 (Gibson *et al.*, 1968) serves as a prototypical model for aromatic hydrocarbon degradation (Zylstra & Gibson, 1989; Zylstra *et al.*, 1988) (Fig. 1). Multiple substrates are capable

of inducing the *tod* operon, although only a subset of these compounds support growth (Cho *et al.*, 2000; Lacal *et al.*, 2006).

In the *tod* operon, initial dioxygenation of toluene is performed by the multi-component toluene dioxygenase, a versatile enzyme encoded by *todC1C2BA* (Jiang *et al.*, 1999, 1996; Yeh *et al.*, 1977; Zylstra & Gibson, 1989). Following dioxygenation, dehydrogenation of the *cis*-dihydrodiol leads to 3-methylcatechol production. TodE (3-methylcatechol 2,3-dioxygenase) catalyses cleavage of this substrate, forming the yellow-coloured *meta*-fission product (MFP) 2-hydroxy-6-oxo-methylhexa-2,4-dienoate (6-methyl HODA), which is then hydrolysed to acetic acid and 2-hydroxypenta-2,4-dienoate by TodF. After hydrolysis, TodG, TodH and TodI further degrade the pentadienoate to form pyruvate and acetate (Zylstra *et al.*, 1988). The entire catabolic *todXFC1C2BADEGIH* operon is transcribed from a single promoter designated P_{todX}. Transcriptional activation of this promoter is mediated by TodS and TodT, a constitutively expressed, two-component system located downstream of

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Abbreviations: 6-vinyl HODA, 2-hydroxy-6-vinylhexa-2,4-dienoate; 6-methyl HODA, 2-hydroxy-6-oxo-methylhexa-2,4-dienoate; C23O, catechol-2,3-dioxygenase; MFP, *meta*-fission product.

Four supplementary figures and a supplementary table detailing the PCR primers used in this study are available with the online version of this paper.

Fig. 1. Pathway of toluene degradation by enzymes of the *tod* operon. Based on a figure by Zylstra *et al.* (1988). See text for details.

todH (Busch *et al.*, 2007; Lacal *et al.*, 2006, 2008; Lau *et al.*, 1997; Mosqueda *et al.*, 1999).

Studies of F1 and related *P. putida* strains expressing the *tod* pathway have implicated TodF (6-methyl HODA hydrolase) as a key determinant of growth substrate specificity, particularly in the metabolism of biphenyl and *n*-alkyl benzenes (Cho *et al.*, 2000; Choi *et al.*, 2003; Furukawa *et al.*, 1993; Ohta *et al.*, 2001; Seah *et al.*, 1998, 2000). In several cases, it has been shown that expression of an alternative MFP hydrolase is sufficient to allow these strains to grow on biphenyl (Choi *et al.*, 2003; Furukawa *et al.*, 1993; Ohta *et al.*, 2001). Analysis of available MFP hydrolase crystal structures has revealed a conserved, structural basis for the strict substrate specificity of these enzymes and underlined their importance in aromatic degradation pathways (Fushinobu *et al.*, 2002; Habe *et al.*, 2003; Nandhagopal *et al.*, 2001).

Mechanism-based inactivation of catechol-2,3-dioxygenases (C23Os) has been implicated as another potential determinant of growth substrate specificity (Bartels *et al.*, 1984; Cerdan *et al.*, 1994; Klecka & Gibson, 1981; Ramos *et al.*, 1987; Rojo *et al.*, 1987; Vaillancourt *et al.*, 2002). Inactivation of C23Os during substrate cleavage appears to involve primarily accidental oxidation of active-site Fe(II) to Fe(III) (Cerdan *et al.*, 1994; Vaillancourt *et al.*, 2002). Mechanism-based inactivation has been demonstrated most often with chlorocatechols, but *para*-substituted alkyl catechols can also exert a similar effect (Vaillancourt *et al.*, 2006). Inactivation of this type has been shown to limit the substrate range of toluates degraded by the TOL pathway, polychlorinated biphenyls degraded by the *bph* pathway and chlorobenzenes degraded by the *tod* pathway (Cerdan *et al.*, 1994; Klecka & Gibson, 1981; Ramos *et al.*, 1987; Rojo *et al.*, 1987; Vaillancourt *et al.*, 2002; Ward *et al.*, 2004).

In vitro, enzymic inactivation of C23Os can be reversed by treatment with reducing agents or incubation under anaerobic conditions with Fe(II) (Vaillancourt *et al.*, 2006). *In vivo*, some organisms appear to utilize separate proteins, [2Fe–2S] plant-like ferredoxins, to reactivate oxidized C23Os and restore proper function. This has been most clearly demonstrated in the case of XylT, a novel plant-like [2Fe–2S] ferredoxin in the TOL pathway that has

been shown to reactivate XylE, a C23O homologous to TodE (Hugo *et al.*, 1998, 2000; Polissi & Harayama, 1993). Similar enzymes are encoded in other aromatic degradation operons including DmpQ, a XylT-like ferredoxin in the *dmp* operon encoding the metabolism of phenols in *Pseudomonas* sp. CF600 (Powlowski & Shingler, 1994). Like XylT, DmpQ is capable of reactivating XylE, suggesting a conserved mechanism of action for [2Fe–2S] ferredoxins (Hugo *et al.*, 2000). By serving as a natural repair system in aromatic degradation pathways, it is likely that [2Fe–2S] ferredoxins play key roles in augmenting the range of growth substrates in numerous organisms.

Despite its structural similarity to ethylbenzene, which can be used by F1 for growth, styrene does not support growth of F1 even though it induces the *tod* operon to moderate levels (Cho *et al.*, 2000; Lacal *et al.*, 2006). The observed accumulation of yellow MFP by F1 exposed to styrene, along with reportedly low levels of F1 MFP hydrolase activity toward the styrene MFP, has previously led to the supposition that TodF functions as an enzymic block in styrene metabolism (Cho *et al.*, 2000). There are, however, no published reports on the kinetics of styrene MFP hydrolysis. Here, we present a kinetic analysis of the styrene MFP hydrolase activity of TodF and provide evidence that its substrate specificity does not limit styrene metabolism as previously suggested. Instead, we demonstrate that 3-vinylcatechol inactivates TodE, the *meta*-cleaving C23O in the *tod* operon, and suggest that substrate-level inactivation of this C23O is the limiting metabolic factor which prevents F1 from growing on styrene. Together, these data suggest an expanded role for C23O inactivation as a key determinant of growth substrate specificity during the degradation of aromatics.

METHODS

Strains and growth conditions. *P. putida* F1 and its plasmid-carrying derivatives were grown at 30 °C in Luria–Bertani medium (LB) supplemented with appropriate antibiotics (25 mg chloramphenicol l⁻¹ or 150 mg ampicillin l⁻¹) or minimal salts medium (MSM) (Focht, 1994) containing suitable growth substrates. Toluene or styrene was provided in vapour phase for growth on agar plates or at a concentration of 5 mM in liquid media. When specified, acetate was supplied at a concentration of 20 mM. *Escherichia coli* JM109

(Yanisch-Perron *et al.*, 1985) and S17 (de Lorenzo & Timmis, 1994) strains were grown at 37 °C in LB containing 15 mg nalidixic acid l⁻¹ and 50 mg streptomycin l⁻¹, respectively. Strains containing pBBR1MCS-5 and derivatives were selected on medium containing 25 mg gentamicin l⁻¹.

Plasmid and strain construction. The plasmid vectors used in this work are described in Table 1. Specific primers for genes in the *tod* and *dmp* operons were designed based on available *P. putida* F1 (GenBank accession no. NC_009512) and *Pseudomonas* sp. CF600 (GenBank accession nos X60835 and X60836) (Shingler *et al.*, 1992) sequences, respectively (Supplementary Table S1, available with the online version of this paper). PCR was performed by using the HotStart Master Mix kit from Qiagen and a PTC-200 DNA Engine thermocycler from MJ Research. PCR fragments were digested with appropriate enzymes and ligated into pBBR1MCS-5 (Gm^R) or pRSET-A (Amp^R) by using T4 DNA ligase (Promega). The resultant plasmids were transformed into appropriate hosts by electroporation. Transformants were selected on LB plates containing antibiotics and screened by PCR.

GC-MS detection of 3-vinylcatechol. F1 was grown for 48 h on MSM styrene+acetate. After removal of cells via centrifugation (10 000 g at 4 °C for 10 min), intermediates in the supernatant were derivatized with 1.5 % K₂CO₃ and 0.5 % acetic anhydride (Baker *et al.*, 1994). Following extraction with chloroform, samples were analysed on a Hewlett Packard 6890 GC coupled to an HP 5973 MS detector. An HP-5MS column (29.2 mm × 0.25 mm × 0.25 µm) was used with a temperature range of 60–300 °C at a ramp of 30 °C min⁻¹.

Biosynthesis of 3-vinylcatechol and MFPs. JM109 (pDTG601) (pTodD) and S17 (pDmpB) were grown overnight in LB and diluted 1:100 into fresh media of the same type. At mid-exponential phase (OD₆₀₀=0.4), IPTG was added to a concentration of 1 mM. Following a 3 h induction, cultures were washed and resuspended in 30 mM K₃PO₄ buffer (pH 7.0). For synthesis of 3-vinylcatechol, a suspension of JM109 (pDTG601) (pTodD) was exposed to 1 mM styrene and incubated for 8 h at 37 °C. The supernatant was then harvested following centrifugation (10 000 g at 4 °C for 15 min), filter-sterilized and kept on ice prior to use. The purity of 3-vinylcatechol was >99 % as determined via GC-MS. For synthesis of 6-methyl HODA and 2-hydroxy-6-vinylhexa-2,4-dienoate (6-vinyl HODA), toluene or styrene was added in the vapour phase to a 1:1 co-culture of JM109 (pDTG601) (pTodD) and S17 (pDmpB) until the suspension turned deep yellow, indicating the presence of concentrated MFP. Final concentrations of 6-methyl HODA and

6-vinyl HODA were determined spectrophotometrically by measuring absorbance at 389 and 425 nm, respectively. A reported absorption coefficient of 11.9 mM⁻¹ cm⁻¹ was used for 6-methyl HODA and verified experimentally, while an absorption coefficient of 10.7 mM⁻¹ cm⁻¹ was calculated for 6-vinyl HODA.

Preparation of cell-free extracts. *P. putida* F1 and its derivatives were grown overnight at 30 °C on MSM acetate with toluene present in the vapour phase as an inducer. JM109 (pTodF) was grown overnight in LB, inoculated 1:100 into fresh medium and induced with 1 mM IPTG at an OD₆₀₀ of 0.4. Following induction, cell suspensions were washed and resuspended in sonication buffer (100 mM Tris/HCl, 10 µM PMSF, 1 µM DTT, pH 8) and lysed through sonication. Supernatants were harvested after centrifugation for 15 min (12 000 g at 4 °C). Protein concentrations of cell-free extracts were determined with a Bradford protein assay (Bio-Rad) following the manufacturer's instructions. Extracts were stored at -20 °C in 50 % glycerol until use.

Purification of His-tagged TodF. *E. coli* BL21(DE3) pLysS (pRSET-A: TodF_{His}) was grown overnight at 37 °C, inoculated 1:50 into 500 ml fresh SOB media (Hanahan, 1983) and induced with 1 mM IPTG at an OD₆₀₀ of 0.4. After 16 h incubation at 37 °C under gentle shaking, cells were harvested by centrifugation (10 000 g at 4 °C) for 10 min and resuspended in lysis equilibration wash (LEW) buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0). Prior to lysis by sonication, the cell suspension was placed on ice and incubated with lysozyme (0.2 mg ml⁻¹) for 30 min with gentle mixing. Crude lysate was collected following centrifugation (12 000 g at 4 °C) for 15 min and added to 0.5 g Prepease (USB) nickel resin. Protein-bound resin was separated through gravity sedimentation and washed three times with 15 ml LEW buffer. Three 5 ml aliquots of LEW buffer containing 250 mM imidazole were used to elute TodF_{His}.

Enzyme assays

MFP degradation. Degradation of MFPs was quantified by measuring the rate of decrease in absorbance at room temperature. Wavelengths of 389 and 425 nm were used for 6-methyl HODA and 6-vinyl HODA, respectively. Concentrations of MFP were estimated by using the appropriate absorption coefficients. Degradation assays were performed at 25 °C in a 96-well plate format by using cell-free extracts in triplicate. Initial degradation rates were measured and standardized to protein content for comparisons between strains.

Determination of kinetic parameters. The kinetic parameters of purified TodF_{His} were determined through measurement of initial

Table 1. Plasmids used in this study

Plasmid	Characteristics	Reference
pBBR1MCS-5	Gm ^R , broad host range cloning vector	Kovach <i>et al.</i> (1995)
pRSET A	Amp ^R , high level protein expression vector for Ni ²⁺ affinity purification	Kroll <i>et al.</i> (1993)
pTodE	Derivative of pBBR1MCS-5 containing <i>todE</i> , TodE expression vector	This study
pDmpB	Derivative of pBBR1MCS-5 containing <i>dmpB</i> , DmpB expression vector	This study
pDmpQB	Derivative of pBBR1MCS-5 containing <i>dmpQB</i> , DmpQB expression vector	This study
pDmpQ	Derivative of pBBR1MCS-5 containing <i>dmpQ</i> , DmpQ expression vector	This study
pTodF	Derivative of pBBR1MCS-5 containing <i>todF</i> , TodF expression vector	This study
pTodD	Derivative of pBBR1MCS-5 containing <i>todD</i> , TodD expression vector	This study
pRSET-A: TodF _{His}	Derivative of pRSET A containing <i>todF</i> for generation of an N-terminal His-tagged fusion protein	This study
pDTG601	Derivative of pKK223-3 containing <i>todC1C2BA</i> , used for generation of 3-vinylcatechol and 3-methylcatechol	Zylstra & Gibson (1989)

degradation rates with varying concentrations of 6-methyl HODA or 6-vinyl HODA. Graphpad Prism 5 (Graphpad Software) was used to calculate K_m and k_{cat} based on a least-squares analysis of Michaelis–Menten plots.

Measuring C23O activity. C23O activity was measured spectrophotometrically as an increase in absorbance at 375 nm following the addition of 2 mM catechol to whole-cell suspensions. For *E. coli* strains harbouring C23Os, C23O activity was also measured by using 3-vinylcatechol in addition to non-substituted catechol as a substrate. In these cases, absorbance increase was measured at 425 nm. Briefly, induced cells were washed and resuspended in 30 mM K_3PO_4 buffer and assayed in triplicate with a Bio-Tek Synergy HT-I microplate reader. For F1 strains, induction was achieved by exposing acetate-grown cells to toluene for 8 h. *E. coli* strains were induced for 4 h with 1 mM IPTG. Following the addition of catechol, initial rates of MFP production were calculated and correlated with protein content. Concentrations of catechol MFP were estimated by using a reported absorption coefficient of $40\text{ mM}^{-1}\text{ cm}^{-1}$ (Seah *et al.*, 1998).

C23O inactivation assays. Induced cells (see above) were collected by centrifugation and washed in 30 mM K_3PO_4 buffer. When working with styrene or toluene, triplicate cultures were exposed to 2 mM of the substrate. With 3-vinylcatechol and 3-methylcatechol, F1 cultures were resuspended in filtered supernatant containing 100 μM substrate. To generate dose–response curves, a range of 3-vinylcatechol concentrations was added to resting cultures. At set time points, 1 ml aliquots were taken, centrifuged at $16\,000\text{ g}$ for 1 min and resuspended in an equal volume of 30 mM K_3PO_4 buffer. C23O activity was measured as described above.

Quantification of growth on styrene. Due to optical interference from coloured metabolites, growth of recombinant F1 strains on styrene was assessed by c.f.u. counts rather than OD_{600} . F1 and related strains were grown overnight in MSM+acetate, washed twice in 30 mM K_3PO_4 buffer and inoculated 1:100 into MSM containing 5 mM styrene. At set time points, 100 μl aliquots were taken and serially diluted in sterile 30 mM K_3PO_4 buffer. Five microlitres of each dilution was spotted onto LB agar and individual colonies were counted after overnight incubation at 30°C .

RESULTS

F1 exposed to styrene accumulates 3-vinylcatechol

When toluene-induced F1 resting cultures were exposed to styrene vapours, a yellow compound was observed in the culture medium (data not shown). The accumulation of a yellow MFP, putatively identified as 6-vinyl HODA, was previously reported when F1 was exposed to styrene (Cho *et al.*, 2000). Spectral analysis yielded an absorbance maximum of approximately 425 nm, similar to the value of 419 nm reported by Cho *et al.* (2000). Its accumulation was transient, however, and after approximately 48 h the culture had turned a deep, opaque brown (Supplementary Fig. S1, inset). In resting cells exposed to toluene, no such colour was evident. Given that catechol polymerization products are typically brown or black, it was hypothesized that 3-vinylcatechol had accumulated in the culture media and subsequently polymerized. GC/MS analysis of chloroform extracts of the acetylated supernatant revealed a

single, large peak with a mass spectrum consistent with acetylated 3-vinylcatechol (Supplementary Fig. S1). No catecholic intermediates were detected in cells exposed to toluene (data not shown). The accumulation of 3-vinylcatechol suggested that TodE, the C23O responsible for cleaving this intermediate, might be inactivated during styrene metabolism. The failure to detect 6-vinyl HODA suggested that this metabolite might be degraded by TodF, although lack of detection could have been due to poor extraction efficiency or inadequate instrument sensitivity. To clarify the role of TodF in styrene metabolism, analyses of F1 cell-free extracts and purified TodF were performed.

TodF catalyses the efficient transformation of 6-vinyl HODA

To determine if the reported inability of TodF to degrade the styrene MFP (Cho *et al.*, 2000) prevented growth on styrene, cell-free extracts of toluene-induced F1 were exposed to biologically synthesized 6-vinyl HODA. Interestingly, F1 degraded 6-vinyl HODA at a considerably higher rate than previously reported (Cho *et al.*, 2000) (Supplementary Fig. S2a). To confirm that TodF was responsible for the observed activity, a *todF* expression vector was constructed (pTodF) and expressed in *E. coli* JM109. Consistent with our initial results, cell-free extracts of induced JM109 (pTodF) rapidly transformed 6-vinyl HODA (Supplementary Fig. S2b). Both the wild-type- and the plasmid-borne copies of *todF* were sequenced and found to be 100% identical to the previously published sequence (GenBank accession no. M64080) (Menn *et al.*, 1991). To assess TodF activity *in vitro*, purified TodF_{His} (Fig. 2, Table 2) was assayed with 6-methyl HODA and 6-vinyl HODA, the ring-fission products of toluene and styrene, respectively. When exposed to both substrates, TodF_{His} followed classical Michaelis–Menten kinetics (Fig. 2). Interestingly, the K_m calculated for 6-vinyl HODA (20 μM) was significantly lower than that for 6-methyl HODA (31 μM), indicating increased enzyme affinity for 6-vinyl HODA. The k_{cat} for 6-vinyl HODA, however, was more than six times lower than that for 6-methyl HODA (4.8 versus 32 s^{-1}). The k_{cat}/K_m values for both substrates indicated that TodF degraded 6-methyl HODA approximately four times more efficiently than 6-vinyl HODA.

C23O activity is abolished in *P. putida* F1 exposed to styrene and 3-vinylcatechol

That TodF was able to degrade 6-vinyl HODA relatively efficiently suggested that this enzyme was not limiting growth of F1 on styrene. Based on the previously observed accumulation of 3-vinylcatechol, it was hypothesized that styrene metabolism resulted in the abrogation of C23O activity in growing F1 cells. To test this hypothesis, resting cultures of toluene-induced F1 were exposed to styrene and its intermediate 3-vinylcatechol, and assayed for C23O activity. Interestingly, within 1 h of exposure to styrene, C23O activity was reduced sevenfold in toluene-induced

Fig. 2. TodF_{His} Michaelis–Menten kinetics. TodF_{His} is capable of degrading 6-vinyl HODA, albeit less efficiently than 6-methyl HODA. These curves represent a least-squares fit of the Michaelis–Menten equation to degradation rates of 6-methyl HODA (a) and 6-vinyl HODA (b). Kinetic parameters for the two substrates are listed in Table 2. (c) SDS-PAGE separation of TodF_{His} purification.

cells (Fig. 3a). Exposure of similarly induced cells to toluene caused no such inactivation. 3-Vinylcatechol, the substrate directly cleaved by TodE, was an even more potent inhibitor and appeared to abolish C23O activity completely within 2 h (Fig. 3a). Inactivation appeared to be concomitant with the appearance of 6-vinyl HODA (data not shown). 3-Methylcatechol generated from toluene served as a control and had no effect on C23O activity.

To determine if 3-vinylcatechol was the actual inhibitor of C23O activity and if metabolism of styrene to 3-vinylcatechol was required for C23O inactivation, a *todE* expression vector (pTodE) was constructed and expressed in *E. coli* S17. The resultant strain was then exposed to either styrene or 3-vinylcatechol. As expected, C23O activity in S17 (pTodE) was abrogated by exposure to 3-vinylcatechol, but was unaffected by styrene (Fig. 3b). The relevant controls of toluene and 3-methylcatechol had no effect on C23O activity.

Expression of DmpQB and TodE *in trans* permits growth on styrene

Given that C23O inactivation was implicated as being responsible for the inability of F1 to grow on styrene, it was hypothesized that increasing the levels of C23O activity through the expression of plasmid-borne genes and/or expression of a plant-like ferredoxin would permit F1 to use styrene as a growth substrate.

F1 has no plant-like ferredoxin of its own, so an expression vector encoding the ferredoxin DmpQ from *Pseudomonas* CF600 (Powlowski & Shingler, 1994), designated pDmpQ, was constructed and expressed in F1. pDmpQB, which expressed DmpQ together with its endogenous C23O partner DmpB, was also created and heterologously expressed in F1. In this way, DmpQ would rescue the C23O activity of DmpB even if, by chance, it did not rescue that of TodE. An F1 strain carrying the TodE expression vector pTodE was also analysed to determine if simply increasing basal levels of the native C23O enzyme of F1 could yield similar results.

Analysis of the growth cultures grown in MSM with styrene yielded surprising results (Fig. 4). Intriguingly, all F1 strains were capable of modest initial growth on styrene in the first 28 h. By 60 h, however, it was apparent that F1 wild-type had experienced cell death with a striking reduction in c.f.u. compared with at time 0. At 60 h, F1 (pDmpQ) and F1 (pDmpB) c.f.u. counts were an order of magnitude larger than F1, but not significantly different from values at time 0 ($P > 0.05$). Although F1 (pDmpQ) was unable to grow on styrene, the increased c.f.u. count at 60 h compared with that for F1 suggested a modest protective effect. Consistent with this observation, analysis of the C23O activity of F1 (pDmpQ) following styrene exposure demonstrated that expression of DmpQ also conferred increased resistance to C23O inactivation compared with F1 (Supplementary Fig. S3). Brown polymerization products were evident in all strains that

Table 2. Kinetic parameters for 6-methyl HODA and 6-vinyl HODA

Values shown are mean $\pm$ SD of three determinations

Substrate	K_m (μ M)	k_{cat} (s^{-1})	V_{max}	$K_{cat}/K_m \times 10^{-5}$ ($M^{-1} s^{-1}$)
6-Methyl HODA	31 ± 3.5	32 ± 0.9	63 ± 1.8	10.3 ± 0.8
6-Vinyl HODA	20 ± 2.1	4.8 ± 0.14	19 ± 2.1	2.4 ± 0.18

Fig. 3. C23O inactivation following exposure to styrene and 3-vinylcatechol. In resting cultures of *P. putida* F1, exposure to either styrene or 3-vinylcatechol resulted in the significant reduction of C23O activity. Neither toluene nor its metabolite 3-methylcatechol had any effect (a). In *E. coli* S17 (pTodE), only 3-vinylcatechol caused C23O inactivation (b). In (a) and (b), C23O activity was measured following the addition of 2 mM catechol to washed cells (see Methods). Error bars, SD ($n=3$).

experienced a reduction in c.f.u. after 28 h and indicated accumulation of 3-vinylcatechol (data not shown).

Neither F1 (pDmpQB) nor F1 (pTodE), however, accumulated 3-vinylcatechol and both strains were able to grow on styrene. F1 (pTodE) grew noticeably slower than for F1 (pDmpQB), although c.f.u. counts from both strains were nearly three orders of magnitude higher than for F1 after 60 h. Importantly, as additional evidence that TodF activity was not limiting growth on styrene, the rates of 6-vinyl HODA degradation in F1 (pDmpQB) and F1 (pTodE) were found to be identical to those for the wild-type (data not shown). Sequencing also revealed that both strains harboured wild-type *todF* alleles.

To gain further insight into the relationship between C23O inactivation and growth, resting cultures of *E. coli* S17 (pDmpQB), S17 (pDmpB) and S17 (pTodE) were exposed to 3-vinylcatechol and analysed for C23O activity. The initial rate of 3-vinylcatechol cleavage was highest in S17 (pDmpQB), with a C23O activity of 920 μM (mg

protein) $^{-1}$ min $^{-1}$, approximately 2.5- and 2.0-fold higher than S17 (pTodE) and S17 (pDmpB), respectively (Fig. 5). As differences in activity could have resulted from differential protein expression or stability, inactivation behaviour following 3-vinylcatechol exposure was analysed. The inactivation behaviours of S17 (pDmpB) and S17 (pTodE) were clearly distinct: exposure to 100 μM 3-vinylcatechol completely abolished C23O activity in S17 (pDmpB), but S17 (pTodE) retained residual activity even after exposure to 200 μM 3-vinylcatechol (Fig. 5). Intriguingly, S17 (pTodE) appeared to be even more resistant to inactivation than S17 (pDmpQB).

Fig. 4. Growth of F1 and recombinant strains on styrene. Although all strains initially grew on styrene, only those strains over-expressing C23Os and/or a ferredoxin were able to avoid the toxicity and cell death associated with 3-vinylcatechol accumulation. All strains were grown on MSM with styrene provided in liquid phase at a concentration of 5 mM. Error bars, SD ($n=3$), cannot be seen because they are smaller than the symbols used.

Fig. 5. C23O activity and inactivation behaviour of *E. coli* strains expressing relevant C23Os. S17 (pDmpQB) (black bars) had the highest initial C23O activity following exposure to 3-vinylcatechol, while S17 (pDmpB) (white bars) and S17 (pTodE) (grey bars) yielded similar activities. Despite having the lowest initial activity, TodE was more resistant than both DmpB and DmpQB to 3-vinylcatechol-mediated inactivation. Measurements were taken following 30 min exposure to 3-vinylcatechol. Error bars, SD ($n=3$). *Absolute values for S17 (pTodE), S17 (pDmpB) and S17 (pDmpQB) were 360, 450 and 920 μM (mg protein) $^{-1}$ min $^{-1}$, respectively.

DISCUSSION

In this report we have demonstrated that the inability of *P. putida* F1 to grow on styrene is not due to the failure of TodF to metabolize the styrene MFP 6-vinyl HODA, as has been suggested previously (Cho *et al.*, 2000). Rather, we found that 3-vinylcatechol accumulates during exposure to styrene and inactivates the C23O TodE. This inactivation prevents the conversion of 3-vinylcatechol to intermediates that can support growth, but more importantly, allows 3-vinylcatechol to accumulate, causing cell death. Finally, we have shown that increasing C23O expression is all that is required to prevent 3-vinylcatechol accumulation and permit F1 to grow on styrene.

Previous studies have demonstrated multiple means to expand the substrate range of aromatic degradation pathways, including mutations in regulators, recruitment of alternative catabolic enzymes and the expression of 'repair' enzymes such as [2Fe–2S] ferredoxins (Cerdan *et al.*, 1994; Cho *et al.*, 2000; Choi *et al.*, 2003; Furukawa *et al.*, 1993; Ramos *et al.*, 1987; Rojo *et al.*, 1987). With respect to the *tod* operon, work has focused on TodF and the expansion of substrate range through the recruitment of alternative MFP hydrolases, BphD (Furukawa *et al.*, 1993) and CmtE (Choi *et al.*, 2003).

In the current study, we specifically found that purified TodF_{His} was able to degrade 6-vinyl HODA at a rate that was slightly lower than, but of the same order of magnitude as, 6-methyl HODA. The observation that TodF degrades 6-vinyl HODA, coupled with our finding that F1 and derivatives all initially grew on styrene (Fig. 4), suggests that enough styrene was metabolized to citric acid cycle intermediates to support growth. This conclusion is also supported by our finding that F1 (pDmpQB) and F1 (pTodE), which grew well on styrene, still harboured wild-type *todF* alleles and activity. Together, these findings are strong evidence that TodF activity is not the factor preventing growth on styrene. Rather, the ability of the F1 derivatives overexpressing C23Os to grow on styrene, together with the effect of purified 3-vinylcatechol on TodE activity, makes it clear that inactivation of TodE is responsible for the accumulation of 3-vinylcatechol, 3-vinylcatechol toxicity and lack of net growth of F1 on styrene after 60 h.

Interestingly, simply increasing the basal 3-vinylcatechol C23O activity in F1 twofold through expression of DmpB was not enough to permit F1 (pDmpB) to grow on styrene, even though a similar level of C23O activity enhancement permitted growth of F1 (pTodE) (Fig. 4). Further analysis suggested that this was because DmpB was actually more sensitive to the deactivating affects of 3-vinylcatechol than TodE even though they had similar initial activity (Fig. 5). When co-expressed with its endogenous ferredoxin DmpQ, DmpB was more resistant to inactivation (Fig. 5) and cleaved approximately twofold more 3-vinylcatechol per milligram protein prior to complete inactivation (Supplementary Fig. S4). These differences in inactivation behaviour are clearly reflected in the growth curve presented in

Fig. 4 and suggest a dose–response relationship between catechol turnover and growth on styrene.

Catechols can initiate toxicity through a wide range of molecular mechanisms, including the production of reactive oxygen species (Schweigert *et al.*, 2001), and it is likely that accumulation of 3-vinylcatechol following C23O inhibition had toxic consequences. Although physiological end points were not a focus of this work, the noted decrease in c.f.u. in strains harbouring the C23Os that were most prone to inactivation (Figs 4 and 5) points to cell death and implicates 3-vinylcatechol as the probable cause of toxicity. In certain aromatic degradation systems, catechol toxicity is avoided by preferentially increasing the rate of catechol-consuming rather than catechol-producing reactions through duplication of catechol dioxygenase genes. In this manner, catechol intermediates do not accumulate and potential toxicity is avoided. It was shown that in *Ralstonia eutropha* JMP134(pJP4), multiple copies of *tfdC* (chlorocatechol 1,2-dioxygenase) were required for efficient 3-chlorocatechol turnover and growth on 3-chlorobenzoic acid (Laemmli *et al.*, 2000; Pérez-Pantoja *et al.*, 2003; Trefault *et al.*, 2004). A reduction in *tfdC* copy number inhibited growth on 3-chlorobenzoic acid, as did an increase in 3-chlorocatechol production. In another study, the predominant organism in a chlorobenzene-contaminated aquifer, *Ralstonia* JS745, was found to utilize a mosaic pathway containing partially duplicated *clcA* genes responsible for chlorocatechol degradation (Müller *et al.*, 2003; van der Meer *et al.*, 1998). Intriguingly, the upper pathway in JS745 was highly homologous to the genes encoding toluene dioxygenase (*todC1C2BAD*) in F1. Although both *tfdC* and *clcA* encode *ortho*- rather than *meta*-cleaving dioxygenases, these studies underline the importance of efficient catechol consumption as a means for substrate expansion. In our current work, increasing the expression of TodE – thus mimicking gene duplication – was enough to confer growth on styrene and reduce toxicity.

Other aromatic pathways may achieve similar results through uncoupled regulation of the upper pathway, which is responsible for initial dioxygenation and catechol production, and of the lower pathway, which is responsible for catechol degradation. Indeed, both the *nah* and *bph* operons for naphthalene and biphenyl degradation, respectively, contain multiple LysR-type regulator binding sites which allow for independent transcription of upper and lower pathway genes (Huang & Schell, 1991; Watanabe *et al.*, 2003, 2000). The importance of regulating relative levels of catechol degradation activity was hinted at by Park & Madsen (2004) who have shown that regulation by NahR, which preferentially up-regulates the lower pathway genes encoding degradation of catechol, was critical for the survival of naphthalene degraders in naphthalene-contaminated soil. This contrasts with the monocistronic nature of the *tod* operon and the absence of a supplementary promoter directly upstream of *todE*.

Our work with styrene suggests that the coupled regulation of the upper and lower pathways in the *tod* operon might

be one factor limiting growth substrate range and suggests why a modular strategy for control of the upper and lower pathways might confer selective advantages on organisms trying to exploit diverse aromatic substrates for growth. Interestingly, in many sphingomonads, which are known for their ability to use a wide range of aromatic substrates for growth, the upper and lower pathways appear as distinct modules and are not co-ordinately regulated. In many cases they are not even found in the same region of the genome (Armengaud *et al.*, 1998; Kim & Zylstra, 1999). The need to avoid the production of deactivating catechols may also have contributed to the evolutionary pressure that resulted in the emergence of an alternative pathway for styrene degradation that relies on a styrene monooxygenase and leads to phenylacetic acid (Mooney *et al.*, 2006). In this pathway, catechol is not produced and the hydroxylated ring is cleaved hydrolytically rather than oxidatively.

This work helps to clarify the role of TodF in styrene metabolism and clearly demonstrates that accumulation and toxicity of 3-vinylcatechol rather than failure to metabolize 6-vinyl HODA is what prevents F1 from growing on styrene. Our results are consistent with the need of cells to avoid catechol accumulation and provide the first evidence for TodE inactivation by 3-alkylcatechols. This was clearly demonstrated by our ability to enhance the range of substrates F1 uses for growth by simply over-expressing C23Os, and may help to explain further the value of modular genetic strategies employed by many bacteria that grow on a wide array of aromatic substrates.

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