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A second chromosomal copy of the *catA* gene endows *Pseudomonas putida* mt-2 with an enzymatic *safety valve* for excess of catechol

by

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1 **ABSTRACT**

2

3 *Pseudomonas putida* mt-2 harbors two different routes for catabolism of catechol, namely one *meta*

4 pathway encoded by the *xyl* genes of the TOL plasmid pWW0 and one *ortho* pathway determined by the

5 chromosomal *ben* and *cat* genes. *P. putida* mt-2 has a second chromosomal copy of the *catA* gene

6 (named *catA2*) located downstream of the *ben* operon that encodes an additional catechol-1,2-

7 dioxygenase. The metabolic and regulatory phenotypes of strains lacking one enzyme, the other and both

8 of them in cells with and without the TOL plasmid were evaluated. The data consistently indicated that

9 induction of the *ortho* pathway by benzoate plasmid-less strain *P. putida* KT2440 led to catechol surplus,

10 the toxicity of which at high concentrations being counteracted by CatA2. Cells carrying pWW0 but

11 lacking *catA2* experienced both a rapid loss of the plasmid when grown on benzoate (a substrate of the

12 *lower* pathway) and a slowdown of their growth rate when cultured with benzylalcohol (a substrate

13 converted to benzoate by the *upper* pathway). These data reveal the role of CatA2 as a type of metabolic

14 *safety valve* for excess catechol that alleviates the metabolic conflict generated by simultaneous

15 expression of the *meta* and *ortho* pathways, thereby facilitating their co-existence.

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18 INTRODUCTION

19

20 The soil bacterium *Pseudomonas putida* mt-2 is able to grow on a variety of aromatic compounds owing

21 to the metabolic capacity endowed by both the TOL plasmid pWW0 (Assinder and Williams, 1990) and a

22 large number of chromosomally encoded biodegradation routes (Jiménez *et al.,* 2002; Jiménez *et al.,*

23 2008; Nogales et al. 2011). One intriguing aspect of such a metabolic diversity in the same cells is the

24 frequent duplication of genes that appear to encode the same functions and are thus subsequently

25 annotated as orthologs (Koonin, 2005). An archetypal example is the redundancy of some of the genes

26 for metabolism of benzoate (Jiménez *et al.,* 2002; Nelson *et al.,* 2002). This compound is converted to

27 catechol by either the TOL enzymes *xylXYZL* or by the chromosomally-encoded and highly similar genes

28 *benABCDE* (Fig. 1), and both gene clusters are induced by the same pathway substrate (benzoate). Then

29 catechol can be channeled towards either a *meta*-cleavage pathway by the TOL enzyme XylE (catechol

30 2,3 dioxygenase, C2,3O; (Nozaki *et al.,* 1963; Nakai *et al.,* 1983), or an *ortho*-cleavage pathway by the

31 CatA enzyme of the *cat* cluster (catechol 1,2 dioxygenase, C1,2O; PP_3713; Nakai *et al.,* 1995). The

1   resulting products of either reaction then follow different fates as shown in Fig. 1. The semialdehyde from

2   *meta* cleavage is processed all the way down to produce pyruvate and acetyl-CoA by the rest of the TOL

3   enzymes, whereas the *cis,cis*-muconate follows its way through the enzymes encoded by the *cat* and the

4   *pca* genes towards succinyl-CoA and acetyl-CoA (Harwood and Parales, 1996). While the metabolic

5   conflict created when *Pseudomonas putida* mt-2 cells are exposed to 3-methylbenzoate (and thus methyl-

6   catechols are generated) is overcome through a regulatory solution (Cowles *et al.,* 2000; Silva-Rocha and

7   de Lorenzo, submitted) the catechol pool produced by the action of the *meta* and the *ortho* pathways on

8   benzoate has to bifurcate into two biochemical itineraries.

9

10   *P. putida* mt-2 and its variant devoid of the TOL plasmid *P. putida* KT2440 contain a second copy of a

11   gene encoding CatA (named thereafter *catA2,* PP_3166), located in the *ben* operon (Fig. 1; Jiménez *et*

12   *al.,* 2002). The biological role of *catA2* is unknown although it encodes a functional copy of the catechol

13   dioxygenase activity (van Duuren *et al.,* 2011) and it shares 76% of identity with its canonical counterpart.

14   Yet, its presence seems to be unnecessary, as all the activities needed for catabolism of catechol in *P.*

15   *putida* KT2440 are fulfilled by the *cat* gene cluster. Furthermore, the location of a *catA2* gene downstream

16   of the catalytic genes of the *ben* cluster often appears in other *P. putida* strains (e.g. the native host of the

17   TOL plasmid). In contrast, a *catA2* it is not present in any of the known benzoate-degradation clusters

18   borne by other *Pseudomonas* species (Fig. S1 and Jiménez *et al.,* 2002).

19

20   In this work we provide genetic and biochemical evidence that the enzyme encoded by *catA2* helps to

21   reduce the intracellular levels of catechol in the bacterial cytoplasm. Furthermore, *catA2* is a key element

22   that enables coexistence of the *xyl, ben* and *cat* pathways in the same cells when bacteria employ

23   benzoate as a growth substrate by mitigating the biochemical conflict mentioned above. This relies on the

24   inherent properties of the enzyme encoded by *catA2,* which acts as a *safety valve* (Danchin, 2009c) for

25   limiting the level of intracellular catechol resulting from the sum of the activities of the *xylXYZL* and

26   *benABCD* gene products. Such a biochemical *patch*, which seems to be specific for *P. putida,* not only

27   illustrates the repertoire of solutions that environmental bacteria have evolved for fixing intricate chemical

28   problems, but also pinpoints that such safety valves must form part of any robust metabolic engineering

29   endeavor.

30

31   RESULTS

1

Coarse metabolic phenotypes endowed by catA and catA2

3

4 As mentioned above, the genome of *P. putida* KT2440 encodes two *catA* orthologs (Fig. 1). To gain a

5 preliminary picture of their relative contribution to catechol metabolism we started by generating mutants

6 in either gene or in both, and the properties of the resulting strains were examined. To this end, we

7 created isogenic strains *P. putida* Δ*catA*, Δ*catA2* and Δ*catA* Δ*catA2* as explained in the Experimental

8 Procedures section. The ability of these strains to use benzoate as the sole carbon source was tested in

9 growth experiments and simultaneous monitoring of the substrate concentration in the culture media. As

10 shown in Fig. 2A, inactivation of *catA2* had no detectable effect in the growth phenotype as compared

11 with the wild type strain. In contrast, the Δ*catA* mutant displayed a significantly delayed growth on

12 benzoate. These data suggested that bacterial growth at the expense of CatA2-only is possible but far

13 from the optimum provided by CatA. As expected, the double mutant entirely lacking C1,2O activity was

14 incapable of using 5 mM benzoate as the sole carbon source (Fig. 2A). Similar results were obtained

15 using 1, 2.5 and 10 mM benzoate (Fig. S2). A second feature revealed during the growth of the mutants

16 on benzoate was the accumulation of catechol, visualized by formation of dark insoluble pigments (Fig.

17 3A, flask 4). These pigments result from the polymeric aggregates of quinonic intermediates that form

18 spontaneously in solution after catechol oxidation (Prieto *et al.,* 1993). As expected, the buildup of a

19 brownish color was evident in the cultures of the double Δ*catA* Δ*catA2* mutant, because this strain

20 produces catechol from benzoate (Fig. 1) but cannot degrade it any further. As a control, the wild-type

21 strain (Fig. 3A, flask 1) did not accumulate any of such insoluble material, thereby accrediting its capacity

22 to deal with all catechol produced during degradation of benzoate. In good agreement with the growth

23 data (Fig. 2A), the single mutant Δ*catA* (which lacks the main C1,2O activity, see next section) also

24 accumulated a considerable level of catechol (Fig. 3A, flask 2), while the Δ*catA2* equivalent (Fig. 3A, flask

25 2) barely generated any insoluble material in the culture. The intensity of the black pigments was

26 compared to that of catechol standards of known concentration added to cell-free media and incubated in

27 parallel to the bacterial cultures. This allowed estimating that $\geq$ 95% of the share of benzoate

28 consumption and catechol processing goes through the canonical enzyme CatA and only < 5% proceeds

29 through the second counterpart, CatA2 (Fig. 3A).

30

31 *catA and catA2 encode the same enzymatic activities with different kinetic properties*

1

2   To have an accurate estimate of the contribution of either CatA or CatA2 to the processing of catechol in

3   *P. putida* KT2440 growing on benzoate, the levels of C1,2O activity were measured in the wild-type strain

4   and each of the single and double mutants. To this end, cells were grown on citrate in the presence of

5   benzoate, protein extracts were recovered from each of the cultures and processed as explained in the

6   Experimental Procedures section for determination of enzymatic activity. The resulting figures are shown

7   in Fig. 2B. As expected, the highest activity was displayed by the wild type strain (1.2 ± 0.2 μmol min^{-1}

8   mg^{-1}), followed by the $\Delta catA2$ mutant (0.96 ± 0.07 μ mol min^{-1} mg^{-1}) and, at a much lower level, strain

9    $\Delta catA$ (0.08 ± 0.02 μmol min^{-1} mg^{-1}). No detectable C1,2O activity was found in the double mutant $\Delta catA$

10    $\Delta catA2$ strain (Fig. 2B). The figure of (relative) specific activity in the crude extract of the mutant lacking

11   CatA2 was in good agreement with the estimation of catechol accumulated in the culture supernatants of

12   the same strain (~5%). Furthermore, the data implied the total level of C1,2O in wild-type cells to be the

13   addition of those of CatA and CatA2. We thus wondered whether such a drastic difference between the

14   two enzyme variants was due to the intrinsic biochemical properties of each of them. To tackle this issue,

15   genes *catA* and *catA2* were cloned and separately over-expressed in the cytoplasm of *E. coli*. Soluble

16   protein extracts of the corresponding strains (Fig. S3) enriched with either protein were then employed for

17   determining their apparent kinetic properties as well as their activity towards alternative substrates. These

18   analyses indicated that CatA and CatA2 have dissimilar affinities for catechol as reflected by their

19   apparent K_m (1.3 ± 0.2 and 7.4 ± 1.4 μM, respectively), although still in the same range. More strikingly,

20   their apparent specific activities (V_{max}) for the same substrate differed by nearly twenty fold, clearly

21   revealing that CatA2 (0.3 ± 0.1 μmol·min^{-1}·mg^{-1}) was considerably less efficient than CatA (6.6 ± 0.3

22   μmol·min^{-1}·mg^{-1}; Fig. S4). In contrast, no significant variation in affinity for the substrate or in dioxygenase

23   activity was appreciated between the two proteins when tested with other compounds, such as 4-

24   methylcatechol, 3-methylcatechol, 4-chlorocatechol and protocatechuate as substrates (see

25   Supplementary Table S1). Taken together, the data above reveal the co-occurrence of two C1,2O

26   enzymes whose major difference seems to be the values of their kinetic parameters. Still, these data did

27   not yet shed any light on the *raison d'etre* of such a redundancy.

28

29   CatA2 *helps keeping intracellular levels of catechol low*

30

1   The high K_m of CatA2 means that the enzyme reaches its maximum activity when the concentration of

2   catechol is also high. Under that circumstance, both CatA and CatA2 could cooperate keeping levels of

3   catechol low, thereby allowing the cells to handle higher concentrations of the growth substrate. To

4   examine this possibility, we evaluated the intracellular accumulation of catechol using a biosensor based

5   on the transcriptional regulator DmpR that activates expression of the *luxAB* reporter genes

6   transcriptionally fused to the *Po* promoter (Sze *et al.,* 1996). DmpR recognizes a broad range of phenolic

7   compounds including catechol, whereas it does not respond to benzoate (Shingler and Moore, 1994). On

8   this basis, we transferred a plasmid encoding the biosensor system to *P. putida* KT2440 as well as to the

9   *cat* mutants and luminescence was recorded in the presence of different concentrations of benzoate. The

10   results show that wild-type *P. putida* produces enough C1,2O activity to metabolize all the catechol

11   generated in the cells, even at the highest concentration of benzoate tested (5 mM; Fig. 2C). The

12   phenotype is, however, quite different in the single mutants. Cells lacking CatA accumulate catechol when

13   exposed to every concentration of benzoate, whereas cells devoid of CatA2 show accumulation only

14   above 1 mM of the substrate (Fig. 2C). This result indicated that the threshold at which CatA becomes

15   saturated by the substrate *in vivo* occurs at benzoate concentrations in the culture medium in the range

16   1.0-2.5 mM. Above this level, the extra C1,2O activity provided by CatA2 helps keeping levels of catechol

17   low, although both activities eventually become saturated at highest benzoate concentrations. The double

18   mutant *catA catA2* was also tested with the DmpR-based biosensor to the same end but surely the levels

19   of intracellular catechol were toxic for the cells, which failed to growth and displayed no significant

20   luminescence.

21

22   Apart of examining catechol levels, we inspected the effect of CatA2 on transcription of the *ben* and *cat*

23   operons, as changes in C1,2O activity could potentially modify benzoate consumption by changing the

24   flow rates of the *ben-cat* pathway. The rationale in this case was that a faster removal of catechol should

25   decrease the transient intracellular level of benzoate and thus be less available for activating BenR, the

26   transcriptional regulator of the P*ben* promoter (Fig. 1). The result should thus be the lowering of

27   expression of the *ben* genes. At the same time, high C1,2O activity should also generate elevated levels

28   of *cis,cis*-muconate, which in turn binds CatR, leading to greater transcription of the *cat* operon. These

29   hypotheses were tested with plasmids bearing transcriptional P*benA*-GFP and P*catB*-GFP reporter

30   fusions. As shown in Supplementary Fig. S5, the loss of *catA* led only to a minor increase in the activity of

31   the P*benA*-GFP construct, but the GFP output remained basically stable in all conditions. The same was

1   true for the *PcatB*-GFP reporter in the wild-type strain and the individual mutants, but not in the strain

2   lacking CatA and CatA2, which is not capable of producing *cis,cis*-muconate. The results suggest that

3   intracellular fluctuations *in vivo* of either benzoate or *cis,cis*-muconate are not reflected in the activity of

4   the corresponding promoters, perhaps because of a very high affinity of the regulators for their respective

5   agonists.

6

7   CatA2 *influences the activity of the meta pathway of the TOL plasmid*

8

9   As mentioned above and shown in Fig. S1, the presence of a *catA2* gene in *ben* operon is a feature

10   unique of *P. putida* strains. According to available literature (Nakazawa, 2002; Regenhardt *et al.,* 2002),

11   strain *P. putida* KT2440 is basically the same as the environmental isolate *P. putida* mt-2 (Assinder and

12   Williams, 1990; Greated *et al.,* 2002) but cured of the TOL plasmid (Bagdasarian *et al.,* 1981; Franklin *et*

13   *al.,* 1981). That the regulatory interplay of the host bacterium and the plasmid is so intricate (Kessler *et*

14   *al.,* 1994) suggests an intimate mutual adaptation of the metabolic blocks encoded by either replicon

15   which could have survived the forced loss of the plasmid. With this notion in mind we passed TOL pWW0

16   plasmid by conjugation (see Experimental Procedures) back to strains *P. putida* Δ*catA, P. putida* Δ*catA2*

17   and *P. putida* Δ*catA* Δ*catA2*. The growth of each of the exconjugants on benzoate as the only C source

18   was then compared to that of *P. putida* mt-2, i.e. *P. putida* KT2440 (pWW0) under identical conditions.

19   Note that benzoate is processed through both the enzymes encoded by *xylXYZL* and *benABCD* into

20   catechol (Fig. 1). But then, the metabolism of this intermediate bifurcates towards either a *meta* pathway

21   by XylE (generating 2-hydroxymuconate-6-semialdehyde, HMS), or an *ortho* route by CatA and CatA2

22   (producing *cis,cis*-muconate; Fig.1). While the growth curves in the presence of different concentrations of

23   benzoate did not show significant differences (Fig. 4A and Fig. S6), inspection of the cultures of *P. putida*

24   Δ*catA2* revealed a transient accumulation of a yellow compound that could be easily identified and

25   quantified spectrophotometrically as HMS (Fig. 3B and Fig. 4B). Since the levels of catechol accumulated

26   in the cell are higher in the absence of CatA2 (Fig. 2C), the accumulation of HMS indicates that the

27   excess of substrate is channeled by the Δ*catA2* mutant into the *meta* route. In contrast, the absence of

28   CatA alone, did not to result in any visual phenotype of the culture and behaved in a way similar to the

29   Δ*catA* Δ*catA2* strain in which benzoate degradation relies exclusively in the *meta* pathway.

30

1   That the *catA2* variant of *P. putida* (pWW0) accumulated HMS could be due to a higher activity of the

2   *meta*-TOL operon. That increased activity could be originated by a surplus of catechol, which then

3   becomes available to the C2,3O of the TOL system. Alternatively, or in addition, the expression levels of

4   the *meta*-TOL genes could increase due to the accumulation of benzoate in the *catA2* mutants. To

5   examine these possibilities we first measured the specific enzymatic activity of XylE in resting cells

6   recovered from cultures of each of the strains growing on benzoate. The results indicated that cells

7   lacking *catA2* had a much higher C2,3O activity ($\geq$ 6-fold) than the wild-type *P. putida* mt-2 (Fig. 5A). A

8   higher level was observed also in the double mutant $\Delta catA$ $\Delta catA2$ and to a lesser extent in the single

9   deletion $\Delta catA$ (Fig. 5A). To investigate whether these changes were the result of differences in

10   expression of *meta* pathway enzymes, we constructed a dedicated *Pm-GFP* reporter plasmid (pGLR1-

11   *Pm*, see Experimental Procedures) and measured its output when transformed in each of the strains

12   under study containing the TOL plasmid. As indicated in Fig. 5B, *Pm* activity followed the same trends as

13   the levels of C2,3O dioxygenase shown in Fig. 5A. Although the differences were not as pronounced as

14   with enzymatic activities, such variations of the *Pm-GFP* fusion sufficed to explain the changes in C2,3O

15   in the various strains exposed to benzoate. In sum, it seems that CatA2 is an actor of the interplay

16   between the *ortho* and the *meta* pathways of *P. putida* cells bearing the TOL plasmid in two possible and

17   not mutually exclusive ways: [i] the loss of *catA2* leads to an excess of catechol that is channeled to the

18   *meta* pathway and [ii] the same mutation increases intracellular accumulation of non-catabolized

19   benzoate which could account for the increase in *Pm* activity. Note that the last is different than the effect

20   of *catA2* in *Pben* activity (Supplementary Fig. S5), thereby identifying new regulatory connections that are

21   beyond the scope of this article. But regardless of the mechanisms involved, what are the practical

22   consequences of this state of affairs?

23

24   CatA2 *modulates simultaneous expression of xyl and ben pathways for benzoate metabolism in P. putida*

25   *mt-2*

26

27   It has been long known that repeated growth of *P. putida* mt-2 in benzoate as the sole C source leads to

28   outgrowth and eventual takeover by the population that has lost the TOL plasmid (Nakazawa and Yokota,

29   1973; Williams and Murray, 1974; Stephens and Dalton, 1987). This hints to a typical fitness scenario in

30   which cells that spontaneously loose the plasmid grow better because the *meta* pathway does not provide

31   an advantage in the conversion of the substrate into biomass compared to the *ortho* pathway, as it is

1 reflected in reported growth rates (Nakazawa and Yokota, 1973). But, alternatively (or in addition) to the

2 extra replication load, cells that get rid of the plasmid may have solved a biochemical toxicity problem that

3 burdens the growth of the plasmid-containing bacteria (Duetz and van Andel, 1991; Duetz *et al.,* 1991).

4 Since the competition of the *ortho* and *meta* dioxygenases for catechol presented above can be the key

5 feature of such a conflict, we set out to either exacerbate the clash between the two routes -or to

6 eliminate it, and examine the effect on the maintenance of the TOL plasmid. To this end, each of strains

7 bearing pWW0 (wild-type, ∆*catA,* ∆*catA2* and ∆*catA* ∆*catA2*) were repeatedly re-grown in minimal

8 medium with benzoate and, after successive passes, the cultures plated on minimal medium with 3-

9 methyl benzylalcohol as sole carbon source for ensuring retention of the entire TOL pathway encoded in

10 the plasmid (i.e. only those cells with the whole complement of *upper* and lower *xyl* genes can grow on

11 such a substrate; Shaw and Harayama, 1990). As a reference, the cell counts were then compared with

12 those of the same cultures plated on benzoate. Two striking features of such analyses become evident in

13 the data shown in Fig. 6. First, cells lacking *catA2* tended to lose the TOL plasmid much faster than the

14 wild-type *P. putida* mt-2 cells, suggesting that this second dioxygenase eases a co-occurrence (sub-

15 optimal as it may be) of both the *ortho* and *meta* pathways in the same cells growing on benzoate. In

16 contrast, cells lacking *catA* retained the plasmid in full indicating that pWW0 becomes very stable when

17 the *ortho* pathway is not fully functional. The same was true for the double ∆*catA* ∆*catA2* strain. This last

18 case provided in fact a positive control for the full maintenance of the *xyl* genes, because the strain lacks

19 an *ortho* route. Taken together, these results suggest that simultaneous activity of the *meta* and *ortho*

20 pathways generates a metabolic conflict that leads to the loss of the TOL plasmid. To further verify this

21 hypothesis we grew the same set of strains on benzylalcohol, a substrate that becomes metabolized to

22 benzoate by the enzymes of the *upper* TOL pathway. In TOL$^+$ cells, conversion of benzylalcohol to

23 benzoate proceeds entirely through the upper route but then the benzoate can again bifurcate between

24 the *meta* and the *ortho* pathways. This means that bacteria growing on benzylalcohol are forced to keep

25 the TOL plasmid. The toxicity associated with the *meta* route and its relief by CatA2 become evident in

26 the results shown in Fig. 7. Increasing concentrations of benzylalcohol leads to faster growth of all strains

27 excepting for the single *catA2* mutant. In contrast, the single *catA* mutant and the double deletion ∆*catA*

28 ∆*catA2* grew faster that the wild type strain at the highest benzylalcohol concentration in a fashion

29 consistent with the loss of the TOL plasmid in benzoate-grown cells shown in Fig. 6. These results

30 support the role of CatA2 in easing the co-existence of the *ortho* and *meta* pathway in TOL$^+$ cells growing

31 in benzoate.

1

2   DISCUSSION

3

4   In this work we have attempted to make some sense out of the presence of a second copy of the *catA*

5   gene in *P. putida* KT2440 which –unlike the canonical copy in the *cat* operon, lays in the *ben* operon and

6   it is coexpressed with the preceding genes in response to benzoate (Fig. 1; van Duuren *et al.,* 2011). The

7   *catA2* sequence is 90% and 88% identical to the *catA* sequences of *P. putida* W619 and *P. entomophila*

8   L48 respectively, where the *ben* and *cat* genes are clustered together (Fig. S1). This suggests that *catA2*

9   may have been acquired by horizontal gene transfer independently from *catA* with which it shares a 76%

10   of identity as it is also suggested by the early independent evolution of the *catA2* subgroup of genes (Fig.

11   S7). Finally, *catA2* seems to be unique of *P. putida* mt-2 and related strains such as F1, ND6, BIRD-1,

12   S16, DOT-T1 and GB-1 (Fig. S1). Interestingly, according to 16S rRNA relationships, the only strain that

13   does not show the same organization, *P. putida* W619, it is also the closest to *P. entomophila* in the

14   phylogeny, suggesting that W619 might not be correctly classified as *P. putida* (Wu *et al.,* 2011).

15

16   In *P. putida* KT2440, CatA2 is dispensable for the growth in benzoate and has a discrete contribution to

17   the C1,2O activity of the cell when assayed in crude extracts (Fig. 2). It also shows poor kinetic

18   parameters when compared to CatA, something that is supported by other works reporting similar

19   differences (Nakai *et al.,* 1988; van Duuren *et al.,* 2011). Furthermore, *catA2* does not have a detectable

20   role in controlling transcription of the *ben* and *cat* operons (Fig S5) and does not affect either the

21   transcriptome (van Duuren *et al.,* 2011) or the proteome (Yun *et al.,* 2011) of *P. putida* KT2440 growing

22   on benzoate as sole carbon source. In contrast, CatA2 clearly helps to keep intracellular concentrations of

23   catechol low, plausibly preventing its toxicity (Fig. 2C). This seems particularly important in the presence

24   of the TOL plasmid when both the *ortho* and *meta* routes for benzoate degradation are present. In this

25   case, CatA2 channels catechol into the *ortho* pathway as revealed by the increased production of HMS in

26   the Δ*catA2* strain (Fig. 4). The same mutant also shows greater transcriptional activation of the *meta xyl*

27   operon and C2,3O activity, surely due to an enhanced expression of the *lower* TOL operon under the

28   same conditions (Fig. 5). Regardless of mechanistic details, the fact of the matter is that *catA2* seems to

29   deal with an excess of catechol when both pathways are fully active in *P. putida* mt-2 growing on

30   benzoate. That the kinetic constants of CatA2 are less efficient that those of CatA suggests that the

1   second enzyme becomes operative only when the levels of catechol go beyond a certain threshold, as is

2   the case of cells bearing the TOL plasmid.

3

4   The situation just described is reminiscent of the notion of a physiological *safety valve* proposed by

5   (Danchin, 2009c) for explaining the high K_m of the lactose exporter encoded by *lacA*, the third and much

6   overlooked gene of the lactose operon. In this case, the safety valve protects cells from the hyper

7   accumulation of the substrate caused by the lactose permease and thus it allows them to cope with the

8   transient buildup of osmotic pressure. By the same token, one could consider a biochemical safety valve

9   in which the buildup of an intermediate toxic metabolite is held-in-check by an enzyme that gets rid of it

10   when a given concentration is surpassed. We believe that this is exactly what happens in the case

11   reported in this work. Metabolic safeguarding systems of this sort are known in other biodegradation

12   systems (Endo *et al.,* 2007), where the relief of the toxicity is reported to take place through the export of

13   the compound. In contrast, as shown above, the relief of catechol toxicity in *P. putida* involves its

14   conversion into an innocuous metabolite –a situation not unlike that of chlorocatechols in *Cupriavidus*

15   *necatur* (Pérez-Pantoja *et al.,* 2003).

16

17   Advances in genome sequencing have revealed a very high incidence of paralogy in the functional

18   complements of environmental bacteria (Bratlie *et al.,* 2010). Gene redundancy is believed to facilitate

19   genetic adaptation or create novel biochemical functions (Reams and Neidle, 2003; Wagner, 2008;

20   Andersson and Hughes, 2009). Yet, the abundance of paralogs in some bacteria is still intriguing, one

21   extreme instance being the various operons for benzoate metabolism born by *Burkholderia xenovorans*

22   LB400 (Denef *et al.,* 2006), the several clusters for benzoate and phthalate in *Rhodococcus jostii* RHA1

23   (Patrauchan *et al.,* 2005) and the redundancy of maleilacetate reductase in haloaromatic compounds

24   degradation in *C. necator* (Pérez-Pantoja *et al.,* 2009). At the same time, redundancy is a source of

25   genetic instability (Treangen and Rocha, 2011), so their retention is indicative of a role in solving

26   regulatory problems and/or originate novel biochemical functions (Ohno, 1970; Andersson and Hughes,

27   2009). On the basis of all the above, we believe that counteracting accumulation of toxic intermediates is

28   not just an oddity of this particular system but a general principle of natural metabolic evolution that

29   should also be incorporated in any sound metabolic engineering design (Danchin, 2012). It is remarkable

30   that the way the problem has been solved in this case is by stitching the pathway with an extra gene that

1   recalls the plug-ins that computer programs incorporate for solving specific software problems (Danchin,

2   2009b, a).

3

4   EXPERIMENTAL PROCEDURES

5

6   *Bacterial strains, culture conditions and general DNA procedures*

7

8   All *P. putida* strains were derived from *P. putida* mt-2 (ATCC 33015) or *P. putida* KT2440 (Bayley *et al.,*

9   1977; Franklin *et al.,* 1981). *E. coli* strains were routinely grown in Luria-Bertani (LB) medium at 37 ºC. *P.*

10   *putida* strains were grown either in LB or in M9 minimal medium (Sambrook and Russell, 2001)

11   supplemented with benzoate (1-10 mM) as sole carbon source. Where appropiate, antibiotics were added

12   to the medium at the following concentrations (μg/ml): kanamycin, 50; gentamycin, 10; chloramphenicol

13   30. Plasmids were transferred from either *E. coli* or *P. putida* mt-2 to *P. putida* KT2440 by tripartite

14   conjugation on membrane filters (MF^{TM}, 0.45 mm, Millipore), including helper strain *E. coli* HB101

15   (pRK600). After 6-8 h incubation at 30 ºC on LB agar, the conjugation mixture was plated on minimal

16   selective media as indicated in each case. For determination of doubling times bacteria were grown in

17   microtiter plates with 200 µl wells containing M9 medium supplemented with the concentration of the

18   substrate indicated in each case. Plates were incubated at 30ºC with continuous radial shaking and the

19   OD_{600} monitored every 15 min. Time points corresponding to at least 3 h of growth in the exponential

20   phase were fit to an exponential growth equation using a non-linear regression method (GraphPad Prism

21   Software), out of which averages and standard deviations from six independent experiments were

22   calculated. DNA constructs were sequenced using BigDye^{TM} Terminator v3.1. All PCR reactions were

23   performed using Taq DNA polymerase (New England Biolabs) and the primers listed in Supplementary

24   Table S2. Mutant strains *P. putida* Δ*catA*, Δ*catA2* and Δ*catA* Δ*catA2* were generated by disruptive

25   homologous recombination using suicide plasmids containing internal regions of the genes *catA* and

26   *catA2*. The region corresponding to *catA* was amplified using oligonucleotides catA5 and catA3. The

27   internal region of *catA2* was amplified using the oligonucleotides catA2int5 and catA2int3. These

28   fragments were cloned into the suicide vectors pK18mob (Km^R; Schäfer *et al.,* 1994) for *catA* or

29   pJQ200SK (Gm^R; Quandt and Hynes, 1993) for *catA2* using *Eco*RI/*Bam*HI restriction sites in both cases.

30   These plasmids can only replicate in *E. coli* strains and when transferred to *P. putida* KT2440 by

31   triparental mating they integrate in the bacterial chromosome by a homologous recombination that breaks

1   the target sequence. Disruptions were confirmed by PCR. For recombinant expression in *E. coli*, *catA* and

2   *catA2* genes were amplified from the *P. putida* KT2440 chromosome by PCR using, respectively,

3   oligonucleotides catA5Nt/catA3ext and catA25/ catA23 (Supplementary Table S2) and they were cloned

4   into the hyperexpression vector pET29 (Promega) using the engineered restriction sites *Nde*I/*Eco*RI,

5   thereby rendering the corresponding plasmids pETCatA and pETCatA2. The *PbenA* and *PcatB* promoters

6   were amplified from chromosomal DNA by PCR using the respectively the pairs of oligonucleotides SacI-

7   PbenA-FW/BamHI-PbenA-RV and EcoRI-catB-FW/BamHI-catB-RV (Supplementary Table S2). The *Pm*

8   promoter was similarly amplified from the TOL plasmid pWW0 using toligonucleotides EcoRI-Pm-FW and

9   BamHI-Pm-RV. The PCR products were digested with *Sac*I and *Bam*HI (*PbenA*) or *Eco*RI and *Bam*HI

10   (*PcatB* and *Pm*) and inserted in the vector pGLR1 containing a *gfp-luxCDABE* bicistronic reporter system

11   (Benedetti *et al.,* 2012) for constructing pGLR-PbenA, pGLR-PcatB and pGLR1-Pm, respectively. These

12   plasmids were transformed by electroporation in *P. putida* derivatives as previously reported (Martínez-

13   García and de Lorenzo, 2012). The TOL plasmid pWW0 was transferred by mating as indicated above

14   using the wild type strain *P. putida* mt-2 as donor and the mutants Δ*catA,* Δ*catA2* and Δ*catA* Δ*catA2* as

15   recipients. Transconjugants were selected in M9 medium supplemented with 5 mM 3-methylbenzyl

16   alcohol as the sole carbon source and the corresponding antibiotic (kanamycin, gentamycin or both).

17

18   *Protein expression and enzymatic assays*

19

20   CatA and CatA2 enzymes were expressed in *E. coli* and *P. putida* as follows. Starting cultures of *E. coli*

21   BL21/DE3 (Promega) harboring plasmids pET29CatA or pET29CatA2 were cultured overnight in LB at 30

22   ºC. Cells were then diluted 20 times in order to inoculate 50 ml of LB supplemented with isopropyl-β-D-1-

23   thiogalactopyranoside (0.5 mM). Expression of CatA and CatA2 proteins took place for 12 h at 30 ºC with

24   shaking. Starting cultures of *P. putida* were obtained by growing cells overnight in M9 medium

25   supplemented with citrate 0.2% as the sole carbon source. The following day, cells were diluted 20 times

26   and grown for 5 h at 30 ºC in 50 ml of M9 containing citrate (0.2%), as the sole carbon source, and

27   benzoate (5 mM), as the inducer, until they reached an optical density at 600 nm (OD_{600}) of 0.5. In all

28   cases crude extracts for C1,2O assays were obtained after harvesting cells by centrifugation, followed by

29   resuspension in 10 ml of Tris-HCl 30 mM buffer pH 8.2 and lysis in a French-Press at 1000 psi. C1,2O

30   activity was monitored at 260 nm corresponding to the formation of the ring cleavage product *cis,cis*-

31   muconate (ε = 16800 $M^{-1} \cdot cm^{-1}$) as described previously (Kojima *et al.,* 1967). Linear regression fits in the

Lineweaver-Burk plots were performed with GraphPad Prism software package. In the case of extracts obtained from *E. coli* BL21, activity levels were corrected estimating the fraction of C1,2O enzymes in a total extract of 5 μg of protein. Protein determination was performed by PAGE-SDS integrating the signals in the different lanes with the ImageJ software package. Substrate specificity of the recombinant enzymes was determined monitoring the oxygen consumption rate using a DW1 Hansa-Tech Oxygen electrode. The resting cells assay for C2,3O was made as described elsewhere (Velázquez *et al.,* 2005) registering the absorbance at 375 nm of the 2-hydroxymuconate-6-semialdehyde (HMS) resulting from the reaction. Benzoate consumption and HMS accumulation in cell free supernatants were monitored measuring their absorbance at 270 (ε = 93200 M^{-1}cm^{-1}) and 375 nm (ε = 46000 M^{-1}cm^{-1}), respectively.

Promoter activity and catechol accumulation

For the analysis of promoter activity, the different *P. putida* derivatives harboring the promoters fused to GFP were inoculated into 2 ml of M9 minimal medium supplemented with 0.2% citrate and allowed to grow for 16 h. The cells were then diluted 20 times in fresh medium in the presence or absence of benzoate at a final concentration of 1 mM. Diluted cells (200 μL) were placed in 96-well microplates (OptiluxTM, BD Falcon) and analysed in a Wallac Víctor II 1420 Multilabel Counter (Perkin Elmer). The OD$_{600}$ and the fluorescence were recorded at time intervals of 15 min. To this end, GFP was excited at 488 nm, and the fluorescence signal was recovered with a 525(40) BP filter. Promoter activity was determined as the ratio between fluorescence and cell density and the results are given in relative units using as a reference the maximal activity reached after 3 h of incubation. Intracellular accumulation of catechol was monitored with the *dmpR/Po-luxAB* biosensor born by plasmid pVI466 (Sze *et al.,* 1996). After transferring this construct to the wild type *P. putida* strain and its *cat* mutants, cells were cultured in citrate 0.2% and benzoate added at the concentrations indicated in each case. When the cultures were in mid-exponential phase an aliquot of 200 μl was transferred to a 96-well plate and used to determine the OD$_{600}$. Right after that 10 μl of a fresh solution of 1% decanal were added to the plate and the luminescence recorded after 10 s of vigorous shaking in the plate reader. Six independent experiments were used to calculate averages and standard deviations.

Curing the TOL plasmid of P. putida cells

1 Starter cultures of the different strains were grown overnight on M9 minimal medium supplemented with

2 citrate (0.2%) as carbon source. These cultures were used to inoculate 20 ml of M9 minimal medium with

3 5 mM benzoate as the sole carbon source with a starting OD_{600} ~ 0.1. Cells were incubated for 12 h at 30

4 ºC until late stationary phase and then reused to inoculate again fresh M9 benzoate medium. This

5 process was repeated in 4 successive steps. After each of these cycles, a small aliquot of the culture was

6 used to track the loss of the plasmid by plating in M9 medium supplemented with either benzoate or 3-

7 methylbenzyl alcohol (both at 5 mM) as the sole carbon source. The loss of the TOL plasmid was

8 determined as the ratio between the colony forming units (CFUs) obtained in 3-methylbenzyl alcohol

9 plates divided by the CFUs on benzoate plates. Average ratios were calculated after three independent

10 experiments with three technical replicas of each.

11

12 *Sequence analyses*

13

14 Open reading frames were identified with the ORF Finder at the NCBI server

15 (http://www.ncbi.nlm.nih.gov/projects/gorf/). Protein sequences were compared with those present in the

16 databases using the BLAST (Altschul *et al.,* 1990) algorithm for known proteins, or the TBLASTN against

17 microbial genomes at the NCBI server (http://blast.ncbi.nlm.nih.gov/). Multiple sequence alignments were

18 performed with CLUSTALW (Thompson *et al.,* 1994) at PBIL server (http://npsa-pbil.ibcp.fr/cgi-

19 bin/npsa_automat.pl?page=npsa_clustalw.html). Protein molecular weights were obtained from their

20 sequences using the Compute pI/Mw tool at ExPASy server (http://web.expasy.org/compute_pi/).

21

22 **Acknowledgments**

23

28

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30

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Figure 1. Genetic, regulatory and biochemical connections of benzoate catabolism in *P. putida* KT2440.
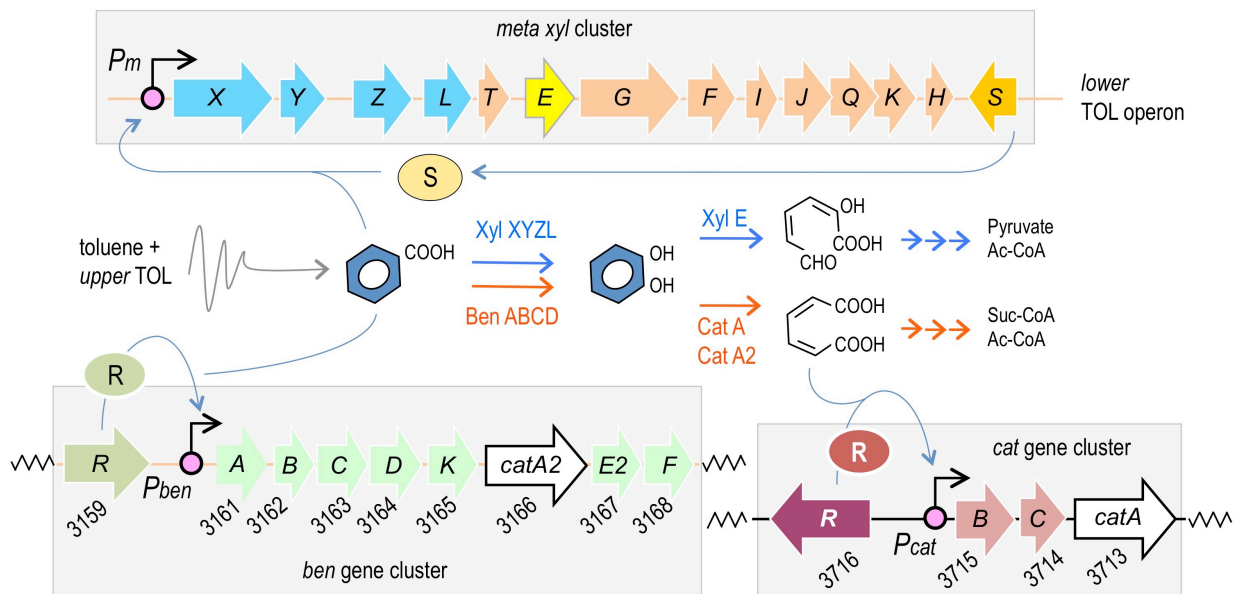
The figure sketches the three sets of reactions involved in the process: the *xyl* genes of the lower TOL operon (top), which are expressed from the *Pm* promoter activated by XylS, the *ben* genes (bottom left) for conversion of benzoate into catechol (activated by BenR) and the *cat* genes for final channeling of catechol into central metabolism. As shown, benzoate can be degraded to central intermediates via the *ortho* pathway encoded in the chromosome in which *ben* and *cat* genes are required (PP coordinates in the genome of KT2440 strain are indicated). In the presence of the TOL plasmid, that converts toluene into benzoate, the latter can be metabolized via the *meta* pathway by the products of the *xyl* genes. Both pathways converge towards catechol and diverge at that point, since this compound can be cleaved between positions 1 and 2 by CatA and CatA2, whereas XylE cleaves between positions 2 and 3. The transcriptional control of this biochemical traffic is carried out by activators that bind their cognate promoters in response to their inducers: benzoate for BenR and XylS and *cis,cis*-muconate for CatR.

Figure 2. The role of *catA* and *catA2* in benzoate metabolism.
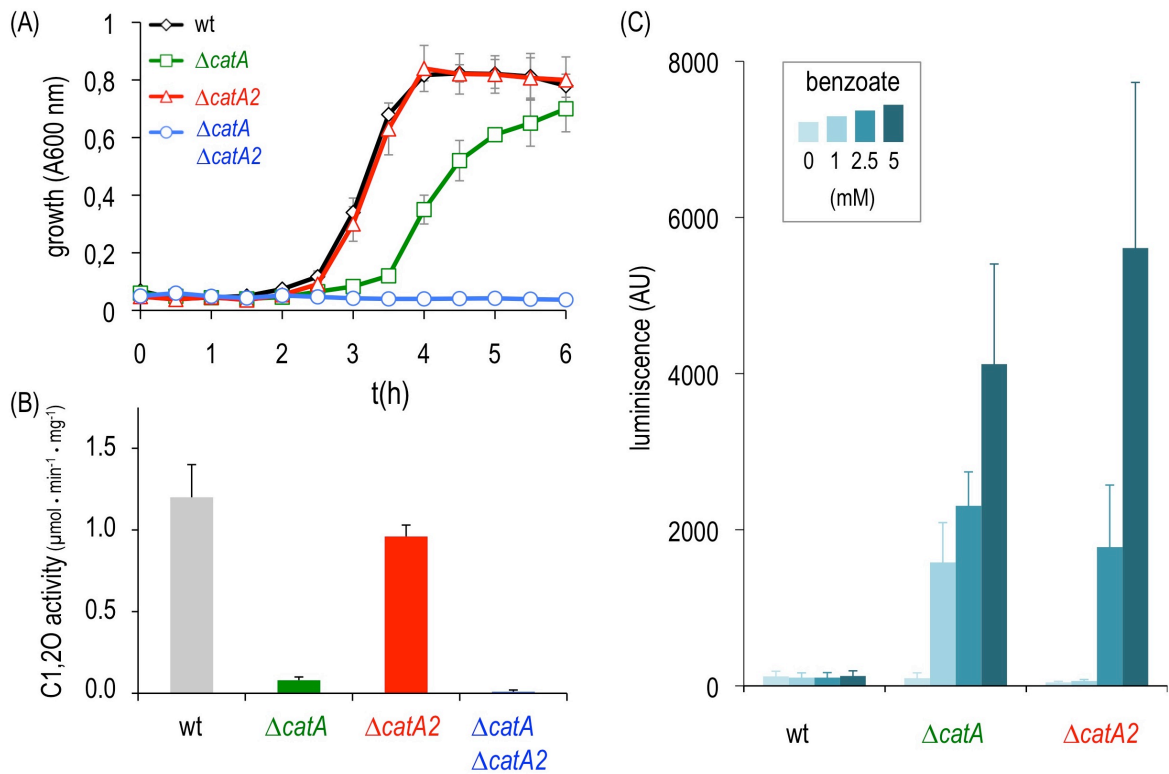
(A) Growth curves of the strain *P. putida* KT2440 and the single and double mutants in benzoate (5 mM) as the sole carbon source. (B) C1,2O activity in crude extracts of *P. putida*. Each of the strains indicated were pregrown in citrate (0.2%) as the major carbon source and supplemented with 5 mM benzoate for induction of *cat* genes. (C) Intracellular levels of catechol exposed with a *lux*-based biosensor containing the regulator DmpR and the promoter *Po*. Cells were grown in different concentrations of benzoate as the sole carbon source as indicated. Both specific activity and luminescence were recorded in mid-exponential phase. Experimental points and error bars shown indicate the average value and the standard deviation of at least four independent experiments.

Figure 3. Accumulation of colored metabolic intermediates of benzoate catabolism in *P. putida cat* mutants.
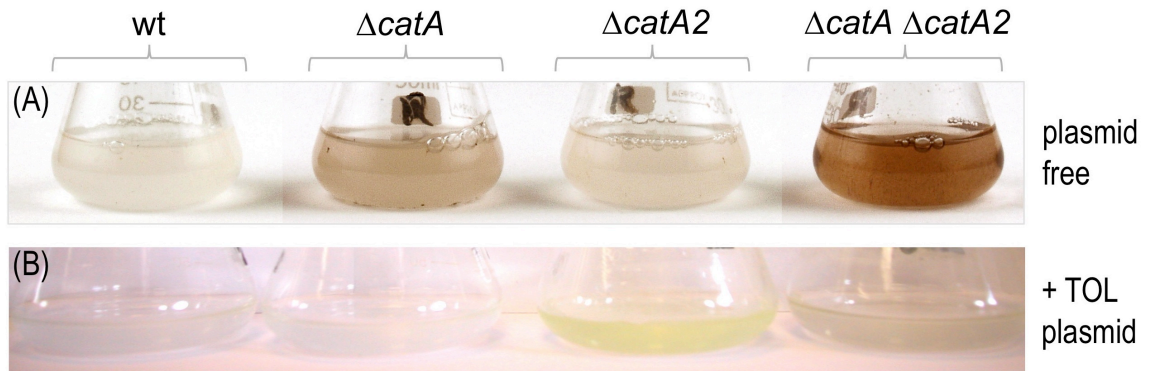
(A) Plasmid-free *P. putida* KT2440 and its Δ*catA,* Δ*catA2* and Δ*catA* Δ*catA2* variants were pre- grown in minimal medium with 0.2% citrate, diluted to $OD_{600} = 0.05$ in benzoate 5 mM and incubated for 8 h. Note dark tars accumulated as a result of spontaneous oxidation and polymerization of catechol. (B) The plasmid pWW0 was transferred to the same strains shown above and the resulting cells were cultured in the same way. In this case the yellow pigmentation corresponds to the accumulation of HMS after 4 h of culture.

Figure 4. Growth and accumulation of HMS by *cat* mutants *P. putida* harboring the TOL plasmid.
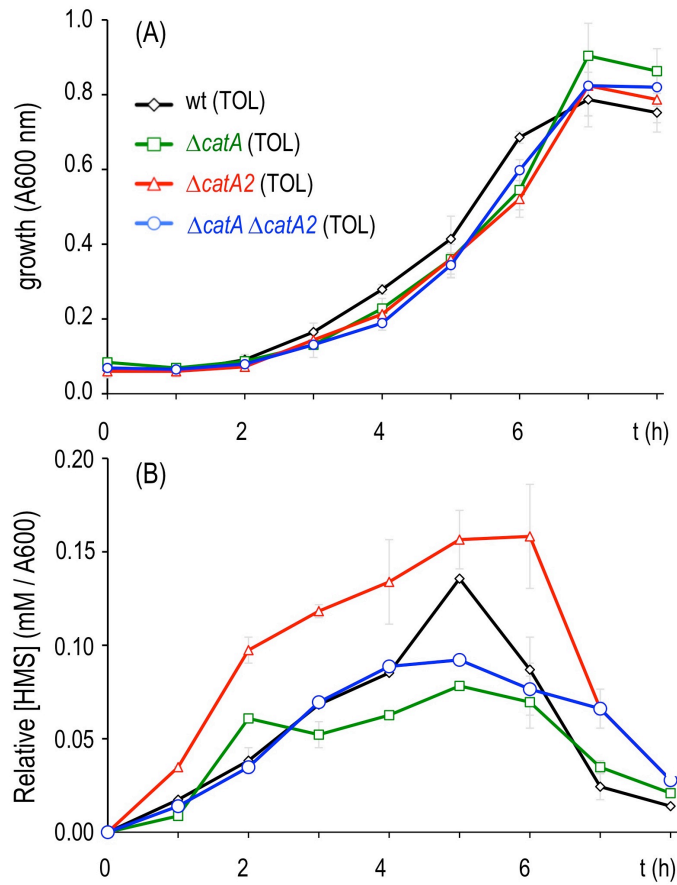
(A) Growth curves of the Δ*catA,* Δ*catA2* and Δ*catA* Δ*catA2* strains bearing the TOL plasmid pWW0 on benzoate (5 mM) as the sole carbon source. (B) HMS accumulation in culture supernatants normalized to the optical density of each culture at the times indicated. The results shown are the average of ≥ 4 independent experiments.

1   **Figure 5**. XylE activity and *Pm* promoter output in response to benzoate.

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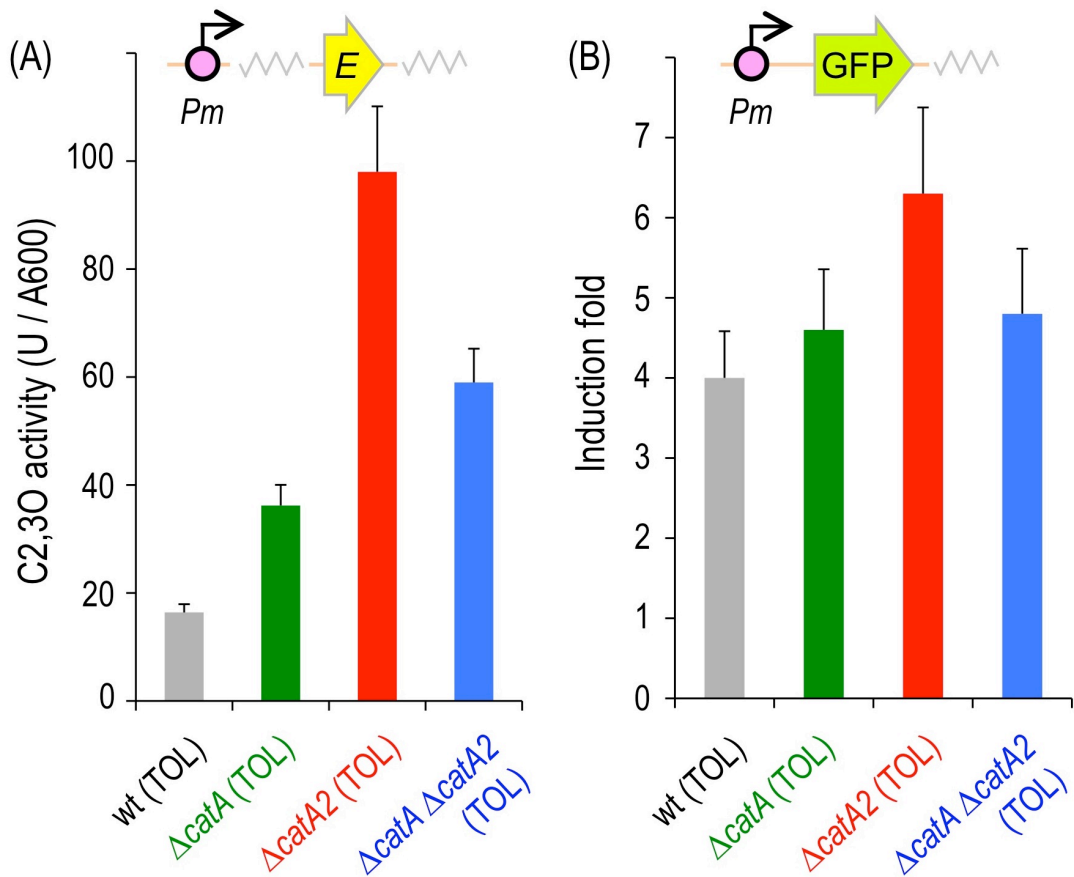
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5   (A) The *P. putida* strains carrying the TOL plasmid pWW0 were pre-grown in 0.2% citrate and transferred

6   to a minimal medium with benzoate as sole carbon source. The figure plots the relative levels of C2,3O

7   (encoded by *xylE*) 3 hour after the transfer. (B) Same pWW0-containing strains but harboring pGLR1-Pm

8   with a transcriptional fusion of the *Pm* promoter to GFP. The induction fold of the Y axis is the ratio

9   between the fluorescence raised by GFP expression (per OD_{600}) in the presence or absence of benzoate

10   after 3 h of culture**.** The average of $\geq$ 4 independent experiments is shown.

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1 **Figure 6.** Loss of TOL plasmid in consecutive cultures in benzoate as the sole carbon source.

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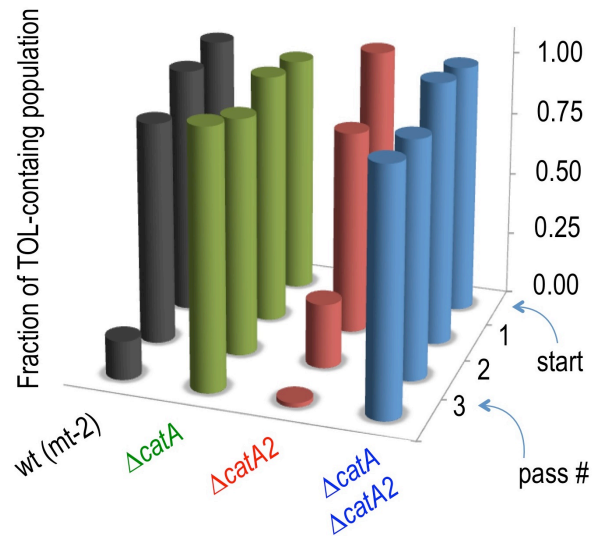
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5 The *P. putida* variants carrying the TOL plasmid were pre-grown in citrate, transferred to a minimal

6 medium with benzoate as sole carbon source and grown for 24 h. After that time, an aliquot of the cells

7 was transferred to fresh media with benzoate. This step was repeated two more times to carry out, in

8 total, four consecutive cultures in benzoate. For differentiating cells that maintained the TOL plasmid from

9 those that had lost it, adequate dilutions of culture samples were plated on minimal with medium 3-

10 methylbenzylalcohol and the CFUs compared to those growing on benzoate at each pass. The bars

11 indicate the average of four independent experiments. Standard deviation (not shown) was systematically

12 $\leq$ 15%.

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Figure 7. Effect of the *ortho* and *meta* pathway usage in the doubling time of the *cat* mutants.
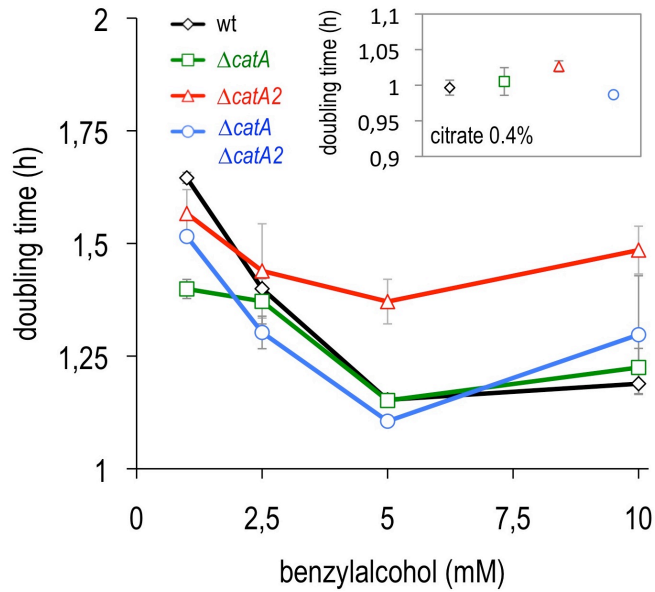
Cells were grown in different concentrations of benzoate or benzylalcohol as the sole carbon sources as indicated. Metabolism of benzylalcohol by the upper TOL pathway renders benzoate that induces simultaneously the *ortho* and *meta* pathways. Since benzylalcohol requires the *upper* pathway in the TOL plasmid to be metabolized, cells are forced to keep the plasmid for growth. The inset shows the doubling time of the mutants growing on citrate (0.4%) as a control, where the difference is negligible (note the different scale in the Y axis).

SUPPLEMENTARY MATERIAL

Supplementary Figure S1. Genomic context of *ben* and *cat* genes in various *Pseudomonas* strains
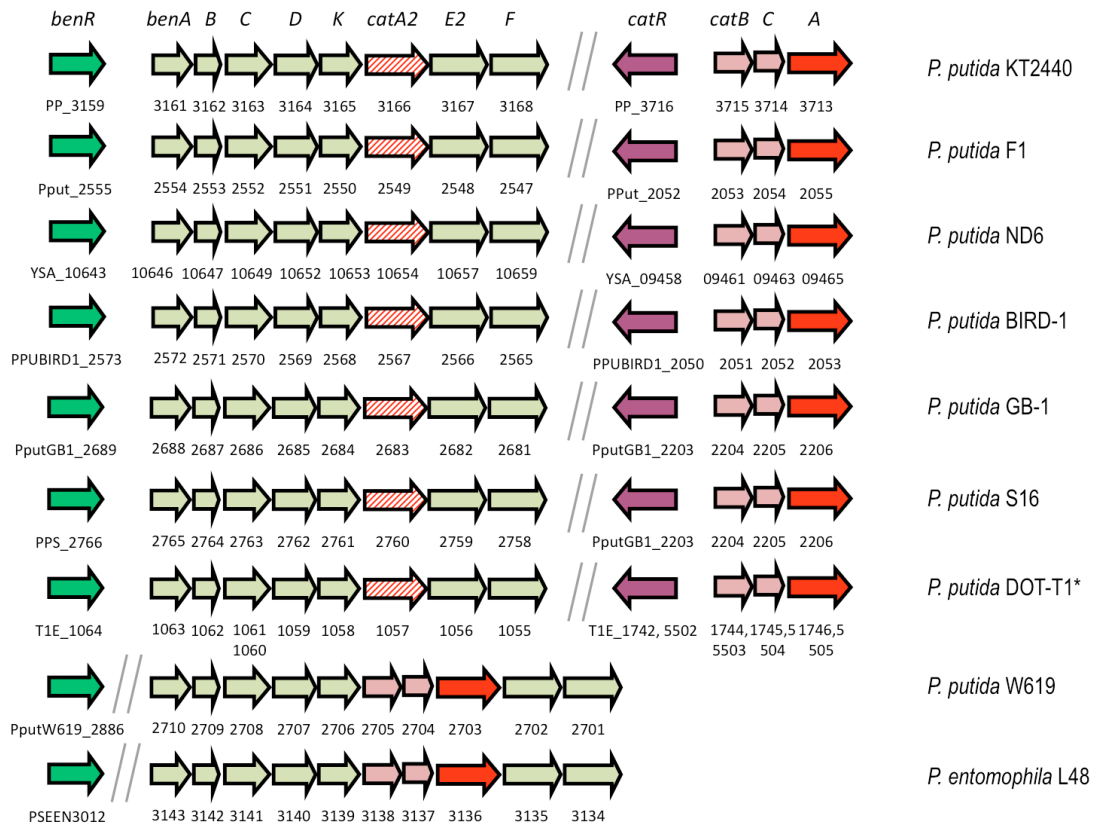
The figure compares the *ben* (green) and *cat* (red) gene clusters that contain *catA* variants. Lighter colors indicate catabolic and transport genes and dark colors designate regulatory genes. ORFs encoding CatA and CatA2 are displayed as solid red and stripped red arrows, respectively. In *P. putida* DOT-T1 (marked *) the *benC* gene is split in two ORFs. Also, two virtually identical *cat* clusters were identified in different positions of the genome.

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3 **Supplementary Figure S2.** Growth phenotypes of *P. putida* KT2440 and *cat* mutants in the presence of

4 different concentrations of benzoate.

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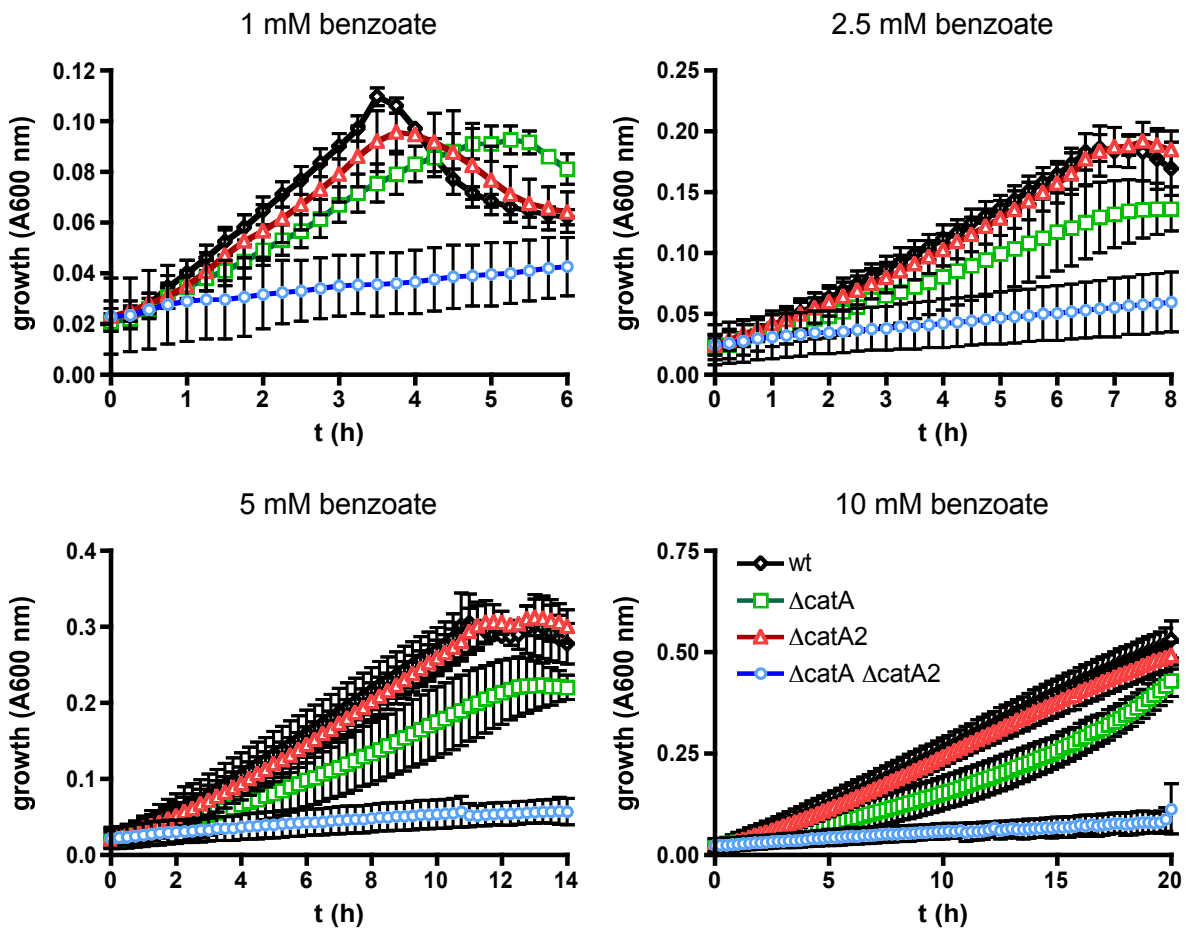
7

8 The figure shows the average absorbance and standard deviation of three culture replicates as a function

9 of time. Experiments were carried out at 30 °C in 96 well plates (200 µl/well) in a plate reader with orbital

10 continuous shaking. The results are qualitatively similar to the growth curves shown in Fig. 2A where the

11 cells were grown in flasks and the values of absorbance at 600 nm determined in a spectrophotometer.

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1 **Supplementary Figure S3.** Protein extracts employed for determination of apparent kinetic

2 parameters of CatA and CatA2 enzymes

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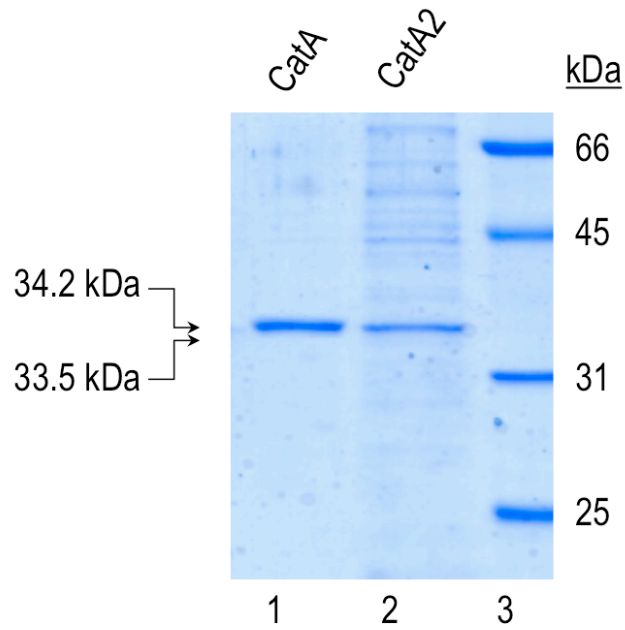
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6
7
8 The image is a representative SDS-PAGE analysis of crude extracts of *E. coli* BL21 expressing CatA and

9 CatA2 as indicated. Gel was loaded with 5 μg of total protein. Broad Range markers (New England

10 Biolabs) were used as molecular weight standards.

11

1

2 **Supplementary Figure S4.** Kinetic assays of enzymatic activity using protein extracts of *E. coli* cells
3 expressing either CatA or CatA2. Insets show the Lineweaver-Burk plots
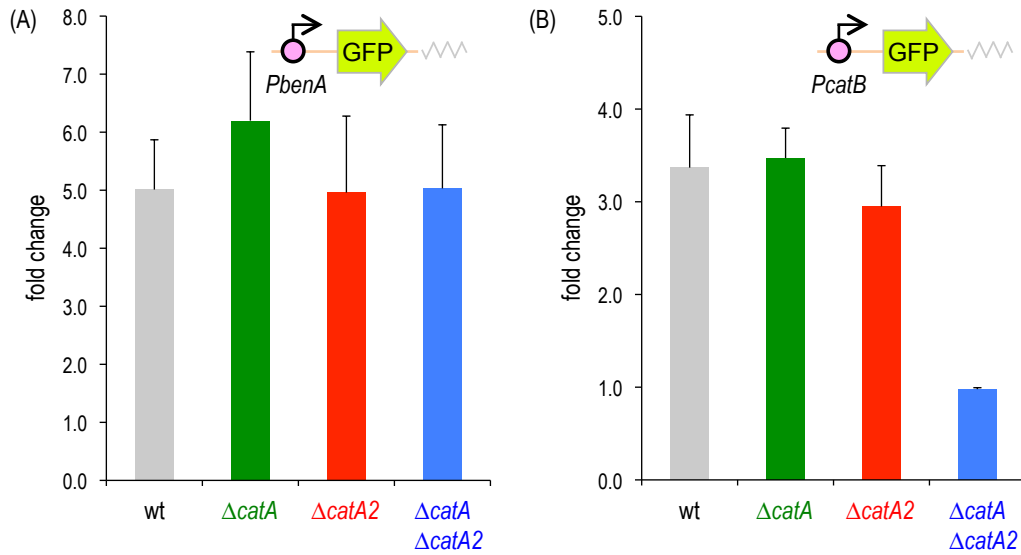4 used to estimate the K_m and V_{max}.
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CatA

CatA2

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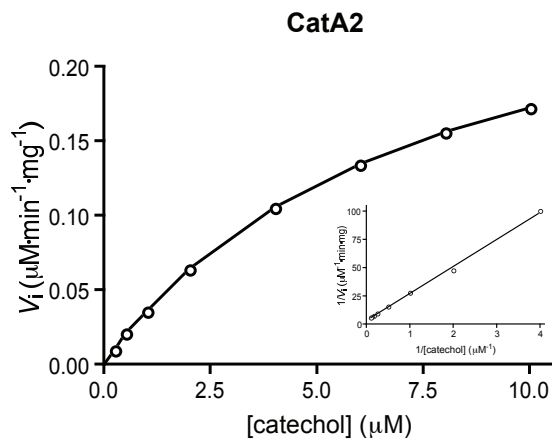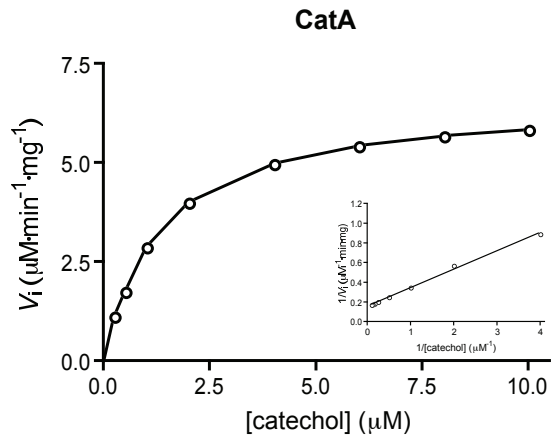
3   **Supplementary Figure S5.** *PbenA* and *PcatB* output in response to benzoate.

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8   *P. putida* wild-type cells and its *cat* mutant derivatives indicated harboring a *PcatB*-GFP transcriptional

9   fusion were grown in citrate (0.2%) as the carbon source and added with benzoate (5 mM) for induction of

10   promoter activity. The induction fold of the Y axis is the ratio between the fluorescence raised by GFP

11   expression (per OD_{600}) in the presence or absence of benzoate after 3 h of culture**.** The average of $\geq$ 4

12   independent experiments is shown.

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Supplementary Figure S6. Growth phenotype of *P. putida* KT2440 and mutants harboring the TOL plasmid in the presence of different concentrations of benzoate.
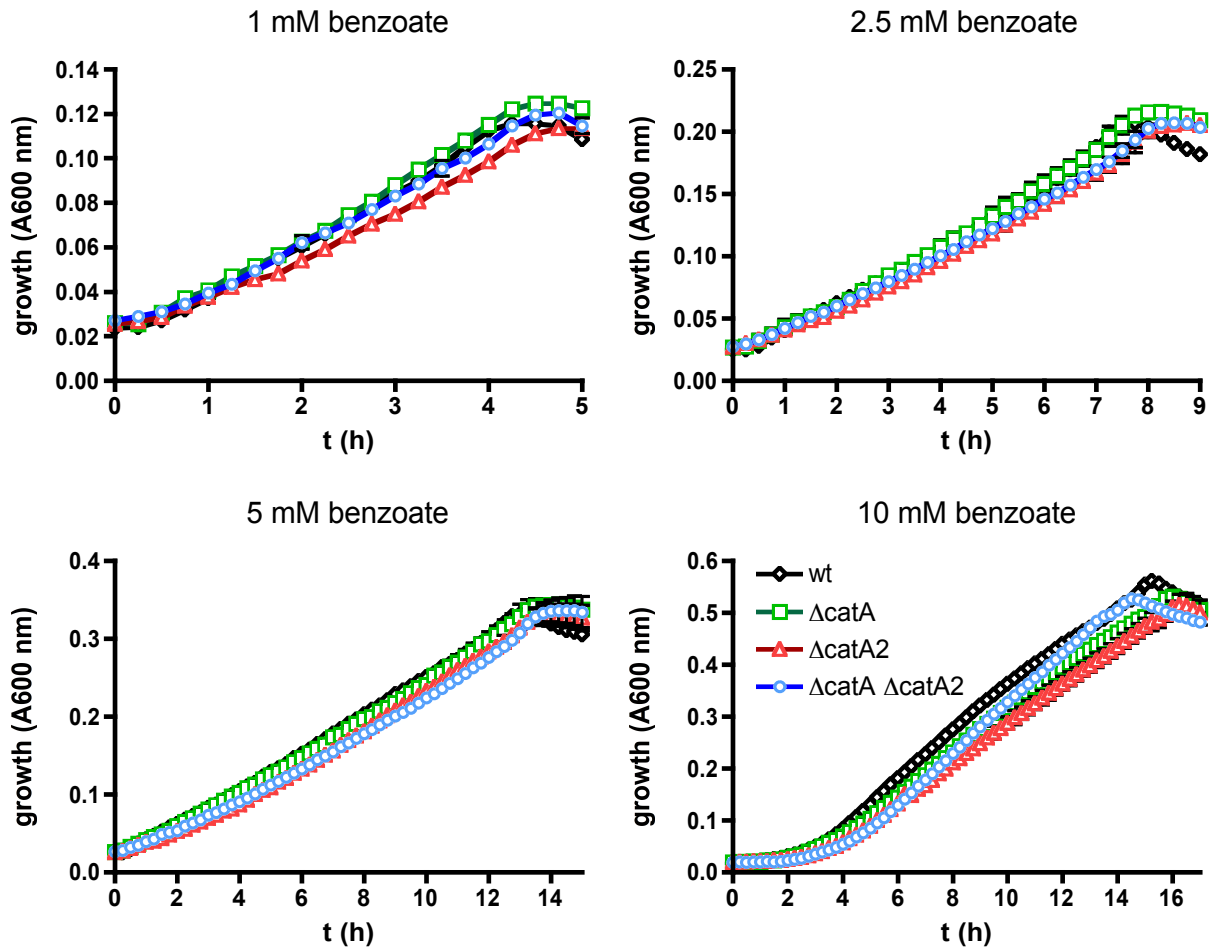
The figure shows the average absorbance and standard deviation of three culture replicates as a function of time. Experiments were carried out in microtiter plates (200 μl/well) in a plate reader with orbital continuous shaking and incubation at 30 °C. The results are qualitatively similar to the growth curves shown in Fig. 2A where the cells were grown in flasks and the values of absorbance at 600 nm determined in a spectrophotometer.

1 **Supplementary Figure S7.** Phylogenetic tree of CatA and CatA2 enzymes in various

2   *Pseudomonas* strains

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6 The figure plots the maximum likelihood phylogenetic tree showing the relatedness among catechol 1,2-

7 dioxygenases of *Pseudomonas* strains harbouring more than a single *catA* gene. The deduced protein

8 sequences were aligned with MUSCLE using default values (Edgar, 2004). The alignment was used to

9 evaluate models of sequence evolution using Prottest v.2.4 (Abascal *et al*., 2005) considering the Akaike

10 information criterion to select the most appropriate evolutionary model. The tree was constructed based

11 on the selected model using MEGA 5.0 (Tamura *et al*., 2011). Predicted gene duplications are shown in

12 blue circles. The larger evolutionary distance among CatA1 and CatA2 in *P. putida* KT2440 and closely

13 related strains indicates a different origin, thereby suggesting that *catA2* is acquired by horizontal gene

14 transfer.

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1 **Supplementary Table S1.** Substrate specifity of CatA and CatA2

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Substrate	Relative activity[a] (%)	
	CatA	CatA2
catechol	100	100
4-methylcatechol	44	59
3-methylcatechol	5	7
4-chlorocatechol	1.7	5
protecatechuate	<1	n.d.

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5 [a]Relative activity is defined as the percentage of the specific activity recorded using catechol as the

6 substrate reaction for each of the enzymes.

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1   **Supplementary Table S2.** Oligonucleotides used in this study

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name	sequence (5' to 3')
catA5	CGGAATTCGCCAGCAAAGTTGATC
catA3	GGGGATCCAAGGATGCCGAAGCC
catA2int5	CGGGATCCAGGAGGCAGGGCGC
catA2int3	GCGAATTCCCCCGACAGGTTTATC
catA5Nt	GCCATATGACCGTGAAAA TTTCCC
catA3ext	CCGAATTCTAGATCACCCGCCCCTG
catA25	CGCATATGACC GTGAACATTTCCC
catA23	CCGAATTCAGGCCTCCTGCAAAGCTC
SacI-PbenA-FW	ATGGAGCTCACCTGGTAGCTGCAAAAGGA
BamHI-PbenA-RV	GTCGGATCCGCCAGGGTCTCCCTTGTTAT
EcoRI-catB-FW	ATCGGAATTCAGCACGGTGCAGGTCTGT
BamHI-catB-RV	AGTCGGATCCGTTGCCAGGTCCCGTCAG
EcoRI-Pm-FW	ATGGAATTCGGCGACGTTCAAGAAGTAT
BamHI-Pm-RV	GTCGGATCCTTGTTTCTGTTGCATAAAGCCTA

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Supplementary references

Abascal, F., Zardoya, R., and Posada, D. (2005) ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **21:** 2104–2105.

Edgar, R.C. (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5:** 113.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28:** 2731-2739.