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Strict and direct transcriptional repression of the *pobA* gene by benzoate avoids 4-hydroxybenzoate degradation in the pollutant degrader bacterium *Cupriavidus necator* JMP134

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Summary

As other environmental bacteria, *Cupriavidus necator* JMP134 uses benzoate as preferred substrate in mixtures with 4-hydroxybenzoate, strongly inhibiting its degradation. The mechanism underlying this hierarchical use was studied. A *C. necator benA* mutant, defective in the first step of benzoate degradation, is unable to metabolize 4-hydroxybenzoate when benzoate is also included in the medium, indicating that this substrate and not one of its catabolic intermediates is directly triggering repression. Reverse transcription polymerase chain reaction analysis revealed that 4-hydroxybenzoate 3-hydroxylase-encoding *pobA* transcripts are nearly absent in presence of benzoate and a fusion of *pobA* promoter to *lacZ* reporter confirmed that benzoate drastically decreases the transcription of this gene. Expression of *pobA* driven by a heterologous promoter in *C. necator benA* mutant, allows growth on 4-hydroxybenzoate in presence of benzoate, overcoming its repressive effect. In contrast with other bacteria, regulators of benzoate catabolism do not participate in repression of 4-hydroxybenzoate degradation. Moreover, the effect of benzoate on *pobA* promoter can be observed in heterologous strains with the sole presence of PobR, the transcriptional activator of *pobA* gene, indicating that PobR is enough to fully reproduce the phenomenon. This novel mechanism for benzoate repression is probably mediated by direct action of benzoate over PobR.

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Introduction

A vast array of aromatic compounds (AC) are found in the environment as amino acids, lignin degradation products, secondary metabolites produced by plants, or pollutants such as petroleum derivatives, herbicides, pesticides, solvents and industrial by-products. A significant number of AC is toxic and resistant to biodegradation as consequence of their chemical stability (Chaudhry and Chapalamadugu, 1991; Symons and Bruce, 2006). However, microorganisms, including aerobic soil bacteria, degrade several types of AC using different biochemical pathways that allow them to grow on these carbon sources (Harwood and Parales, 1996; Cao *et al*., 2009). Most of the current knowledge on AC biodegradation has been obtained with single AC growth tests. This is more explained for the sake of the simplicity and better control of variables provided by such experimental setting than to better reflex the natural situation where AC mixtures, often complex, are commonly found (Gibson and Subramanian, 1984). Several examples that binary AC mixtures are hierarchically degraded have been reported, i.e. one AC is a preferred substrate that is catabolized first in the mixture delaying degradation of the second component (Nichols and Harwood, 1995; Ampe *et al*., 1998; Brzostowicz *et al*., 2003; Choi *et al*., 2007; Zhan *et al*., 2009). The presence of a favourite, preferred substrate whose catabolism represses the expression of genes involved in degradation of the alternative, non-preferred substrate is a phenomenon known as catabolic repression by carbon (CRC). The regulatory mechanism has been most intensively studied in Enterobacteria such as *Escherichia coli* and Firmicutes as *Bacillus subtilis*, where CRC molecular basis for sugar mixtures have been well established (Deutscher, 2008; Görke and Stülke, 2008). However, the CRC mechanisms for mixtures including non-sugar substrates in proteobacteria have been shown to be different from those already reported. In the α-proteobacterium *Sinorhizobium meliloti*, succinate mediates CRC of sugar utilization through a two-component regulatory system (Garcia *et al*., 2010). In the γ-proteobacteria *Pseudomonas putida* and *Acinetobacter baylyi*, CRC in mixtures of AC plus organic acids involves a global regulator called

catabolite repression control (Crc) protein, which is a RNA-binding protein that acts post-transcriptionally (Zimmermann *et al*., 2009; Rojo, 2010). Finally, in the β-proteobacteria *Acidovorax* sp. KKS102, a two-component system is proposed to be involved in the CRC of succinate over biphenyl degradation (Ohtsubo *et al*., 2006), indicating that multiple CRC mechanisms are present in bacteria.

The molecular studies on substrate combinations containing only AC have been focused mostly on the model mixture composed by benzoate (Bz) and 4-hydroxybenzoate (4-HB) that commonly are metabolized by different branches of the widespread β-ketoadipate pathway (Harwood and Parales, 1996; Fig. 1A). Bz is the preferred substrate in Bz/4-HB mixtures allowing growth of the γ-proteobacteria *P. putida* PRS2000 (Nichols and Harwood, 1995; Cowles *et al*., 2000) and *A. baylyi* ADP1 (Brzostowicz *et al*., 2003). In both cases, the CRC mechanism is mainly based on repression of *pcaK* gene, encoding a 4-HB transporter, and involves the transcriptional activators of the Bz degradation: BenR in the case of strain PRS2000, an AraC-type regulator (Cowles *et al*., 2000), and CatM/BenM in strain ADP1, both belonging to the LysR-type regulator family (Romero-Arroyo *et al*., 1995; Collier *et al*., 1998). In the case of strain ADP1, interaction of CatM and BenM over transcriptional start of *pcaU* gene, encoding the regulator of the *pca* genes (Gerischer *et al*., 1998), inhibits activation of *pca* genes, including *pcaK* gene and lowering the PcaK-mediated uptake of 4-HB (Brzostowicz *et al*., 2003).

The β-proteobacterium *Cupriavidus necator* JMP134 (Don and Pemberton, 1981) uses more than 50 different AC as sole carbon source, including Bz and 4-HB, and its genome encodes most of the AC catabolic pathways described so far in proteobacteria (Pérez-Pantoja *et al*., 2008; Lykidis *et al*., 2010) and shows a high degree of genetic redundancy (Pérez-Pantoja *et al*., 2008; 2009). Several other proteobacterial strains show broad catabolic abilities towards AC (Pérez-Pantoja *et al*., 2010a,b), making highly relevant the elucidation of the strategies followed by such catabolically versatile species when they are exposed to AC mixtures. We investigated such strategies choosing as study model the metabolization of the Bz/4-HB mixture by *C. necator* JMP134. In this bacterium, Bz and 4-HB are degraded through the catechol and protocatechuate *ortho* ring cleavage branches of the β-ketoadipate route respectively (Pérez-Pantoja *et al*., 2008; Fig. 1A). Bz is degraded using the enzymes encoded by the *ben*/*cat* gene clusters controlled by transcriptional regulators CatR1 and CatR2, whereas 4-HB is metabolized by the gene products of the *pob*/*pca* cluster mastered by transcriptional regulators PobR and PcaQ (Fig. 1B). We report here a novel mechanism for Bz/4-HB hierarchical utilization in *C. necator* JMP134 not relying on the regulators of Bz pathway as described in other bacteria, and supporting the diversity of CRC mechanisms evolved in environmental bacteria to deal with mixtures of several substrates.

Results and discussion

Bz inhibits degradation of 4-HB by C. necator *in Bz/4-HB mixtures*

As in the γ-proteobacteria *P. putida* PRS2000 and *A. baylyi* ADP1, growth of *C. necator* JMP134 on Bz/4-HB mixtures revealed preference for Bz. When tested as single substrates, both 4-HB and Bz supported abundant and fast growth of *C. necator* (Fig. 2A and B), as complete utilization of both AC was observed after 12 h of culture. However, degradation of 4-HB was completely arrested when provided in an equimolar mixture with Bz, whereas the latter was used as growth substrate as efficiently as in single cultures (Fig. 2C). This result suggests that Bz and/or its catabolites suppress 4-HB degradation as removal of the latter compound started only after complete consumption of Bz as preferred substrate (Fig. 2C). To distinguish if Bz or its intermediates produced this effect, the same growth tests were carried out with a *C. necator benA* mutant. This mutant lacks a functional benzoate 1,2-dioxygenase, enzyme that performs the conversion of Bz to 1,2-dihydroxy-cyclohexa-3,5-diene-1-carboxylate (Fig. 1A) (Yamaguchi and Fujisawa, 1982). This mutant grows on 4-HB as the wild type (Fig. 2D), but it is unable to grow on and consume Bz (Fig. 2E), even if the bacterium was cultured for 72 h (data not shown). The latter discards growth based on utilization of *box* genes encoded functions (Pérez-Pantoja *et al*., 2008), which would allow growth on benzoate through CoA derivatives, and revealing the major role of *ben-cat* pathway for growth on this compound in *C. necator* JMP134. Surprisingly, when this mutant was cultivated in the Bz/4-HB mixture it completely failed to grow (Fig. 2F), indicating that Bz is the main, if not the only responsible of growth suppression on 4-HB, and that its metabolization is not necessary for the repressive effect. The *benA* mutant grew efficiently on mixtures of Bz with 2-hydroxybenzoate or 3-hydroxybenzoate (data not shown), therefore discarding toxicity effects of non-metabolized Bz. The repression of 4-HB catabolism was dose-dependent as growth inhibition in the *C. necator benA* mutant was observed when Bz was present at levels over 0.5 mM (Fig. S1A). Further support for Bz being the molecule directly involved in repression came from the observed immediate growth arrest on 4-HB of the *benA* mutant after addition of Bz to a 6 h culture (Fig. S1B).

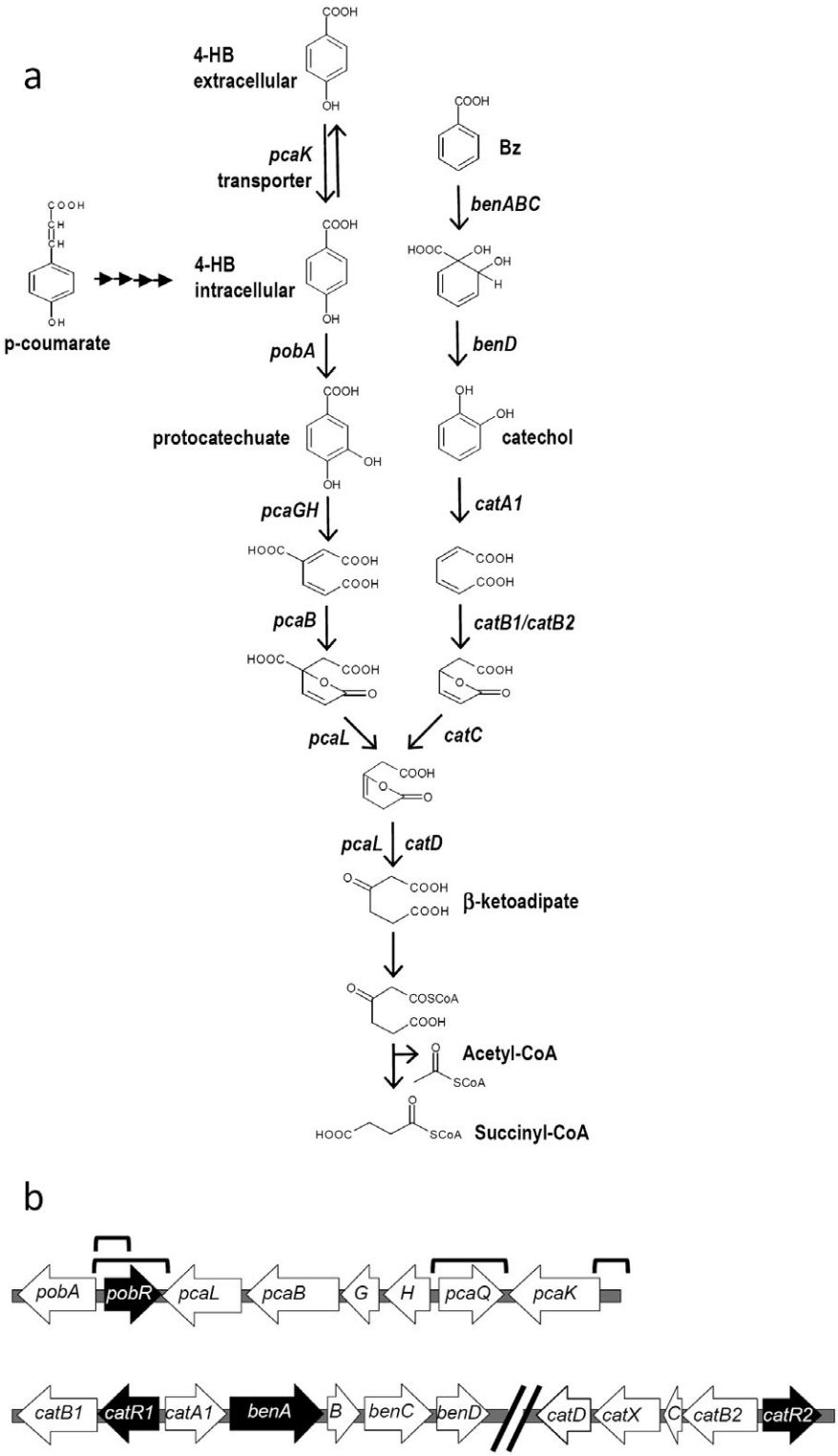

Fig. 1. Catabolic route and genes for degradation of benzoate and 4-hydroxybenzoate in *Cupriavidus necator*.
A. Degradation of benzoate and 4-hydroxybenzoate in *C. necator* JMP134 via the catechol and protocatechuate branches of β-ketoadipate pathway respectively. Peripheral reactions for *p*-coumarate producing 4-hydroxybenzoate as intermediate are also depicted.
B. Gene clusters involved in 4-hydroxybenzoate (top) and benzoate (bottom) catabolism are shown. Black boxes indicate genes that were mutated by chromosomal disruption. Brackets indicate promoter sequences from *pobA, pcaH* and *pcaK* genes cloned to construct *lacZ* transcriptional fusions.

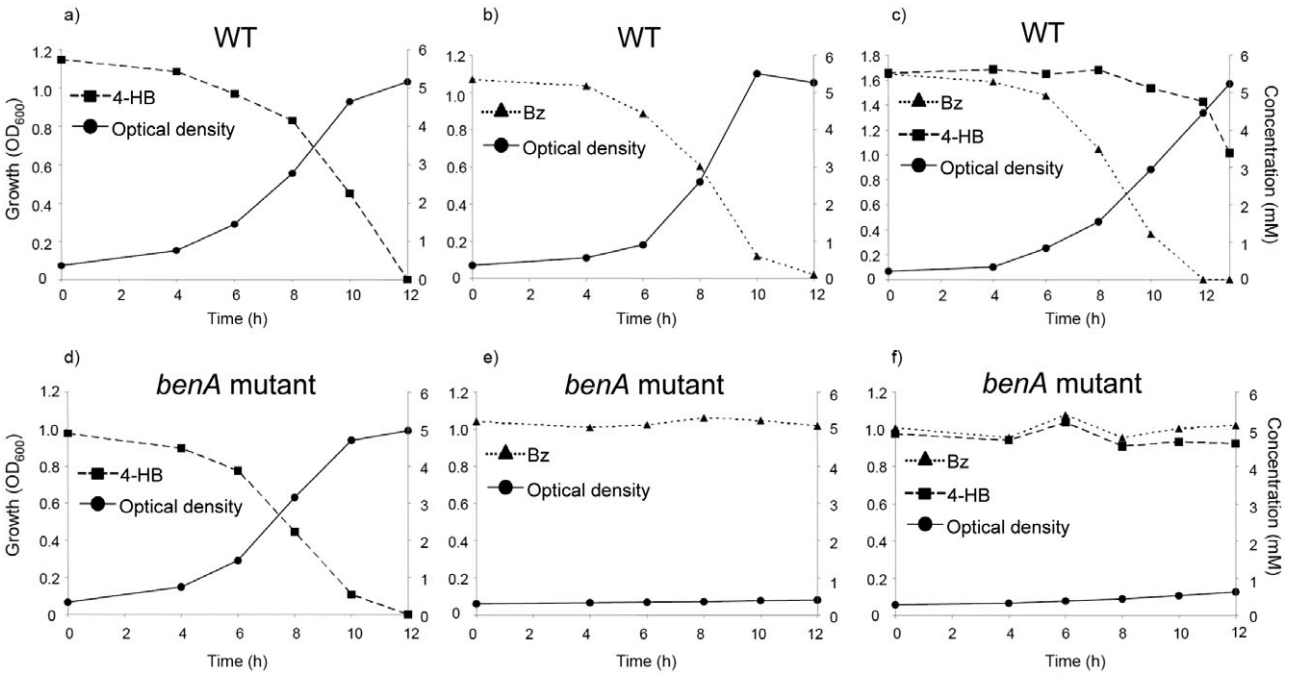

Fig. 2. Catabolic preference for benzoate during growth of *Cupriavidus necator* on benzoate/4-hydroxybenzoate mixtures. Growth of *C. necator* and a *C. necator benA* mutant on 5 mM 4-hydroxybenzoate (A, D), 5 mM benzoate (B, E) or a mixture of both (C, F). Optical density at 600 nm (closed circles) and concentrations of 4-hydroxybenzoate (closed squares) and benzoate (closed triangles) are shown.

Bz decreases pob *and* pca *genes expression*

In order to identify the target of Bz repression, the growth of the *C. necator benA* mutant in mixtures of Bz plus *p*-coumarate or protocatechuate was evaluated. During *p*-coumarate catabolism, 4-HB is intracellularly produced by *C. necator*, therefore bypassing 4-HB transport, whereas protocatechuate is the first downstream intermediate in degradation of 4-HB, thus not requiring the enzyme catalysing the first step in 4-HB catabolism (Pérez-Pantoja *et al*., 2008; Fig. 1A). The *C. necator benA* mutant grew well on both *p*-coumarate or protocatechuate as single substrates, but growth on the *p*-coumarate/Bz mixture was severely repressed (Fig. S2A). As *p*-coumarate behaves as 4-HB in mixtures with Bz, the repressive effects of Bz are not targeted to 4-HB transport but probably are focused on enzymes belonging to 4-HB catabolic pathway. However, an inhibition of *p*-coumarate conversion by benzoate cannot be completely ruled out. In turn, growth on the protocatechuate/Bz mixture was only slightly decreased (Fig. S2B), indicating that the main target for Bz effect occurs upstream formation of protocatechuate. It was envisaged that the putative target would be the enzyme 4-HB 3-hydroxylase that catalyses formation of protocatechuate from 4-HB and it is encoded by *pobA* gene (Fig. 1A) (Pérez-Pantoja *et al*., 2008). To test if Bz affects *pobA* expression, reverse transcription polymerase chain reaction (RT-PCR) experiments were performed on different inducing conditions. The *pobA* transcript was clearly identified when cells of *C. necator* were exposed to 4-HB but was undetected in incubations with Bz/4-HB, as in the non-induced condition (Fig. 3). The expression of *benA* induced by Bz was not affected under the same conditions, indicating that the cells are fully responsive to AC as inducers (Fig. 3). In order to evaluate if the absence of *pobA* transcript is due to a transcriptional or post-transcriptional regulatory mechanism, the activity of *pobA* promoter was tested by β-galactosidase assays in cells of *C. necator* containing

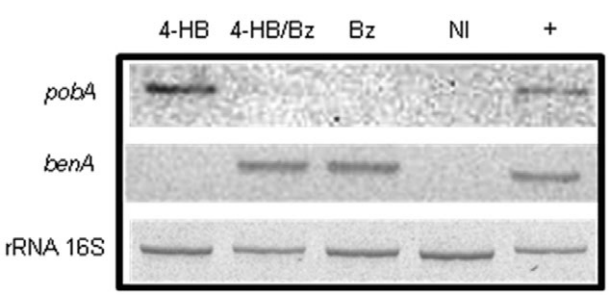

Fig. 3. Transcriptional repression by benzoate of *pobA* expression in *Cupriavidus necator*. Agarose gel electrophoresis of reverse transcription PCR products from gene transcripts encoding 4-hydroxybenzoate 3-hydroxylase (*pobA*) or benzoate 1,2-dioxygenase alpha subunit (*benA*) are shown. 16S rRNA gene transcripts were used as internal control. *C. necator* cells were exposed for 1 h to 4-hydroxybenzoate (lane 1), benzoate (lane 2), 4-hydroxybenzoate plus benzoate (lane 3) or none inducer (lane 4). Positive controls of PCR amplification (lane 5).

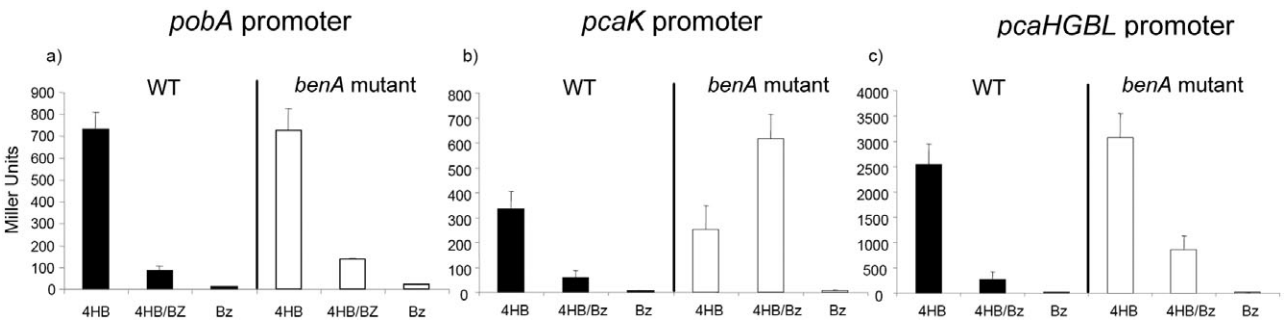

Fig. 4. Repression by benzoate of the promoter activity of *pobA, pcaK* and *pcaHGBL* genes from *Cupriavidus necator*. β-galactosidase activity levels obtained from transcriptional fusions to *pobA* (A), *pcaK* (B) and *pcaHGBL* (C) promoters exposed to 4-hydroxybenzoate, benzoate or a mixture of both in the wild type (closed bars) or in the *benA* mutant (open bars). Values are the average of three replicates. Vertical bars represent standard deviation from the mean.

transcriptional fusions to *lacZ* reporter exposed to Bz and/or 4-HB. A high activity level of *pobA* promoter was detected in presence of 4-HB, but a sharp decrease was noticed when cells were exposed to the Bz/4-HB mixture (Fig. 4A). Exactly the same activity pattern was determined in the *C. necator benA* mutant (Fig. 4A). These results strongly indicate that Bz lowers the levels of *pobA* transcripts repressing the activity of its promoter. Additional effects of Bz at the transcriptional level were tested with other two promoters involved in 4-HB degradation: the promoters of *pcaHGBL* and *pcaK* genes, encoding the conversion of protocatechuate to β-ketoadipate and 4-HB transport respectively (Pérez-Pantoja *et al*., 2008, Fig. 1B). The presence of Bz decreased activity of these two promoters with respect to the level reached with 4-HB as single substrate (Fig. 4B and C). However, in the *C. necator benA* mutant the repressive effect was observed only for *pcaHGBL* promoter (Fig. 4C) as the activity levels of *pcaK* promoter are not lower in the Bz/4-HB mixture than in 4-HB as single substrate (Fig. 4B). The repression of *pcaHGBL* expression by Bz is probably provoked by an indirect effect because of the absence of protocatechuate in Bz/4-HB mixtures, just because of the strongly reduced levels of 4-HB 3-hydroxylase in presence of Bz. It should be noted that protocatechuate is the inducer of the *pcaHGBL* promoter in strain JMP134 (data not shown) in a similar way as described for protocatechuate degradation in *A. baylyi* (Gerischer *et al*., 1998), and the activity of this promoter remains unaffected by the presence of Bz in a Bz/protocatechuate mixture (data not shown). On the other hand, repression of *pcaK* promoter would be triggered by a Bz metabolite because the promoter activity in *C. necator benA* mutant was not inhibited (Fig. 4). In this context, *cis,cis*-muconate, a benzoate degradation metabolite, did not produce any detectable effect on *pcaK* promoter activity (data not shown). Taken together, the results reported here indicate that *pobA* promoter activity is the key target for Bz repression but seems to be accompanied by inhibition of *pcaK* promoter activity involving a Bz metabolite, thus controlling both uptake and degradation of 4-HB.

Bz repression of 4-HB degradation is fully overcome when pobA *is heterologously expressed*

To further explore the role of Bz on *pobA* expression, the *pobA* gene from *C. necator* was cloned under the control of the inducible and arabinose-responsive heterologous P_{BAD} promoter, and this construct was introduced in the *C. necator benA* mutant for growth tests. This derivative containing the heterologously expressed *pobA* gene grew normally on 4-HB, and kept unable to grow on Bz (not shown). When arabinose was added as inducer of P_{BAD} promoter this *benA* mutant derivative grew in the Bz/4-HB mixture at growth yields similar to those obtained on 4-HB as single substrate (Fig. 5A, compare with Fig. 2D). In contrast, this *benA* mutant derivative did not proliferate on the Bz/4-HB mixture in absence of arabinose (Fig. 5B). It should be noted that complete degradation of 4-HB (but not Bz) was achieved (Fig. 5A), indicating that Bz repression was fully overcome when *pobA* expression is driven by a heterologous promoter, thus further supporting that the key Bz target is the *pobA* promoter.

The Bz catabolism regulators CatR1 and CatR2 do not have a role in the repression of 4-HB degradation

As indicated above, regulators of Bz degradation in *A. baylyi* ADP1 (CatM and BenM, LysR-type) and *P. putida* PRS2000 (BenR, AraC-type) are key players in Bz repression over 4-HB catabolism (Nichols and Harwood, 1995; Gaines *et al*., 1996; Cowles *et al*., 2000; Brzostowicz *et al*., 2003). *C. necator* JMP134 possesses two homologous regulators of Bz catabolic pathway: CatR1 and CatR2, belonging to LysR family, which presumably use Bz and/or *cis,cis*-muconate to activate Bz degradation (Pérez-Pantoja *et al*., 2008; Trefault *et al*., 2009). To

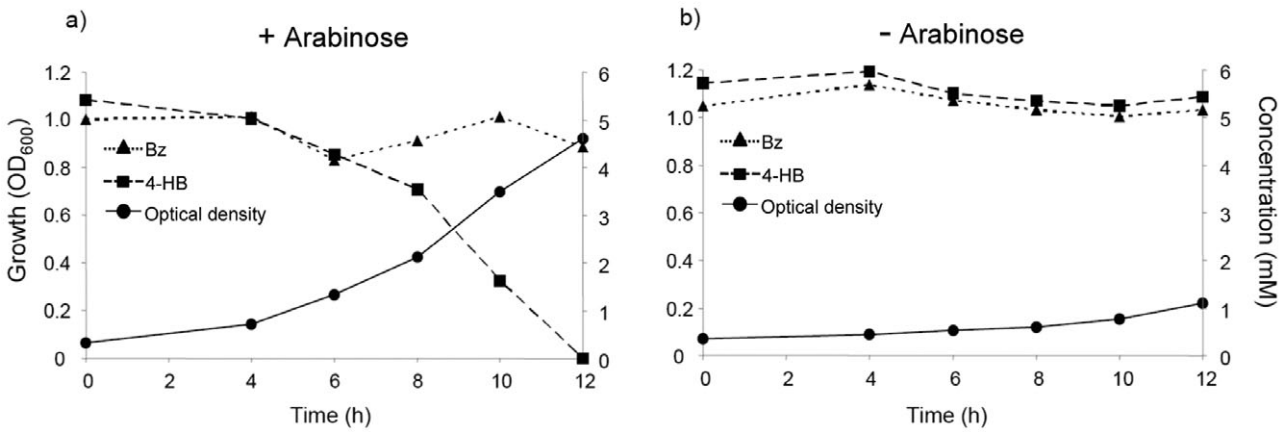

Fig. 5. Growth on a benzoate/4-hydroxybenzoate mixture of a *Cupriavidus necator benA* mutant expressing *pobA* gene driven by a heterologous P_{BAD} promoter. Growth of a *C. necator benA* mutant on benzoate and/or 4-hydroxybenzoate in the presence (A) or absence (B) of L-arabinose. Optical density at 600 nm (closed circles) and concentrations of benzoate (closed triangles) and 4-hydroxybenzoate (closed squares) are shown.

assess if these regulators are also involved in the mechanism explaining Bz preference in *C. necator*, a double *catR1-catR2* mutant was constructed, just because single mutants of each regulator only show slight effects in Bz growth indicating functional redundancy (data not shown). As expected, this double mutant did not grow on Bz. Unexpectedly, and in contrast to the γ-proteobacterial strains ADP1 and PRS2000, the regulators of *C. necator* do not participate in the repression that Bz exerts on 4-HB catabolism as deduced by the fact that the double mutant *catR1-catR2* still shows Bz repression over 4-HB degradation (Fig. S3). These results clearly indicate that CatR regulators are not acting in the repression of *pobA* promoter by Bz in *C. necator* JMP134.

PobR mediates Bz repression of pobA *expression in* C. necator

A key aspect in several CRC phenomena is the amount of transcriptional regulator activating the targeted promoter that can be post-transcriptionally modulated, as shown for BenR in *P. putida* (Moreno *et al*., 2007), or counteracted by a negative controlling factor interfering with its activating function, as reported for PhlR in *P. putida* (Müller *et al*., 1996). In *C. necator*, PobR is the putative regulator able to activate *pobA* expression in the presence of 4-HB (Pérez-Pantoja *et al*., 2008; Fig. 1B). Accordingly, a *C. necator pobR* mutant was completely unable to grow on 4-HB (data not shown). The introduction of several copies of *pobR*, under the control of the P_{BAD} promoter, in the *C. necator benA* mutant does not revert the repression generated by Bz, as such derivative was still unable to grow on the Bz/4-HB mixture (data not shown), thus suggesting that this phenomenon is not mediated by competing repressors that would be titrating the activating function of PobR over *pobA* promoter, or by mechanisms downregulating the amount of PobR. To obtain further evidence that no other regulators are participating in Bz repression in strain JMP134, two heterologous bacterial hosts: the β-proteobacterium *Burkholderia phytofirmans* and the γ-proteobacterium *E. coli* were chosen to reconstitute the Bz repression mechanism in a different genomic background. Both strains lack PobR homologues (Fig. S4), and therefore the introduction of *pobR* gene is required for induction of *pobA* promoter in response to 4-HB. Transcriptional fusions to *lacZ* driven by *pobA* promoter with or without *pobR* gene were introduced in both hosts and β-galactosidase activity levels were determined in the presence of 4-HB and/or Bz. In the absence of PobR, cells of *B. phytofirmans* containing the transcriptional fusion to *pobA* promoter did not produce significant levels of β-galactosidase even after exposure to 4-HB, Bz or both (Fig. 6B). However, when the *pobA* promoter was tested under the control of PobR, a clear induction profile by 4-HB was observed, being repressed by Bz in a similar way as described in *C. necator* (Fig. 6A, compare with Fig. 4A). Essentially the same pattern was observed for *pobA* promoter activity in *E. coli*, although the repression effect of Bz in the Bz/4-HB mixture was lower (Fig. 6C and D). These results clearly show that PobR regulator from *C. necator* is enough to fully reproduce the observed repressive effect in other bacterial hosts.

Taking together, these results allow speculation that Bz would be directly modulating the activity of PobR regulator as a negative effector. This mechanism would be highly specific because structurally similar AC as 2-hydroxybenzoate, 3-hydroxybenzoate or protocat-

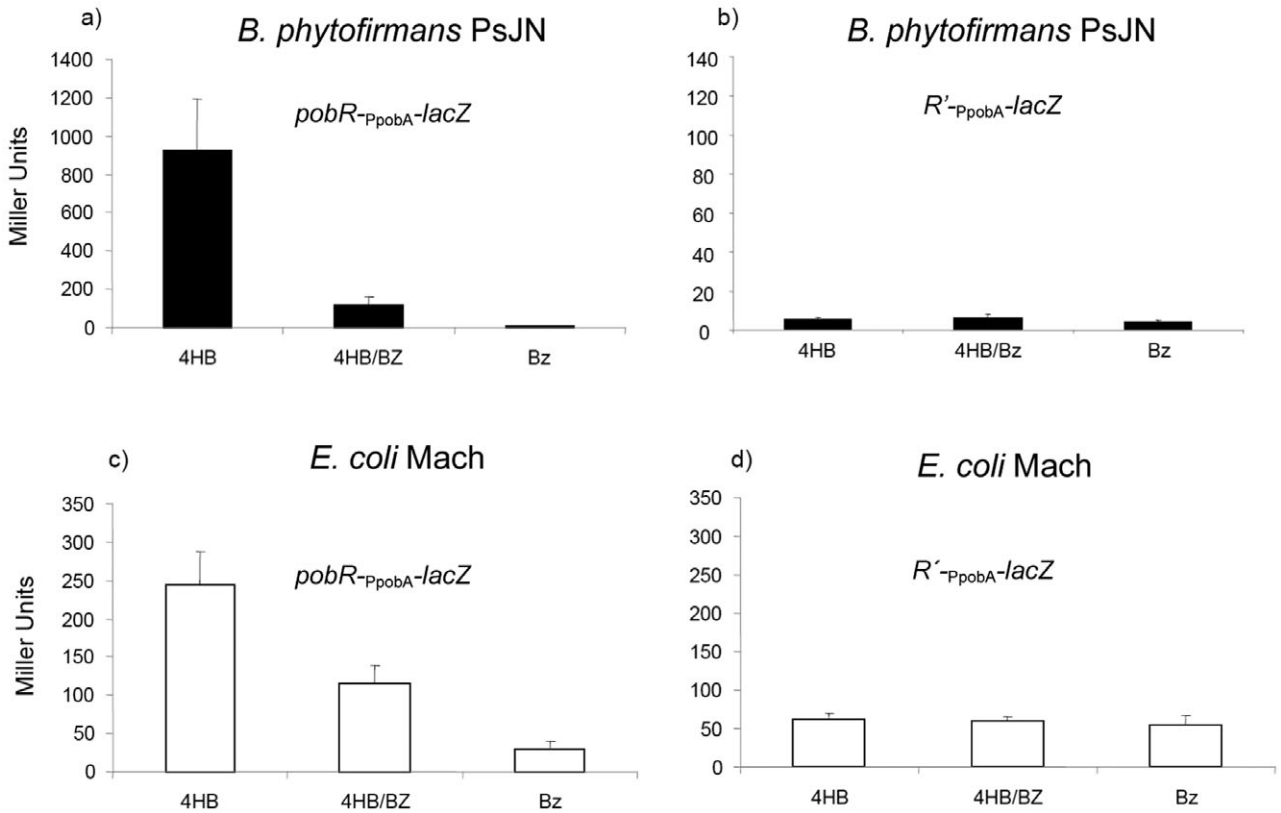

Fig. 6. Repression by benzoate of *pobA* promoter activity in *Burkholderia phytofirmans* and *Escherichia coli*, in the presence or absence of *pobR* gene. β-galactosidase activity levels obtained from transcriptional fusions to *pobA* promoter including (A, C) or not (B, D) the *pobR* gene from *C. necator*, in cells of *B. phytofirmans* PsJN (closed bars) or *E. coli* Mach (open bars) exposed to 4-hydroxybenzoate, benzoate or a mixture of both (5 mM each). R'-PpobA-*lacZ* corresponds to the complete sequence of the *pobA* promoter plus part of *pobR* gene (see Fig. 1B, top). Values are averages for three replicates. Vertical bars represent standard deviation from the mean.

echuate are unable to repress the activation of *pobA* by PobR (Fig. S5), at levels found for Bz; and it is additionally supported by the inverse relation between the Bz : 4-HB ratio and the induction level of *pobA* promoter (Fig. S6), suggesting that both compounds are antagonist competitors for a same regulator. The effect of Bz on PobR requires further investigation through detailed *in vitro* studies (DNA–protein interactions, inducer binding, etc.).

Although transcriptional activators usually respond to only one molecule able to modulate their binding to the promoter region, it has been described that in some cases multiple effectors can regulate the activating function of regulators (Ezezika *et al*., 2007; Lorca *et al*., 2007; Manso *et al*., 2009). Remarkably, most of these examples have been described in transcriptional regulators controlling catabolism of AC, suggesting that their structural similarity would be suitable for recognition of multiple effectors by regulators. One example of antagonist effectors such as the mechanism proposed for PobR here, has been recently described for the IclR regulator in *E. coli*, although the metabolites involved, glyoxylate and pyruvate, are non-aromatics (Lorca *et al*., 2007), and proposed for the aromatic regulator MhpR in phenol degradation by *Acinetobacter calcoaceticus* PHEA-2 (Zhan *et al*., 2009).

Concluding remarks

Our study shows that the mechanism for Bz preference in the β-proteobacterium *C. necator* JMP134 is clearly different from those reported in γ-proteobacterial species. In contrast to the mechanism described in *A. baylyi* ADP1 involving *cis-cis*-muconate, an intermediate of Bz catabolism, as the molecule mediating the repression, Bz itself is triggering the phenomenon in *C. necator*. A second mechanistic difference is the fact that 4-HB transporter has been proposed as the main (although some effects on *pobA* gene expression are also reported) target of repression in *A. baylyi* and *P. putida* (Cowles *et al*., 2000; Brzostowicz *et al*., 2003), which is in contrast to 4-HB 3-hydroxylase (PobA) as the key controlled element in *C. necator*. The latter is strongly supported by RT-PCR analysis, β-galactosidase assays and the heterologous

expression of *pobA*. A third fundamental difference relies on the lack of role for transcriptional regulators controlling catabolism of Bz in *C. necator* JMP134, CatR1 and CatR2 (Pérez-Pantoja *et al*., 2008), which do not participate in the repressive mechanism operating in this bacterium as deduced by the phenotype shown by the *catR1-catR2* mutant.

How frequent is the Bz repression mechanism proposed in *C. necator* among bacteria? One clue to solve this question comes from a genomic survey for *C. necator*-type PobR homologues in bacteria. It should be noted that PobR of *C. necator* belongs to the AraC-type family of transcriptional regulators (Pérez-Pantoja *et al*., 2008) in contrast to PobR of *A. baylyi*, which is a member of the IclR-type family (Molina-Henares *et al*., 2006). Based on amino acid identities, more than 100 *C. necator* PobR homologues were retrieved from the Integrated Microbial Database of DOE Joint Genome Institute (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi), belonging all of these sequences to species from α-, β- and γ-proteobacterial classes (Fig. S4). More importantly is the fact that the majority of these bacterial species possess *pobR/pobA* genes organization very similar to that found in *C. necator* (Fig. S4). In addition, benzoate 1,2-dioxygenase encoding genes are found in several of these bacteria (Fig. S4), suggesting that they would have the ability to degrade both Bz and 4-HB, and thus may possess a similar mechanism for Bz preference.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacteria and plasmids are listed in Table S1. *C. necator* and its derivatives were grown at 30°C in mineral salts medium (Dorn *et al*., 1974), supplemented with 5 mM Bz, 4-HB, protocatechuate, *p*-coumarate or fructose (10 mM), plus the appropriate antibiotics: kanamycin (Km, 100 μg ml^{-1}), gentamycin (Gm, 30 μg ml^{-1}) or spectinomycin (100 μg ml^{-1}). *B. phytofirmans* and its derivatives were grown under the same conditions. *E. coli* Mach (Invitrogen, Carlsbad, CA, USA) was grown at 37°C in Luria–Bertani (LB). Growth was measured at optical density at 600 nm (OD600). At least three replicates were performed for each growth measurement.

Chromosomal disruption of gene sequences in C. necator

Internal fragments of *benA, pobR, catR2* and the complete *catR1* gene sequence were amplified by PCR, using the primer pairs mutbenAFW-mutbenARV, mutpobRFW-mutpobRRV, mutcatR2FW-mut*catR2*RV and mutcatR1FW-mutcatR1RV respectively. All primer pairs are shown in Table S2. The PCR products were cloned using the pCR2.1-TOPO system (Invitrogen) to generate plasmids pCR2.1*benA*, pCR2.1*pobR*, pCR2.1*catR2* and p*catR1*. For single mutant construction, pCR2.1*benA* and pCR2.1*pobR* plasmids were electroporated in *C. necator* to get one-recombination-event disruption of the target gene, obtaining *C. necator benA* and *C. necator pobR* mutants, which were selected on LB agar containing 100 μg ml^{-1} Km. p*catR1* was digested with SalI and ligated to the Gm resistance of pBSL202 (Alexeyev *et al*., 1995), digested with XhoI, to obtain p*catR1*-Gm, which possesses *catR1* gene interrupted with a Gm resistance. Then, suicidal plasmid p*catR1*-Gm was electroporated in *C. necator* to obtain two recombination events, generating a replacement of the functional gene by the interrupted gene. *C. necator catR1* mutant was selected on LB agar containing 30 μg ml^{-1} Gm. Finally, suicidal plasmid pCR2.1*catR2* was electroporated in *C. necator catR1* mutant to obtain a double mutant *catR1-catR2* derivative that was selected on LB agar containing 30 μg ml^{-1} Gm and 100 μg ml^{-1} Km. Correct insertions in all mutant strains were confirmed by PCR and sequencing.

Detection of transcripts by RT-PCR

Cells of *C. necator* were grown overnight in minimal medium with fructose to inoculate a fresh culture medium, grow the cells until OD600 = 0.7, and then supplement this culture with 5 mM of Bz, 4-HB or a mixture of both and incubate for 1 h. Total RNA was obtained from 4 ml of each culture, using RNA protect bacteria reagent and RNeasy Mini Kit (QIAGEN, Chatsworth, CA, USA). The RNA was quantified using a GeneQuant 1300 Spectrophotometer (GE Healthcare, Piscataway, NJ, USA) and treated with TURBO DNase Kit (Ambion, Austin, TX, USA) to remove DNA contamination. The RT-PCR was performed using ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA) with 1 μg of RNA in 20 μl reactions. After reverse transcription, PCR amplifications of *pobA, benA* and 16S rRNA genes were performed in a 25 μl mixture, which contained 1 μl of total cDNA, 50 pmol of each primer, 50 μM of each deoxynucleoside triphosphate, 1 mM MgCl2, and 0.5 U of *Taq* DNA polymerase, prepared in the reaction buffer supplied by the manufacturer. The temperature programs were as follows (conditions for primer pair 8F-1392R shown in parenthesis): initial denaturation at 95°C for 5 min, then 30 (25) cycles of 30 (45) s at 95°C, 30 s (1 min) at 60 (56) °C, and 60 (2 min) s at 72°C, with a final extension at 72°C for 7 min. Negative control reactions were performed in the same way, except that reverse transcriptase was omitted. PCR products were detected by agarose gel electrophoresis using SYBR safe DNA gel stain (Invitrogen).

Construction of lacZ *reporter fusions*

Putative promoter regions were fused to the *lacZ* gene of pKGWP0 (Marín *et al*., 2010). A PCR product comprising the 3–1023 and 2–1101 nt region upstream of the translational starts of the *pobA* or *pcaH* genes, respectively, including *pobR or pcaQ* gene (Fig. 1B), and a PCR product comprising the 3–238 and 1–492 nt regions upstream of the translational start of *pobA* and *pcaK* genes (Fig. 1B), respectively, were obtained. The amplified DNA fragments were cloned into the XhoI-XbaI *or* XhoI-KpnI restriction site of pKGWP0 forming

pobR-P_{pobA}-lacZ, P_{pobA}-lacZ, *pcaQ*-P_{pcaH}-lacZ and P_{pcaK}-lacZ, and transferred to *C. necator, C. necator benA, E. coli* and *B. phytofirmans* and selected in minimal medium supplemented with spectinomycin. β-galactosidase assays were performed according to standard protocols (Miller, 1972) after 4 h of incubation, using a mixture of 4-HB (2.5 mM) as inducer and Bz (2.5 mM) as repressor, or using single compounds.

Construction of plasmid derivatives expressing pob *genes*

The *pobA* and *pobR* genes were PCR amplified using primer pairs pobAFW-pobARV and pobRFW-pobRRV, respectively, and cloned using pCR8/GW/TOPO TA Cloning Kit (Invitrogen) and resulting plasmids were electroporated in *E. coli*. Then, gene sequences were transferred from pCR8/GW/TOPO derivatives to pBS1 destination vector (Bronstein *et al.*, 2005) by recombination-based transfer of the PCR product using the Gateway LR Clonase II Enzyme Mix (Invitrogen). The recombinant plasmids were electroporated in *C. necator benA*, obtaining *C. necator benA* (*pobA*) and *C. necator benA* (*pobR*). For expression of *pobA* or *pobR* genes driven by the heterologous PBAD promoter, these derivatives were exposed to L-arabinose (5 mM), which is not a carbon source for *C. necator*.

Analytical methods

The presence of 4-HB and Bz was determined by high-performance liquid chromatography using cell-free supernatants from cells grown on single compounds or in the mixture of Bz and 4-HB (5 mM each). Samples (20 μl) were obtained at different times of the growth curve and injected into a JASCO liquid chromatograph equipped with a Kromasil 100-3.5 C18 4.6 μm diameter column. A methanol-H_2O (60:40) mixture containing 0.1% (vol/vol) phosphoric acid was used as the solvent, at a flow rate of 1 ml min^{-1}. The column effluent was monitored at 232 (Bz) and 256 nm (4-HB). Retention times for Bz and 4-HB were 3.5 min and 2 min respectively.

Chemicals

4-HB, *p*-coumarate and protocatechuate were purchased from Sigma-Aldrich (Steinheim, Germany). Bz and L-arabinose were purchased from Merck (Darmstadt, Germany).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Dose-dependent benzoate inhibition of growth on 4-hydroxybenzoate of a *Cupriavidus necator benA* mutant. Growth curves of a *C. necator benA* mutant on (A) 5 mM 4-hydroxybenzoate with 0 mM (■), 0.5 mM (□), 1 mM (▲), 2.5 mM (△) or 5 mM (●) benzoate; (B) upon addition of 5 mM benzoate (○), or an equivalent volume of liquid medium (●), after 6 h growing on 5 mM 4-hydroxybenzoate.

Fig. S2. Growth of a *Cupriavidus necator benA* mutant on mixtures of *p*-coumarate/benzoate and protocatechuate/benzoate. Growth curves of a *C. necator benA* mutant on (A)

p-coumarate (□) or *p*-coumarate with benzoate (■); (B) protocatechuate (△) or protocatechuate with benzoate (▲). All growth substrates were provided at 5 mM.

Fig. S3. Growth of a *Cupriavidus necator catR1/catR2* double mutant on benzoate/4-hydroxybenzoate mixture. Optical density at 600 nm (●) and concentrations of 4-hydroxybenzoate (■) and benzoate (▲).

Fig. S4. Dendrogram of *Cupriavidus necator* PobR homologues. The dendrogram was obtained by the neighbour-joining method using MEGA 4.0 software based on sequence alignments calculated by CLUSTAL W, using default settings. PobR homologues were retrieved from the Integrated Microbial Genomes database (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) displaying at least ~30% amino acid identity to PobR from *C. necator* JMP134. Except otherwise is indicated all these bacterial genomes possess a *pobR/pobA* cluster organization similar to *C. necator*. (●) A *pobA* homologue is present in this genome, but not in the same gene cluster as *pobR* homologue; (○) A cluster of *benABC* homologues encoding benzoate 1,2-dioxygenase is present in this genome.

Fig. S5. Influence of different compounds on the promoter activity of *pobA, pcaK* and *pcaHGBL* genes in *C. necator*. β-galactosidase activity levels obtained from transcriptional fusions to *pobA* (top), *pcaK* (middle) and *pcaHGBL* (bottom) promoters exposed to (0.5 mM) 4-hydroxybenzoate or mixtures of (0.5 mM) 4-HB with (2.5 mM) benzoate (Bz), *cis,cis*-muconate (CCM), 3-hydroxybenzoate (3-HB), 2-hydroxybenzoate (2-HB) or protocatechuate PCA. NI (no inducer). Values are the average of three replicates.

Fig. S6. Influence of ratio 4-HB/Bz on the promoter activity of *pobA* gene in *C. necator*. β-galactosidase activity levels obtained from transcriptional fusions to *pobA* promoter exposed to (top) Bz invariable concentration with increasing 4-HB concentrations and (bottom) 4-HB invariable concentration with increasing Bz concentrations. Values are the average of three replicates.

Table S1. Bacterial strains and plasmids used in this work.

Table S2. Primer pairs used in this work.

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