Hierarchy of carbon source utilization in soil bacteria: Hegemonic preference for benzoate in complex aromatic compound mixtures degraded by *Cupriavidus pinatubonensis* JMP134

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Abstract

Cupriavidus pinatubonensis JMP134, like many other environmental bacteria uses a range of aromatic compounds as carbon sources. Previous reports have shown the preference for benzoate when this bacterium grows on binary mixtures composed of this aromatic compound and 4-hydroxybenzoate or phenol. However, this observation has not been extended to other aromatic mixtures resembling a more archetypal context. We addressed a systematic study on the substrate preference by *C. pinatubonensis* JMP134 growing on representative aromatic compounds channelled through different catabolic pathways described in aerobic bacteria. Growth tests in nearly the entire set of binary combinations and in mixtures composed of 5 or 6 aromatic components showed that benzoate and phenol were always the preferred and deferred growth substrates respectively. This pattern was supported by kinetic analyses that showed shorter times to initiate consumption of benzoate in aromatic compound mixtures. Gene expression analysis by Real Time RT-PCR showed that, in all mixtures, the repression by benzoate over other catabolic pathways was mainly exerted at the transcriptional level. Additionally, inhibition of benzoate catabolism suggests that its multiple repressive actions are not mediated by a sole mechanism as, suggested by dissimilar requirements of benzoate degradation for effective repression in different aromatic compound mixtures. The hegemonic preference for benzoate over multiple aromatic carbon sources is not explained on the basis of growth rate and/or biomass yield on each single substrate nor by obvious chemical or metabolic properties of these aromatic compounds.

Introduction

Aromatic compounds (AC) are widespread in the environment, displaying a heterogeneous structural diversity. They can be naturally originated by biotic and abiotic processes or released as pollutants into the environment. AC primarily can be found as aromatic amino acids, secondary products abundantly generated by plants, structural components of the very complex lignin heteropolymer in woody plants, and xenobotic compounds: biocides, industrial by-products, and petroleum derivatives, among others. Microorganisms may degrade hundreds of different AC using specialized biochemical pathways that allow them to grow on these carbon sources (1-3). Typically, bacteria should deal with AC being part of complex mixtures in naturally occurring organic compounds, such as those found in plant exudates (4), soils (5), and even in dissolved organic matter from freshwater and seawater (6). Therefore, microorganisms are concurrently exposed to several structurally heterogeneous AC as potential substrates, which raise the question of whether the components of these mixtures are used simultaneously or in a sequential manner. In the case of the sequential utilization pattern, characterized by the diauxic growth, one compound inhibits degradation of the other by exerting metabolite toxicity (7), competitive inhibition of enzymes (8-9), depletion of electron acceptors (10-11), or carbon catabolite repression (12-13). The last phenomenon implies that the presence of the preferentially utilized compound represses the expression of genes involved in degradation of the alternative non-preferred substrate, and has been extensively studied using sugars, amino acids and organic acids as representative of preferred carbon sources in aerobic bacteria (12-13); and most recently reported in anaerobic species, too (14-15). The hierarchical utilization of binary mixtures of AC has also been studied but much less extensively and mostly

67 focused in substrates that are metabolized by closely related catabolic pathways. The
68 degradation of mixtures of benzoate (Bz) and phenol (Phe), both converted into
69 catechol to be subsequently channelled into the β-ketoadipate pathway by *ortho* ring-
70 cleavage, has been studied in *Acinetobacter* species (16-17), Pseudomonads (18), and
71 *Ralstonia eutropha* (19), showing a sharp pattern for the preferential utilization of Bz.
72 The molecular mechanism underlying the inhibition of Phe consumption in this mixture
73 has not been clarified yet. Moreover, molecular studies on the hierarchical utilization of
74 mixtures of AC have been performed mostly on Bz and 4-hydroxybenzoate (4-Hb)
75 mixtures, where different branches of the β-ketoadipate pathway are used to metabolize
76 these single components (20). The inhibition of 4-Hb degradation by Bz has been
77 studied in *Pseudomonas putida* PRS2000 (21-22) and *Acinetobacter baylyi* ADP1 (23),
78 clearly establishing that the repression is acting at the transcriptional level in these γ-
79 proteobacteria. In both species it has been suggested that catabolite repression would
80 be mediated by transcriptional regulators of Bz degradation and focused on the *pcaK*
81 gene, encoding the 4-Hb permease (21, 23). The repression of 4-Hb degradation by Bz
82 has also been reported in the β-proteobacterium *Cupriavidus pinatubonensis* JMP134,
83 opening new opportunities to study the use of mixtures of AC in metabolically versatile
84 bacteria (24).

85 *Cupriavidus pinatubonensis* JMP134 (25), formerly *Alcaligenes eutrophus,*
86 *Ralstonia eutropha, Wautersia eutropha*, and *C. necator*, utilizes roughly sixty different
87 AC as a sole energy and carbon source and its genome encodes nearly the whole set
88 of degradation pathways of AC reported in Proteobacteria (26, 29). This is remarkable
89 but not so unusual since several other Proteobacteria and Actinobacteria possess

numerous catabolic abilities (3, 27), and particularly several members of the *Burkholderiales* group encode an amazingly large number of AC catabolism genes (27, 28). On comparative basis, *C. pinatubonensis* and related ß-proteobacterial strains have a relatively restricted potential to degrade sugars and small organic acids compared to their capability to degrade AC (29), and therefore the question on the preference in mixtures of AC is even more relevant with these specialized strains. The study of the Bz preference over 4-Hb in this strain revealed some interesting features of the catabolite repression phenomena in mixtures of AC, given that it targets the *pobA* gene, encoding the first step in 4-Hb degradation, and is mediated by an interaction between Bz and PobR, the transcriptional activator of *pobA* (24). One of the most striking traits of the reported regulatory circuit is that no Bz turnover is required to exert a strong repression over the target promoter, suggesting that this phenomenon is triggered by a signal unrelated to the metabolism of Bz. This mechanism is also noticeably different from the one mentioned above for the γ-proteobacterial species *P. putida* and *A. baylyi* (21, 23).

The unquestionable and marked preponderance of Bz as preferred substrate in the previously reported systems prompted us to study whether such a prevailing role is extended to binary mixtures with AC catabolized by routes other than the β-ketoadipate pathway, in order to establish if such a hierarchy is only a peculiar trait of this route or a global attribute in metabolically versatile bacteria. In addition, studies to clarify if patterns of sequential consumption are maintained in complex mixtures of AC must be addressed since this is a more realistic approach to understanding bacterial catabolism in nature, where the pool of available substrates is usually highly diverse in composition.

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We performed a systematic analysis of substrate preferences in *C. pinatubonensis* with eight AC degraded via the main AC catabolic pathways reported in Proteobacteria (3, 26, 28; Fig. 1): Bz, 4-Hb, Phe, phenylacetate (Pac), 3-hydroxybenzoate (3-Hb), 4-hydroxyphenylacetate (4-Hpa), tyrosine (Tyr), and 2,4-dichlorophenoxyacetate (2,4-D), establishing the hierarchical relations among them, assessing their transcriptional patterns and glimpsing at the mechanisms supporting these interactions.

Materials and Methods

Bacterial strains and growth conditions. *C. pinatubonensis* JMP134 and its Δ*benA* mutant (24) were grown at 30°C in mineral salts medium (30), supplemented with 5 mM Bz, 4-Hb, 3-Hb, Pac, 4-Hpa, Tyr; 2 mM 2,4-D, Phe or 10 mM fructose. These concentrations of AC were used in single or binary cultures. Some growth tests were performed at other concentrations, or with five or six member mixtures. Controls without AC were routinely run. *E. coli* Mach™ (Invitrogen, Carlsbad, CA) was grown at 37ºC in a Luria-Bertani medium. Growth was measured at optical density at 600 nm (OD_{600nm}). At least three replicates were performed for each growth test.

Quantification of transcript levels by quantitative Real-Time RT-PCR. Cells of *C. pinatubonensis* JMP134 were grown overnight in a minimal medium with fructose to inoculate a fresh culture medium, further growing the cells until OD_{600nm} was equal to 0.7. Then they were induced with 5 mM of Bz, 3-Hb, 4-Hb, 4-Hpa, Pac, Tyr, or 2 mM of Phe and 2,4-D, or a mixture of Bz and the other AC, and incubated for 1 h. Controls without the addition of AC were also performed. Then, total RNA was obtained from four

ml cultures of cells, using RNAprotect® bacteria reagent and the RNeasy® Mini Kit (QIAGEN, Chatsworth, CA, USA). The RNA was quantified using an EON Microplate spectrophotometer (Biotek, Winooski, VT, USA) and treated with the TURBO DNase Kit (Ambion, Austin, TX, USA) to remove DNA contamination. The reverse transcription was performed using the ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, WI, USA) with 1 µg of RNA in 20 µL reactions. Real Time RT-PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and the Eco Real-Time PCR detection system (Illumina, San Diego, CA, USA). The PCR mixture (15 µL) contained 3.0 µL of template cDNA (diluted 1:10) and 0.2 µM of each primer. Amplification was performed under the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s followed by a melting cycle from 55 to 95°C. Relative gene expression calculations were conducted as described in the software manufacturer's instructions: an accurate ratio between the expression of the gene of interest (GOI) and the housekeeping (HK) reference gene was calculated according to the following expression: $2^{-(\Delta CtGOI - \Delta CtHK)}$. Then, gene expression levels were normalized to the average value of the gene expression determined in the non-induced treatment. The *rps7* gene (Reut_A3184) was used as HK. Primer pairs for *benA* (Reut_B4403), *pobA* (Reut_B5020), *mhbM1* (Reut_B5861), *mhbM2* (Reut_B5805), *hmgA* (Reut_B3923), *tfdA* (Reut_D6479), *tyrB* (Reut_B4503), *hpaX* (Reut_B4218), *paaK1* (Reut_B3741), *paaA1* (Reut_B3735), *paaA2* (Reut_A3206), *paaK2* (Reut_A3017), *phlK1* (Reut_A1700), and *phlK2* (Reut_B5680) genes are listed in Table 1. All experiments were performed in two biological and two technical replicates.

160 **Analytical methods.** AC were detected by high-performance liquid chromatography
161 using cell-free supernatants from cells grown on single AC or mixtures of AC. Samples
162 (20 µL) were obtained at different times of the growth curve and injected into a Hitachi
163 LaChrom Elite chromatograph equipped with a L-2130 pump, a L-2455 diode array
164 detector, a L-2200 auto sampler, and a Kromasil 100-3.5 C18 4.6-µm-diameter column.
165 For binary and unitary curves, a methanol-H_2O (60:40) mixture (except mixtures Bz/Pac,
166 4-Hb/3-Hb, 3-Hb/4-Hpa [40:60], and 4-Hb/4-Hpa [20:80]) containing 0.1% (vol/vol)
167 phosphoric acid was used as the solvent at a flow rate of 1 mL min^{-1}. The column
168 effluent was monitored at 210 nm for all AC. Retention times for the methanol-H_2O
169 (60:40) mixture were: Bz 3.2 min, 4-Hb 1.8 min, 3-Hb 2.0 min, Pac 3.1 min, 4-Hpa 1.9
170 min, 2,4-D 8.0 min, Phe 2.6 min, and Tyr 1.4 min. For the methanol-H_2O (40:60)
171 mixture, retention times were: Bz 3.8 min, 4-Hb 2.8 min, 3-Hb 3.4 min, Pac 3.5 min, and
172 4-Hpa 2.7 min. Retention times for the methanol-H_2O (20:80) mixture were 4-Hb 8.8 min
173 and 4-Hpa 10.0 min respectively. The six and five mixture samples of AC were eluted
174 with a mobile phase of 15% methanol, 20 mM acetate, and milliQ water, at pH 3.3.
175
176 **Chemicals.** AC were purchased from Aldrich Chemical (Milwaukee, WI), except Bz, Tyr
177 and fructose that were purchased from Merck (Darmstadt, Germany).
178
179 **Statistical analysis.**

180 Data were statistically analysed using one-way analysis of variance. When analysis of
181 variance showed significant effects, the Tukey's HSD (Honestly Significant Difference; p
182 < 0.05) test was applied.

Results

Bz and Phe are always the preferred and the deferred growth substrate, respectively, when *C. pinatubonensis* grows in AC binary mixtures.

To extend the study on the substrate preference of *C. pinatubonensis* to binary mixtures other than the previously reported Bz/4-Hb combination, catabolized through catechol and protocatechuate *ortho* ring cleavage pathways, respectively, six additional AC were selected, which are degraded through other key catabolic pathways reported in bacteria (Fig. 1). The substrates were 3-Hb (catabolized through the gentisate pathway), Pac (catabolized via the phenylacetyl-CoA pathway), 4-Hpa (channelled via 1-hydroxylation into the homogentisate ring cleavage pathway), 2,4-D (degraded via the chlorocatechol *ortho* ring cleavage pathway), Phe (degraded via the catechol *meta*- and *ortho*- ring cleavage pathways), and Tyr (channelled via 4-hydroxyphenylpyruvate into the homogentisate ring cleavage pathway). The corresponding twenty-eight binary mixtures were scored for bacterial growth and % of substrate removal. Phe and 2,4-D were tested at 2 mM to prevent toxic effects. Phe itself, its catabolic intermediate catechol, and the 2,4-D intermediates, 2,4-dichlorophenol and chlorocatechols, have been reported to be toxic through mechanisms such as oxidative phosphorylation uncoupling, enhancement of Fenton reaction, DNA adduct formation, among others (19, 31-34). Controls with the respective single AC were carried out to make proper kinetic comparisons.

Bz was always the preferred substrate in every mixture (Fig. 2A shows results for Bz-Pac as an example; all of the other mixtures containing Bz are shown in Fig. S1); i.e. only once Bz was substantially degraded (as judged by % of removal), the degradation of the other AC started. This lag in onset degradation by this strain was better observed

when the time course of removal in the mixture was compared with those of the respective single cultures of AC (Fig. 2A, and Fig. S1). In contrast, Phe was always the deferred growth substrate in these binary mixtures (Fig. 2B shows results for the 4-Hb/Phe combination as an example; see the other Phe containing mixtures in Fig. S1). All other combinations, i.e. those where Bz or Phe were absent, showed no clear preference for any member of the binary mixture (Fig. 2C shows results for the 3-Hb/4-Hpa combination; see the remaining mixtures in Fig. S1, as an example).

To better determine growth substrate removal in mixtures, two parameters were defined and tracked: i) degradation time overlaps, expressed as the percentage of the total degradation time of the growth substrate whose removal began first, when the catabolism of the other substrate was also occurring, and ii) degradation start time, corresponding to the time when removal of each compound was first detected. Overlaps in Bz degradation times in binary mixtures with 4-Hb, 3-Hb, Pac or Phe were near or lower than 10%, i.e. only in the last 10% of the Bz degradation period were the other AC also degraded (Fig. S2A). On the hand, in every mixture containing Phe the degradation of this substrate never started before the partner of AC (no values in the Phe row in Fig. S2A). Bz exerted the strongest preference over Phe catabolism (12% degradation time overlap) compared with the other six AC that were favoured by *C. pinatubonensis*. In the mixtures of AC where Bz and/or Phe were absent, around 50% or higher overlap values were always observed (Fig. S2A), indicating that no preference was the more common metabolic trait. Finally, a nondescript behaviour was observed with 2,4-D, given that whether or not its degradation ended before (4-Hb, 3-Hb, Pac, or 4-Hpa) or after (Bz, Phe, Tyr) (Fig. S1), > 50% overlap values were always found (Fig. S2A).

In addition, the analysis of degradation start times shows that Bz consistently delayed start time of the other AC catabolism. Delays ranged from 1.6-fold for Tyr to 3.4-fold for 3-Hb (Fig. S2B, compare values of the first column (Bz) with those of the single AC supplemented mixtures, grey boxes). In contrast, the Phe degradation start time (3.2 h as single compound) was always retarded (from 1.8-fold with Bz to 2.5-fold with Tyr, Fig. S2B). Tyr also delayed the degradation start time of other AC 2 whereas, in general, the other AC did not significantly modify the degradation start time of the other member of the binary mixtures (Fig. S2B).

The Bz/Phe mixture was further explored, determining the range of Bz concentrations (0.1 to 4 mM) that the delayed use of a fixed Phe concentration (1 mM). Results indicated that as few as 0.25 mM Bz were enough to fully express the preference for Bz, given that only in the 0.1 mM Bz/1.0 mM Phe mixture, Phe degradation started when about half of initial Bz was still present (data not shown).

Bz exerts different repression levels on transcription of the genes encoding initial steps of degradation of AC in binary mixture cultures of *C. pinatubonensis.*

To determine if the mechanism involved in Bz preference was based on transcriptional control, we then carried out a Real Time RT-PCR analysis to compare the expression of key degradation genes of AC, in the absence/presence of Bz. The experimental setup consisted of *C. pinatubonensis* cells previously grown on a non-repressive carbon source (fructose, data not shown) and exposed to Bz, to the partner of AC, and to the mixture of both. Transcript levels were normalized to the *rps7* housekeeping reference gene. As previously reported (24), the presence of Bz completely inhibited *pobA* gene expression (Fig. 3A), encoding the initial

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monooxygenase converting 4-Hb into protocatechuate (Fig. S3A), reflecting that Bz preference is based on transcriptional repression. Bz also completely repressed transcription of *phlK1* and *phlK2* genes (Fig. 3B and 3C), encoding homologous subunits of Phe hydroxylases performing the conversion of Phe to catechol (Fig. S3B) that is further channelled into *ortho* and *meta* ring cleavage pathways respectively (Fig. S3B). The promoter activities of both genes, measured in *lacZ* transcriptional fusions, were determined at two Bz concentrations (0.2 and 2.0 mM): one exerting loose and the other tight Bz preference over Phe (see previous section). Bz (2.0 mM) significantly prevented expression of both promoters in *C. pinatubonensis* cells growing on Bz/Phe mixtures, whereas 0.2 mM Bz slightly did (Fig. S4).

Bz also repressed expression of the *mhbM1* gene (Fig. 3D), encoding the monooxygenase transforming 3-Hb into gentisate (Fig. S3C). The paralogous *mhbM2* gene (Fig. S3C) was not induced by 3-HB (data not shown) and was therefore not tested. Partial repression by Bz was also observed for expression of *hpaX* gene (Fig. 3E), encoding the channelling of 4-Hpa into the homogentisate pathway (Fig. S3D). Bz did not decrease the expression of the *tyrB* gene (statistically significant) (Fig. 3F), encoding the initial transformation of Tyr through the homogentisate pathway (Fig. S3D); furthermore, Bz did not diminish the expression of the *hmgA* gene (data not shown), encoding the homogentisate dioxygenase (Fig. S3D) in Bz/4-Hpa or Bz/Tyr mixtures. Similarly, Bz did not affect the expression of the *tfdA* gene (data not shown), encoding the initial step in 2,4-D degradation. Finally, there was no decrease in expression levels of the *paaK1* gene (Fig. 3G) nor the *paaA1* gene (Fig. 3H), encoding the first and the second step of the phenylacetyl-CoA ring cleavage pathway, respectively (Fig. S3E). The paralogous *paaK2* and *paaA2* (Fig. S3E) were induced at

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low levels by Pac (data not shown) and, similar to *paaK1* and *paaA1* genes, their transcript levels were not affected by Bz (data not shown).

To determine if AC, whose degradations were delayed by Bz, were exerting some activation on the expression of the *benA* gene, thus favouring Bz degradation, the corresponding quantitative Real-Time PCR analysis, was performed. As expected, Bz triggered *benA* gene transcription when present in mixtures with the other AC (Fig. 4A). Apparent increases in *benA* mRNA levels observed in Pac/Bz and Phe/Bz mixtures were not statistically significant, and the rest of the AC tested did not increase *benA* mRNA levels to at any extent (see Fig. 4A and zoomed data in Fig. 4B).

More than one mechanism is probably underlying Bz preference by *C. pinatubonensis*.

As reported previously, inhibition in activity of key regulator PobR by Bz molecule *per se* provokes transcriptional repression over the *pobA* gene controlling the first step in 4-Hb degradation by *C. pinatubonensis* (24). To see if analogous mechanisms also explained Bz preference over the other AC, we performed growth tests using a *C. pinatubonensis* mutant Δ*benA*, completely unable to grow and to remove Bz due to its lack of the initial dioxygenase involved in Bz degradation. As expected, the non-degraded Bz fully prevented degradation of 4-Hb and therefore growth on Bz/4-Hb mixtures (Fig. 5A). Remarkably, the *C. pinatubonensis* Δ*benA* mutant exhibited a significant delay in growth on Bz/Phe mixtures (Fig. 5B), although final biomass yields for the single compound and the binary mixture cultures were essentially similar. It should be noted that toxic effects may explain delayed growth of this mutant and final yields in these Bz/Phe mixtures would be recovered by adaptation or mutation. A third

behaviour was found given that the *C. pinatubonensis* Δ*benA* mutant showed a low but noticeable growth delay with Bz/4-Hpa (Fig. 5C), Bz/3-Hb, Bz/Pac and Bz/Tyr mixtures (data not shown). This mutant in 2,4-D or the Bz/2,4-D mixture did not show any difference in lag phase or final biomass yields (data not shown), indicating that different molecular mechanisms may underlie the preference for Bz, because the exclusive presence of non metabolized Bz was not always enough for full repression by this compound (with the exception of 4-Hb), and suggesting that the formation of Bz intermediates, and/or the operation of downstream enzymes of the catabolic route, may be required for this phenomenon.

C. pinatubonensis also prefers Bz in multiple members, low concentration mixtures.

To better characterize the preference for Bz by *C. pinatubonensis*, six member mixtures of AC (Bz, 4-Hb, 3-Hb, Pac, 2,4-D, and Tyr) were used to test growth and degradation. Three low concentration levels (50, 100, or 250 μM, each mixture components) were used to prevent toxicity of summed individual concentrations of AC, but more importantly to test if Bz preference was also observed at lower concentration levels. At these concentration levels, single cultures of AC showed low, but measurable growth; bacterial cell yields were essentially proportional to the amount of the carbon source added, and complete removals of AC were recorded (data not shown). The low concentration binary mixtures containing Bz still showed preference for this compound, but this behaviour was more clearly observed in 250 μM than in 100 μM mixtures, and practically absent in 50 μM mixtures (data not shown). Notably, the preference for Bz was also clearly observed in six member mixture cultures at 100 μM (Fig. 6A) and 250

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326 μM (data not shown), but no clear trend was observed with 50 μM mixtures (data not
327 shown). Five member mixture cultures lacking Bz showed no preference for any AC at
328 250 μM (Fig. 6B), 100 μM or 50 μM mixtures (data not shown).

329 It is worth noting that compared with single cultures of AC, half-lives of Bz, 4-Hb,
330 and 3-Hb decreased in low concentration six member mixtures containing Bz (although
331 such effect was not observed at 250 μM), whereas those of Pac, Tyr, and 2,4-D
332 increased (Table S1). This suggests that Bz negatively affects the degradation of the
333 other AC at lower concentrations, especially at 250 μM. In concordance, five member
334 mixture cultures lacking Bz showed that the five AC exhibited quite similar and constant
335 half-lives (Table S1). Six member mixture cultures of the *C. pinatubonensis* Δ*benA*
336 mutant at low concentration levels showed that the continuous presence of Bz again
337 increased their half-lives again while significantly decreasing those of Tyr at 50 μM
338 (Table S1, grey rows). The absence of Bz in five member mixture cultures of this mutant
339 tends to homogenize half-lives in a similar way observed for the five member mixture
340 cultures of the wild type strain (Table S1), suggesting a role for Bz itself in these effects.
341 In addition, chemostat cultures fed with this six member mixtures (200 μM each)
342 showed essentially complete and similar degradation levels at different dilution rates
343 (Table S2), excepting the highest dilution rate (condition #3) close to system washout
344 (condition #4), where only Bz and Tyr were fully degraded.
345
346 **Discussion**

347 This work consistently showed that Bz is always degraded preferentially and that,
348 in contrast, Phe is permanently the deferred substrate in mixtures including AC

representing catabolic pathways other than that of β-ketoadipate (3, 27, 28). No clear preference of substrate utilization was found in combinations of AC lacking Bz or Phe, indicating that simultaneous catabolism is the prevailing trait when *C. pinatubonensis* is exposed to mixtures of AC. A key factor to be considered in the study of sequential aromatic catabolism is the component amounts in the mixtures of AC since the utilization profile would be substrate concentration-dependent. In this bacterium, the identified preferences were observed in a range of concentrations higher than those that can be found in most natural, non-polluted environments (35-38). However, it is still possible that preference also takes place at lower concentrations and that the utilized growth and degradation tests do not have enough resolution to detected it. In any case, the results reported here indicate a key nutritional and/or regulatory role of Bz for microbial communities in natural environments.

Bz preference over other AC has been shown in several cases (16, 17, 21-24, 39, 40). In addition, Bz is also degraded first by *C. pinatubonensis* in mixtures with acetate (41) and is preferred over succinate in "*Aromatoleum aromaticum*" EbN1 (42) and over 4-methylbenzoate and succinate in *Magnetospirillum* sp. pMbN1 (43). Succinate catabolism repression in strain EbN1 was specific for Bz and not a general feature of AC, since Pac and 4-Hb were simultaneously degraded in mixtures with this C_4-compound (42). Altogether, it seems to be clear that Bz is a distinctive preferred substrate for several soil bacteria. There are, of course, examples of simultaneous degradation in Bz containing mixtures (14, 44), including synergic interactions such as those described in *Sagittula stellata* E-37, a member of *Roseobacter* lineage, where increased growth rates are found for Bz/4-Hb mixtures (45).

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There are several possible explanations for the Bz preference over AC, none of them fully satisfactory. It can be proposed that Bz is more toxic than the other AC, so cells need to degrade it faster for detoxification purposes. However, the comparison of toxicity data of the AC used here indicates that Bz has toxic levels quite similar to all other AC, except 2,4-D and Phe, Table S3). Even if toxic catabolic intermediates are taken into consideration, such a possibility does not seem to be the case since catechol formed during Bz catabolism (Fig. 1, and Fig. S3A) –the most toxic of the intermediates generated (31, 33, 34)- is also produced from Phe (Fig. 1 and Fig. S3B).

Another possibility is that Bz interferes with the inward transport of the other AC. Such a mechanism of degradation repression is operative in *P. putida* and *A. baylyi* (21, 23). However, this is not probable because uptake of AC takes place without the need of specific transporters at the concentration levels used here (20, 46, 47). Bz control by activation of a putative outward transport system (e.g. an efflux pump) is also a possibility but the little available evidence seems to indicate a role in antibiotic resistance, at least for a salicylic acid efflux pump (48). Permeability comparisons (XlogP3 values, see Table S3) do not provide any additional clues on differences that might explain the substrate preference through a transport mechanism.

Our results do not show a direct relation among growth parameters and substrate preferences (compare single compounds half-lives in Table S1). Growth yields determined as mg of cells / mmoles of added carbon in 0.25 - 8 mM cultures did not show any significant difference for Bz seeing that its growth yield averages were essentially the same as of Tyr and Pac and somewhat higher (15-35%) than those of the other AC except 2,4-D (Table S3). Consistently, the formation of energy yielding aerobic metabolism intermediates, assuming channelling through the tricarboxylic acids

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cycle, did not provide any favourable condition for Bz (Table S3), given that it produces the same intermediates such as 4-Hb and 2,4-D, and has even less potential to obtain NADH and ATP than 3-Hb, Pac, 4-Hpa, and Tyr. Consistently, the formation of energy yielding intermediates (Table S3), provides only a slightly favorable condition for Bz over 4-Hb because the metabolism of the latter consumes an extra reducing equivalent at the PobA step compared to the BenABC/BenD steps (Fig. S3A); but compared with 3-Hb, Pac, 4-Hpa, and Tyr, Bz has even less potential to obtain NADH and ATP.

Another explanation for Bz preference would be based on the ecological niche. If some AC are more available than others, it would make sense that bacteria exposed to it evolve more proficient metabolic routes (increased uptake, sensitive gene induction, robust and efficient enzymes, better detoxification mechanisms, and fine-tuned metabolic fluxes, among others) than for the less abundant AC, thus improving its competitiveness in such habitats. It should be noted that in seawater, a natural environment not expectedly rich in AC, bacteria would be degrading Bz and 4-Hb simultaneously as reported in *Sagittula stellata* E-37, a member of the very abundant marine *Roseobacter* lineage (45). There are contrasting aspects concerning a putative, relatively high natural abundance for Bz in terrestrial environments. Bz may be more chemically stable in oxygenated habitats, because it does not possess reactive hydroxyl groups in contrast to Phe, 3-Hb, 4-Hb, 4-Hpa, and Tyr, which facilitates the formation of polymeric, less available structures. On the other hand, the Bz unit seems to be not the more abundant plant-related aromatic residue seeing as compounds with substituted rings and C6-C3 or C6-C2 structured aromatics are more frequently generated as monomers during lignin decay (49, 50).

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No matter what the reason(s) would be to explain Bz preference, results reported here suggest that at least more than one molecular mechanism is underlying this preference in *C. pinatubonensis*. The first key to be considered is that most of biodegradation inhibition is taking place at the transcriptional level, as it is revealed by gene expression studies, discarding more simplistic scenarios such as the competitive inhibition of enzymes (8, 9) or depletion of electron acceptors (10, 11). Transcriptional repression of the catabolic pathway for the deferred substrate has been previously demonstrated for the Bz/4-Hb mixture (24), where Bz is the main, if not the only, compound required to block induction of the target *pobA* gene through a possible interaction with the regulatory protein PobR. As a result the *C. pinatubonensis* mutant Δ*benA* cannot grow on the Bz/4-Hb mixture since Bz itself exerts absolute repression over the 4-Hb catabolism (24, this work, Fig. 5A). In contrast, all other Bz containing mixtures of AC allowed growth of this mutant, but with different delays (Fig. 5), indicating that Bz preference in combinations of AC other than Bz/4-Hb would require some Bz catabolic intermediate, as it is reported for *P. putida* and *A. baylyi* (21, 23) and/or an unknown metabolic signal as operation of downstream enzymes, a particular redox status or tricarboxylic acids cycle fluxes. It should be noted, however, that these differences might be also explained if Bz itself (assuming essentially the same repression mechanism for 4-Hb) would have lower affinities for the corresponding target regulators than for PobR, or that mutants arise that allow delayed growth in the presence of Bz. The possible occurrence of more than one mechanism involved in Bz preference is however further supported by the varied extents of repression (Fig. 3), since full, partial and no reduction of the expression of AC degradation genes were detected. It is necessary to indicate, however, that no or partial repression may be

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443 explained by the wrong choice of target genes. In principle, tested genes encode initial,
444 presumably regulated catabolic steps (26), but the possibility that the expression of
445 other catabolic genes and/or uptake genes is the target of a putative transcriptional
446 repression system cannot be ruled out.

447 Although four ways to degrade Bz have been reported in bacteria: one aerobic
448 and the other three under oxygen limiting conditions (1), only two of them (encoded by
449 the *ben*/*cat* pathway and the *box* pathway genes) are present in the *C. pinatubonensis*
450 genome (26). Only the *ben*/*cat* pathway is active under the conditions used in this work,
451 since the *C. pinatubonensis* Δ*benA* mutant does not grow on Bz and accumulates Bz
452 (24). This dismisses possibilities such as benzoyl-CoA type intermediates (generated
453 during Box pathway operation), repressing the expression of genes involved in the
454 degradation of some of the other AC tested here, which has been demonstrated for Box
455 intermediates controlling the degradation of gentisate in *Comamonas testosteroni* (51).

456 Contrasting the hegemonic role of Bz, Phe is consistently the deferred substrate
457 in binary mixtures. Phe has also been reported as the non-preferred substrate in other
458 species (16, 17, 52). The elucidation of the molecular basis for this is clearly required,
459 and some hints from this report can help future work. Possible explanations for no
460 preference are its toxicity, since along with 2,4-D, Phe is the only AC with lower yields at
461 higher concentrations (Table S3), which may reflect its scored toxicity levels (Table S3)
462 and the potentially lower energy yielding intermediates when *meta* ring cleavage is used
463 (Table S3). Concerning possible molecular mechanisms, Bz clearly represses
464 expression of *phlK*1 and *phlK*2 genes through diminishing its promoter activities (Fig.
465 3B, 3C and Fig. S4), but additional work is required to determine if these genes are also
466 affected by the other AC, and if other genes are targets for repression of AC, such as

promoters of *phlB* and *catA*2 genes encoding the initial steps of *meta* and *ortho* ring cleavage pathways, respectively (Fig. S3B). Interestingly, Phe is the only AC tested here whose degradation is controlled by sigma factor σ^{54}, which has been frequently reported as the transcription initiation factor involved in expression of catabolic operons highly regulated in response to environmental cues (53).

The pattern of catabolic pathways influenced by preferential utilization of Bz would reflect the evolutive track of *C. pinatubonensis*. As pointed out by Cases and de Lorenzo (54), the phenomenon of catabolite repression is the result of adaptive regulatory mechanisms specifically evolved during the 'natural history' of each bacterium, considering habitat lifestyle and degree of specialization. It can be hypothesized that the current genome of the metabolically-versatile *C. pinatubonensis* has been shaped as a copiotroph (lifestyle) living in soils (habitat) containing Bz as one of the more abundant carbon sources but also including several other additional substrates (generalist, low degree of specialization). During its evolutive track, *C. pinatubonensis* has gained novel catabolic functions, such as 2,4-D biodegradation, a strain-specific trait (25, 55), but the regulatory mechanisms may not yet be fully adapted to this new capacity allowing a less constrained expression even in the presence of Bz, as it is reported here.

Acknowledgements

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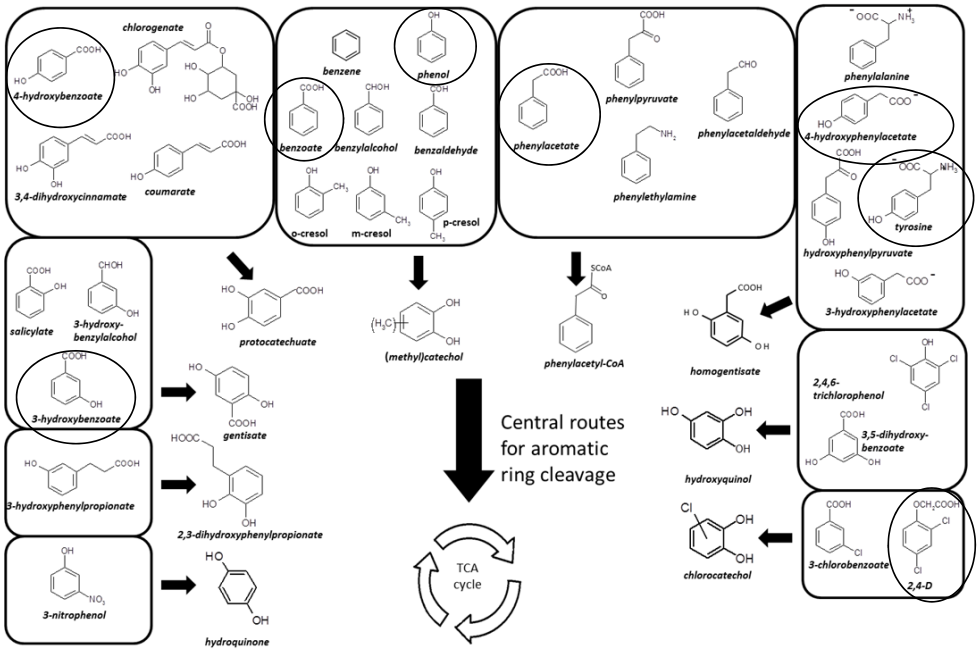
655 degradation pathways. Environ Microbiol **6:**655-668.
656

657 **Figure 1. Pérez-Pantoja, Leiva-Novoa *et al.***

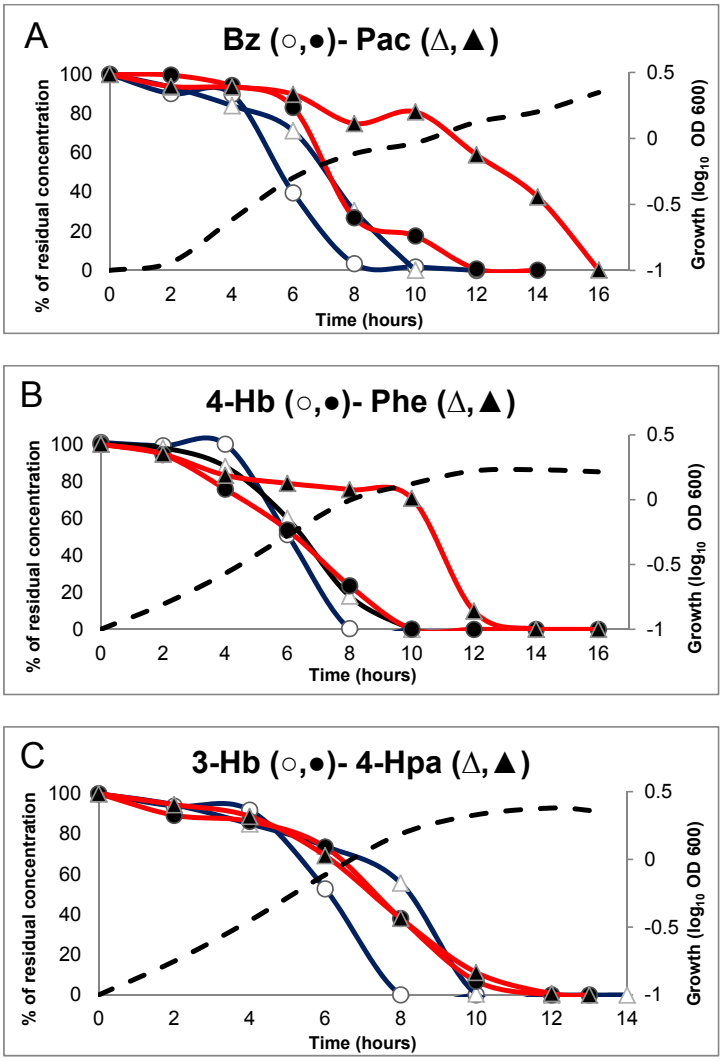


658

659 Main aromatic compounds catabolic pathways reported in Proteobacteria. A
660 selection of aromatic compounds that are used as carbon and energy source by the
661 β-proteobacterium *Cupriavidus pinatubonensis* JMP134 (26) is shown in squares, as
662 well as the corresponding aromatic intermediates that are further metabolized by the
663 respective ring cleavage pathways reported in Proteobacteria (3, 26, 28). Encircled
664 structures correspond to aromatic compounds used in this study.

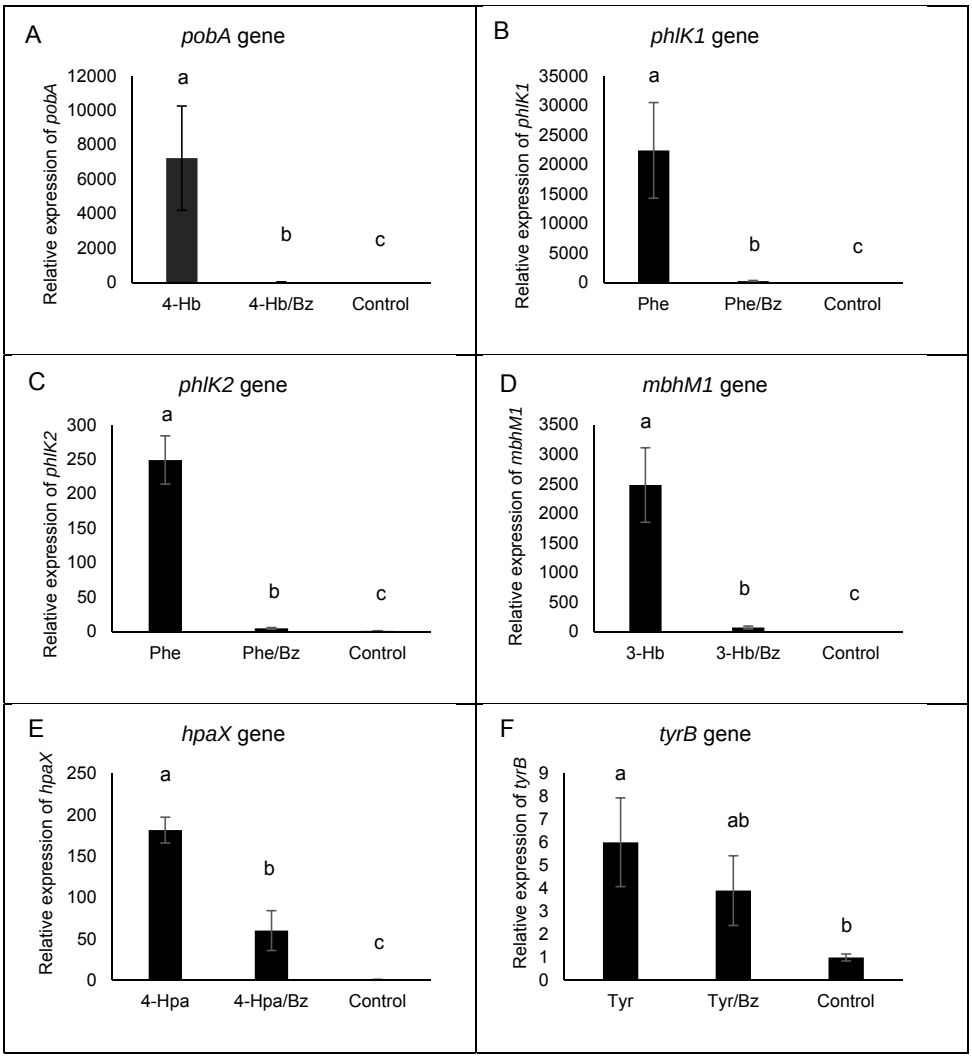
665

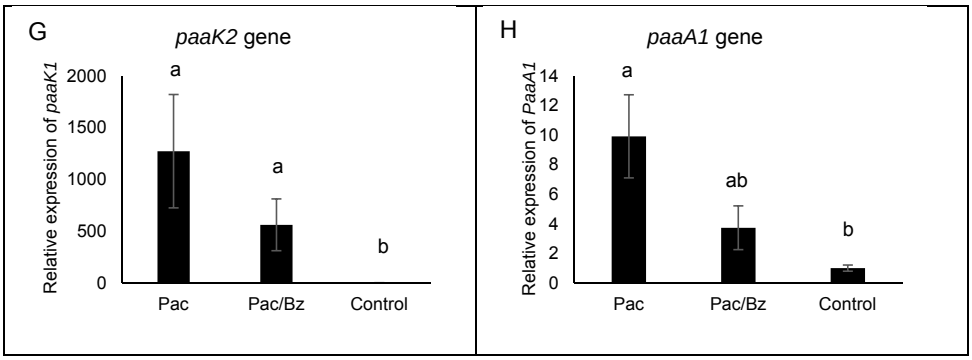
666 **Figure 2. Pérez-Pantoja, Leiva-Novoa *et al*.**



667 Growth and carbon source degradation curves of *Cupriavidus pinatubonensis* JMP134
668 grown on binary mixtures of aromatic compounds and the corresponding carbon source
669 degradation in the respective single compound cultures. Except phenol (2 mM), all other
670 aromatic compounds were tested at 5 mM in single compounds and binary mixture cultures.
671 Note that final growth yields reflect different amounts of added carbon. Open and closed
672 symbols represent substrate removal in single compounds and binary mixture cultures,
673 respectively. Dashed lines represent binary mixtures growth levels determined by OD_{600nm}
674 measurements. Plots correspond to a representative curve from 4-6 biological replicates.
675 Standard deviations of technical replicates were lower than 5% and are not shown for clarity.

676 **Figure 3: Pérez-Pantoja, Leiva-Novoa *et al*.**

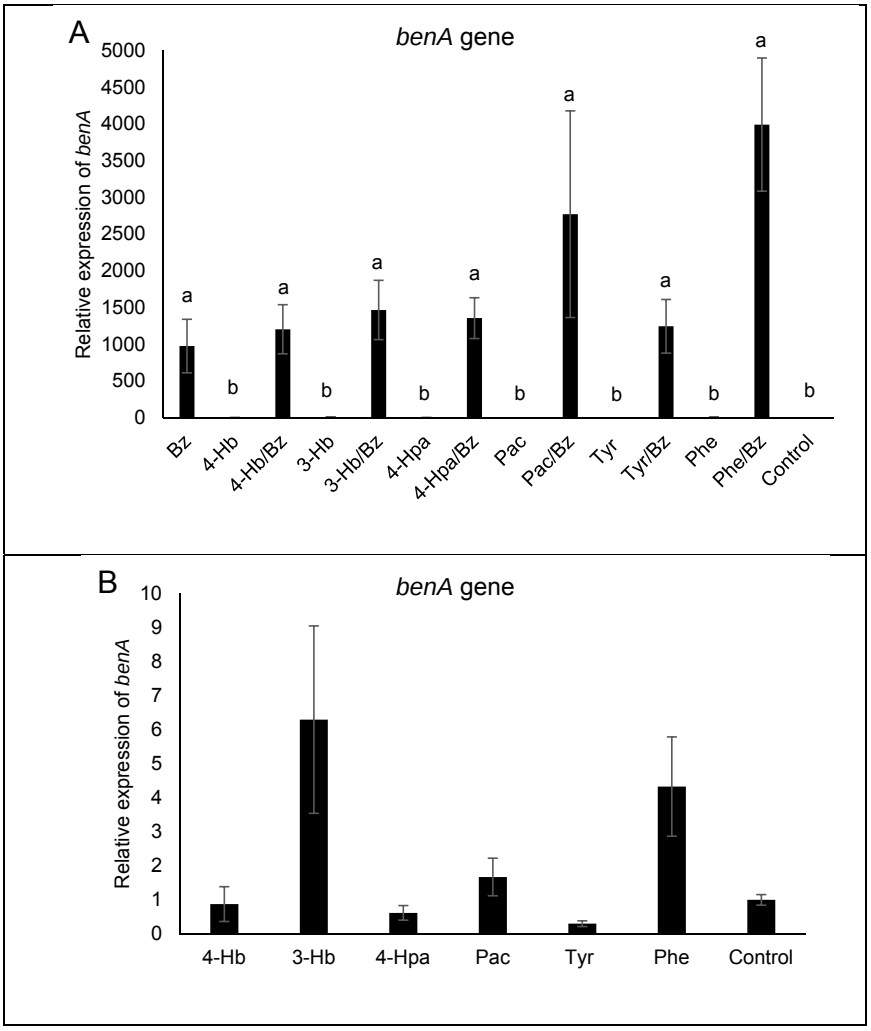




677 Relative transcript levels of key genes encoding initial steps in degradation of
678 aromatic compounds by *Cupriavidus pinatubonensis* JMP134. Fructose grown cells
679 ($OD_{600\ nm}$= 0.7) were exposed to each single compound (5 mM of Bz, 3-Hb, 4-Hb, 4-
680 Hpa, Pac or Tyr, 2 mM of phenol or 2,4-D) of the binary mixture and the
681 corresponding binary mixture (same individual concentrations indicated above), for 1
682 h. Values (three biological and two technical replicates) correspond to the average
683 plus standard error, normalized with respect to the *rps7* housekeeping reference
684 gene. Values from inductions with benzoate were negligible and are not shown for
685 clarity purposes. No aromatic compounds were added in controls. Note that different
686 scales are used. Different letters indicate statistically significant differences between
687 values (One way ANOVA Tukey's HSD tests; $p < 0.05$).

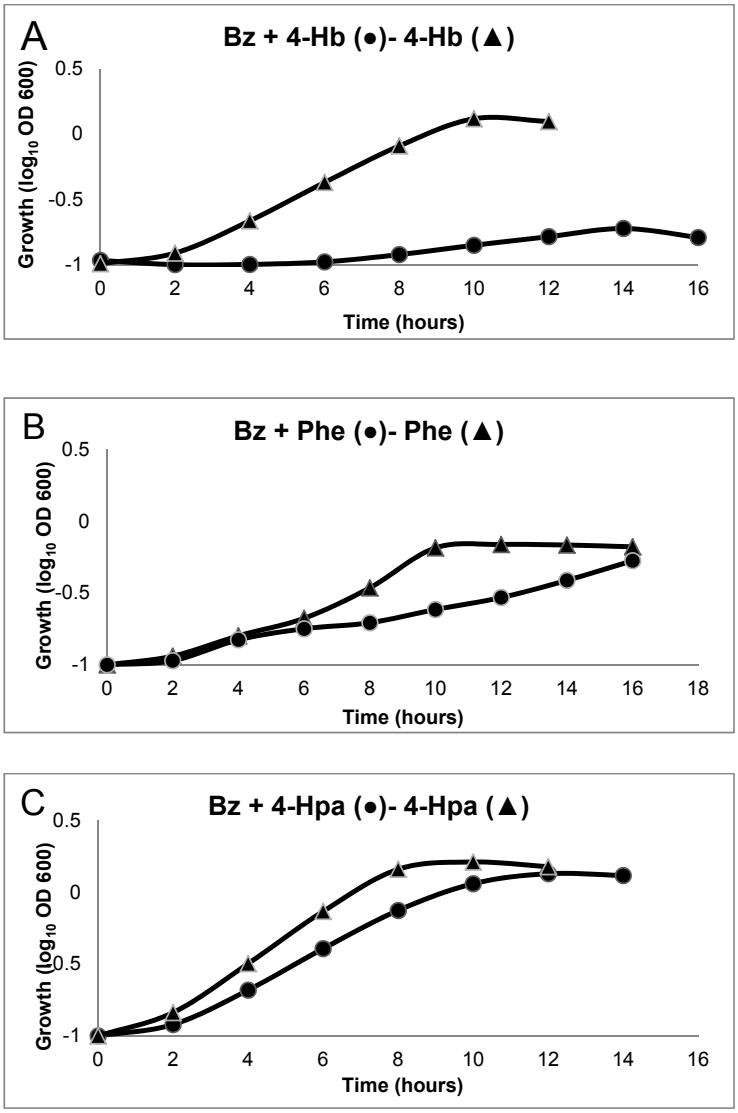
688

689 **Figure 4: Pérez-Pantoja, Leiva-Novoa *et al*.**



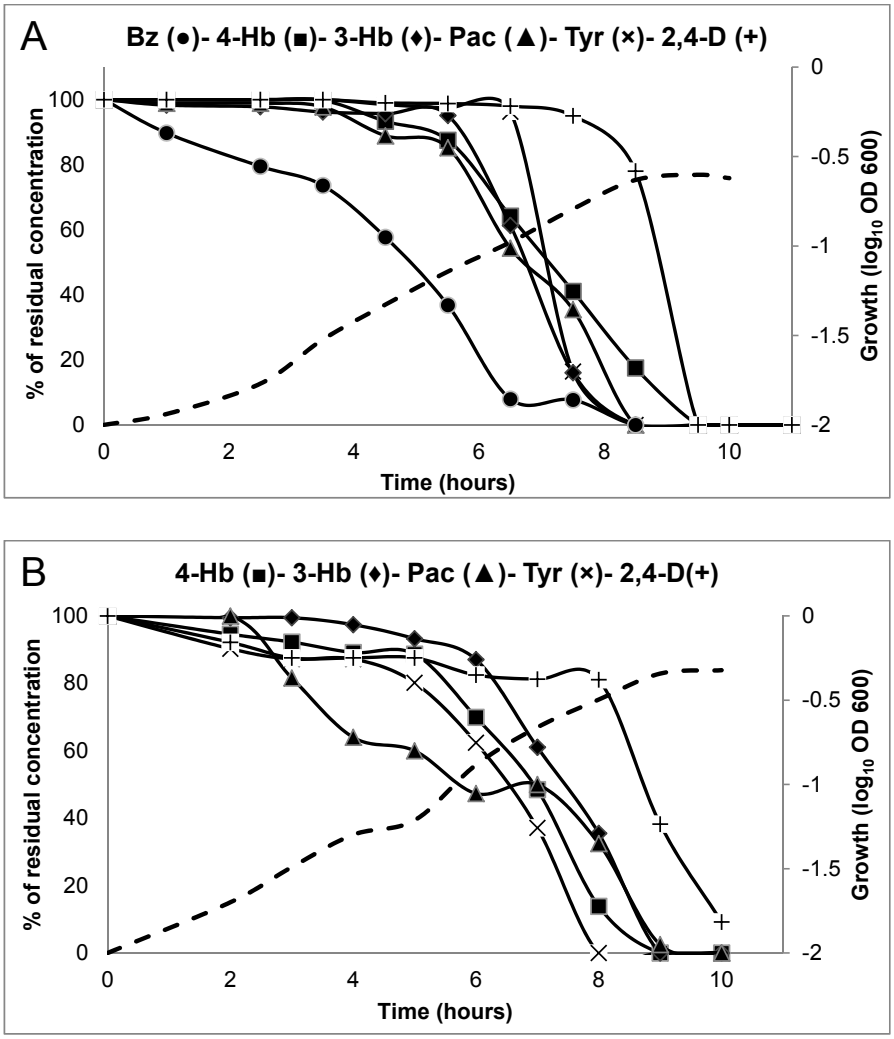
690 Relative transcript levels of *benA* gene encoding the initial step of benzoate (Bz)
691 degradation by *Cupriavidus pinatubonensis* JMP134, exposed to binary Bz
692 containing mixtures of aromatic compounds (AC) or single AC (A). Zoomed data
693 for single AC other than Bz are shown in (B). Fructose grown cells (OD_{600nm}=
694 0.7) were exposed to the binary mixture (5 mM each single AC, except 2 mM
695 phenol) or the single AC (5 mM, except 2 mM phenol) for 1 h. Values (three
696 biological and two technical replicates) correspond to the average plus standard
697 error, normalized with respect to the *rps7* housekeeping reference gene.
698 Different letters indicate statistically significant differences between values (One
699 way ANOVA Tukey's HSD tests; $p < 0.05$).

700 **Figure 5: Pérez-Pantoja, Leiva-Novoa *et al*.**



701 Growth curves of the *Cupriavidus pinatubonensis* JMP134 Δ*benA* mutant grown on
702 benzoate containing binary mixtures of aromatic compounds [2 mM for phenol; 5
703 mM for 4-hydroxybenzoate and 4-hydroxyphenylacetate] (circles), and the
704 corresponding other single compound [same concentrations indicated above]
705 (triangles). Note that final growth yields reflect different amounts of added carbon.
706 Plots correspond to a representative curve from at least three biological replicates.

Figure 6: Pérez-Pantoja, Leiva-Novoa *et al*.



Growth and carbon source degradation by *Cupriavidus pinatubonensis* JMP134 grown on 5, or 6 member mixtures of aromatic compounds. The six member mixture (A) consisted of benzoate, 3-, and 4-hydroxybenzoate, phenylacetate, tyrosine and 2,4-dichlorophenoxyacetate (100 µM each), whereas the five member mixture (B) [250 µM each], lacked benzoate. Continuous lines represent substrate removal of each aromatic compound. Dashed line represent growth levels determined by OD_{600nm} measurements. The curve is representative of 3-5 different experiments. Standard deviations of technical replicates were lower than 5% and are not shown for clarity.

717 **Table 1. Primer pairs used for Real Time RT-PCR.**

Primer name	Sequence (5'→3')	Primer name	Sequence (5'→3')
tfdAFw	CATCTCGAACGTCAGTCTCG	tfdARv	GGTTGCTGAAAGGAGCTGTC
benAFw	GGCTCGTCCACCTACACCTA	benARv	GCTCGTTCTGCTGCTTGC
pobAFw	ATCTACGCCAGCCACGAA	pobARv	GCGAGTTCATCCCAGAAGC
mhbM1Fw	GCCGTCTTTACCGACTACCT	mhbM1Rv	CGAGAATCGACAGATGGATG
mhbM2Fw	AGACATGCCAAAGGACTTGC	mhbM2Rv	TGACGACGAGGTTGTACTGC
paaK1Fw	GCCAGACCGAGAAACAGG	paaK1Rv	GCCGAAGATGCCAACCTT
paaA1Fw	ACGCACAGTTGGTGGATACC	paaA1Rv	CAGTCCTTCGCTTCGATCTT
paaK2Fw	ACTATCCGTTCGGCATGTTC	paaK2Rv	CGATGTCCTTGAGCGTGTAA
paaA2Fw	CGGACACGGCCTCTATCTCT	paaA2Rv	CGTCGGGTAGTTGAAGATCG
hpaFw	GCTCGCTTATGTGCTGGAC	hpaRv	CTCTTGGGCGTGATGAAATC
phlL1Fw	AACCTGGAGTTCGTGGAGAA	phlL1Rv	GGAGCCAAAGCCATACGC
phlK2Fw	CGGAGTTGAGCGTAGACCTG	phlK2Rv	TCAGTTTTCCAGCCTTGTCA
tyrBFw	CGATCCTTTCGCTCAACG	tyrBRv	CCTTTTCGACAGCTTCCATG
hmgAFw	AAGTTCCAGGGCAATCTGTG	hmgARv	TACTTGTACGGCGCGAAGTT
rpS7Fw	GAAAAGAAGGCAGGCAAGG	rpS7Rv	TTCGACCGGAACCTGATAGT

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