REVISED VERSION

SUPPLEMENTAL MATERIAL

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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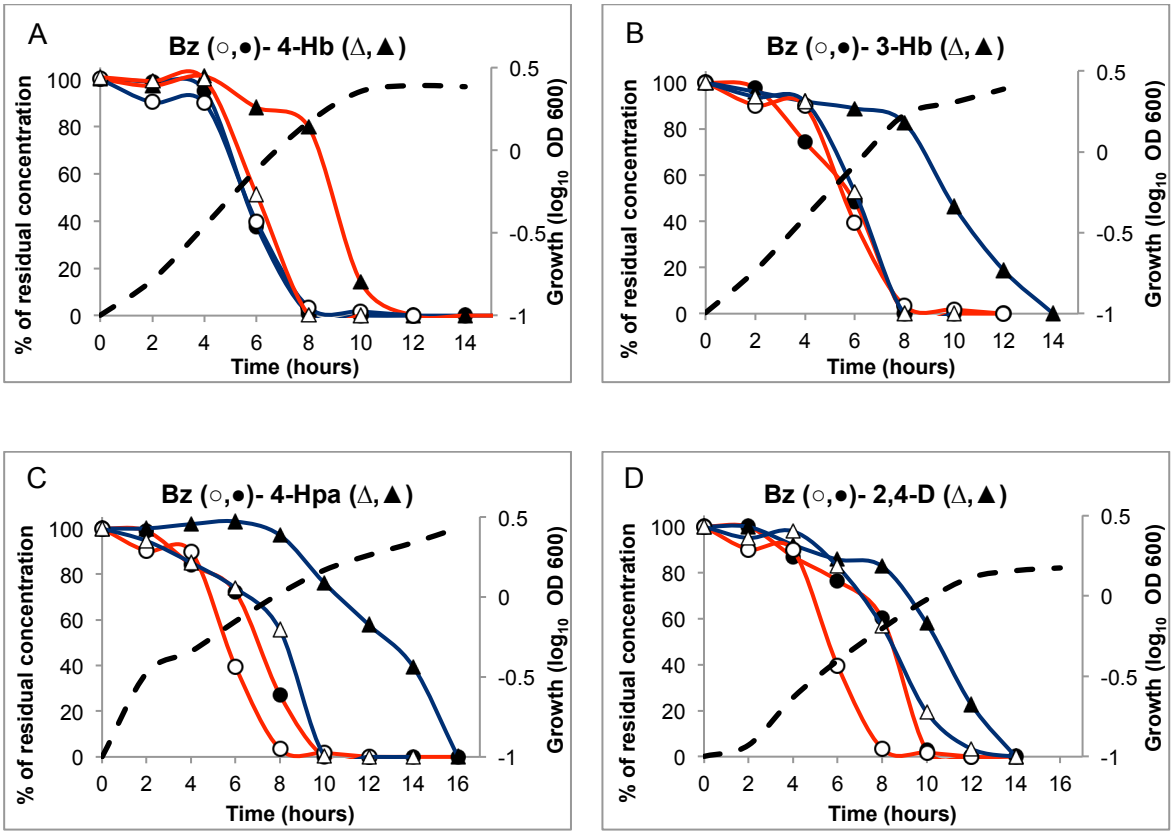
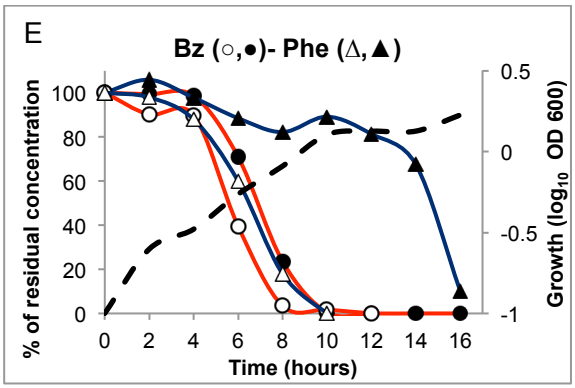
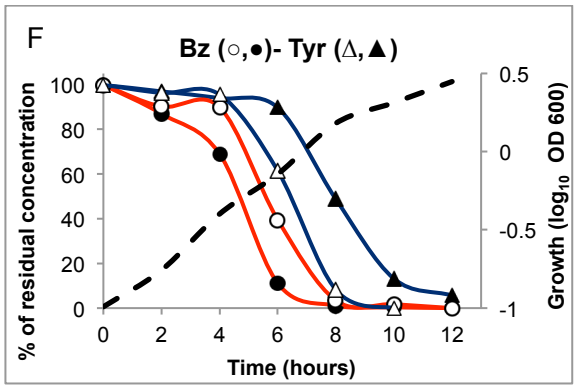
Figure S1A-Y: Pérez-Pantoja, Leiva-Novoa *et al*.




E
Bz (○,●)- Phe (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)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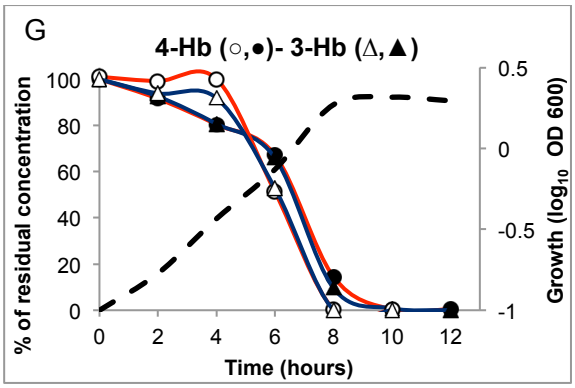
Bz (○,●)- Tyr (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)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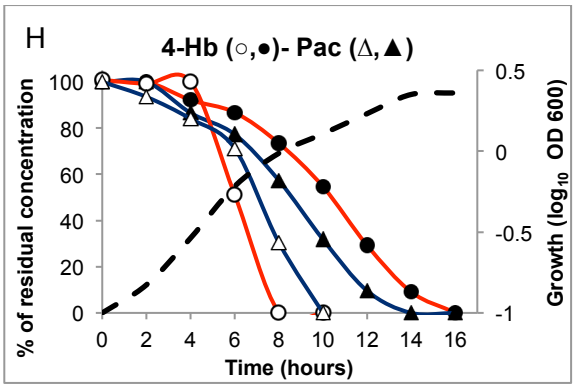
4-Hb (○,●)- 3-Hb (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)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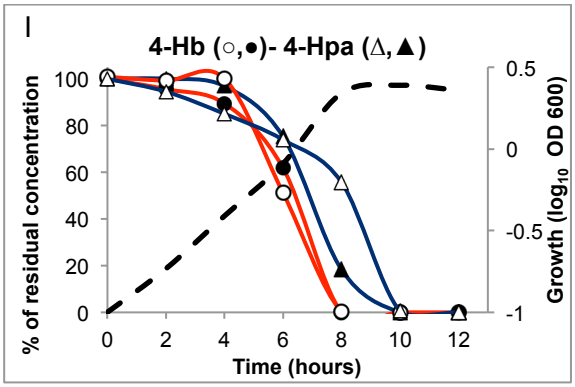
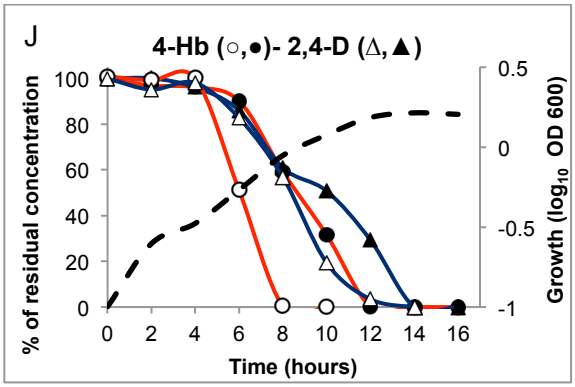
4-Hb (○,●)- Pac (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)


I
4-Hb (○,●)- 4-Hpa (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)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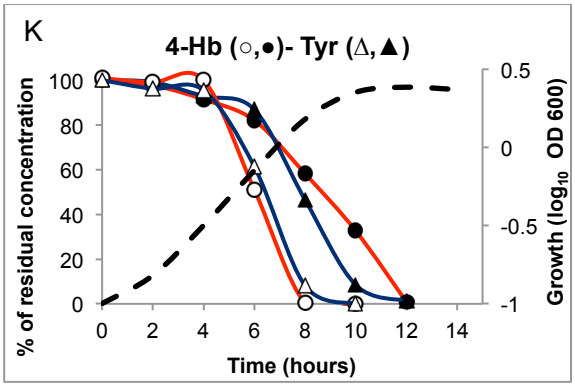
4-Hb (○,●)- 2,4-D (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)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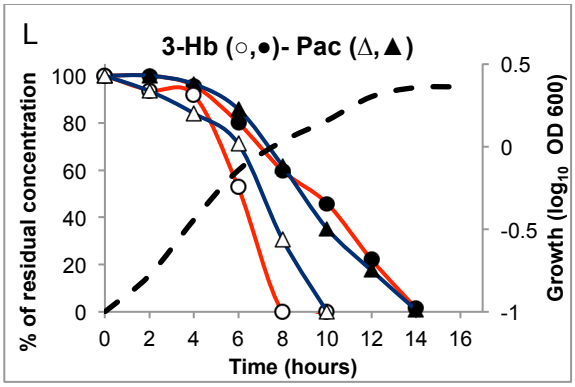
4-Hb (○,●)- Tyr (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)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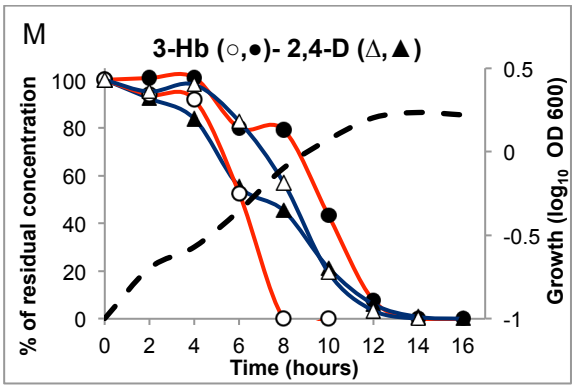
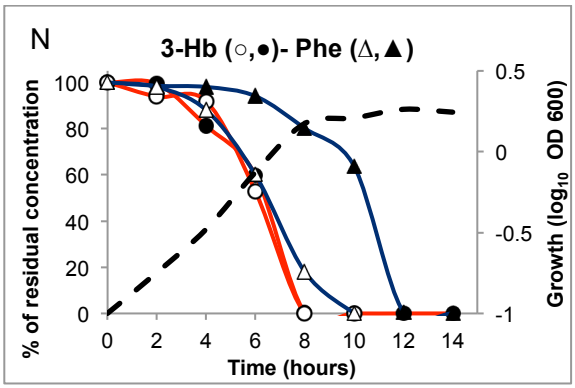
3-Hb (○,●)- Pac (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)


M
3-Hb (○,●)- 2,4-D (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)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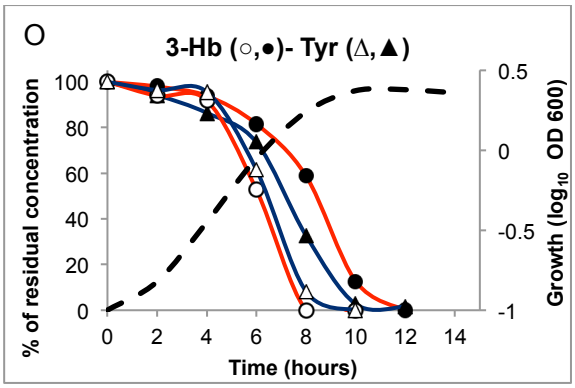
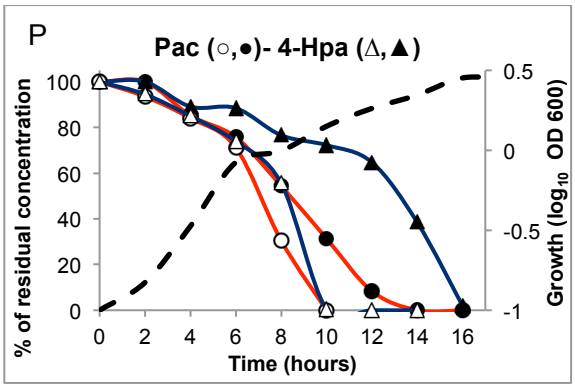
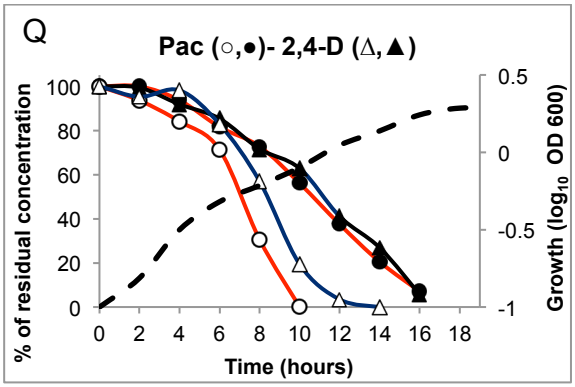
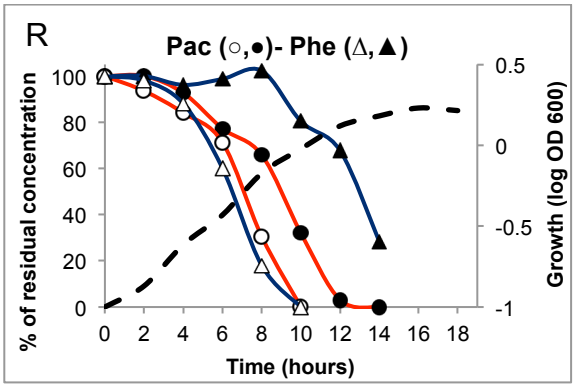
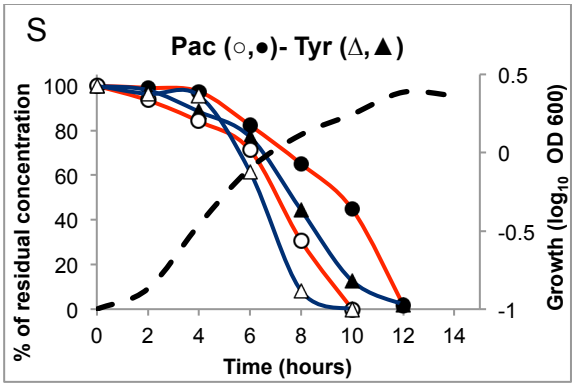
3-Hb (○,●)- Phe (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)

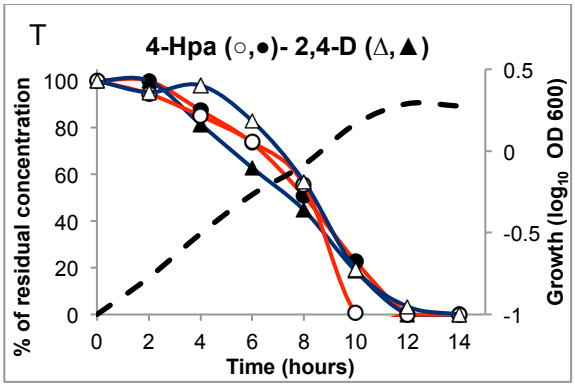

O
3-Hb (○,●)- Tyr (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)

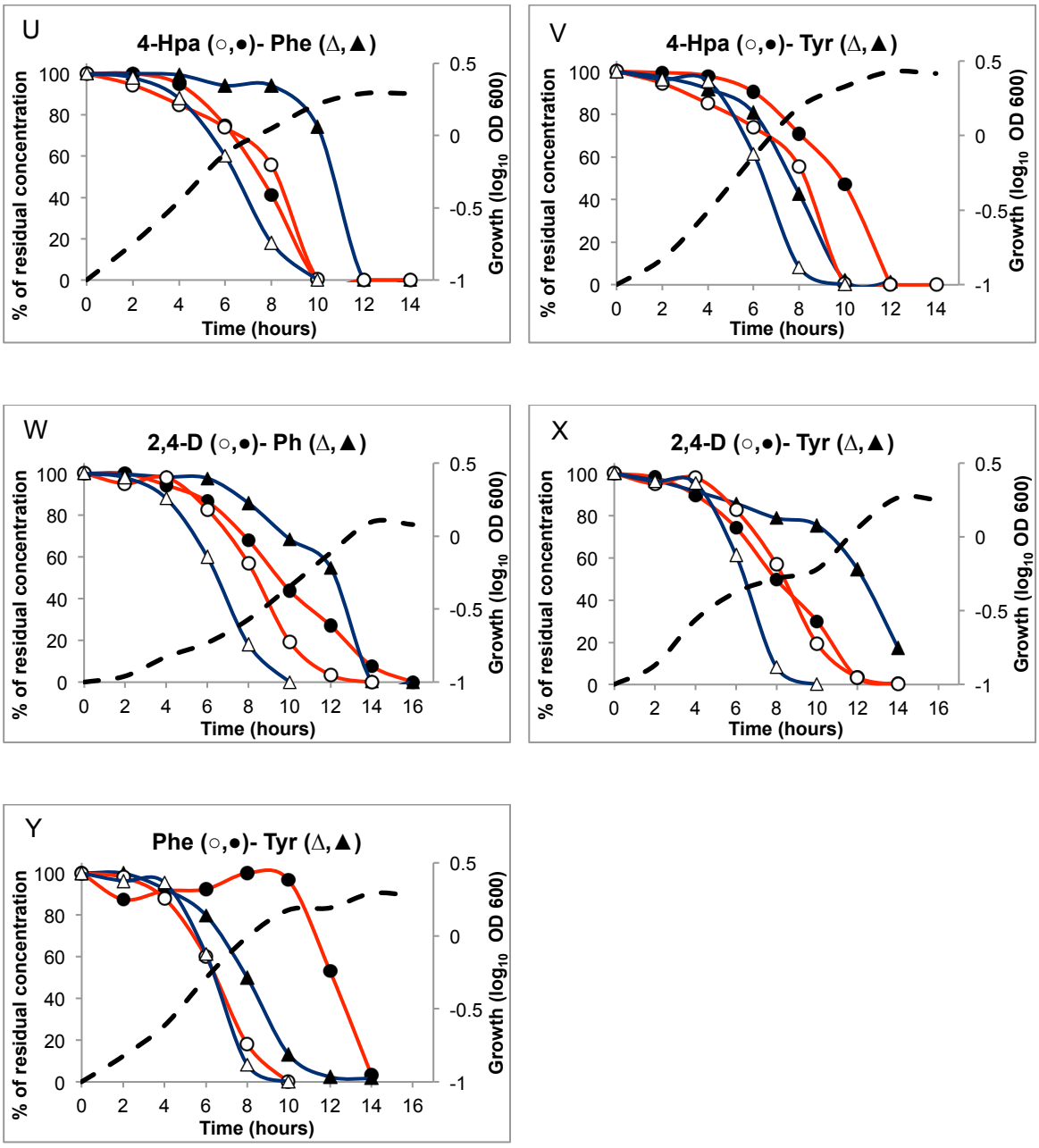

P
Pac (○,●)- 4-Hpa (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)


Q
Pac (○,●)- 2,4-D (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)


R
Pac (○,●)- Phe (Δ,▲)
% of residual concentration
Growth (log OD 600)
Time (hours)


S
Pac (○,●)- Tyr (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)


T
4-Hpa (○,●)- 2,4-D (Δ,▲)
% of residual concentration
Growth (log10 OD 600)
Time (hours)



Growth and carbon source degradation curves of *Cupriavidus pinatubonensis* JMP134 grown on binary mixtures of aromatic compounds, and the corresponding carbon source removal, in the respective single compound cultures. Except phenol and 2,4-dichlorophenoxyacetate (2 mM), all other aromatic compounds were tested at 5 mM in single compounds and binary mixture cultures. Note that final growth yields of mixtures containing phenol or 2,4-dichlorophenoxyacetate reflect the lower amount of added carbon. Open and closed symbols represent substrate removal in

single compounds and binary mixture cultures, respectively. Dashed lines represent binary mixture growth levels determined by OD_{600nm} measurements. Plots correspond to a representative curve from 4-6 biological replicates. Standard deviations of technical replicates were lower than 5% and are not shown for clarity.

Figure S2. Pérez-Pantoja, Leiva-Novoa *et al*.

A

	Bz	4-Hb	3-Hb	Pac	4-Hpa	2,4-D	Phe	Tyr
Bz		8%	3%	1%	26%	58%	12%	50%
4-Hb	8%		82%	73%	73%	75%	40%	76%
3-Hb	3%	82%		89%	87%	54%	27%	57%
Pac	1%	73%	89%		48%	75%	68%	71%
4-Hpa	26%	73%	87%	48%		63%	45%	57%
2,4-D	58%	75%	54%	75%	63%		60%	64%
Phe	12%	40%	27%	68%	45%	60%		25%
Tyr	50%	76%	57%	71%	57%	64%	25%	

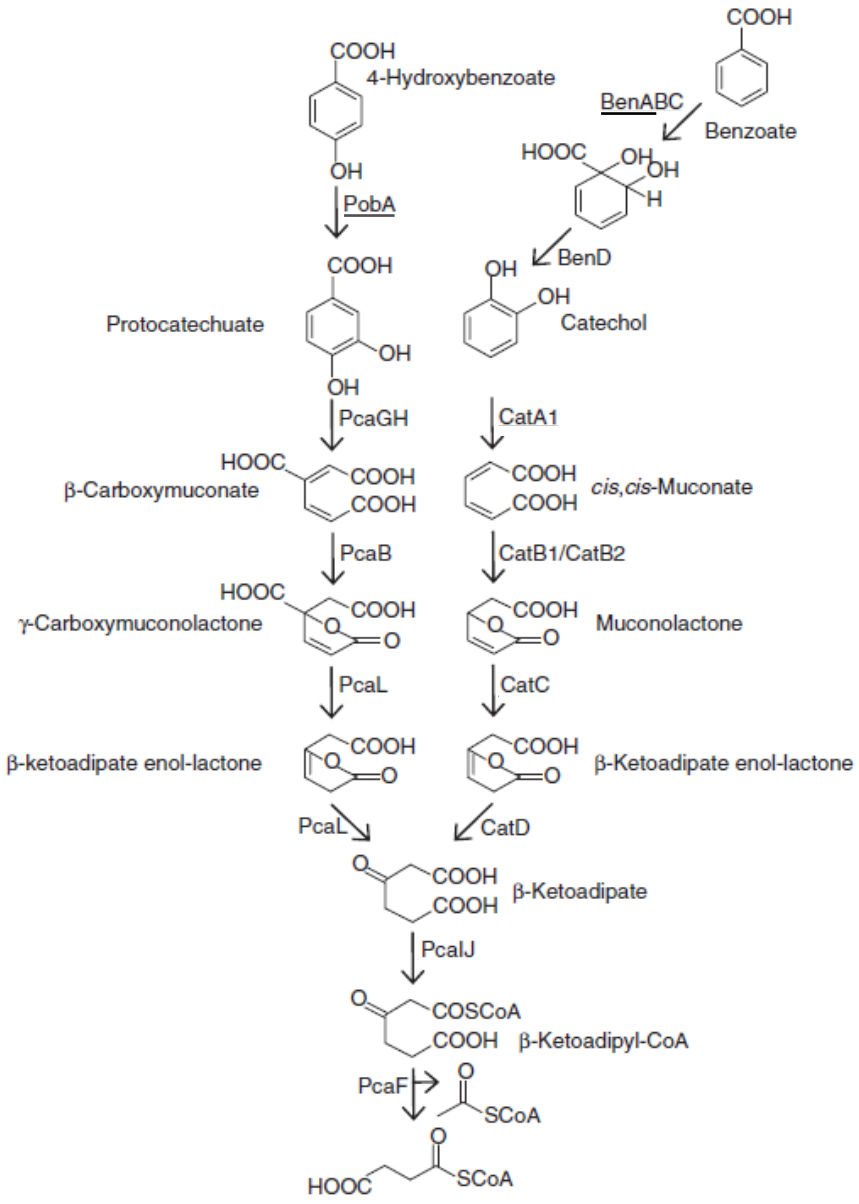
B

	Bz	4-Hb	3-Hb	Pac	4-Hpa	2,4-D	Phe	Tyr
Bz	2.4	2.5	3.9	4.6	1.7	4.1	2.8	4.3
4-Hb	9.4	4.1	4.1	6.3	3.8	3.8	3.4	5.6
3-Hb	8.5	4.7	2.5	4.7	3.6	5.2	2.7	7.4
Pac	9.9	5.0	5.1	4.6	4.4	5.9	6.1	5.6
4-Hpa	5.8	4.6	3.9	7.5	3.2	5.1	4.2	6.7
2,4-D	5.9	3.4	2.6	5.6	2.8	2.6	5.2	5.5
Phe	5.8	7.0	6.8	6.1	7.1	6.9	3.2	7.9
Tyr	6.3	5.0	5.3	5.6	5.1	5.7	5.6	3.8

Growth substrate degradation time overlaps, and degradation start times in *Cupriavidus pinatubonensis* grown in binary mixtures (see legend of Figure S1 for details). A) Degradation time overlap percentages determined as the fraction of the complete degradation time of the AC whose degradation started first (left column) when the degradation of the second growth substrate (top row) also took place. Values around and higher than 50% reflect no significant preference for any member of the binary mixture. Black boxes indicate not observed situations. B) Degradation start times (hours) in single AC cultures (grey-shaded boxes) and binary mixtures. Values correspond to the start degradation time of the growth substrate indicated in the left column in the presence of the growth substrate listed in the top row. The two halves are not equal because two start degradation values (one from each member) were determined in each binary mixture. Kinetic calculations based on Breidt *et al*., 1994.

Breidt F, Romick TL, Fleming HP. 1994. A rapid method for the determination of bacterial growth kinetics. J Rapid Meth Automat Microbiol **3:**59-68.

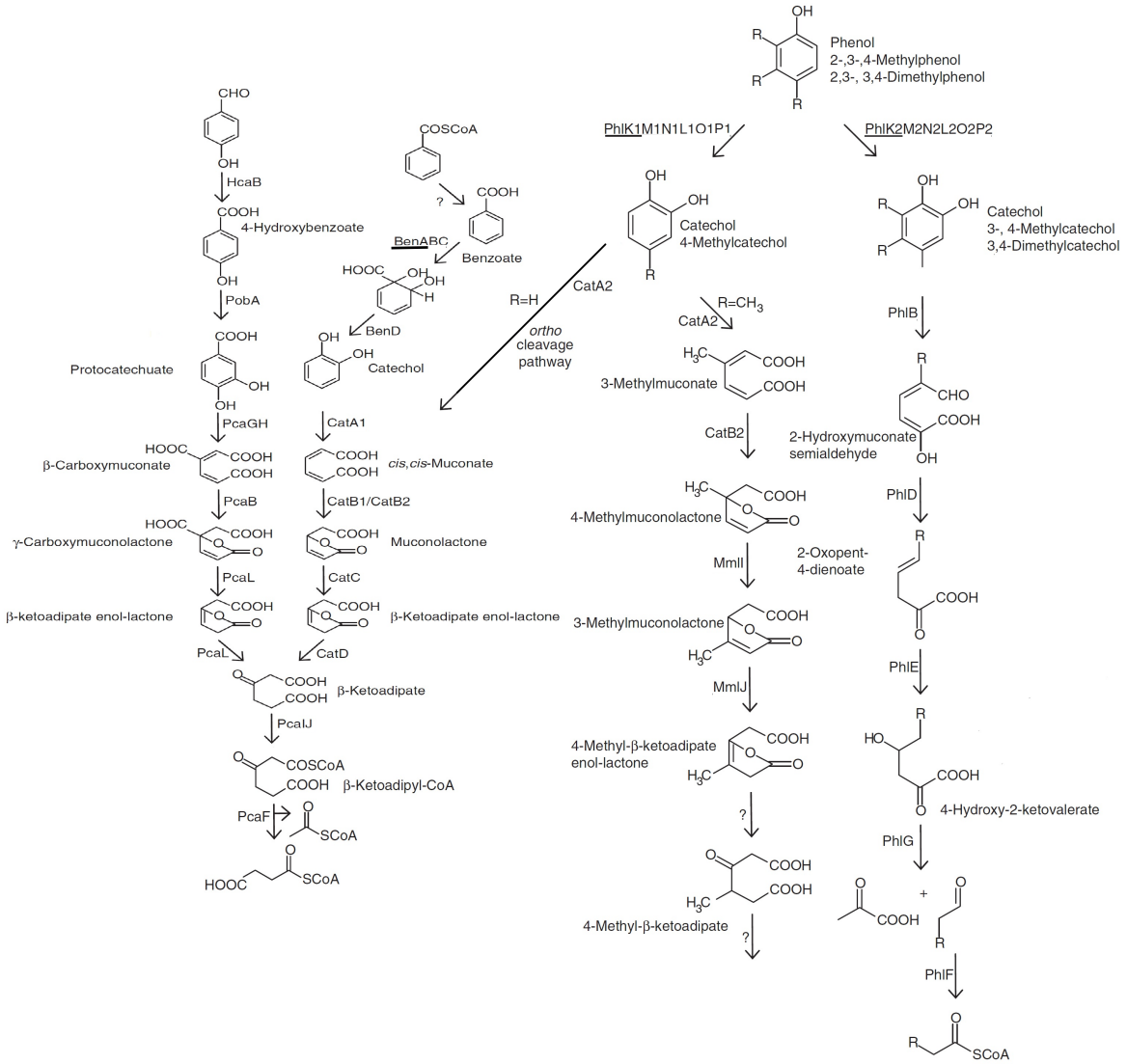
Figure S3A. Pérez-Pantoja, Leiva-Novoa *et al*.



Catabolic routes of benzoate and 4-hydroxybenzoate in *Cupriavidus pinatubonensis* (Pérez-Pantoja *et al*., 2008). Here, and in all Figure S3, target gene products for Real Time RT-PCR analysis are underlined.

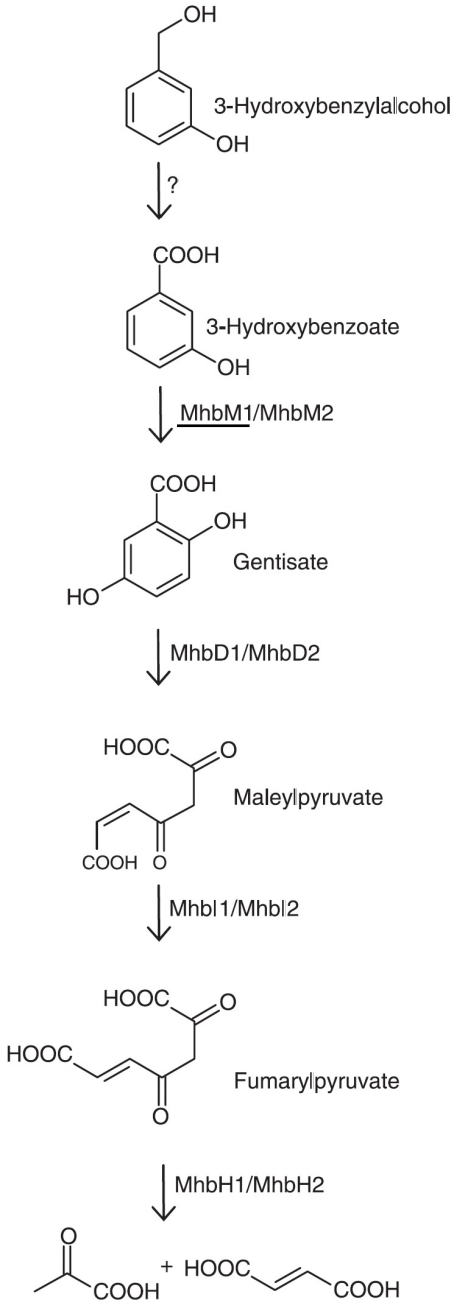
Pérez-Pantoja D, De la Iglesia R, Pieper DH, González B. 2008. Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus necator* JMP134. FEMS Microbiol Rev **32:**736–794.

Figure S3B. Pérez-Pantoja, Leiva-Novoa *et al*.



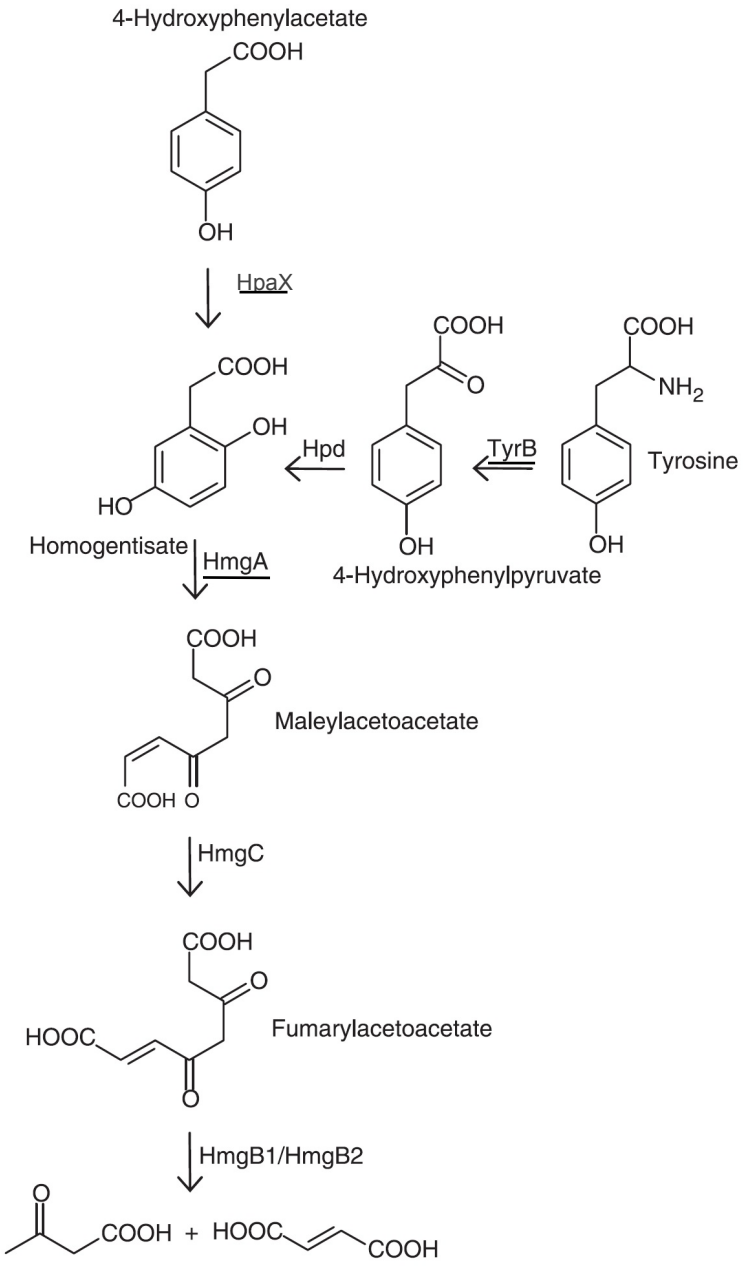
Catabolic routes for phenol in *Cupriavidus pinatubonensis* JMP134 (Pérez-Pantoja *et al*., 2008).

Figure S3C. Pérez-Pantoja, Leiva-Novoa *et al*.



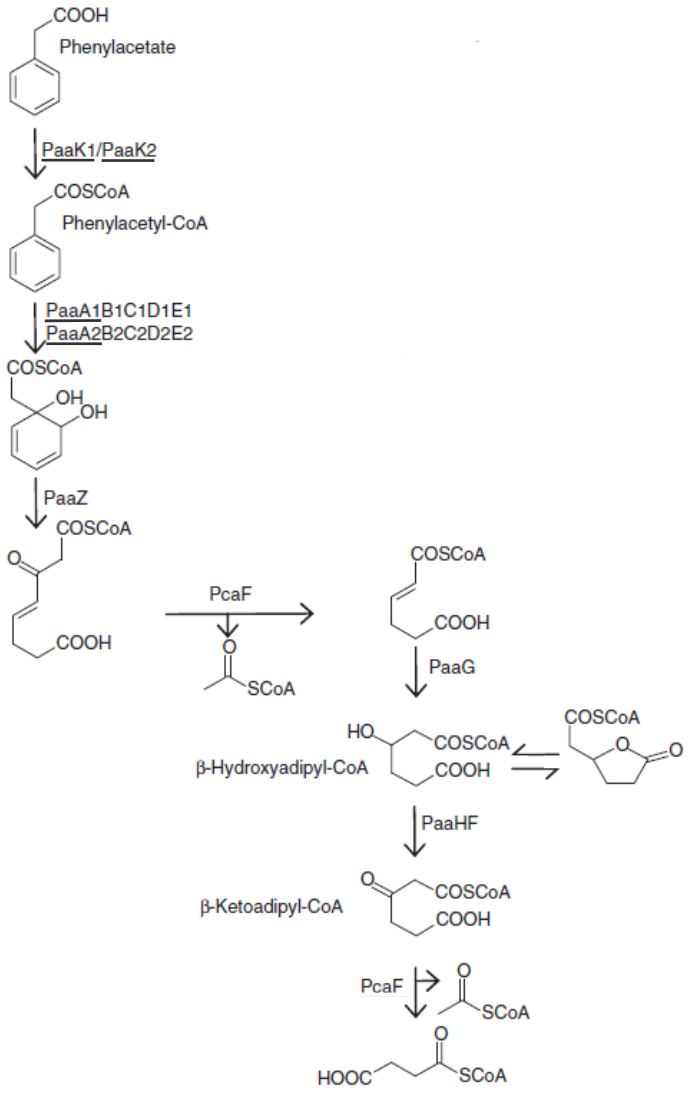
Catabolic route of 3-hydroxybenzoate in *Cupriavidus pinatubonensis* (Pérez-Pantoja *et al*., 2008).

Figure S3D. Pérez-Pantoja, Leiva-Novoa *et al*.



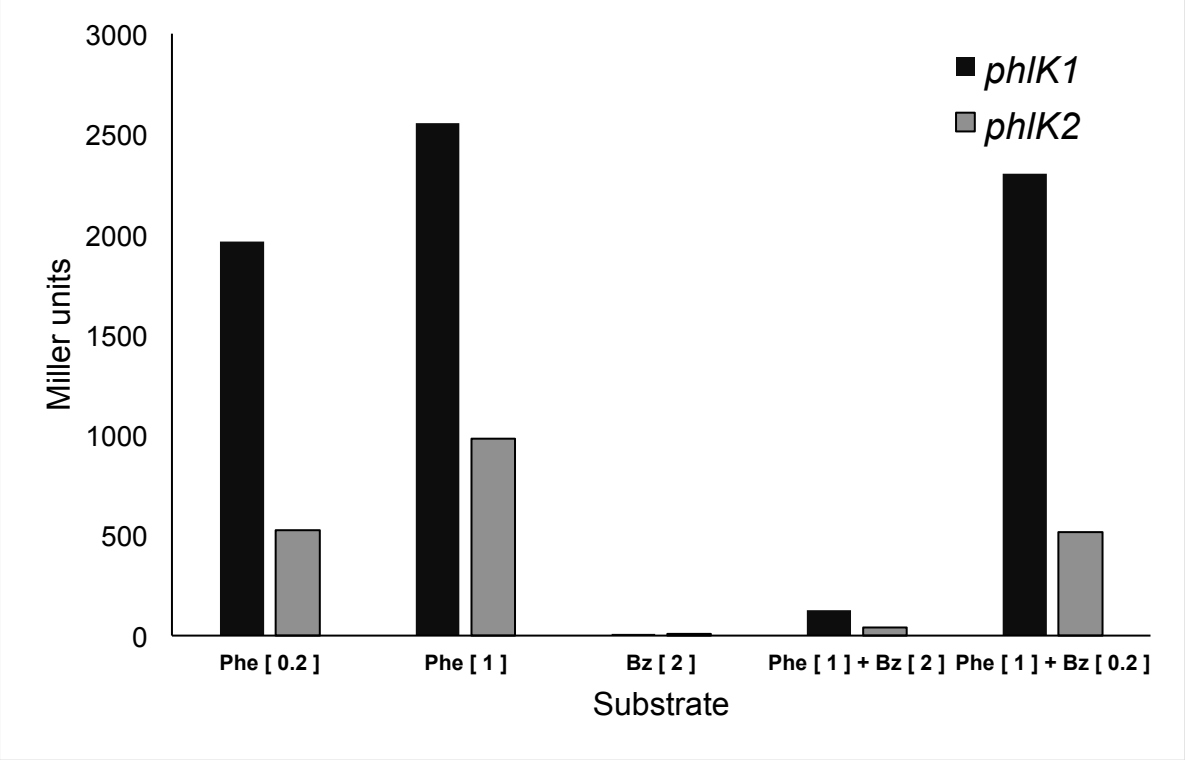
Catabolic route for 4-hydroxyphenylacetate and tyrosine, through homogentisate in *Cupriavidus pinatubonensis* JMP134 (Pérez-Pantoja *et al*., 2008).

Figure S3E. Pérez-Pantoja, Leiva-Novoa *et al*.



Catabolic route for phenylacetate in *Cupriavidus pinatubonensis* JMP134 (Pérez-Pantoja *et al*., 2008).

Figure S4. Pérez-Pantoja, Leiva-Novoa *et al*.



Beta galactosidase levels obtained from transcriptional fusions of promoters of genes encoding *PhlK1* and *PhlK2* phenol hydroxylases in *Cupriavidus pinatubonensis* cells previously grown on benzoate/phenol (Bz/Phe) mixtures. Numbers in brackets indicate the concentration of Bz and Phe. Miller units were calculated based on Miller (1972). Values are averages of three biological replicates. Standard deviations were lower than 10% (not shown).

Promoter regions were fused to the *lacZ* gene of pKGWP0 plasmid (Marin *et al*, 2010). PCR products comprising 450 and 500 nt upstream of the translational initiation codon of *phlK1* and *phlK2* genes, respectively, were obtained. The amplified DNA fragments were purified, digested and ligated into the *XhoI-KpnI or XbaI-KpnI* restriction sites of previously digested pKGWP0 plasmid to form *phlK1*-lacZ, and *phlK2*-lacZ, and subsequently transformed by electroporation into wild-type *C. pinatubonensis*, or *C. pinatubonensis* Δ*benA* (Donoso *et al*, 2011). Transformed colonies were selected in minimal medium supplemented with 100 ug/mL spectinomycin.

ß-galactosidase assays were performed according to standard protocols (Miller, 1972) after 6 h of incubation. Bz/Phe mixtures or single compounds were used as inducers. Bz levels were almost completely removed and most of Phe remained untouched after 6 h.

Donoso RA, Pérez-Pantoja D, González B. 2011. Strict and direct transcriptional repression of the *pobA* gene by benzoate avoids 4-hydroxybenzoate degradation in the pollutant degrader *Cupriavidus necator* JMP134. Environ Microbiol **13:**1590-1600.

Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Marín M, Pérez-Pantoja D, Donoso RA, Wray V, González B, Pieper DH. 2010. Modified 3-oxoadipate pathway for the biodegradation of methylaromatics in *Pseudomonas reinekei* MT1. J Bacteriol **192:**1543-1552.

Table S1: Half-lives of AC used as single compounds or in mixtures for growth of *Cupriavidus pinatubonensis* JMP134 wild type or the Δ*benA* mutant (shaded rows).

Compound/mixture	Bz	4-Hb	3-Hb	Pac	Tyr	2,4-D
Single (50 µM)	5.8±0.4	9.2±1.1	9.1±0.1	4.3±0.4	3.1±0.1	3.5±0.6
Single (100 µM)	6.3±0.3	8.3±0.1	9.2±0.9	5.0±0.1	3.8±0.5	3.8±0.4
Single (250 µM)	5.5±0.1	6.5±0.5	8.1±0.7	5.5±0.6	3.8±0.6	4.1±0.2
Bz/4-Hb/3-Hb/Pac/Tyr/2,4-D (50 µM)	3.2±0.1	6.1±0.3	3.9±0.4	6.2±0.2	4.2±0.2	7±0.1
Bz/4-Hb/3-Hb/Pac/Tyr/2,4-D (100 µM)	3.1±0.9	6.2±0.2	5.9±0.5	6.1±0.3	6.1±0.6	7.4±0.7
Bz/4-Hb/3-Hb/Pac/Tyr/2,4-D (250 µM)	4.1±0.2	7.9±0.2	7.9±0.9	8.3±1.1	7.1±0.4	7.4±0.7
4-Hb/3-Hb/Pac/Tyr/2,4-D (50 µM)	n.a.	5.9±0.2	5.9±0.3	6.1±0.2	5.1±0.5	6.4±0.3
4-Hb/3-Hb/Pac/Tyr/2,4-D (100 µM)	n.a.	5.3±0.4	5.8±0.3	6.0±0.3	5.4±0.4	6.5±0.2
4-Hb/3-Hb/Pac/Tyr/2,4-D (250 uM)	n.a.	6.2±0.2	6.5±0.1	6.0±0.4	5.6±03	7.5±0.1
Bz/4-Hb/3-Hb/Pac/Tyr/2,4-D (50 µM)	∞	6.3±0.2	5.7±0.2	6.1±0.3	1.2±0.4	9.1±0.6
Bz/4-Hb/3-Hb/Pac/Tyr/2,4-D (100 µM)	∞	6.2±0.4	5.9±0.1	5.5±0.4	3.8±0.3	6.7±0.2
Bz/4-Hb/3-Hb/Pac/Tyr/2,4-D (250 µM)	∞	8.3±0.5	5.9±0.4	7.3±0.6	6.1±0.1	8.3±0.3
4-Hb/3-Hb/Pac/Tyr/2,4-D (50 µM)	n.a.	4.2±0.1	4.5±0.6	4.6±0.5	3.9±0.1	4.7±0.3
4-Hb/3-Hb/Pac/Tyr/2,4-D (100 µM)	n.a.	5.2±0.5	5.7±0.3	6.1±0.5	4.4±0.2	6.4±0.8
4-Hb/3-Hb/Pac/Tyr/2,4-D (250 µM)	n.a.	5.8±0.3	6.3±0.4	6.5±0.2	4.6±03	6.4±0.4

∞: No degradation of Bz at recorded time (at least 36 h). n.a.: not applied. Half-lives (h^{-1}) were calculated based on Breidt *et al*. (1994).

Breidt F, Romick TL, Fleming HP. 1994. A rapid method for the determination of bacterial growth kinetics. J Rapid Meth Automat Microbiol **3:**59-68.

Table S2: Growth and degradation parameters of a continuously fed culture of *Cupriavidus pinatubonensis* JMP134 with a six members mixture of benzoate, 3- and 4-hydroxybenzoate, phenylacetate, tyrosine and 2,4-dichlorophenoxyacetate (200 µM each).

#	Flux (ml/min)	µ (h^{-1})	Doubling time (h)	Cell density ($OD_{600\ nm}$)	Bz (µM)	4-Hb (µM)	3-Hb (µM)	Pac (µM)	Tyr (µM)	2,4-D (µM)
1	4.35	0.348	2.0	0.54	0	0	0	0	0	8
2	4.55	0.364	1.9	0.59	0	0	0	0	0	30
3	4.80	0.384	1.8	0.56	0	11	15	25	0	84
4	5.02	0	0	0.05	162	189	208	145	177	202

Continuous culture performed in a chemostat BioFlo 110 (New Brunswick Scientific, Edison, NJ). Culture volume: 0.75 L; dilution rate: variable; aeration rate: 0.16 L h^{-1}; agitation rate: 250 rpm; temperature: 30ºC. Output concentration and cell density values were recorded after five retention times.

Table S3: Some metabolic features of aromatic compounds used in this work

Compound	Curve type [a]/ Yield [b]	XlogP3[c]	Energy yielding intermediates [d]	Toxicity [e] LD50 (mg/kg)/MIC (mM)
Benzoate	A / 4.4	1.9	Succinate, acetate	1600/66
4-hydroxybenzoate	A / 3.2	1.6	Succinate, acetate	2200/68
3-hydroxybenzoate	A / 3.2	1.5	Pyruvate, fumarate	2000/91
Phenylacetate	A / 4.2	1.4	Acetate, succinate	2250/>178
4-hydroxyphenylacetate	A / 3.8	0.8	Fumarate, acetoacetate	3500****/112
2,4-dichlorophenoxyacetate	B / 2.9	2.8	Succinate, acetate*	350/25
Phenol	B / 3.6	1.5	Succinate, acetate or Pyruvate, acetate**	270/16
Tyrosine	A / 4.5	-2.3	Fumarate, acetoacetate***	>1450****/51

[a] Growth was tested at 0.5, 1.0, 2, 4 and 8 mM. Curve type: A= growth yields essentially proportional to the substrate concentration; B= growth yields lower at higher substrate concentrations.

[b] Growth yield averages expressed as mg of cells / mmoles of added carbon (Optical density=1.0 equivalent to 1 mg of cells, based on average bacterial mass is 10^{-12} g).

[c] XlogP3 values based on computation of octanol/water partition coefficients as described by Cheng *et al*. (2007). Data retrieved from http://pubchem.ncbi.nlm.nih.gov.

[d] Energy yielding intermediates in *C. pinatubonensis* based on available info on biochemical routes (Pérez-Pantoja *et al*., 2008).

[e] Toxicity. Lethal dose, LD50= values (mg/kg), mouse (oral dose, except otherwise is indicated) reported in Material Safety Data Sheets. Minimal inhibitory concentrations (MIC) determined in Luria-Bertani cultures of *C. pinatubonensis* JMP134, *Pseudomonas syringae* DC3000 and *Escherichia coli* Mach, grown on Luria-Bertani cultures in the presence of 0, 4, 16, 32, 44, 64, 72, 128 and 178 mM of the respective AC.

*: The first reaction requires alpha ketoglutarate but produces succinate and glyoxal, the latter is probably not metabolized.

**: Assuming that *ortho* or *meta* ring cleavage may take place and that acetaldehyde produced by the *meta* ring cleavage pathway is oxidized to acetate.

***: Acetoacetate can be converted into two acetate molecules.

**** Intraperitoneal dose.

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