

Expanding the current knowledge and biotechnological applications of the oxygen-independent *ortho*-phthalate degradation pathway

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Summary

ortho-Phthalate derives from industrially produced phthalate esters, which are massively used as plasticizers and constitute major emerging environmental pollutants. The *pht* pathway for the anaerobic bacterial biodegradation of *o*-phthalate involves its activation to phthaloyl-CoA followed by decarboxylation to benzoyl-CoA. Here, we have explored further the *pht* peripheral pathway in denitrifying bacteria and shown that it requires also an active transport system for *o*-phthalate uptake that belongs to the poorly characterized class of TAXI-TRAP transporters. The construction of a fully functional *pht* cassette combining both catabolic and transport genes allowed to expand the *o*-phthalate degradation ecological trait to heterologous hosts. Unexpectedly, the *pht* cassette also allowed the aerobic conversion of *o*-phthalate to benzoyl-CoA when coupled to a functional box central pathway. Hence, the *pht* pathway may constitute an evolutionary acquisition for *o*-phthalate degradation by bacteria that thrive either in anoxic environments or in environments that face oxygen limitations and that rely on benzoyl-CoA, rather than on catecholic central intermediates, for the aerobic catabolism of aromatic compounds. Finally, the recombinant *pht* cassette was used both to screen for functional aerobic box pathways in bacteria and to engineer recombinant biocatalysts for *o*-phthalate bioconversion into sustainable bioplastics, e.g., polyhydroxybutyrate, in plastic recycling industrial processes.

Introduction

Phthalic acids are benzoic acids with one additional carboxylic group in either *ortho* (*o*-phthalate, PA), *meta* (isophthalate) or *para* (terephthalate) position. Although these compounds are generated as intermediates during the bacterial degradation of polycyclic aromatic hydrocarbons found in fossil fuels, the main source of phthalic acids is the chemical industry (Junghare *et al.*, 2016). Phthalic acids are produced massively as essential constituents of plastics, either as part of the polymeric structure, e.g., terephthalate in polyethylene terephthalate (PET) plastic, or as non-covalently bound dissolved additives (softeners or plasticizers) (Gao and Wen, 2016). Most synthetic plasticizers are di-esters of PA (called phthalates) and they are classified as xenobiotics produced industrially only since the 1960s (Benjamin *et al.*, 2015). As PA esters are not covalently bound to the plastic, they can easily diffuse out of the polymer itself, leading to contamination in nearly every environment. PA esters are one of the most frequently detected emerging organic contaminants in the environment (Gao and Wen, 2016), and they have been listed as major man-made priority pollutants due to their hepatotoxic, teratogenic, carcinogenic and endocrine disrupting (anti-androgenic) properties (Annamalai and Namasivayam, 2015). Hence, it becomes urgent to remove phthalates from the environment effectively and economically. On the other hand, and within the current strategies for plastic recycling as a sustainable plastic waste management, the treatment of the PA esters constitutes a major challenge (Brandon and Criddle, 2019; Boll *et al.*, 2020).

Compared to abiotic degradation, microbial degradation of PA esters is a more efficient, cost-effective and environmental-friendly strategy in the ecosystem. In most cases, biodegradation is initiated by non-specific esterases that release the free PA moiety and the side chain alcohols (Keyser *et al.*, 1976; Eaton and Ribbons, 1982; Liang *et al.*, 2008). The latter are being utilized by microorganisms while PA is quite resistant and persists in the environment (Gao and Wen, 2016). However, certain microorganisms can mineralize PA, as well as the other two phthalate isomers, under aerobic conditions. In all cases, the three phthalate isomers are converted to a

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dihydroxybenzoic acid by the action of ring-hydroxylating dioxygenases and subsequent decarboxylation. Protocatechuate, in most cases, or 2,3-dihydroxybenzoate are then attacked by ring-cleavage dioxygenases and converted to central intermediates either via the *ortho*- or *meta*-cleavage pathways (Ribbons and Evans, 1960; Keyser *et al.*, 1976; Eaton and Ribbons, 1982; Boll *et al.*, 2020; Kasai *et al.*, 2019).

In contrast to the well-studied aerobic biodegradation of PA, anaerobic biodegradation of PA was reported later in the literature (Nozawa and Maruyama, 1988). Only very recently some studies based on differential proteomics and *in vitro* enzyme assays allowed the identification and characterization of the genes (hereafter named as *pht*) and enzymes involved in the anaerobic peripheral pathway for the conversion of PA to benzoyl-CoA (Boll *et al.*, 2020), which is further degraded through the anaerobic benzoyl-CoA central pathway (*bzd* genes) (Carmona *et al.*, 2009) (Fig. 1A). The *pht* peripheral pathway has been studied in closely related denitrifying β -proteobacteria, such as *Azoarcus* sp. PA01 (Junghare *et al.*, 2016), *Thauera chlorobenzoica* 3CB-1, *Aromatoleum aromaticum* EbN1 and *Azoarcus evansii* KB740 (recently reclassified within the *Aromatoleum* genus; Ebenau-Jehle *et al.*, 2017; Rabus *et al.*, 2019), and in an obligate anaerobic sulfate-reducing δ -proteobacterium, *Desulfosarcina cetonica* (Geiger *et al.*, 2019). Whereas in denitrifiers, the PA is first activated to phthaloyl-CoA by a heterodimeric class III succinyl-CoA-dependent PA CoA transferase (SPT) (Junghare *et al.*, 2016; Mergelsberg *et al.*, 2018) (Fig. 1A), in sulfate-reducing bacteria, the activation is carried out by an ATP-dependent PA CoA ligase

(Geiger *et al.*, 2019). Nevertheless, both in denitrifying and sulfate-reducing bacteria, *o*-phthaloyl-CoA is subsequently decarboxylated to benzoyl-CoA by a phthaloyl-CoA decarboxylase (PCD). PCD belongs to the UbiD enzyme family of (de)carboxylases that contain a prenylated flavin adenosine mononucleotide (prFMN) cofactor generated by an associated UbiX-type prenyltransferase (Fig. 1A) (Junghare *et al.*, 2016; Mergelsberg *et al.*, 2017; Geiger *et al.*, 2019; Marshall *et al.*, 2019). A similar biochemical strategy has been recently described for the anaerobic biodegradation of isophthalate in the fermenting bacterium *Syntrophorhabdus aromaticivorans* (Junghare *et al.*, 2019).

All PA-induced *pht* gene clusters described so far contain at least four genes (three genes in obligate anaerobes) encoding the enzymes SPT (*phtSaSb*), PCD (*phtDa*) and its associated FMN prenyltransferase (*phtDb*), as well the two additional genes *phtTaTb* proposed to encode a PA transporter (Boll *et al.*, 2020), but whose implication in PA transport has not been demonstrated so far (Fig. 1).

In the aerobic degradation of PA, uptake into bacterial cells appears to require an active transport system, i.e., either mono-component permeases of the major facilitator superfamily (MFS) of transporters or multicomponent solute-binding protein (SBP) dependent transport systems that rely on a periplasmic SBP protein which interacts with the substrate with high affinity (Chang *et al.*, 2009; Hara *et al.*, 2010; Fukuhara *et al.*, 2010; Hosaka *et al.*, 2013), rather than its passive diffusion across the inner membrane which only may work at low external pH when the carboxylic groups are

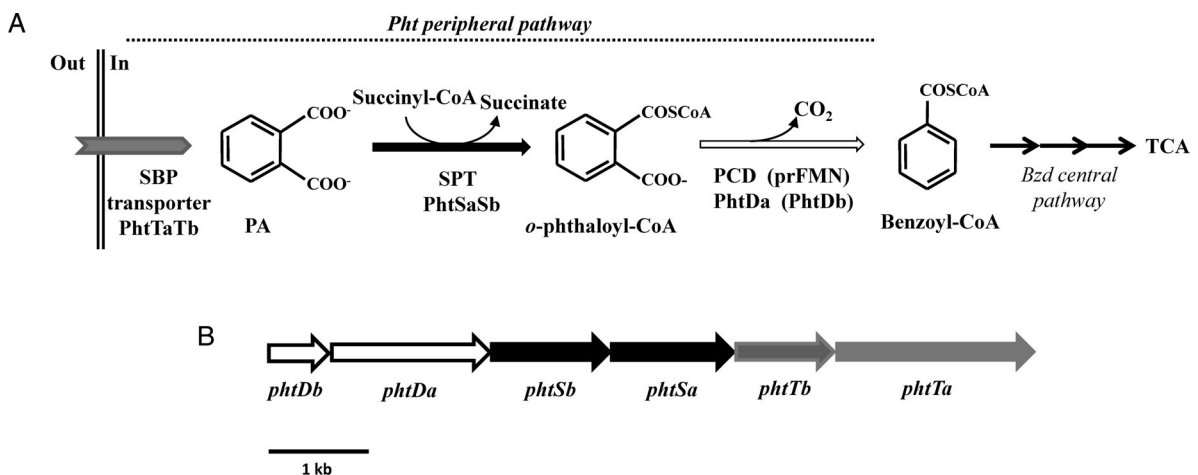


Fig 1. Scheme of the biochemistry and associated genetic determinants of the *pht* peripheral pathway for PA degradation in denitrifying bacteria. A. Uptake of PA inside the bacterial cell by a putative solute-binding protein (SBP) transporter (PhtTaTb) is indicated with a grey arrow. Activation of PA to *o*-phthaloyl-CoA by the SPT CoA transferase (PhtSaSb) is shown with a black arrow. Decarboxylation of *o*-phthaloyl-CoA to benzoyl-CoA by a PCD decarboxylase (PhtDa and its associated PhtDb FMN prenyltransferase) is shown with a white arrow. Benzoyl-CoA is finally de-aromatized and funnelled into the tricarboxylic acid cycle (TCA) via the anaerobic *bzd* central pathway.

B. General organization of the PA-induced *pht* gene cluster. The colour code of the genes corresponds to that of the functions indicated in panel A. In obligate anaerobes, the *pht* gene cluster shows a similar organization but the *phtSaSb* genes are replaced by a single gene encoding a PA CoA ligase.

protonated. However, there is no experimental demonstration that an active uptake is also essential for anaerobic PA degradation.

The initial aim of this work was to experimentally demonstrate for the first time that PA uptake in denitrifying bacteria requires an SBP-dependent secondary transporter (PhtTaTb) that becomes essential for anaerobic PA degradation through the peripheral pht pathway. The construction of a fully functional cassette to confer PA degradation to heterologous hosts has allowed us to discover that unexpectedly the pht pathway also allows the aerobic conversion of PA to benzoyl-CoA opening a new view about the biological significance and evolution of this route primarily classified as a strict anaerobic pathway. Finally, using the newly acquired knowledge on the pht pathway, we have been able of engineering novel recombinant organisms to channel the degradation of PA, a major feedstock from plastic recycling processes, towards the industrial production of more sustainable bioplastics.

Results and discussion

Anaerobic PA degradation requires an active uptake system

The peripheral pht pathway for the anaerobic degradation of PA in certain denitrifying bacteria involves only two enzymatic steps catalysed by the SPT and PCD enzymes (Fig. 1A) (Junghare *et al.*, 2016; Mergelsberg

et al., 2017; Mergelsberg *et al.*, 2018). To check whether the expression of the four *phtDbDaSbSa* catabolic genes (Fig. 1B) would confer to other denitrifying bacteria the ability to use PA as sole carbon an energy source, we cloned these genes from *A. aromaticum* EbN1 strain and expressed them in *Escherichia coli* from a pIZ1016 broad-host range plasmid (Table 1) under the control of the *Ptac/lacI^R* regulatory couple. The resulting pIZPHT plasmid (Table 1) was then introduced into *Azoarcus* sp. CIB, an anaerobic benzoate/benzoyl-CoA degrader that lacks the *pht* genes and, hence, is naturally unable to degrade PA (Martín-Moldes *et al.*, 2015). Interestingly, *Azoarcus* sp. CIB (pIZPHT) was unable to grow on PA as sole carbon source, even after the induction of the *pht* genes by the addition of IPTG to the cultures. This result suggested that the peripheral pht pathway for PA degradation may involve functions other than those strictly responsible for its activation/decarboxylation to benzoyl-CoA. In this sense, it is known that the aerobic degradation of phthalates requires their uptake into the bacterial cell through a specific transport system (Chang *et al.*, 2009; Hara *et al.*, 2010; Fukuhara *et al.*, 2010; Hosaka *et al.*, 2013), and all the PA-induced *pht* clusters reported so far contain a couple of genes (*phtTaTb*) predicted to encode an SBP-dependent secondary transporter (Boll *et al.*, 2020) (Fig. 1B). Moreover, additional genes located near the *pht* genes in the genomes of some denitrifying bacteria encode a putative three-component primary ATP-dependent (ABC) transporter that could also be involved in PA uptake (Junghare

Table 1. Bacterial strains and plasmids used in this work.

Strains	Relevant genotype and main characteristics ^a	Reference or source
<i>E. coli</i> strains		
DH10B	<i>F'</i> , <i>mcrA</i> , Δ (<i>mrr hsdRMS-mcrBC</i>), Φ 80/ <i>lacZ</i> Δ M15, Δ <i>lacX74</i> , <i>deoR</i> , <i>recA1</i> , <i>araD139</i> , Δ (<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (Sm ^r), <i>endA1</i> , <i>nupG</i>	Life Technologies
S17- λ pir	Tp ^r Sm ^r <i>recA thi hsdR</i> M ⁺ RP42::Tc::Mu::Km Tn7 λ pir phage lysogen	de Lorenzo and Timmis (1994)
<i>Azoarcus</i> / <i>Aromatoleum</i> strains		
<i>Azoarcus</i> sp. CIB	Wild-type CIB strain, PA ⁻	López-Barragán <i>et al.</i> (2004)
<i>Aromatoleum evansii</i>	Wild-type strain, PA ⁺	Anders <i>et al.</i> (1995)
<i>Cupriavidus</i> strains		
<i>Cupriavidus necator</i> H16	Wild-type strain, PA ⁻	Pohlmann <i>et al.</i> (2006)
<i>Cupriavidus pinatubonensis</i> JMP134	Wild-type strain, PA ⁻	Don and Pemberton (1981)
<i>Pseudomonas</i> strains		
<i>Pseudomonas putida</i> KT2440	Wild-type strain, PA ⁻	Franklin <i>et al.</i> (1981)
Plasmids		
pIZ1016	Gm ^r , pBBR1MCS-5 broad-host range cloning vector	Moreno-Ruiz <i>et al.</i> (2003)
pIZPHT	Gm ^r , pIZ1016 derivative harbouring the catabolic <i>pht</i> genes for the peripheral PA degradation pathway from <i>Aromatoleum aromaticum</i> EbN1 strain	This work
pIZPHTRAP	Gm ^r , pIZ1016 derivative harbouring the <i>pht</i> cassette for the peripheral PA degradation pathway	This work
pIZPHTRAP Δ phtDa	Gm ^r , pIZPHTRAP derivative lacking the <i>phtDa</i> gene encoding the PCD decarboxylase	This work

^aThe abbreviations used are as follows: Sm^r, streptomycin-resistant; Gm^r, gentamicin-resistant; PA, *o*-phthalate degradation.

et al., 2016; Ebenau-Jehle *et al.*, 2017; Boll *et al.*, 2020). Therefore, all these observations suggested that an active PA uptake could be essential for the anaerobic degradation of PA through the *pht* pathway.

To get some insights into PA transport in denitrifying bacteria that naturally express the *pht* peripheral pathway, we used *A. Evansii* as model system. Cells grown anaerobically on MC medium containing 3 mM benzoate (control condition) or 3 mM PA were incubated with 25 μ M [14 C]-PA, and a time-course of the amount of [14 C]-PA incorporated into the cells was determined. Whereas cells grown on PA showed uptake of this aromatic acid (uptake rate of about 670 pmol min $^{-1}$ mg protein $^{-1}$) cells grown on benzoate did not show [14 C]-PA uptake (Fig. 2A). This result suggested that a specific PA transporter is induced when cells grow on this aromatic compound.

To determine whether the uptake of PA requires an active transport system, *A. Evansii* cells grown on PA were exposed to 2,4-dinitrophenol (DNP), an ionophore which disrupts the proton motive force, or to 1,3-dicyclohexylcarbodiimide (DCCD), which blocks ATP synthesis by acting on the ATPase complex (Chae and Zylstra, 2006), before the transport assay was performed. Whereas cells treated with DCCD retained the PA transport capacity, cells exposed to DNP lacked completely the ability to transport [14 C]-PA (Fig. 2B). Thus, these results indicate that PA uptake in *A. Evansii* cells requires an active transport system that is mainly dependent on the proton motive force rather than on the ATP pool, hence suggesting the involvement of an SBP-dependent

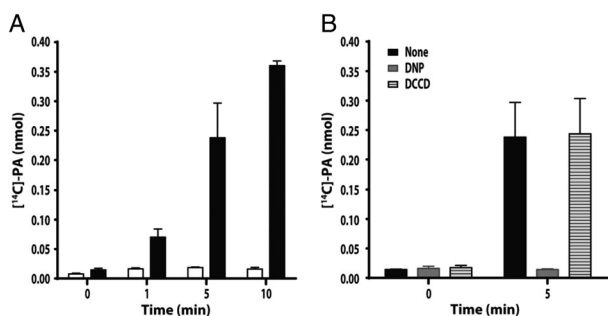


Fig 2. Uptake of PA by *A. Evansii* cells grown under nitrate-reducing conditions.

A. The [14 C]-PA uptake assays were performed with cells grown on benzoate (white bars) or PA (black bars). Graphed values are the average of three independent experiments (error bars indicate standard deviations).

B. Effect of energetic inhibitors on [14 C]-PA uptake in *A. Evansii* cells grown anaerobically on PA. Cells were grown and then exposed or not (None) to 1 mM DNP (proton-motive force blocking agent 2,4-dinitrophenol) or 1 mM DCCD (ATP synthase inhibitor dicyclohexylcarbodiimide) before performing the [14 C]-PA uptake assays. Graphed values are the average of three independent experiments (error bars indicate standard deviations).

secondary transporter instead of a primary ABC-type transport mechanism (Mulligan *et al.*, 2011).

Engineering a fully functional *pht* pathway

Once it was shown that an active secondary transport system is induced when cells grow anaerobically on PA, we engineered a new recombinant *pht* cassette containing the four *pht* catabolic genes and the closely associated *phtTaTb* genes, under control of the *Ptac/lacI^q* regulatory couple generating the broad-host range pZPHTRAP plasmid (Fig. 3A and Table 1). *Azoarcus* sp. CIB (pZPHTRAP) acquired the ability to use PA as sole carbon and energy source under anaerobic conditions (Fig. 3B), hence demonstrating that both catabolic genes (*phtDbDaSbSa*) and the putative transport genes (*phtTaTb*) are essential for the anaerobic degradation of PA via benzoyl-CoA.

We show here that an active transport system is essential for the peripheral *pht* pathway to metabolize PA via CoA-derived intermediates. Although transport genes are usually found associated to clusters for anaerobic degradation of polar dissociated aromatic compounds, e.g., aromatic acids (Egland *et al.*, 1997; Carmona *et al.*, 2009; Trautwein *et al.*, 2012; Juárez *et al.*, 2013), and some SBPs have been studied *in vitro* (Pietri *et al.*, 2012; Salmon *et al.*, 2013) nobody had demonstrated their physiological role so far. Thus, our results constitute the first experimental demonstration on the key role of an active transport system, that of PA, in the anaerobic catabolism of aromatic compounds.

Interestingly, the GC content of the *phtTaTb* genes from *Aromatoleum/Azoarcus/Thauera* strains (about 60% GC) is significantly lower than that of the *pht* catabolic genes (about 65% GC), which fits with the average GC content of the complete genome. Moreover, in some *pht* clusters, such as that of *A. aromaticum* EbN1, the transport genes are separated from the catabolic ones by insertion sequences (Ebenau-Jehle *et al.*, 2017). These observations suggest that *pht* transport genes may have been recruited through horizontal gene transfer during the recent ongoing evolution of *pht* clusters in bacteria that have been adapted to benefit from unusual substrates introduced by humans into the current ecosystems.

Characterization of the TAXI-TRAP transporter for PA uptake

To further confirm that the *phtTaTb* genes encode a PA transporter system and to determine its biochemical properties, we performed PA uptake experiments at different [14 C]-PA concentrations in *Azoarcus* sp. CIB (pZPHT), i.e., control cells that contain only the catabolic *pht*

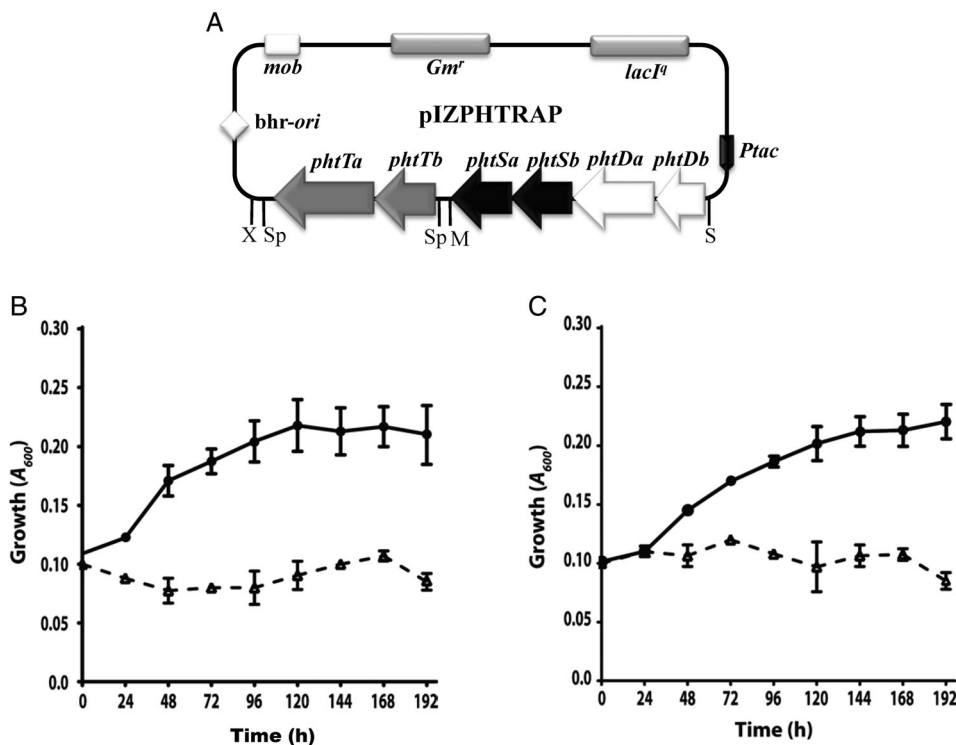


Fig 3. Expression of the *pht* cassette in *Azoarcus* sp. CIB cells.

A. Scheme of the *pht* cassette cloned into the broad-host range pIZPHTRAP plasmid. The *pht* genes are indicated by white, black and grey thick arrows. Some restriction sites are indicated: M, *Mfe*I, S, *Sgr*DI, Sp, *Spe*I, X, *Xba*I. The gentamicin resistance gene (*Gm*^r), mobilization (*mob*), broad-host range origin of replication (*bhr ori*), and the regulatory couple *lacI*^q repressor gene (*lacI*^q) and *Ptac* promoter are shown.

B. Growth curves of *Azoarcus* sp. CIB (pIZPHTRAP) (filled circles) and *Azoarcus* sp. CIB (pIZPHTRAP Δ phtDa) (open triangles). Cells were grown anaerobically on MC minimal medium containing 3 mM PA and 10 mM nitrate as sole donor and electron acceptors, respectively. Gentamicin ($7.5 \mu\text{g ml}^{-1}$) was added to assure plasmid maintenance. Bacterial growth was monitored by measuring A_{600} .

C. Growth curves of *Azoarcus* sp. CIB (pIZPHTRAP) (filled circles) and *Azoarcus* sp. CIB pIZPHTRAP Δ phtDa (open triangles) cultivated aerobically on MC minimal medium containing 3 mM PA. Values are the mean of three different experiments. Error bars indicate standard deviations.

genes, and in *Azoarcus* sp. CIB (pIZPHTRAP), i.e., cells that express the catabolic *pht* genes as well as the *phtTaTb* transport genes. Whereas *Azoarcus* sp. CIB (pIZPHT) cells did not show [¹⁴C]-PA uptake, *Azoarcus* sp. CIB (pIZPHTRAP) cells showed a maximum uptake rate of about $156 \pm 14 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$, with a saturation kinetics at about 20 μM PA and a transport affinity (K_m) of 15 μM (Fig. 4A).

As expected for an SBP-dependent secondary transporter, the [¹⁴C]-PA uptake was completely inhibited when cells were pre-incubated with DNP, but it did not decrease when cells were pre-incubated with the ATP synthase inhibitor DCCD (Fig. 4B). These results taken together confirm that PhiT_aT_b constitutes a secondary transporter responsible of PA uptake.

[¹⁴C]-PA uptake rates were tested in the presence of non-radioactive phthalate isomers as competitive inhibitors. As a control, when 250 μM unlabelled PA was used as competitor, the uptake rate of the 25 μM [¹⁴C]-PA radioactive compound was undetected (Fig. 4B). Interestingly, when 250 μM unlabelled isophthalate or

terephthalate were used, the [¹⁴C]-PA uptake rate was reduced to 50% or 60%, respectively, of the rate in the absence of a competitor substrate (Fig. 4B). These results suggest that although the transporter has a high affinity for PA, it shows a low substrate specificity for the other two phthalate isomers.

The main type of SBP-dependent secondary transporters are TRAP-transporters, which are usually constituted by an SBP belonging to the DctP family, and by large (DctM) and small (DctQ) integral membrane proteins (Mulligan *et al.*, 2011; Pietri *et al.*, 2012). TRAP-transporters involved in the transport of aromatic compounds have already been characterized (Fig. 5) (Chae and Zylstra, 2006; Salmon *et al.*, 2013). Interestingly, the SBP (PhiT_b) protein of the two-component PA transporter does not belong to the classical TRAP class but to a new class called TAXI-TRAP (Fig. 5). TAXI-TRAP transporters contain an SBP that does not show similarity to the SBP of classical TRAP transporters and is being called TAXI (TRAP associated extracytoplasmic immunogenic) protein. Moreover, TAXI-TRAP transporters usually have the

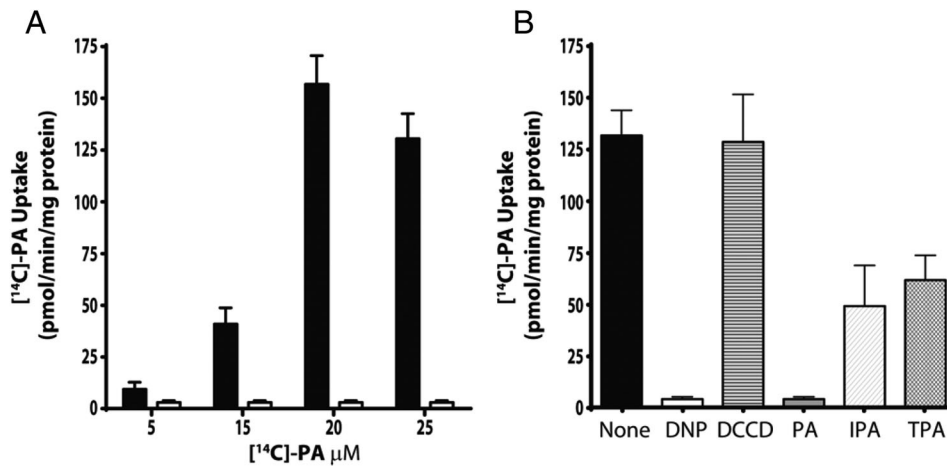


Fig 4. Uptake of PA by the PhtTaTb TAXI-TRAP transporter.

A. [^{14}C]-PA uptake assays were performed with *Azoarcus* sp. CIB (pIZPHTRAP) cells expressing the PhtTaTb transporter (black bars) and with *Azoarcus* sp. CIB (pIZPHT) control cells that do not express the PhtTaTb transporter (white bars), by using increasing concentrations (5–25 μM) of [^{14}C]-PA.

B. The uptake rates of [^{14}C]-PA were examined by pre-incubating cells for 10 min with either 1 mM DNP or 1 mM DCCD previous to the transport assay. PA uptake rates were also examined by adding 250 μM non-labelled PA, isophthalate (IPA), or terephthalate (TPA) as competitors to the transport assay containing 25 μM [^{14}C]-PA. Values are the mean of three different experiments. Error bars indicate standard deviations.

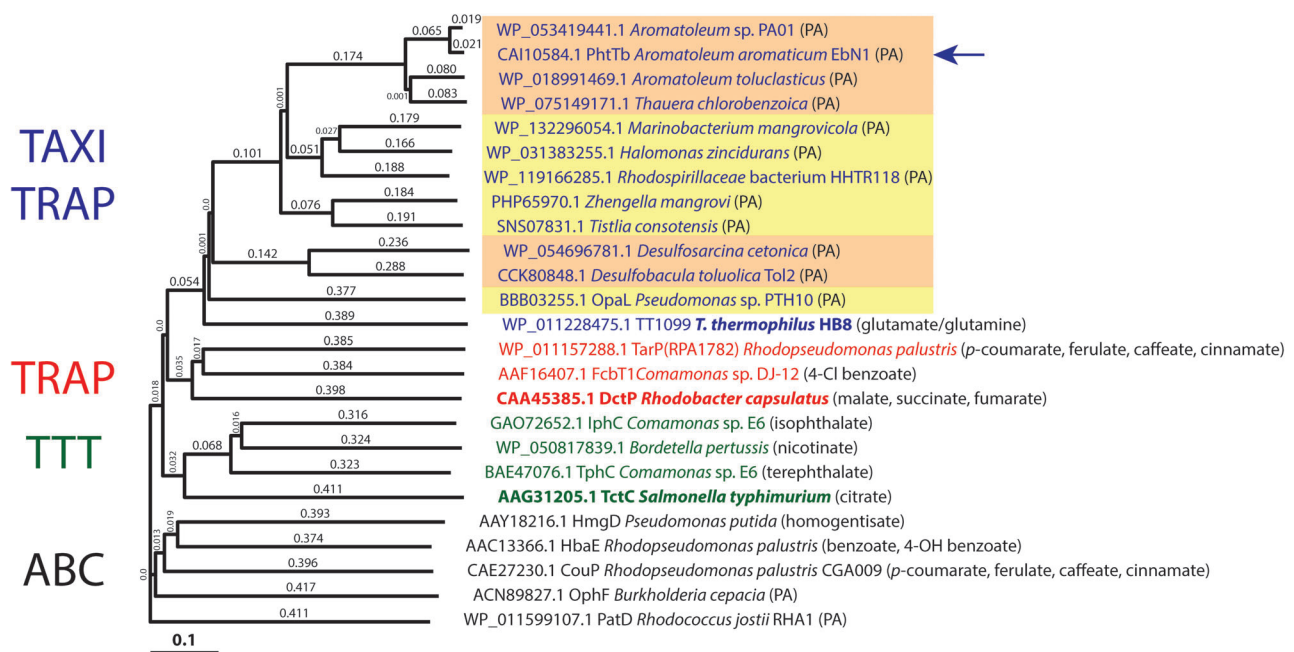


Fig 5. Phylogenetic tree of the solute binding-protein (SBP) of different primary (ABC-type) and secondary (TRAP-, TTT-, TAXI-TRAP-type) transporters of aromatic compounds. Multiple sequence alignment of the different proteins and the phylogenetic tree were obtained as detailed in Experimental Procedures. The bar represents 0.1 substitutions per amino acid. Protein names, GenBank codes and the name of the microorganism harbouring the corresponding protein are indicated. The substrate of each transporter system is also indicated in parentheses. The prototype SBP of TRAP, TTT and TAXI-TRAP transporters is shown in bold. Among TAXI-TRAP transporters (in blue), the PhtTb protein from *A. aromaticum* EbN1 studied in this work is indicated with an arrow. Orange shadowed are PhtTb-like proteins present in *pht* clusters; yellow shadowed are predicted PhtTb-like proteins present in putative clusters for PA degradation via classical aerobic degradation pathways.

DctQ and DctM membrane subunits of standard TRAP transporters fused into a single integral membrane protein (Mulligan *et al.*, 2011; Rosa *et al.*, 2018). Accordingly, the PhtTa protein (657 aa) of the PA transporter contains

15 predicted transmembrane segments (TM) in its structure, which are organized as 10 TM at the N-terminus (corresponding to a DctM-like large subunit), 4 TM at the C-terminus (corresponding to a DctQ-like small subunit),

and an additional TM fusion segment (Supporting Information Fig. S1). The only structure available for a TAXI protein, that of TT1099 from the bacterium *Thermus thermophilus* (Takahashi *et al.*, 2004), reinforced its predicted role as putative glutamate transporter (Rabus *et al.*, 1999). Examination of the genome context of TAXI genes in archaea also suggests that they may be involved in transport of glutamate/glutamine or closely related C5-dicarboxylates such as 2-oxoglutarate (Mulligan *et al.*, 2011; Rosa *et al.*, 2018). However, to our knowledge, the PhtTaTb transporter described in this work represents the first TAXI-TRAP transporter whose biological function has been characterized.

It is worth noting that *phtTaTb* orthologues can be also identified in clusters involved in phthalate degradation via classical aerobic pathways (Fig. 5). Thus, the *opa* cluster for PA degradation via 2,3-dihydroxybenzoate in *Pseudomonas* sp. strain PTH10 encodes the *opaL* and *opaN* gene products (Kasai *et al.*, 2019) that show 28% and 42% identity to the *phtTb* and *phtTa* gene products, respectively. Moreover, the genomes of certain strains of *Halomonas* and *Marinobacterium* species (γ -proteobacteria), and *Tistlia*, *Zhengella* and a Rhodospirillaceae bacterium (α -proteobacteria), contain *phtTb* orthologues (Fig. 5) within clusters that are likely responsible for aerobic PA degradation via the classical protocatechuate pathway. Therefore, the predicted role of TAXI-TRAP systems for the uptake of PA in bacteria belonging to different phylogenetic groups provides further insights into the functionality of this poorly characterized class of secondary SBP-dependent transporters. Moreover, this work extends the current view on the diversity of transport systems for the uptake of phthalates by bacteria, which spans from monocomponent inner membrane MFS transporters, e.g. OphD and MopB (Saint and Romas, 1996; Chang *et al.*, 2009), to multi-component SBP-dependent transporters of all major families, i.e., primary ABC-type (e.g. OphFGH, PatDABC) (Chang *et al.*, 2009), and secondary TTT-type (TpiABTphC for terephthalate, TpiABlphC for isophthalate) (Fukuhara *et al.*, 2010; Hosaka *et al.*, 2013), and TRAP-type (PhtTaTb) (this work) (Fig. 5).

The peripheral pht pathway allows aerobic degradation of PA in denitrifying bacteria

The *pht* pathway was reported to be an anaerobic peripheral pathway (Ebenau-Jehle *et al.*, 2017; Mergelsberg *et al.*, 2017; Boll *et al.*, 2020). However, we unexpectedly observed that *Azoarcus* sp. CIB (pIZPHTRAP) expressing the complete *pht* cassette was also able to grow under aerobic conditions when using PA as sole carbon and energy source (Fig. 3C). These PA-grown cells showed a significant induction of the endogenous *boxB* gene encoding a key enzyme of the aerobic hybrid pathway for

benzoyl-CoA degradation (Supporting Information Fig. S2) (Valderrama *et al.*, 2012). Therefore, these results strongly suggest that the *pht* pathway could allow the aerobic metabolism of PA via benzoyl-CoA when the cells are equipped with an aerobic box pathway.

To further confirm this assumption and to discard that an enzyme from the CIB strain expressed in aerobically-grown *Azoarcus* sp. CIB (pIZPHTRAP) cells could functionally replace the PCD decarboxylase activity (which was shown to be oxygen sensitive in *in vitro* assays (Ebenau-Jehle *et al.*, 2017; Mergelsberg *et al.*, 2017), we constructed a mutant *pht* cassette without an active *phtDa* gene. When the resulting plasmid pIZPHTRAP Δ *phtDa* was transferred to *Azoarcus* sp. CIB, the recombinant strain failed to grow on PA either in the presence or absence of oxygen (Fig. 3B and C), strongly suggesting that *phtDa* is essential and that no other enzyme encoded in the genome of the CIB strain can replace this function. In agreement with these findings, we have additionally confirmed that *A. Evansii*, which is also equipped with a box pathway (Gescher *et al.*, 2005), was also able to use PA as sole carbon and energy source in the presence of oxygen (Supporting Information Fig. S3).

Classical aerobic PA pathways rely on ring-hydroxylating dioxygenases which require oxygen tensions that may quickly become limiting in many natural ecosystems or when bacteria grow on surfaces forming biofilms (Boll *et al.*, 2020). The results presented here unravel a new function for the *pht* peripheral pathway as an alternative route for aerobic PA degradation in denitrifying bacteria that thrive in environments that face oxygen limitations or fluctuating oxygen tensions.

Aerobic degradation of PA via the pht pathway requires a functional box central pathway

When *Azoarcus* sp. CIB grows on the presence of oxygen, benzoyl-CoA can only be degraded through the hybrid box pathway (Valderrama *et al.*, 2012). However, conversion of benzoyl-CoA to free benzoate by the action of specific or unspecific thioesterase and/or CoA-transferase activities (Ismail, 2008) could be proposed also as an alternative mechanism for aerobic benzoyl-CoA degradation in bacteria that harbour a classical aerobic ben (oxidizes benzoate to catechol)-cat (converts catechol to central metabolism) pathway (Fig. 6A) (Dagley *et al.*, 1960; Stanier and Ornston, 1973; Harwood and Parales, 1996; Pérez-Pantoja *et al.*, 2008; Díaz *et al.*, 2013; Nogales *et al.*, 2017).

To explore which should be the catabolic requirements of a bacterial host to allow aerobic mineralization of PA via the *pht* peripheral pathway, we transferred plasmid pIZPHTRAP to different bacterial species unable to degrade PA and that contain different combinations of hybrid (*box*) and/or classical (*ben-cat*) benzoate-degradation clusters. Thus, we

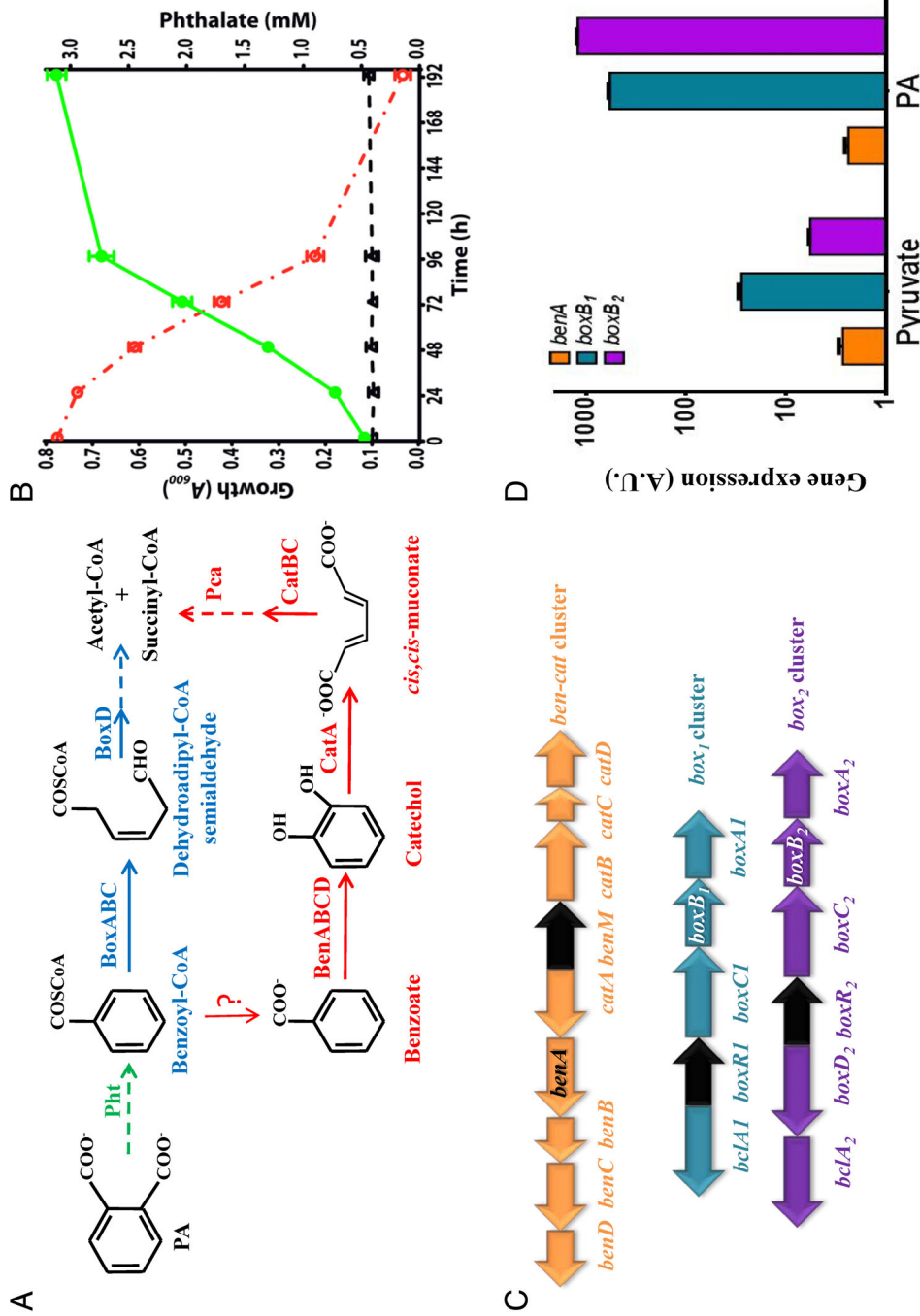


Fig 6. Expressing the *pht* cassette in *C. necator* H16 cells.

A. Scheme of the putative PA degradation pathways in *C. necator* H16 cells expressing the Pht enzymes that convert PA to benzoyl-CoA. The aerobic hybrid pathway (box pathway) and the classical aerobic pathway (ben-cat pathway) are indicated in blue and red respectively. Main enzymes and intermediates are shown. Discontinuous arrows indicate that more than one enzymatic step is involved.

B. Growth curves of *C. necator* H16 cells (black line) and *C. necator* H16 (pZPHTRAP) cells (green line) on MC minimal medium containing 3 mM PA. Bacterial growth was monitored by measuring A_{600} . Consumption of PA is also indicated (red line). Values are the mean of three different experiments. Error bars indicate standard deviations.

C. Organization of the *ben-cat* cluster and the two *box* clusters in the genome of strain H16. The *benA*, *boxB*₁, and *boxB*₂ genes used as probes for monitoring the expression of the *ben-cat*, *box1* and *box2* clusters, are highlighted. Black arrows show the regulatory genes.

D. Expression of the *benA*, *boxB*₁, and *boxB*₂ genes when *C. necator* H16 (pZPHTRAP) cells are grown aerobically on 18 mM pyruvate or 3 mM PA. Total RNA was isolated and the expression of the genes was measured by real-time RT-PCR. The relative expression level of the housekeeping *recA* gene. Each value is the average from three separate experiments; error bars indicate standard deviations.

generated recombinant strains of *P. putida* KT2440, which contains only the *ben-cat* genes (Jiménez *et al.*, 2002), *Cupriavidus pinatubonensis* JMP134, which contains a set of *ben-cat* genes and a set of *box* genes (Pérez-Pantoja *et al.*, 2008), and *C. necator* H16 (formerly known as *Ralstonia eutropha* H16), which contains a set of *ben-cat* genes in chromosome 1, a *box* cluster in chromosome 1 (*box*₁), and a second *box* cluster in chromosome 2 (*box*₂) (Pohlmann *et al.*, 2006) (Table 1).

Whereas *P. putida* KT2440 (pIZPHTRAP) and *C. pinatubonensis* JMP134 (pIZPHTRAP) grew on benzoate, they did not grow on PA. However, *C. necator* H16 (pIZPHTRAP) was able to consume PA and grow aerobically on this aromatic compound as sole carbon source (Fig. 6B). These results suggested that the classical *ben-cat* pathway was not able to metabolize the benzoyl-CoA generated by the peripheral *pht* pathway (in agreement with the observation that a putative benzoyl-CoA thioesterase encoding gene is lacking in the genome of these strains), and that only cells expressing a functional *box* pathway could allow aerobic mineralization of PA via benzoyl-CoA (Fig. 6A). In this sense, it should be mentioned that although strain JMP134 contains a *box* cluster in its genome, this cluster might not encode a functional *box* pathway since it lacks the *boxD* gene (Gescher *et al.*, 2006; Pérez-Pantoja *et al.*, 2008) and does not allow growth on benzoate to a JMP134Δ*benA* mutant strain (Donoso *et al.*, 2011; Pérez-Pantoja *et al.*, 2015).

To confirm that *C. necator* H16 (pIZPHTRAP) was degrading PA via the *box* pathway, we analysed the expression of some marker genes of the classical *ben-cat* pathway, e.g. *benA* gene, and the hybrid *box* pathway, e.g., *boxB*₁ (for cluster *box*₁) and *boxB*₂ (for cluster *box*₂) (Fig. 6C). Real-time RT-PCR gene expression studies of *C. necator* H16 (pIZPHTRAP) cells grown aerobically on PA were compared with those of cells grown on pyruvate (control). Whereas the basal expression of the *benA* gene was similar in pyruvate and PA, the *boxB*₁ and *boxB*₂ genes were induced in PA around 40- and 400-fold, respectively. These results suggest that *C. necator* H16 (pIZPHTRAP) cells grown on PA cannot generate free benzoate which has been shown to be the inducer of the *ben* genes in *Cupriavidus* strains (Donoso *et al.*, 2011; Pérez-Pantoja *et al.*, 2015). On the contrary, the results presented strongly suggest that an efficient metabolic flux for PA degradation via the *pht* pathway requires the coupling to a hybrid *box* pathway rather than to a classical *ben-cat* pathway.

Whereas the classical *ben-cat* benzoate degradation pathway has been extensively studied in a wide variety of bacteria, the aerobic hybrid *box* pathway has been characterized more recently (Fuchs *et al.*, 2011). Although *in silico* studies suggest that *box* genes are spread among α-, β- and some δ-proteobacteria (Valderrama *et al.*, 2012;

Suvorova and Gelfand, 2019), there are only few reports demonstrating an active *box* pathway in certain strains and none of them belong to the *Cupriavidus* genus (Gescher *et al.*, 2005; Gescher *et al.*, 2006; Deneff *et al.*, 2006; Bains *et al.*, 2009; Valderrama *et al.*, 2012; Chen *et al.*, 2014). Therefore, the results presented here with *Cupriavidus* strains reveal that the *pht* cassette becomes a useful genetic tool to screen for functional *box* pathways in bacteria by monitoring growth on PA.

Moreover, this work provides additional clues to the physiological role of *box* genes present in aerobic microorganisms endowed with classical benzoate degradation pathways (*ben-cat* genes). It was generally thought that in these microorganisms the *box* pathway allows growth on benzoate under reduced oxygen tensions (transition to stationary phase) or when the main *ben* pathway is mutated (Deneff *et al.*, 2005; Deneff *et al.*, 2006). We propose that the *box* pathway is also an adaptive trait of ecological value when cells acquire metabolic modules that lead to the formation of benzoyl-CoA. We have shown this here with the *pht* cassette as a proof of concept, but the natural location of *pht* genes either in plasmids or in chromosomal regions flanked by transposases and inverted repeats (Ebenau-Jehle *et al.*, 2017; Boll *et al.*, 2020), might also facilitate their horizontal transfer and the spread of the PA degradation capability among recipient bacteria that harbour a functional *box* pathway.

The pht cassette as a biotechnological tool to valorize PA

As indicated above, PA is emerging as a feedstock generated in high amounts from the plastics additives. Phthalates account for about 60% of the world consumption of plasticizers that is predicted to be of 12.1 M tons by 2024 (Wei and Zimmermann, 2017; Brandon and Criddle, 2019; Boll *et al.*, 2020). Therefore, the use of PA as feedstock for the microbial synthesis of value-added biodegradable polymers, such as polyhydroxyalkanoates (PHAs), could be a promising approach in the up-coming plastic recycling industrial processes (Prieto, 2016; Wackett, 2019; *A European Strategy for Plastics in a Circular Economy*, https://ec.europa.eu/environment/waste/plastic_waste.htm). PHAs are an environmentally friendly alternative to chemically synthesized plastics due to the fact that they are bio-based, biodegradable, biocompatible, and their material properties are similar to those of petroleum-based plastics (Prieto, 2016; Mozejko-Ciesielska and Kiewisz, 2016; Chen and Jiang, 2018). A common type of PHAs is polyhydroxybutyrate (PHB), which contains only the 3-hydroxybutyrate monomer in its composition (Rehm, 2003). Here we aimed to show the use of the recombinant *pht* cassette as a biotechnological tool for the bioconversion of PA to PHB.

C. necator H16 is an industrially relevant microorganism generally used for its high production of PHB that can account up to 80% of the total cell dry weight (Anderson and Dawes, 1990; Linko *et al.*, 1993). PHB production from acetyl-CoA involves the *phaAB* and *phaC* gene products (Fig. 7A) (Pohlmann *et al.*, 2006; Brigham *et al.*, 2012). Although the H16 strain has been shown to accumulate PHB granules when grown on a wide range of substrates, including certain aromatic compounds such as 4-hydroxybenzoate, 3-hydroxybenzoate, protocatechuate, gentisate, (Tomizawa *et al.*, 2014; Kumar *et al.*, 2019), the strain has never been tested for bioconversion of phthalates. Since we have shown above that the *pht* pathway is functional in strain H16, we tested whether *C. necator* H16 (pIZPHTRAP) cells grown aerobically on PA were able to accumulate PHB. Interestingly, PHB granules were observed inside the recombinant H16 cells (Fig. 7B). PHB monomer composition and cellular PHB content of lyophilized cells were determined by gas chromatography-tandem mass spectrometry (GC-MS) of the methanolysed polyester. Cells were able to accumulate PHB that accounted for 8%–10% of the total cell dry weight. A similar percentage of PHB (14%) was accumulated when *C. necator* H16 (pIZPHTRAP) cells were grown on benzoate as sole carbon source. The composition of PHB was determined by GC-MS, and a single peak corresponding to the 3-hydroxybutyrate monomer was observed (Supporting Information Fig. S4). Thus, all

these results taken together reveal that *C. necator* H16 (pIZPHTRAP) strain constitutes a biotechnologically relevant biocatalyst to valorize PA towards the synthesis of PHB. Moreover, our work highlights that the aerobic hybrid box pathway becomes a suitable alternative to the classical aerobic degradation pathways (ben-cat pathways) for channelling benzoate and benzoate-generating aromatic compounds to the production of PHAs (Fig. 7A).

On the other hand, we checked whether the oxygen-independent *pht* cassette allowed also PA valorization to PHB under anaerobic conditions. Since *Azoarcus* sp. CIB has been shown to accumulate PHB when degrading aromatic hydrocarbons through the anaerobic benzoyl-CoA pathway (*bzd-pim-gcd* genes) (Zamarro *et al.*, 2017), we used this strain as host for testing anaerobic valorization of PA. *Azoarcus* sp. CIB (pIZPHTRAP) strain growing anaerobically on PA was shown to produce PHB, although with a 5-fold lower efficiency than that of the recombinant *C. necator* H16 strain, suggesting that these cells were able to re-route part of the 3-hydroxybutyryl-CoA generated in the anaerobic benzoyl-CoA pathway as substrate of the PhaC synthase that generates PHB (Fig. 7A).

Conclusions

We have demonstrated that PA degradation in denitrifying bacteria via the *pht* peripheral pathway requires

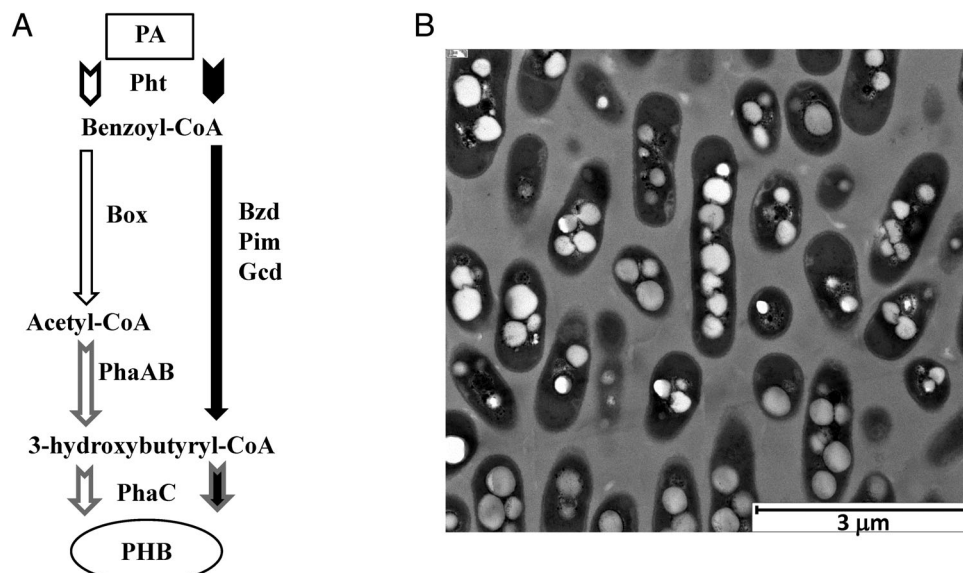


Fig 7. Funnelling PA toward the synthesis of PHB in biocatalysts expressing the *pht* cassette.

A. Scheme of the metabolic pathways involved in the aerobic (white arrows) and anaerobic (black arrows) conversion of PA to PHB. Abbreviations: Pht, Pht peripheral pathway; Box, hybrid aerobic pathway; Bzd, anaerobic benzoyl-CoA central degradation pathway; Pim-Gcd, benzoyl-CoA lower pathway; PhaAB, enzymes involved in the conversion of acetyl-CoA into the monomeric precursor of PHB, i.e., 3-hydroxybutyryl-CoA. PhaC, PHB synthase.

B. Representative transmission electron microscopy (TEM) view of *C. necator* H16 (pIZPHTRAP) cells grown aerobically on 3 mM PA. PHB granules can be observed as white spheres inside the cells.

an SBP-dependent secondary transporter (PhtTaTb) for the uptake of PA inside the bacterial cell. The presence of *phtTaTb* genes associated to putative PA degradation clusters in bacteria belonging to different phylogenetic groups reinforces the physiological role and ecological significance for bioremediation of this transporter that belongs to the poorly characterized class of TAXI-TRAP transporters. The *pht* pathway has been successfully transferred to heterologous hosts unable to degrade PA. Despite *pht* was initially characterized as an anaerobic pathway for the conversion of PA to benzoyl-CoA (Boll *et al.*, 2020), our results expand its function as an alternative peripheral route for the aerobic degradation of PA when coupled to a functional box central pathway. It is worth mentioning that during the evaluation of this work a similar conclusion has been achieved by Ebenau-Jehle *et al.* (2020) using a biochemical approach, thus reinforcing our results. Hence, the *pht* pathway may constitute an evolutionary acquisition for PA degradation by bacteria that thrive either in anoxic environments, or in environments that face oxygen limitations and that rely on benzoyl-CoA, rather than on catecholic central intermediates, for the aerobic catabolism of aromatic compounds. The aerobic and anaerobic valorization of PA towards the biosynthesis of bioplastics, e.g., PHB, in suitable recombinant biocatalysts expressing a *pht* cassette was demonstrated as a proof of concept. Nevertheless, further optimization of the bioconversion efficiencies is needed for industrial applications.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* and *Pseudomonas putida* cells were grown at 37°C or 30°C, respectively, on lysogeny broth (LB) medium (Miller, 1972). *Azoarcus* and *Aromatoleum* strains were grown anaerobically at 30°C on MC minimal medium (López-Barragán *et al.*, 2004) using the indicated carbon source(s) and 10 mM nitrate as the terminal electron acceptor. *Azoarcus*, *Aromatoleum* and *Cupriavidus* strains were grown aerobically at 30°C on NB medium (Difco, 234,000) or on MC minimal medium supplemented with the indicated carbon source(s) but without nitrate. When needed, gentamicin (7.5 µg ml⁻¹) and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (to induce heterologous *pht* expression) were added.

Molecular biology techniques

Standard molecular biology techniques were performed as previously described (Sambrook and Russell, 2001). Plasmid DNA was prepared with a High Pure plasmid isolation

kit (Roche Applied Science). DNA fragments were purified with Gene-Clean Turbo (Q-BIOgene). Gibson Assembly was performed following the published methods (Gibson *et al.*, 2009). All cloned inserts and DNA fragments were confirmed by DNA sequencing through an ABI Prism 377 automated DNA sequencer (Applied Biosystems Inc.). Oligonucleotides employed are listed in Supporting Information Table S1. Transformation of *E. coli* cells was carried out by using the RbCl method or by electroporation (Gene Pulser; Bio-Rad) (Sambrook and Russell, 2001). Plasmids were transferred from *E. coli* S17-1λpir (donor strain) into *Azoarcus*, *Cupriavidus* and *P. putida* recipient strains by biparental filter mating as described previously (López-Barragán *et al.*, 2004). The protein concentration in cell extracts was determined by the method of Bradford (Bradford, 1976) by using bovine serum albumin as the standard.

Construction of the *pht* cassette and derivatives

The pIZPHT plasmid was constructed by amplifying the region 66,351–71,015 bp of plasmid 2 from *A. aromaticum* EbN1 with the EbN1 113 FW/ EbN1 102 RV primers (Supporting Information Table S1). The DNA fragment was further digested with *HindIII/SpeI* and ligated to the pIZ1016 vector (*HindIII/SpeI* digested) by T4 ligase treatment. The pIZPHTRAP plasmid was constructed by amplifying the region 61,439–65,196 bp of plasmid 2 from *A. aromaticum* EbN1 with the 97 FW Gibson/ 95 Rv Gibson primers (Supporting Information Table S1). The DNA fragment was introduced into pIZPHT (*SpeI* digested) by Gibson Assembly protocol (Gibson *et al.*, 2009). The pIZPHTRAPΔ*phtDa* plasmid was constructed from *SnaBI/SphI* double-digested pIZPHTRAP plasmid (which releases a 591-bp internal fragment of *phtDa* gene) which was blunt-ended with Klenow enzyme and then circularized after T4 ligase treatment.

Sequence data analyses

A homology search of *phtTaTb*-like genes in finished and unfinished microbial genome databases was performed with the TBLASTN algorithm (Altschul *et al.*, 1990) at the NCBI server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignment of amino acid sequences from solute binding-protein (SBP) of different primary (ABC-type) and secondary (TRAP-, TTT-, TAXI-TRAP-type) transporters was performed using Clustal Omega alignment tool with default parameters (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Phylogenetic analysis was performed using the neighbour-joining statistical method based on the Poisson correction model, and a tree was reconstructed using the neighbour-joining method at the EBI server (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

The prediction of transmembrane segments in the PhtTa protein was performed by using the TMHMM 2.0 program at the DTU Health Tech server from Technical University of Denmark (<http://www.cbs.dtu.dk/services/TMHMM/>).

PA uptake assays

A. Evansii cells were grown on MC medium with 3 mM benzoate or 3 mM PA as sole carbon source for 24 h. *Azoarcus* sp. CIB cells containing plasmids pIZPHT or pIZPHTRAP (Table 1) were grown on NB medium with 1.0 mM IPTG, to induce the expression of the cloned *pht* genes, for 16 h until reaching the stationary phase (A_{600} of 1.8). Cells were harvested by centrifugation, washed twice and suspended into saline solution to an A_{600} of 4.0. The standard PA uptake assay contained 50 μ l of cells pre-warmed at 30°C for 5 min, and 5 μ l of 250 μ M [carboxyl- 14 C]-PA (50 μ Ci mmol^{-1} ARC 1057; American Radiolabeled Chemicals). Samples were incubated with shaking for 5 min at 30°C, and the assay was stopped by adding 1 ml of ice-cold saline solution and by filtrating the cells through a 0.22 μ m pore-size GSWP nitrocellulose filter (Millipore). Concentrated cells were washed with 5 ml of ice-cold saline solution, the [14 C]-PA accumulated inside the cells was determined by scintillation counting in an LKB Wallac liquid scintillation counter as previously described (Prieto and García, 1997). Where indicated, different amounts of [14 C]-PA or incubation times were used. Uptake rates were calculated relative to the amount of cells (biomass was determined by total protein in crude cell extracts) used in the transport assays.

RNA extraction and RT-PCR assays

C. necator H16 and *Azoarcus* sp. CIB cells harbouring plasmid pIZPHTRAP were grown aerobically on PA-, benzoate- or pyruvate-containing MC medium until the culture reached the end of the exponential phase. Cells were centrifuged, lysed for 10 min at 37°C in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 50 mg ml^{-1} lysozyme. Total RNA was extracted using a High Pure RNA isolation kit (Roche), and DNase-treated with a Turbo DNase kit (Ambion). The concentration and purity of the RNA samples were assessed using the Nanophotometer Pearl (Implen), according to the manufacturer's protocol, and by testing the absence of DNA (*recA* gene) through PCR amplification.

The synthesis of total cDNA was performed by using the Transcriptor First Strand cDNA Synthesis kit (Roche) in 20- μ l reactions containing 1 μ g of RNA, 1 mM concentration of each dNTP, 10 units of reverse transcriptase, 20 units of Protector RNase Inhibitor, and 60 μ M random hexamers as primers in the buffer provided by the manufacturer. The RNA and hexamers were initially heated at

65°C for 10 min and following the addition of the rest of the components, samples were incubated at 25°C for 10 min and then at 55°C for 30 min. Reactions were terminated by incubation at 85°C for 5 min. In standard RT-PCR reactions, the cDNA was amplified with 1 unit of AmpliTaq DNA polymerase (Biotoools) and 0.5 μ M concentrations of the corresponding primer pairs of the *boxB* gene from *Azoarcus* sp. CIB (Supporting Information Table S1). Control reactions in which reverse transcriptase was omitted from the reaction mixture ensured that DNA products resulted from the amplification of cDNA rather than from DNA contamination. Real-time PCR assays were performed as described previously (Martín-Moldes *et al.*, 2016), in a LightCycler®480 II real-time PCR system (Roche). The analysis was performed in three technical replicates from three biological samples. Reactions (20 μ l) contained 1 μ l (2.5 ng) of cDNA, 0.25 μ M of each of the two target-specific primers, and 10 μ l of SYBR Green I Master Mix (Roche). Oligonucleotides used to amplify transcripts from the *benA*, *boxB*₁, *boxB*₂ and the *recA* gene of strain H16, the last one used as housekeeping (internal standard), are listed in Supporting Information Table S1. PCR amplifications were carried out with one denaturation cycle (95°C for 5 min), followed by 30 cycles of amplification (95°C for 10 s, 60°C for 10 s, and 72°C for 10 s). After amplification, 7 melting curves were generated to confirm amplification of a single product. For relative quantification of the fluorescence values, a calibration curve was constructed by sevenfold serial dilutions of a *C. necator* H16 genomic DNA sample ranging from 1 ng to 0.5 $\times 10^{-7}$ ng. This curve was then used as a reference standard for extrapolating the relative abundance of the cDNA target within the linear range of the curve.

Transmission electron microscopy studies

Bacterial cells were harvested, washed twice in saline solution and fixed in 5% (w/v) glutaraldehyde. Afterwards, cells were suspended in 2.5% (w/v) OsO₄ for 1 h, gradually dehydrated in ethanol solutions [30%, 50%, 70%, 90% and 100% (v/v); 30 min each] and propylene oxide (1 h), and embedded in Epon 812 resin. Ultrathin sections were cut with a microtome using a Diatome diamond knife. The sections were picked up with 400 mesh copper grids coated with a layer of carbon, and observed using a Jeol-1230 transmission electron microscope.

Analysis of PHB content and monomer composition

To determine the PHB content of *Azoarcus* sp. CIB and *C. necator* H16 strains harbouring plasmid pIZPHTRAP, cells were grown at 30°C on MC medium with 3 mM benzoate or 3 mM PA under anaerobic or aerobic conditions,

respectively, until they reached the stationary phase. PHB production was quantified by GC–MS of the methanolysed polyester and shown as the percentage of PHB monomer with respect to the total cell dry weight (CDW). Methanolysis procedure was carried out by suspending 5–10 mg of lyophilized cells in 2 ml of methanol containing 3% sulfuric acid and 2 ml of a solution of 0.5 mg ml⁻¹ of 3-methylbenzoic acid (internal standard) in chloroform, and then incubated at 100°C for 5 h in a sealed tube under stirring. After cooling, 1 ml of demineralized water was added, the mixture was vigorously stirred, and two phases were obtained (this step was repeated twice). The organic phase containing the resulting methyl esters of monomers was analysed by GC–MS (de Eugenio *et al.*, 2010). An Agilent series 7890A gas chromatograph coupled with 5975C MS detector (EI, 70 eV) and a split/splitless injector were used for analysis. An aliquot (1 µl) of the organic phase was injected into the gas chromatograph at a split ratio 1:20. Separation of compounds was achieved using a DB-5ht (–60°C–400°C: 30 m × 0.25 mm × 0.1 µm film thickness) column. Helium was used as carrier gas at a flow rate of 1 ml min⁻¹. The injector and transfer line temperature were set at 275°C and 280°C, respectively. The oven temperature program was initial temperature 60°C for 2 min, then from 60°C up to 65°C at a rate of 5°C min⁻¹, and then from 65°C up to 125°C at a rate of 10°C min⁻¹. EI mass spectra were recorded in full scan mode (m/z 40–550).

Consumption of PA in bacterial cultures

The presence of residual PA was quantified by HPLC (Agilent Series 1260 Infinity II, Agilent, CA, USA) using a Mediterranean™ sea18 (TEKNOKROMA) (15 cm × 0.46 cm, 5 µm particle size) column, at room temperature with a flow rate of 1 ml min⁻¹ and an injection volume of 25 µl. The mobile phase was 0.1% trifluoroacetic acid both in water (A) and acetonitrile (B). The elution program was used as follows: at the start, 100% A; after 5 min, the percentage of B was linearly increased to 100% in 20 min. After that, it was ramped to the original composition (100% A) in 2 min, and then equilibrated for 5 min. The retention time of PA was 13.1 min. A standard curve of PA was done (1–5 mM). To demonstrate that the medium does not contain any interfering substance in the chromatogram, the medium without PA was injected as control.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Oligonucleotides used in this work.

Fig. S1. Primary structure of the PhtTa protein showing the predicted hydrophobic transmembrane segments.

A. Transmembrane segments (TM) are indicated with red bars (TM1 to TM10) corresponding to a DctM-like large subunit, blue bars (TM12 to TM15) corresponding to a DctQ-like small subunit, and a green bar (TM11) corresponding to a connecting segment of the DctM-like and DctQ-like domains.

B. Probability of each amino acid of the PhtTa protein to be oriented towards the cytoplasm (blue line), membrane (red line) or periplasm (pink line), according to the prediction based on the use of the TMHMM 2.0 program at the DTU Health Tech server from Technical University of Denmark (<http://www.cbs.dtu.dk/services/TMHMM/>).

Fig. S2. Expression of the *boxB* gene in aerobically-grown *Azoarcus* sp. CIB (pIZPHTRAP) cells. Cells were grown under oxic conditions on MC minimal medium with 3 mM benzoate (lane 1), 0.2% pyruvate (lane 2) or 3 mM PA (lane 3) as sole carbon source. Total RNA was isolated from cells grown until the end of the exponential phase, and the expression of the *boxB* gene was monitored by RT-PCR. Agarose gel electrophoresis of RT-PCR products is shown. Lane M, molecular size markers (Quick-Load™ 100 bp DNA Ladder from New England Biolabs).

Fig. S3. Growth curve of *A. Evansii* cultivated aerobically on MC minimal medium containing 3 mM PA as sole carbon source. Bacterial growth was monitored by measuring A_{600} . Values are the mean of three different experiments. Error bars indicate standard deviations.

Fig. S4. Characterization of the composition of the PHB produced by *C. necator* H16 (pIZPHTRAP) cells. Cells were grown aerobically on MC minimal medium with 3 mM benzoate (A) or 3 mM PA (B) as sole carbon source. PHB was isolated, purified and hydrolyzed as detailed in Experimental Procedures. The resulting methyl esters of monomers were analyzed by GC–MS. The GC chromatograms showing the peaks that correspond to methyl 3-hydroxybutyrate are shown. The insets show the corresponding mass spectra of the peaks. C. Mass spectrum of standard methyl 3-hydroxybutyrate (NIST Mass Spectrometry Data Center).