Benzoyl-CoA pathway-mediated metabolism of meta-hydroxy-aromatic acids in *Rhodopseudomonas palustris*

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ABSTRACT

Photoheterotrophic metabolism of two meta-hydroxy-aromatic acids, meta-, para-dihydroxybenzoate (protocatechuate) and meta-hydroxybenzoate, was investigated in *Rhodopseudomonas palustris*. When protocatechuate was the sole organic carbon source, photoheterotrophic growth in *R. palustris* was slow relative to cells using compounds known to be metabolized by the benzoyl-CoA pathway. *R. palustris* was unable to grow when meta-hydroxybenzoate was provided as a sole source of organic carbon under photoheterotrophic growth conditions. However, in cultures supplemented with known benzoyl-CoA pathway inducers (para-hydroxybenzoate, benzoate, or cyclohexanoate), protocatechuate and meta-hydroxybenzoate were taken up from the culture medium. Further, protocatechuate and meta-hydroxybenzoate were each removed from cultures containing both meta-hydroxy-aromatic acids at equimolar concentrations in the absence of other organic compounds. Analysis of changes in culture optical density and in the concentration of soluble organic compounds indicated that the loss of these meta-hydroxy-aromatic acids was accompanied by biomass production. Additional experiments with defined mutants demonstrated that enzymes known to participate in dehydroxylation of para-hydroxybenzoyl-CoA (HbaBCD) and reductive dearomatization of benzoyl-CoA (BadDEFG) were required for metabolism of protocatechuate and meta-hydroxybenzoate. These findings indicate that, under photoheterotrophic growth conditions, *R. palustris* can degrade meta-hydroxy-aromatic acids via the benzoyl-CoA pathway, apparently due to the promiscuity of the enzymes involved.
INTRODUCTION

A combination of human and plant activities give rise to a variety of aromatic compounds in the environment. Among these compounds are aromatic carboxylic acids that contain hydroxyl groups disposed meta, ortho, or para (m, o, or p) to the carboxyl. Aromatic acids such as benzoate, para-hydroxybenzoate (pHB), meta-hydroxybenzoate (mHB) and meta,para-dihydroxybenzoate (protocatechuic) can serve as carbon sources for some bacteria. In aerobic pathways, molecular oxygen (O2) is used as an electrophilic co-substrate for α-electron destabilization and aromatic ring fission (1). In contrast, strict and facultative anaerobes use O2-independent mechanisms for aromatic ring cleavage (2). In these O2-independent pathways, benzoyl-Coenzyme-A (benzoyl-CoA) thioesters are formed and reductively transformed into essential precursors of central metabolism (3, 4). This work sought to gain additional insight into anaerobic metabolism of hydroxylated aromatic compounds by the facultative photoheterotroph Rhodopseudomonas palustris.

Anaerobic metabolism of aromatic compounds has been most extensively studied in R. palustris and the denitrifying bacterium Thauera aromatica (5). In R. palustris and T. aromatica, catabolism of pHB (Figure 1A) proceeds via benzoyl-CoA, an intermediate that is also used for metabolism of benzoate (6-8). In both organisms, specific CoA-dependent ligases catalyze the ATP-dependent synthesis of pHB-CoA (HbaA, EC 6.2.1.27) (9-11) and benzoyl-CoA (BadA, EC 6.2.1.25) (12-14). The pHB-CoA thioester is subsequently dehydroxylated by pHB-CoA reductase (HbaBCD, EC 1.3.7.9) to produce benzoyl-CoA (15-17). Benzoyl-CoA dearomatization is catalyzed by benzoyl-CoA reductase (BadDEFG, EC 1.3.7.8) forming aliphatic intermediates [1,5-diene-cyclohexanoyl-CoA in T. aromatica (18, 19).
and 1-ene-cyclohexanoyl-CoA in *R. palustris* (20)] that serve as carbon sources and reducing power in catabolic pathways that ultimately yield acetyl-CoA, CO₂, and NADH (21-24).

Despite the striking similarities between the metabolic routes through which pHB and benzoate are catabolized in *T. aromatica* and *R. palustris*, there are significant differences in the ways these two organisms metabolize *meta*-hydroxy-aromatic acids. *R. palustris* is not reported to grow photoheterotrophically with mHB as a sole organic carbon source whereas protocatechuate serves only as a poor organic carbon source relative to the known benzoyl-CoA pathway-inducing substrates, benzoate, pHB, and cyclohexanoate (25-28). In contrast, *T. aromatica* is known to activate both protocatechuate and mHB via enzymatic activity with mHB-CoA ligase (EC 6.2.1.37) to form the cognate benzylic thioester (29, 30). In *T. aromatica*, metabolism of *meta*-hydroxy-aromatic acids proceeds through intermediate mHB-CoA (not benzoyl-CoA). Derived as a product of benzoyl-CoA reductase activity on mHB-CoA, the aliphatic thioester 1,5-diene-3-hydroxycyclohexanoyl-CoA (Figure 1B) is further transformed to ultimately yield metabolic intermediates and reducing equivalents necessary for growth (21, 29, 30).

In this work, we analyzed the ability of *R. palustris* to grow on *meta*-hydroxy-aromatic acids. Our results provide evidence for benzoyl-CoA pathway-mediated metabolism of *meta*-hydroxy-aromatic acids when cells are grown under conditions known to induce expression of benzoyl-CoA pathway enzymes. We provide genetic evidence for the requirement of the benzoyl-CoA pathway for the anaerobic metabolism of *meta*-hydroxy-aromatic acids. Based on this, we present a model to explain why metabolism of *meta*-hydroxy-aromatic acids requires or is enhanced by conditions that induce expression of the benzoyl-CoA pathway enzymes.
MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are described in Table 1. *R. palustris* strain CGA009 and its derivatives were grown in pre-sterilized photosynthetic media (PM) supplemented with carbon sources at selected concentrations (25). As needed, 100 μg mL⁻¹ kanamycin was added to media for maintenance of *R. palustris* mutant strains. Photoheterotrophic cultures were grown at 30°C and illuminated with incandescent light bulbs at ~10 W m⁻². *R. palustris* growth was monitored using a Klett-Summerson photoelectric colorimeter (Klett MFG Co., NY).

For aromatic acid- and cyclohexanoate-supported growth experiments, the initial media pH was set to 7.0, except when protocatechuate was used as an organic carbon source where the initial pH was adjusted to pH 6.5 using phosphoric acid to prevent photochemical degradation of protocatechuate. At the time of inoculation, culture media was sparged with argon gas to ensure anaerobic conditions at the onset of each experiment. Replicate (two to four) 21-mL *R. palustris* cultures were inoculated with ~250 μL of succinate-grown cells and supplemented with 30 mM NaHCO₃ as well as 4.4 to 4.6 mM total aromatic acid, unless specified otherwise. Final aromatic substrate concentrations were chosen to maintain equal total reducing equivalents for all cultures tested at one gram of chemical oxygen demand (COD) per liter of media.

**Analytical methods.** Samples were aseptically removed from cultures by removing 200-μL aliquots while adding argon gas to maintain headspace atmospheric pressure. Samples were filtered through 0.22-μm hydrophilic durapore PVDF membranes (Merck KGaA, Darmstadt,
Germany) and acidified with phosphoric acid (0.5% v/v) before analyzing by gas chromatography (GC) or high-pressure liquid chromatography (HPLC).

**GC analyses.** Acetate, butyrate, and cyclohexanoate concentrations were quantified using a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. Compounds were separated using a Stabilwax®-DA 0.32-mm I.D. × 30-m capillary free fatty acid phase column (Agilent Technologies, Wilmington, DE) using helium as the mobile phase. Ramping of the column temperature began 2 min after the time of injection, increasing from 60°C at a rate of 12°C min⁻¹ and held at 240°C for 2 min. The injector and detector temperatures were 250°C.

**HPLC analyses.** Aromatic acid concentrations were quantified using an LC-10ATVP solvent delivery module HPLC system (Shimadzu, Kyoto, Japan) equipped with an SPD-M10AVP diode array detector (Shimadzu, Kyoto, Japan). Aromatic compounds were separated using an Ultra Aqueous (Restek Corporation, Bellefonte, PA) C₁₈-reversed stationary phase column (5 μm particle size; 120 mm × 4 mm I.D.) and an isocratic aqueous mobile phase of methanol (30% w/v), acetonitrile (6% w/v), and 5mM formic acid in water (64% w/v) at a flow rate of 0.8 mL min⁻¹ (31). Aromatic acids were detected by UV absorption at 280 nm. Concentrations of aromatic compounds were calculated from linear regressions created from standards of known concentration.

**COD analyses.** Culture supernatants were filtered through 0.22-μm membranes prior to measuring the concentration of soluble organic compounds using a HACH High Range (0-1,500 mg per liter) COD kit (HACH Company, Loveland, CO). COD is a standard test specific for organic substrates (32) and measures the amount of oxygen required to fully oxidize and
organic substrate to CO₂. As such, it has been used to understand the fate of reducing equivalents in photoheterotrophic cultures (33). The theoretical COD of various carbon sources used in this study are as follows (mg COD/m mole substrate): acetate, 64; benzoate, 240; butyrate, 160; caffeate, 288; cyclohexanoate, 288; mHB, 224; pHB, 224; protocatechuate, 208; bicarbonate, 0; succinate, 112.

RESULTS

Conditions to induce meta-hydroxy-aromatic acid metabolism in wild type R. palustris.

The goal of this study was to identify culture conditions that allowed anaerobic metabolism of meta-hydroxy-aromatic compounds by wild type R. palustris strain CGA009. Consistent with previous findings (16, 26), photoheterotrophic cultures of strain CGA009 incubated with either mHB or protocatechuate as a sole organic carbon source were incapable of doubling at rates similar to cells grown with benzoate, pHB, or cyclohexanoate (0.4-day, 0.6-day, and 0.4-day doubling times, respectively) (Table 2). The presence of mHB (2.2 mM) as a sole organic carbon source did not support detectable growth of wild type cultures even after an extended incubation period under photoheterotrophic conditions. However, protocatechuate (2.3 mM) supported very slow photoheterotrophic growth of R. palustris. For all experiments during which growth was observed, measurements of media COD indicated greater than 82% removal of the organic substrates as biomass increased, demonstrating that growth was associated with the degradation of the substrates, even in the protocatechuate-fed cultures. By-products from the degradation of the different substrates were not detected by either GC or HPLC analyses. However, a small accumulation of undetected by-products in these growth conditions cannot be ruled out, given the incomplete removal of COD from the culture medium.
We also found that strain CGA009 was able to metabolize \( m \)HB or protocatechuate when either benzoate, \( p \)HB, or cyclohexanoate (for \( m \)HB only) were also supplied as an additional co-substrate (Table 3). Under these conditions, cultures exhibited a biphasic mode of growth, where benzoate, \( p \)HB, or cyclohexanoate were degraded first (defined here as primary substrates) with apparent doubling times similar to those observed when these substrates were sole growth substrates (Table 2). After the primary substrate was consumed, meta-hydroxy-aromatic acid co-substrates, either \( m \)HB or protocatechuate (defined here as secondary substrates), were subsequently removed from the media and the cultures exhibited longer doubling times (average 1.6 days and 19 days, respectively). Notably, in comparison to when benzoate or \( p \)HB was the sole organic carbon source (Table 2), growth with benzoate and \( p \)HB as primary substrates was slower when protocatechuate was the secondary substrate (Table 3), perhaps as an effect of competition between protocatechuate and the primary substrate for required enzymes. Although it was not an enhancer of protocatechuate metabolism, cyclohexanoate was utilized as a primary substrate when \( m \)HB was degraded as the secondary substrate, but growth during \( m \)HB uptake was slower (8.7-day doubling time). The inability of strain CGA009 to degrade protocatechuate as the secondary substrate when cyclohexanoate was the co-substrate may be due to the additional enzymes (e.g., HbaBCD) that are expected to be needed for protocatechuate degradation when compared to \( m \)HB metabolism (Figure 1B). In these two-substrate experiments, the primary and secondary substrates were added in equimolar concentrations and each corresponded to about 50% of the initial COD in the media, so we found approximately equal increases in biomass during growth on the primary and secondary substrate (between 100 and 120 Klett units per substrate). The COD removal efficiency when \( m \)HB was the secondary substrate was between
75.0% and 81.8% (Table 3), demonstrating that mHB was utilized for growth. When protocatechuate was the secondary substrate COD removal efficiencies were 86.7% and 76.9% when biphasic growth was observed, also demonstrating that protocatechuate was utilized as a carbon source for growth. In contrast, when cyclohexanoate was used as the primary substrate and neither biphasic growth nor protocatechuate degradation was subsequently observed, the COD removal efficiency was only 47.9%, in agreement with protocatechuate not being used for growth under this condition. Similar to the experiments with single substrates, by-products of these transformations were not detected but small accumulations of metabolites cannot be ruled out since the COD removal was incomplete in these cultures.

To further investigate the utilization of mHB and protocatechuate, we performed another set of growth experiments with a molar excess of the secondary substrates. For example, when strain CGA009 was incubated in media with an approximately 5-to-1 molar excess of mHB (3.7 mM) to pHB (0.7 mM), pHB was depleted first, followed by mHB (Figure 2A). Under these conditions, the contribution of pHB to the total COD in the media was 16.6% whereas the measured COD removal in the experiment was 71.5%. This confirms that mHB is being metabolized by the culture. Furthermore, there is an approximately 4-fold increase in optical density as the mHB is degraded compared to that observed when pHB was degraded (178 Klett units compared to 50 Klett units), consistent with the excess mHB providing most of the organic carbon for growth. Similarly, in experiments where protocatechuate (3.9 mM) was present at a 5-to-1 molar ratio over benzoate (0.8 mM) in the media (Figure 2B), benzoate degradation and concomitant growth preceded subsequent consumption of protocatechuate and slower cell growth, with an ~4-fold increase in optical density during the period of
protocatechuate degradation. In this case, media COD concentrations were reduced by an average of 66.5%, whereas benzoate represented only 18.7% of the initial COD. Therefore, the results of these experiments also confirmed that protocatechuate was used as a source of carbon and reducing equivalents to support cell growth in these cultures, without excluding the possibility of some undetected metabolites accumulated in the media.

Surprisingly, we found that strain CGA009 also grew when provided with mHB and protocatechuate as co-substrates at equimolar concentrations, with cells utilizing mHB as the primary substrate at short doubling times and protocatechuate as the slowly-degraded secondary substrate (Figure 3A) supporting growth at relatively longer doubling times (Table 3). A COD removal efficiency of 67.8% and a nearly two fold increase in optical density after mHB had been depleted (Figure 3A) confirmed the utilization of both substrates for growth, although the accumulation of metabolites cannot be ruled out given the low COD removal efficiency.

We also found that neither mHB nor protocatechuate (2.2 mM) were removed from cultures of wild-type cells when either acetate (7.8 mM) or butyrate (3.1 mM) was added as a co-substrate (data not shown). This indicates that catabolism of the meta-hydroxy-aromatic acids was not supported by other organic carbon sources and suggests that meta-hydroxy-aromatic acid metabolism requires the presence of one or more benzoyl-CoA pathway enzymes, expression of which are induced by the presence of benzoate, pHB, or cyclohexanoate (25, 34-36).

Consistent with previous findings (26), in cultures where caffeate (an analog of protocatechuate with a 3-carbon aliphatic side-chain) was the sole organic carbon source (2.2 mM), this compound was taken up from the medium concomitantly with an increase in the medium’s protocatechuate concentration. In this case, the protocatechuate concentration rose...
to ~2.2 mM (the same as the initial caffeate concentration; data not shown), suggesting that the aliphatic side-chain of caffeate was being used to support growth without significant metabolism of the meta-hydroxy-aromatic acid moiety. Combined, these results suggest that utilization of the meta-hydroxy-aromatic compounds requires the activity of benzoyl-CoA pathway enzymes.

Role of benzoyl-CoA pathway enzymes in meta-hydroxy-aromatic acid metabolism. There are reported differences in the pathways used for the anaerobic metabolism of meta-hydroxy-aromatic acids by *R. palustris* and *T. aromatica*. Thus, we tested if previously characterized genes that encode benzoyl-CoA pathway enzymes are needed for the observed metabolism of meta-hydroxy-aromatic acids in *R. palustris*. It was previously reported that *R. palustris* cells lacking pHB-CoA reductase (HbaBCD) activity (CGA506; Table 1) grow at wild-type rates while using benzoate but exhibit a growth defect when pHB is the organic carbon source under photoheterotrophic conditions (16). In media containing equimolar benzoate and protocatechuate (2.2 mM), strain CGA506 grew with wild type photoheterotrophic generation times while benzoate was present in the media (Figure 4B, Table 3). However, once benzoate was consumed, growth of strain CGA506 was impaired and protocatechuate in the media decayed slowly (Figure 4B). Further, compared to wild-type cultures incubated under identical conditions (Figure 4A), the COD removal efficiency was lower in the CGA506 culture at the end of the experiment (Table 3). This, plus the lower overall cell yield of CGA506 under these conditions support the hypothesis that pHB-CoA reductase activity is required for protocatechuate metabolism in *R. palustris*. In further support of this notion, we found that when strain CGA506 is incubated with equimolar concentrations of mHB and protocatechuate (2.3 mM), mHB supported growth with wild-type generation times but protocatechuate
metabolism was inhibited (Figure 3B) compared to the wild-type strain (Figure 3A, Table 3). Taken together, our findings suggest that the protocatechuate thioester (protocatechuyl-CoA), or its potential m-dehydroxylated intermediate (pHB-CoA), is subject to p-dehydroxylation by pHB-CoA reductase (Figure 1), and that this activity is required for protocatechuate-supported growth by *R. palustris*. In addition, pHB-CoA reductase is not required for degradation of mHB.

To test the role of benzoyl-CoA reductase (BadDEFG) in the metabolism of meta-hydroxymethoxyaromatic acids, we used a mutant (CGA606) lacking benzoyl-CoA reductase activity (7). In this case, we analyzed growth of strain CGA606 on cyclohexanoate and mHB since cyclohexanoate enters the benzoyl-CoA pathway downstream of benzoyl-CoA reductase activity (Figure 1A). When incubated with equimolar concentrations of cyclohexanoate and mHB (2.0 mM), strain CGA606 grew with wild type doubling times as long as cyclohexanoate was present in the medium (Figure 5B; Table 3). However, growth ceased after cyclohexanoate was depleted from the medium and there was no net removal of mHB (Figure 5B), indicating that the loss of benzoyl-CoA reductase activity blocked the ability of cells to metabolize mHB. Further, the amount of COD removed by strain CGA606 was lower than in wild type cells under this growth condition (Table 3), consistent with the hypothesis that BadDEFG is required for mHB degradation. These data suggest that benzoyl-CoA reductase is likely facilitating the dearomatization of mHB-CoA or its potential m-dehydroxylated intermediate (benzoyl-CoA), once it is formed in wild-type cells (Figure 1).

We also found that strain CGA606 was capable of only slow growth (>60-day generation times) with protocatechuate as the sole carbon source (2.2 mM), similar to the observations that had been made when testing the wild-type strain (Table 2). This observation may be due to both strains making use of protocatechuate photochemical degradation products as growth
substrates, given our observation that protocatechuate degrades at approximately the same rate under light in abiotic tubes and in cultures where protocatechuate is the sole organic carbon source (data not shown). These data also leave open the possibility that a yet-to-be-characterized pathway for anaerobic aromatic metabolism that has been proposed in previous studies (11, 26) may exist in strain CGA009 and contribute to protocatechuate metabolism under these conditions. However, the potential contribution of this pathway is apparently minimal relative to that of the benzoyl-CoA pathway when cells are grown in the presence of substrates that induce activity of benzoyl-CoA pathway enzymes.

**DISCUSSION**

The ability to utilize aromatic compounds with one or more ring substitutions is integral to the biodegradation of natural or xenobiotic aromatic compounds. For example, lignin is a significant portion of plant lignocellulose and is a polymer of aromatic subunits containing an aliphatic side-chain, a *para*-substitution, and up to two substitutions in the *meta*-positions (relative to the aliphatic side-chain). In addition, there are many toxic products of human activity that contain functional group substitutions on the aromatic ring. Thus, there is considerable interest in identifying microbial pathways to either degrade ring-substituted aromatics or convert them into high-value products. The data in this paper predicts that HbaBCD and BadDEFG have previously unreported roles in the degradation of aromatic compounds containing *meta*-hydroxy-aromatic functional groups.

**Benzoyl-CoA pathway-inducing substrates enhance *meta*-hydroxy-aromatic acid catabolism in *R. palustris*.** Aromatic-supported photoheterotrophic growth by *R. palustris* was first described with benzoate as a sole organic carbon source (7, 12). It was subsequently
shown that *R. palustris* reductively transforms para-hydroxyl ring substitutions via the benzoyl-CoA pathway (Figure 1A) using much of the same enzymology employed during utilization of benzoate (10, 11, 16, 37), and that benzoate, *p*HB, and cyclohexanoate are inducers of this pathway (25-28). Here, we demonstrate that under conditions known to induce expression of benzoyl-CoA pathway genes (34), *R. palustris* also utilizes the meta-hydroxy-aromatic acids protocatechuate and *m*HB during photoheterotrophic growth.

A comparison of growth characteristics of *R. palustris* in single-substrate- and two-substrate-fed cultures reveals new aspects of aromatic acid metabolism by the benzoyl-CoA pathway. *R. palustris* strain CGA009 was unable to utilize *m*HB for growth either when it was the sole photoheterotrophic carbon source or when the media also contained rapidly-metabolized non-aromatic organic acids as co-substrates (butyrate or acetate). Protocatechuate was inefficiently utilized when supplied as a sole organic carbon source. However, degradation of both meta-hydroxy-aromatic compounds was observed in cultures that were either (a) supplemented with one of the three known inducers of benzoyl-CoA pathway gene expression: cyclohexanoate (for *m*HB only), benzoate (for *m*HB or protocatechuate), or *p*HB (for *m*HB or protocatechuate), or (b) fed both *m*HB and protocatechuate as co-substrates. The means by which (a) the three known benzoyl-CoA pathway inducers enhance metabolism of one or both meta-hydroxy-aromatic acids or (b) the combination of *m*HB and protocatechuate as co-substrates enhances the metabolism of each meta-hydroxy-aromatic acid remains to be determined as pathway expression is affected by both transcriptional (35-37) and post-translational mechanisms (38, 39).

**Benzoyl-CoA pathway enzymes mediate meta-hydroxy-aromatic acid metabolism in *R. palustris***. The benzoyl-CoA pathway enzymes in *T. aromatica* are known to be both
expressed and catalytically active with \( \text{meta} \)-hydroxy-aromatic substrates (6, 27, 29, 30).

However, it is not known whether \( T. \) \textit{aromatica} requires the activity of benzoyl-CoA pathway enzymes for \( \text{meta} \)-hydroxy-aromatic acid degradation or if other pathways contribute to metabolism in this organism. In this work, we show that photoheterotrophic metabolism of protocatechuate or \( m \text{HB} \) is only observed in \( R. \) \textit{palustris} under conditions that increase benzoyl-CoA pathway activity. Further, our data provides direct genetic evidence that metabolism of \( \text{meta} \)-hydroxy-aromatic compounds requires the activity of benzoyl-CoA pathway enzymes in \( R. \) \textit{palustris}.

For example, mutants lacking BadDEFG activity (7) are known to be incapable of utilizing benzoate as a sole organic carbon source under photoheterotrophic growth conditions. Whereas wild-type cells were capable of using \( m \text{HB} \) to support growth after cyclohexanone was removed from culture medium (Figure 5A), cells lacking benzoyl-CoA reductase activity (strain CGA606) were unable to grow using \( m \text{HB} \) under identical conditions (Figure 5B). This is direct evidence that BadDEFG activity is necessary for utilization of \( m \text{HB} \) as a growth substrate by \( R. \) \textit{palustris} under photoheterotrophic conditions. Similarly, the properties of the \( hbaB \) mutant (strain CGA506; Table 3) indicates that HbaBCD activity is needed for metabolism of protocatechuate (Figure 3; Figure 4). Collectively, our data predict a potentially new role for \( R. \) \textit{palustris} HbaBCD and BadDEFG enzyme activities in \( \text{para} \)-dehydroxylation and reductive dearomatization of \( \text{meta} \)-hydroxylated metabolic intermediates during photoheterotrophic growth with \( \text{meta} \)-hydroxy-aromatic acids as organic carbon sources. While HbaBCD and BadDEFG may function directly in \( \text{p} \)-dehydroxylation of protocatechuyl-CoA and deearomatization of \( m \text{HB-CoA} \), respectively (Figure 1B), the possibility remains that other uncharacterized enzymes function in reductive \( m \)-dehydroxylation, producing alternative
intermediates $p$HB-CoA and benzoyl-CoA as substrates for HbaBCD and BadDEFG (Figure 1A).

**Inducers of benzoyl-CoA pathway gene expression enhance metabolism of additional substrates.** Given the various conditions that enhanced meta-hydroxy-aromatic acid metabolism, we present a model for benzoyl-CoA pathway expression that enables *R. palustris* to utilize non-inducing compounds as growth substrates. In experiments in which one of the inducers of benzoyl-CoA pathway expression ($p$HB, benzoate, or cyclohexanoate) was used to enhance metabolism of one of the meta-hydroxy-aromatic acids ($m$HB or protocatechuate) the ability to metabolize the new substrate may be a result of differing substrate specificities of the proteins in the pathway. Whereas the known inducers are allosteric effectors of the benzoyl-CoA transcription factors (35, 36, 40) leading to the up-regulated expression of pathway genes, it is likely that the meta-hydroxy-aromatic acids either serve as very weak inducers, or do not induce any expression of the benzoyl-CoA pathway. However, we hypothesize that, as is the case in *T. aromatica*, CoA ligases, HbaBCD, and BadDEFG exhibit broad substrate specificities (28, 30) and, once expressed, facilitate catabolism of substrates that do not induce their expression and lead to growth in *R. palustris*. A slight variation of this model can help describe the results observed in cultures incubated with equimolar concentrations of protocatechuate and $m$HB as co-substrates (Figure 3). Although we did not expect CGA009 to grow under this condition, a review of previous findings provides some explanation of these results. Earlier observations (25) showed that, although protocatechuate (and its metabolic derivatives) does not induce expression of the entire benzoyl-CoA pathway needed for exponential growth, it does induce expression of CoA-ligase enzymes required for thioesterification of aromatic acids at the onset of the pathway (Figure 1). Thus, the CoA ligase
expressed in the presence of protocatechuate may have a sufficiently high affinity for mHB (versus protocatechuate) to allow formation of mHB-CoA – as a potential inducer for the expression of other benzoyl-CoA pathway genes (e.g., hbaBCD and badDEFG). Thereafter, the products mHB catabolism may induce expression of pathways needed for subsequent catabolism of protocatechuate. We hypothesize that each substrate (and its cognate intermediates) induces expression of only a subset of genes needed for growth, and those genes not induced by it or its intermediates are compensated by expression induced by the other meta-hydroxy-aromatic acid under this condition.

**Different factors control benzoyl-CoA pathway metabolism in** *R. palustris* **and** *T. aromatica*. Our results provide evidence of (a) similar roles played by benzoyl-CoA pathway enzymes in *T. aromatica* and *R. palustris* for the degradation of meta-hydroxy-aromatic acids, and (b) regulatory control of benzoyl-CoA pathway expression being restricted to a narrower set of allosteric effector molecules in *R. palustris* when compared to *T. aromatica*. It appears that, although broad substrate specificity may be characteristic of the metabolic enzymes in both *T. aromatica* and *R. palustris*, the latter organism is lacking regulatory elements needed for protocatechuate- or mHB-supported photoheterotrophic growth. One unique feature of anaerobic aromatic metabolism in *R. palustris* is that the transcription factors controlling expression of benzoyl-CoA pathway genes (35, 36) apparently respond to only a narrow set of allosteric effectors, but trigger up-regulation of benzoyl-CoA pathway enzymes, which exhibit catalytic activity toward a broader set of aromatic growth substrates. Here, we demonstrated that protocatechuate and mHB comprise a group of benzoyl-CoA substrates that are not able to support rapid growth unless other compounds metabolized by this pathway are present as co-substrates. A third aromatic acid, *meta*-chlorobenzoate, may also be a member of this
group as it has been reported to support photoheterotrophic growth of *R. palustris* in the presence of benzoate, but does not support growth as a sole organic carbon source (41, 42).

The findings presented in this paper also provide insight into how selective pressures in nature may influence evolution of systems for metabolism of chemically related compounds. For example, it is possible that the observed behavior of *R. palustris* reflects the fact that *meta*-hydroxy-aromatic acids are typically found in nature in environments where a known inducer of the benzoyl-CoA pathway is also present. If this were the case, the common presence of both classes of aromatic acids in nature has apparently not required *R. palustris* to evolve systems to induce benzoyl-CoA pathway expression in the presence of only *meta*-hydroxy-aromatic acids.

In sum, this study identified conditions that enable metabolism of *meta*-hydroxy-aromatic acids in *R. palustris*. We also provided genetic evidence that benzoyl-CoA pathway enzymes are needed for metabolism of protocatechuate and mHB in *R. palustris*. Our findings leave open the possibility of *R. palustris* metabolizing other aromatic compounds that are found in either plant lignin, the environment, or as a product of industrial activity in the presence of one or more benzoyl-CoA pathway inducers.

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**FIGURE LEGENDS**

Figure 1 - Benzoyl-CoA pathway-mediated transformation of aromatic acids. Genes are shown for previously characterized reactions and correspond to annotations in the *R. palustris* genome sequence (7, 43). Reactions and intermediates that have been previously characterized in *R. palustris* are shown within the dashed outline (7, 13, 16, 20). Reactions and intermediates that have been previously characterized in *T. aromatica* are indicated by shading (4, 29, 30). Reactions that have yet to be demonstrated *in vivo* or *in vitro* are denoted with an asterisk and question mark (*?*). (A) Benzoyl-CoA pathway functions used in metabolism of *para*-hydroxybenzoate, benzoate and cyclohexanoate. (B) Proposed benzoyl-CoA pathway functions used in metabolism of protocatechuate and *meta*-hydroxybenzoate.

Figure 2 – Photoheterotrophic growth of *R. palustris* wild type strain CGA009 supplemented with excess of *meta*-hydroxy-aromatic acid to benzoyl-CoA pathway inducing substrate (5-to-1 molar ratio). Cell density (●) is reported in Klett units (right Y-axis). Media benzoate (◇), *para*-hydroxybenzoate (∆), *meta*-hydroxybenzoate (□), and protocatechuate (○) concentrations (left Y-axis). Error bars represent the standard deviation of replicate cultures. (A) *para*-Hydroxybenzoate (0.7 mM) + *meta*-hydroxybenzoate (3.7 mM)-supported photoheterotrophic growth of strain CGA009. (B) Benzoate (0.8 mM) + protocatechuate (3.9 mM)-supported photoheterotrophic growth of strain CGA009.

Figure 3 - *meta*-Hydroxybenzoate (2.3 mM) + protocatechuate (2.3 mM)-supported photoheterotrophic growth of *R. palustris* strains. Cell density (●) is reported in Klett units (right Y-axis). Media *meta*-hydroxybenzoate (□) and protocatechuate (○) concentrations (left Y-axis). Error bars represent the standard deviation of replicate cultures. (A) *meta*-Hydroxybenzoate-
and protocatechuate-grown cells of wild-type strain CGA009. (B) meta-Hydroxybenzoate-grown cells of strain CGA506, deficient in pHB-CoA reductase activity. Protocatechuate decrease in this culture is due to photochemical decay.

Figure 4 – Benzoate (2.2 mM) + protocatechuate (2.2 mM)-supported photoheterotrophic growth of *R. palustris* strains. Cell density (●) is reported in Klett units (right Y-axis). Media benzoate (●) and protocatechuate (○) concentrations (left Y-axis). Error bars represent the standard deviation of replicate cultures. (A) Benzoate- and protocatechuate-grown cells of wild-type strain CGA009. (B) Benzoate-grown cells of strain CGA506, deficient in pHB-CoA reductase activity. Protocatechuate decrease in this culture is due to photochemical decay.

Figure 5 – Cyclohexanoate (2.0 mM) + meta-hydroxybenzoate (2.0 mM)-supported photoheterotrophic growth of *R. palustris* strains. Cell density (●) is reported in Klett units (right Y-axis). Media cyclohexanoate (×) and meta-hydroxybenzoate (□) concentrations (left Y-axis). Error bars represent the standard deviation of replicate cultures. (A) Cyclohexanoate and meta-hydroxybenzoate grown cells of wild-type strain CGA009. (B) Cyclohexanoate-grown cells of strain CGA606, deficient in benzoyl-CoA reductase activity.
Table 1 – *R. palustris* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/Relevant characteristic(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGA009</td>
<td>Wild-type; spontaneous Cm&lt;sup&gt;r&lt;/sup&gt; derivative of CGA001</td>
<td>(25)</td>
</tr>
<tr>
<td>CGA506</td>
<td>hbaB::lacZ-Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(16)</td>
</tr>
<tr>
<td>CGA606</td>
<td>badE::lacZ-Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(7)</td>
</tr>
</tbody>
</table>

* Cm, chloramphenicol; Km, kanamycin; lacZ, promoterless lacZ gene
Table 2 – Doubling times ($T_d$) of wild type *R. palustris* strain CGA009 and COD removal efficiency (%COD) during photoheterotrophic growth in cultures containing a single source of organic carbon.

<table>
<thead>
<tr>
<th>Growth Substrate</th>
<th>$T_d$ a</th>
<th>%COD b</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 mM benzoate</td>
<td>0.4 (0.0)</td>
<td>88.4 (1.0)</td>
</tr>
<tr>
<td>2.2 mM pHB</td>
<td>0.6 (0.0)</td>
<td>86.6 (3.6)</td>
</tr>
<tr>
<td>2.0 mM cyclohexanoate</td>
<td>0.4 (0.0)</td>
<td>89.1 (1.5)</td>
</tr>
<tr>
<td>2.2 mM mHB</td>
<td>NDG</td>
<td>-</td>
</tr>
<tr>
<td>2.3 mM protocatechuate</td>
<td>&gt; 60</td>
<td>82.9 (5.1)</td>
</tr>
</tbody>
</table>

a Doubling times (days) are reported as the average of replicate (two or more) cultures incubated with indicated concentrations of carbon sources; standard deviations are shown in parentheses. NDG, no detectable growth.

b COD removal efficiencies are reported as the average of at least two cultures from which the soluble COD was measured before inoculation and after visible growth of the culture had stopped; standard deviations are shown in parentheses. -, condition not tested.
Table 3 – Doubling times (Td) of *R. palustris* strains and COD removal efficiency during photoheterotrophic growth in cultures containing equimolar combinations of primary substrates (PS) and secondary substrates (SS).

<table>
<thead>
<tr>
<th>Two-substrate combination tested</th>
<th>Doubling times (Td, in days)(^a) on primary and secondary substrates(^b) and net percent of soluble COD removed (%COD)(^c):</th>
<th>Photoheterotrophic <em>palustris</em> strain tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combination of Two Co-Substrates:</td>
<td>CGA009 (wild type)</td>
<td>CGA009 (badE::lacZ-Kmr)</td>
</tr>
<tr>
<td></td>
<td>CGA606 (badE::lacZ-Kmr)</td>
<td>CGA606 (badE::lacZ-Kmr)</td>
</tr>
<tr>
<td>Primary substrate (PS)</td>
<td>Secondary substrate (SS)</td>
<td>Td (PS)</td>
</tr>
<tr>
<td>2.2 mM benzoate</td>
<td>2.2 mM (m)-HB</td>
<td>0.4 (0.0)</td>
</tr>
<tr>
<td>2.2 mM (p)-HB</td>
<td>2.2 mM (m)-HB</td>
<td>0.7 (0.2)</td>
</tr>
<tr>
<td>2.0 mM cyclohexanoate</td>
<td>2.0 mM (m)-HB</td>
<td>0.4 (0.0)</td>
</tr>
<tr>
<td>2.2 mM benzoate</td>
<td>2.2 mM protocatechuate</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td>2.2 mM (p)-HB</td>
<td>2.2 mM protocatechuate</td>
<td>1.1 (0.0)</td>
</tr>
<tr>
<td>2.0 mM cyclohexanoate</td>
<td>2.0 mM protocatechuate</td>
<td>0.4 (0.0)</td>
</tr>
<tr>
<td>2.3 mM (m)-HB</td>
<td>2.3 mM protocatechuate</td>
<td>2.1 (0.3)</td>
</tr>
</tbody>
</table>

\(^a\) Doubling times (days) are reported as the average of replicate (two or more) cultures incubated with indicated concentrations of carbon sources; standard deviations are shown in parentheses. NDG, no detectable growth; -, condition not tested.

\(^b\) HPLC analysis of media (as in Figures 1-5) showed that primary substrates were consumed prior to significant utilization of secondary substrates during each experiment.

\(^c\) Percentages of soluble COD removed are averages of at least two cultures from which the soluble COD concentration was measured before inoculation and after visible growth of the culture had stopped; standard deviations are shown in parentheses. -, condition not tested.

\(^d\) Observed growth was attributed to utilization of protocatechuate because primary substrate concentrations did not change during the course of the experiments.