Phototrophic 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, phototrophic growth in *R. palustris* was slow relative to cells using compounds known to be metabolized by the benzoyl coenzyme A (benzoyl-CoA) pathway. *R. palustris* was unable to grow when meta-hydroxybenzoate was provided as a sole source of organic carbon under phototrophic 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 the dehydroxylation of para-hydroxybenzoyl-CoA (HbaBCD) and reductive deamination of benzoyl-CoA (BadDEFG) were required for metabolism of protocatechuate and meta-hydroxybenzoate. These findings indicate that, under phototrophic growth conditions, *R. palustris* can degrade meta-hydroxy-aromatic acids via the benzoyl-CoA pathway, apparently due to the promiscuity of the enzymes involved.
yl-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 the present study are described in Table 1. R. palustris strain CGA009 and its derivatives were grown in presterilized photosynthetic media (PM) supplemented with carbon sources at selected concentrations (25). As needed, 100 μg of kanamycin ml⁻¹ was added to media for the maintenance of R. palustris mutant strains. Phototrophic 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., New York, NY).

For aromatic acid- and cyclohexanoate-supported growth experiments, the initial medium 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, the culture medium 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

![FIG 1](image_url) 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.

![TABLE 1](table_url) R. palustris strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and/or relevant characteristic(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGA009</td>
<td>Wild type; spontaneous Cm' derivative of CGA001</td>
<td>25</td>
</tr>
<tr>
<td>CGA506</td>
<td>hbaB::lacZ-Km'</td>
<td>16</td>
</tr>
<tr>
<td>CGA606</td>
<td>badE::lacZ-Km'</td>
<td>7</td>
</tr>
</tbody>
</table>

*Cm, chloramphenicol; Km, kanamycin; lacZ, promoterless lacZ gene.
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 1 g of chemical oxygen demand (COD) per liter of medium.

**Analytical methods.** Samples were aseptically removed from cultures by removing 200–300 μl aliquots while adding argon gas to maintain head-space atmospheric pressure. Samples were filtered through 0.22-μm pore-size hydrophilic Durapore polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany) and acidified with phosphoric acid (0.5% [vol/vol]) before analysis 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 capillary free fatty acid-phase column (0.32 mm [inner diameter] by 30 m; 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-M10A VP diode array detector (Shimadzu, Kyoto, Japan). Aromatic compounds were separated using an Ultra Aqueous (Restek Corp., Bellefonte, PA) C₁₈-reversed stationary phase column (5 μm particle size; 120 mm × 4 mm I.D.) and an isotropic aqueous mobile phase of methanol (30% [wt/vol]), acetonitrile (6% [wt/vol]), and 5 mM formic acid in water (64% [wt/vol]) 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 pore-size membranes prior to measuring the concentrations of soluble organic compounds using a HACH High-Range (0 to 1,500 mg/liter) COD kit (HACH Company, Loveland, CO). COD is a standard test specific for organic compounds (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 phototrophic cultures (33). The theoretical COD values for various carbon sources used in the present study are as follows (in mg of COD/mmol of substrate): acetate, 64; benzoate, 240; butyrate, 160; caffeate, 288; cyclohexanoate, 288; mHB, 224; pHb, 224; protocatechuatic, 208; bicarbonate, 0; and succinate, 112.

**RESULTS**

**Conditions to induce meta-hydroxy-aromatic acid metabolism in wild-type *R. palustris*.** The goal of the present 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), phototrophic cultures of strain CGA009 incubated with either *mHB* or protocatechuatic as a sole organic carbon source were incapable of doubling at rates similar to cells grown with benzoate, pHb, or cyclohexanoate (0.4-, 0.6-, 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 phototrophic conditions. However, protocatechuatic (2.3 mM) supported very slow phototrophic growth of *R. palustris*. For all experiments during which growth was observed, measurements of medium COD indicated >82% removal of the organic substrates as the biomass increased, demonstrating that growth was associated with the degradation of the substrates, even in the protocatechuatic-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 *mHB* or protocatechuatic when either benzoate, pHb, or cyclohexanoate (for *mHB* only) were also supplied as an additional cosubstrate (Table 3). Under these conditions, cultures exhibited a biphasic mode of growth, where benzoate, pHb, 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 cosubstrates, either *mHB* or protocatechuatic (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 pHb was the sole organic carbon source (Table 2), growth with benzoate and pHb as primary substrates was slower when protocatechuatic was the secondary substrate (Table 3), perhaps as an effect of competition between protocatechuatic and the primary substrate for required enzymes. Although it was not an enhancer of protocatechuatic metabolism, cyclohexanoate was utilized as a primary substrate when *mHB* was degraded as the secondary substrate, but growth during *mHB* uptake was slower (8.7-day doubling time). The inability of strain CGA009 to degrade protocatechuatic as the secondary substrate when cyclohexanoate was the cosubstrate may be due to the additional enzymes (e.g., HbaBCD) that are expected to be needed for protocatechuatic degradation compared to *mHB* metabolism (Fig. 1B). In these two-substrate experiments, the primary and secondary substrates were added in equimolar concentrations, and each corresponded to ca. 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 *mHB* was the secondary substrate was between 75.0 and 81.8% (Table 3), demonstrating that *mHB* was utilized for growth. When protocatechuatic was the secondary substrate, the COD removal efficiencies were 86.7 and 76.9% when biphasic growth was observed, also demonstrating that protocatechuatic was utilized as a carbon source for growth. In contrast, when cyclohexanoate was used as the primary substrate and

**TABLE 2**

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Conc (mM)</th>
<th>Avg (SD)</th>
<th>T₉₀*(days)</th>
<th>%COD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate</td>
<td>2.2</td>
<td>0.4 (0.0)</td>
<td>88.4 (1.0)</td>
<td></td>
</tr>
<tr>
<td>pHb</td>
<td>2.2</td>
<td>0.6 (0.0)</td>
<td>86.6 (3.6)</td>
<td></td>
</tr>
<tr>
<td>Cyclohexanoate</td>
<td>2.0</td>
<td>0.4 (0.0)</td>
<td>89.1 (1.5)</td>
<td></td>
</tr>
<tr>
<td>mHB</td>
<td>2.2</td>
<td>NDG</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Protocatechuatic</td>
<td>&gt;60</td>
<td>82.9 (5.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The doubling time (T₉₀) is reported as the average of replicate (two or more) cultures incubated with the indicated concentrations of carbon sources. NDG, no detectable growth.

* The COD removal efficiency (%COD) is 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. ~ Condition not tested.

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
neither biphasic growth nor protocatechuic degradation was observed. The COD removal efficiency was only 47.9%, in agreement with protocatechuic acid not being utilized 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 protocatechuic acid, 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 (Fig. 2A). Under these conditions, the contribution of pHB to the total COD in the medium 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 ~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), finding consistent with the excess mHB providing most of the organic carbon for growth. Similarly, in experiments where protocatechuic acid (3.9 mM) was present at a 5-to-1 molar ratio over benzoate (0.8 mM) in the medium (Fig. 2B), benzoate degradation and concomitant growth preceded subsequent consumption of protocatechuic acid and slower cell growth, with an ~4-fold increase in optical density during the period of protocatechuic acid degradation. In this case, medium 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 protocatechuic acid 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 protocatechuic acid as cosubstrates at equimolar concentrations, with cells utilizing mHB as the primary substrate at short doubling times and protocatechuic acid as the slowly degraded secondary substrate (Fig. 3A), supporting growth at relatively longer doubling times (Table 3). A COD removal efficiency of 67.8% and a nearly 2-fold increase in optical density after mHB had been depleted (Fig. 3A) confirmed the utilization of both substrates for growth, although the accumulation of metabolites cannot be ruled out given the low COD removal efficiency.

![Fig 2](image-url) Photoheterotrophic growth of the *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). Medium benzoate (▲), para-hydroxybenzoate (□), meta-hydroxybenzoate (●), and protocatechuic acid (●) concentrations (left y axis) are indicated by the corresponding symbols. Error bars represent the standard deviations of replicate cultures. (A) para-Hydroxybenzoate (0.7 mM) plus meta-hydroxybenzoate (3.7 mM)-supported photoheterotrophic growth of strain CGA009; (B) benzoate (0.8 mM) plus protocatechuic acid (3.9 mM)-supported phototrophic growth of strain CGA009.
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 cosubstrate (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, the expression of which is 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 whether 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 (Fig. 4B and Table 3). However, once benzoate was consumed, the growth of strain CGA506 was impaired, and protocatechuate in the media decayed slowly (Fig. 4B). Further, compared to wild-type cultures incubated under identical conditions (Fig. 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, supports 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 sup-

FIG 3 meta-Hydroxybenzoate (2.3 mM) plus protocatechuate (2.3 mM)-supported photoheterotrophic growth of R. palustris strains. The cell density (●) is reported in Klett units (right y axis). Medium meta-hydroxybenzoate (□) and protocatechuate (○) concentrations (left y axis) are represented by the corresponding symbols. Error bars represent the standard deviations 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.

FIG 4 Benzoate (2.2 mM) plus protocatechuate (2.2 mM)-supported photoheterotrophic growth of R. palustris strains. The cell density (●) is reported in Klett units (right y axis). Medium benzoate (◇) and protocatechuate (○) concentrations (left y axis) are indicated by the corresponding symbols. Error bars represent the standard deviations 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.
In the case of strain CGA606, we analyzed growth on cyclohexanoate metabolism of downstream of benzoyl-CoA reductase activity (Fig. 1A). When wild-type cells were grown under this condition (Table 3), a finding was that the amount of COD removed by strain CGA606 was lower than in wild-type cells (Fig. 1A). This may be due to both strains making use of cyclohexanoate as growth substrates, given our observation that proteocatechuate degrades at approximately the same rate under light in abiotic tubes and in cultures where proteocatechuate 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 proteocatechuate 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 the 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 the present study 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 (Fig. 1A) using much of the same enzymology used during utilization of benzoate (10, 11, 16, 37) and that benzoate, PHB, 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 proteocatechuate and mHB 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 mHB for growth either when it was the sole photoheterotrophic carbon source or when the media also contained rapidly metabolized nonaromatic organic acids as cosubstrates (butyrate or acetate). Proteocatechuate 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 (i) supplemented with one of the three known inducers of benzoyl-CoA pathway gene expression—cyclohexanoate (for mHB only), benzoate (for mHB or protocatechuate), or pHB (for mHB or protocatechuate)—or (ii) fed both mHB and protocatechuate as co-substrates. The means by which (i) the three known benzoyl-CoA pathway inducers enhance metabolism of one or both meta-hydroxy-aromatic acids or (ii) the combination of mHB and protocatechuate as co-substrates enhances the metabolism of each meta-hydroxy-aromatic acid remains to be determined since pathway expression is affected by both transcriptional (35–37) and posttranslational (38, 39) mechanisms.

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 meta-hydroxy-aromatic substrates (6, 27, 29, 30). However, it is not known whether {T. aromatica} requires the activity of benzoyl-CoA pathway enzymes for meta-hydroxy-aromatic acid degradation or if other pathways contribute to metabolism in this organism. In the present study, we show that photolithotrophic metabolism of protocatechuate or mHB is only observed in {R. palustris} under conditions that increase benzoyl-CoA pathway activity. Further, our data provide direct genetic evidence that the metabolism of meta-hydroxy-aromatic compounds requires the activity of benzoyl-CoA pathway enzymes in {R. palustris}.

For example, mutants lacking BadDEFG activity (7) are known to be incapable of utilizing benzoate as a sole organic carbon source under photolithotrophic growth conditions. Whereas wild-type cells were capable of using mHB to support growth after cyclohexanoate was removed from culture medium (Fig. 5A), cells lacking benzoyl-CoA reductase activity (strain CGA606) were unable to grow using mHB under identical conditions (Fig. 5B). This is direct evidence that BadDEFG activity is necessary for utilization of mHB as a growth substrate by {R. palustris} under photolithotrophic conditions. Similarly, the properties of the hbaB mutant (strain CGA506; Table 3) indicates that HbaB activity is needed for metabolism of protocatechuate (Fig. 3; Fig. 4). Collectively, our data predict a potentially new role for R. palustris HbaBCD and BadDEFG enzyme activities in para-dehydroxylation and reductive deamoratization of meta-hydroxyylated metabolite intermediates during photolithotrophic growth with meta-hydroxy-aromatic acids as organic carbon sources. Although HbaBCD and BadDEFG may function directly in p-dehydroxylation of protocatechuy-CoA and deamoratization of mHB-CoA, respectively (Fig. 1B), the possibility remains that other characterized enzymes function in reductive m-dehydroxylation, producing alternative intermediates pHb-CoA and benzoyl-CoA as substrates for HbaBCD and BadDEFG (Fig. 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 noninducing compounds as growth substrates. In experiments in which one of the inducers of benzoyl-CoA pathway expression (pHB, benzoate, or cyclohexanoate) was used to enhance the metabolism of one of the meta-hydroxy-aromatic acids (mHB 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 upregulated 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 mHB as cosubstrates (Fig. 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 thiosterification of aromatic acids at the onset of the pathway (Fig. 1). Thus, the CoA ligase expressed in the presence of protocatechuate may have a sufficiently high affinity for mHB (versus protocatechuate) to allow the 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 the expression of pathways needed for subsequent catabolism of protocatechuate. We hypothesize that each substrate (and its cognate intermediates) induces the expression of only a subset of genes needed for growth, and those genes not induced by it or its intermediates are compensated for 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 (i) similar roles played by benzoyl-CoA pathway enzymes in {T. aromatica} and {R. palustris} for the degradation of meta-hydroxy-aromatic acids and of (ii) the regulatory control of benzoyl-CoA pathway expression being restricted to a narrower set of allosteric effector molecules in {R. palustris} 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 photolithotrophic growth. One unique feature of anaerobic aromatic metabolism in {R. palustris} is that the transcription factors controlling the expression of benzoyl-CoA pathway genes (35, 36) apparently respond to only a narrow set of allosteric effectors but trigger upregulation 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 cosubstrates. A third aromatic acid, meta-chlorobenzoate, may also be a member of this group as it has been reported to support photolithotrophic 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 here 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...
pathway is also present. If this was the case, the common presence of both classes of aromatic acids in nature has apparently not required \textit{R. palustris} to evolve systems to induce benzoyl-CoA pathway expression in the presence of only \textit{meta}-hydroxy-aromatic acids.

In sum, we identified here conditions that enable the metabolism of \textit{meta}-hydroxy-aromatic acids in \textit{R. palustris}. We also provided genetic evidence that benzoyl-CoA pathway enzymes are needed for metabolism of protocatechuate and mHB in \textit{R. palustris}. Our findings leave open the possibility of \textit{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.

**ACKNOWLEDGMENTS**

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**REFERENCES**


