Simultaneous catabolism of plant-derived aromatic compounds results in enhanced growth for members of the Roseobacter lineage

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Running title
Simultaneous aromatic catabolism in roseobacters

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Abstract

Plant-derived aromatic compounds are important components of the dissolved organic carbon pool in coastal salt marshes, and their mineralization by resident bacteria contributes to carbon cycling in these systems. Members of the roseobacter lineage of marine bacteria are abundant in coastal salt marshes and several characterized strains, including *Sagittula stellata* E-37, utilize aromatic compounds as primary growth substrates. The genome sequence of *S. stellata* contains multiple, potentially competing, aerobic ring-cleaving pathways. Preferential hierarchies in substrate utilization and complex transcriptional regulation have been demonstrated to be the norm in many soil bacteria that also contain multiple ring-cleaving pathways. The purpose of this study was to ascertain whether substrate preference exists in *S. stellata* when provided a mixture of aromatic compounds that proceed through different ring-cleaving pathways. We focused on the protocatechuate (*pca*) and the aerobic benzoyl-CoA (*box*) pathways and the substrates known to proceed through them, *p*-hydroxybenzoate (POB) and benzoate, respectively. When these two substrates were provided at non-carbon limiting concentrations, temporal patterns of cell density, gene transcript abundance, enzyme activity, and substrate concentrations indicated *S. stellata* simultaneously catabolized both substrates. Furthermore, enhanced growth rates were observed when *S. stellata* was provided both compounds simultaneously compared to cells grown singly with an equimolar concentration of either substrate alone. This simultaneous catabolism phenotype was also demonstrated in another lineage member, *Ruegeria pomeroyi* DSS-3. These findings challenge the paradigm of sequential aromatic catabolism reported for soil bacteria and contribute to the growing body of physiological evidence demonstrating the metabolic versatility of roseobacters.
Introduction

The structural diversity of aromatic compounds in the environment is influenced by the various mechanisms that produce them, including naturally occurring abiotic (1) and biotic processes (2, 3) as well as those of anthropogenic origin (4). Regardless of the source, the dissolved organic carbon pool containing aromatic compounds in nature is typically structurally heterogeneous (5, 6). This is an important consideration for microbial degradation and has received significant attention in studies examining the catabolism of aromatic compound mixtures classified as environmental pollutants (7). Considerably less attention has focused on microbial physiology of mixtures of naturally occurring aromatic compounds, such as those derived from lignin, the structural component of vascular plants (8).

Microbial mineralization of aromatic compounds plays an important role in global carbon cycling and bioremediation. Bacterial aromatic catabolism is described as “catabolic funneling” where upper (also called peripheral) pathways transform a diverse suite of aromatic compounds into one of a limited number of intermediates that are then subject to ring cleavage (9). The \( \beta \)-ketoadipate pathway is one such pathway and is a paradigm for aerobic catabolism of plant-derived aromatics (10, 11). In organisms possessing one or both its branches, peripheral pathways generate dihydroxylated intermediates, either catechol or protocatechuate. Alternative aerobic ring-cleaving mechanisms are also present in bacteria, including epoxidation of CoA-thioesterified aromatics, as occurs in the benzoyl-CoA (box) pathway for benzoate degradation (12). These catabolic pathways are typically under tight transcriptional regulation (13, 14) and subject to catabolite repression (15-17).
Previous studies reveal substrate preferences are the norm when bacterial strains are presented with a mixture of aromatic compounds (9). This phenomenon has been best characterized for select soil-derived bacteria provided mixtures of benzoate and p-hydroxybenzoate (18-20). Each of these compounds is ultimately processed through parallel ring-cleavage branches of the β-ketoacid pathway, referred to as the catechol (cat) and protocatechuate (pco) branches (21), respectively. The hierarchical nature of substrate utilization profiles has been mechanistically explained as cross-regulation and typically involves transcriptional regulation by pathway metabolites or regulatory proteins (e.g., (22, 23)). However, the extent to which similar hierarchies exist in environmentally relevant microbes is not yet clear. Furthermore, as environmental bacteria are dependent upon growth substrate pools that are highly heterogeneous in composition (24, 25), mixed substrate studies may provide the foundation for a better understanding of bacterial catabolism in nature.

The Roseobacter lineage of marine bacteria is numerically abundant and active in the world’s oceans (26, 27). Group members are most dominant in coastal environments, including salt marshes heavily influenced by lignocellulosic vascular plant material (28). Roseobacter ubiquity and success in the oceans has been attributed, in part, to their ability to use a large repertoire of growth substrates, including aromatic compounds (29-31). Genome analyses have identified several ring-cleaving pathways in roseobacters, including the box and pco pathways (30). Yet, it is unknown whether roseobacters show evidence of substrate preference when presented with mixtures of aromatic compounds representative of compounds derived from vascular plants abundant in coastal marine habitats.
Materials and Methods

Media and growth conditions. A marine basal medium (MBM) containing 1.5% (w/v) sea salts [Sigma-Aldrich, St. Louis, MO] with 225 nM K2HPO4, 13.35 µM NH4Cl, 71 mM Tris-Cl (pH 7.5), 68 µM Fe-EDTA, trace metals, and vitamins was used to culture *Ruegeria pomeroyi* DSS-3 and *Sagittula stellata* E-37 at 30°C (32). *Variovorax paradoxus* EPS was also cultured at 30°C in M9 basal media (33). All growth experiments used cells preconditioned on 3.5 or 7 mM acetate at early stationary phase to match the carbon concentration of the new medium. Initial inocula were ≤1% transfer volume (~10⁵ cells ml⁻¹). Benzoic acid and *p*-hydroxybenzoic acid were obtained from Sigma-Aldrich, and sodium acetate was obtained from Fisher Scientific [Waltham, MA]. All glassware was combusted minimally for 6 h at 450°C to remove trace carbon, and negative (non-carbon amended) controls of cells in basal media were also performed.

Nucleic acid isolation. For RNA preservation and isolation, approximately 10⁸ cells were pelleted at 5000 x g for 5 min, resuspended in 1 ml of RNAlater [Ambion, Austin, TX], and preserved for 1 h at room temperature. RNAlater was removed by aspiration following 6000 x g centrifugation for 5 min, and the cells were flash frozen in liquid N₂ and stored at -70°C until processed. Cells were lysed by agitation in the presence of low-binding 200 µm zirconium beads [OPS Diagnostics, L L C., Lebanon, NJ], and RNA was extracted using the RNaseasy Mini Kit [Qiagen, Valencia, CA]. Genomic DNA was removed using the vigorous Turbo DNase (4 U) [Ambion] method as described in the product manual. Nucleic acids were quantified and purity was assessed with an ND-1000 spectrophotometer [NanoDrop Technologies Inc., Wilmington, DE]. Reverse transcription was carried out in 60 µl volumes containing 180 ng RNA, 600 U M-
MLV reverse transcriptase [Invitrogen, Carlsbad, CA], 500 µg ml⁻¹ random hexamers [Promega, Madison, WI], 120 U RNaseOUT [Invitrogen], 500 µM dNTPs [Promega], and 10 mM DTT [Invitrogen]. Initially the random hexamers in the presence of dNTPs were annealed to the RNA for 5 min at 65°C, followed by an immediate transfer to ice to maintain the binding interaction. Next, to protect the mRNA and increase full-length cDNA yields, DTT and RNaseOUT were added and incubated for 2 min at 37°C. Finally cDNAs were generated from the RNA by M-MLV reverse transcriptase by first activating for 10 min at 25°C followed by 50 min of synthesis at 37°C. The enzymes were denatured and inactivated for 15 min with 70°C, and the remaining cDNA was stored at -20°C.

Gene transcription assays. RT-qPCR was used to assess relative gene expression. Transcripts diagnostic of the benzoyl-CoA pathway (boxA) and the protocatechuate branch of the β-ketoadipate pathway (pcaH) were measured and normalized to the expression of three reference genes (alaS, map, and rpoC). Primers were designed for each of these 5 genes and are shown in Table S1. All primer sets were optimized for quantitative (qPCR) using the following method. In 25 µl qPCR volumes, a matrix of forward and reverse primer concentrations (ranging from 100-1500 nM final concentrations) was used along with a fixed concentration of E-37 genomic DNA (2.5×10⁵ genomes reaction⁻¹) and 1X SYBR Premix Ex Taq (Perfect Real Time) [Takara Bio Inc., Otsu, Japan]. The qPCR amplification included an initial 95°C denaturation for 15 min, followed by 40 cycles of amplification (95°C denaturation for 45 s, 58°C annealing for 45 s, and 72°C elongation and fluorescence detection for 15 s), and a final 72°C extension for 5 min. A melting curve from 50°C to 100°C read every 1°C was performed after each reaction to ensure only a single product melted around 90°C. Within each primer set matrix, the combination of
forward and reverse primer concentrations that yielded the lowest Cq (quantification cycle) was used in the subsequent RT-qPCR.

The 25 µl qPCR volumes consisted of 15% cDNA template, 577 µM oligonucleotide primers, and SYBR Premix Ex Taq (Perfect Real Time) [Takara]. Each cDNA template sample was amplified with primer sets to quantitate *alaS*, *boxA*, *map*, *pcaH*, and *rpoC*. Technical qPCR triplicates were performed for each of the five primer sets used to amplify the three biological triplicates at five time points. The cycling conditions were also the same as described for the qPCR optimization. Non reverse-transcribed aliquots were also performed as negative controls to ensure the RT-qPCR measurements represented cDNA concentration and other nucleic acids’ Cq were negligible (>5 difference). Reference genes for normalization remained unchanged during the sampling time points and have been successfully applied as reference genes to another roseobacter in our laboratory (34). All normalized *boxA* and *pcaH* RT-qPCR data were relativized to their basal expression of E-37 cells grown on 7 mM acetate according to calculations described by Hellemans *et al.* (35). Technical replicate errors were propagated with a truncated first-order Taylor series expansion.

**Protocatechuate 3,4 dioxygenase (PcaHG) enzyme assays.** Approximately 10^{10} cells were washed in a 4°C solution containing 1.5% sea salts [Sigma-Aldrich] and 50 mM Tris-acetate (pH 7.5). Rinsed and pelleted cells were suspended in 396 µl of Bugbuster Protein Extraction Reagent [Novagen, Inc., Madison, WI]. Lysozyme (4 µl) was added (to a final concentration of 0.1 ng ml^{-1}) and the cells were incubated at 30°C for 15 min. After the cell debris was removed via centrifugation (21000 x g for 30 min at 4°C), crude cell lysates were assayed for
protocatechuate 3,4-dioxygenase (PcaHG, EC 1.13.11.3) activity by measuring the kinetic loss of protocatechuate at 290 nm with a DU 800 UV/Vis spectrophotometer [Beckman Coulter, Inc., Brea, CA] (36). Briefly, the 1 ml assays included 400 µM protocatechuate and 50 mM Tris-acetate (pH 7.5) along with ≥3 different volumes of lysate tested below the saturation point.

Protein concentrations were determined with the Coomassie Plus Protein Assay Reagent Kit [Thermo Scientific Pierce, Rockford, IL] (37). The specific activity for each sample was calculated using the Δε of εprotocatechuate and εβ-carboxymuconolactone (2280 cm⁻¹ M⁻¹). E-37 cells grown solely on 7 mM acetate and on 2 mM protocatechuate served as negative and positive controls.

**HPLC-PDA analysis of substrate concentrations.** Aromatic substrates in the spent media were separated with a Waters 2695 high-performance liquid chromatography (HPLC) instrument containing a reverse-phase 3.9 x 150 mm Novak-Pak C₁₈ column [Waters Corp., Milford, MA] coupled to a Waters 2996 photodiode array (PDA) detector. For the spent MBM, an isocratic elution of 0.8 ml min⁻¹ at 25°C with the mobile phase containing 30% MeCN(aq) and 0.07% phosphoric acid(aq) produced distinct peaks for benzoate (3.62 min) and POB (2.12 min). The same separation conditions were used for M9 spent media with the exception of increased (2.5%) phosphoric acid(aq). A ten-point serial dilution curve of authentic standards was used to determine the concentration of each compound at their λmax in the MBM solution that were 230.3 and 256.2 nm for benzoate and POB. The peak area of each eluate was calculated with ApexTrack’s integration tool using the Empower 2 Pro software package [Waters] for each of the technical (HPLC-PDA machine) triplicates performed on each sample. Linear regression of the temporally paired substrate concentrations was used to assess the statistical correlation between the
catabolic/disappearance rate of each substrate using SigmaPlot 11.0 [Systat Software, Inc., Chicago, IL].

Ash-free dry mass (AFDM) measurements. E-37 cells grown at 30°C with 200 rpm agitation in 250 ml baffled flasks containing 100 ml of 14 mM carbon in MBM were used to estimate the total carbon biomass yields on different growth substrates. Cells grown to early stationary phase on 2 mM benzoate, on 2 mM POB, and on 1 mM benzoate + 1 mM POB were harvested by centrifugation at 6000 x g for 10 min. To capture any cells that did not pellet, supernatants were passed through pre-ashed and -weighed glass fiber (GF/F) filters with a nominal 0.7 µm pore size [Whatman Ltd., Maidstone, ME]. Cell pellets were resuspended in 1 ml of spent media and filtered onto their respective GF/F filters. All biomass was dried on the filters for 24 h at 60°C, after which their masses were measured. The dried organic material was then combusted for 4 h at 450°C. The total carbon biomass for each population was determined taking into account the combusted filter masses. Three biological replicates were performed for each growth substrate, and a one-way ANOVA was performed with Tukey’s post-hoc tests in SigmaPlot 11.0. One-tailed Student’s t-tests were performed with MS Excel 2010 [Microsoft Corp., Redmond, WA].

Genome analyses. Using the NCBI Genomes database (http://blast.ncbi.nlm.nih.gov/), co-occurrences of box and pca pathways were identified in cultured taxa. Protein sequences from organisms with experimental data were used as tBLASTn queries: BenA (benzoate 1,2-dioxygenase alpha subunit; YP_046122.1) and BenB (benzoate 1,2-dioxygenase beta subunit; YP_046123.1), CatA (catechol 1,2-dioxygenase; YP_046127.1) and CatB (muconate cycloisomerase I; YP_046131.1), BoxB (benzoyl-CoA 2,3-epoxidase; Q9AIX7.1) and BoxC
(2,3-epoxybenzoyl-CoA dihydrolase; Q84HH6.1), PcaG (protocatechuate 3,4-dioxygenase alpha subunit; YP_046376.1) and PcaH (protocatechuate 3,4-dioxygenase beta subunit; YP_046375.1), and AraC-type PobR (transcriptional regulator; YP_299213.1) and IclR-type PobR (transcriptional regulator; YP_046382.1).

Results

Sagittula stellata E-37, the representative Roseobacter selected for these studies, is a coastal seawater strain that can catabolize a variety of plant-derived sugars as well as aromatic compounds representative of lignin breakdown products (32, 38, 39). Furthermore, it has been shown to selectively attach to lignocellulose particles as well as transform and partially mineralize synthetic lignin (32). Its genome contains six ring-cleaving pathways (aerobic benzoyl-CoA [box], gentisate [gtd], homogentisate [hmg], homoprotocatechuate [hpa], phenylacetate [paa], and protocatechuate [pca]) (30), making it an especially attractive model system to examine mixed substrate growth during aromatic compound catabolism. In order to facilitate comparisons with previous studies conducted in soil bacteria, we focused our efforts here on the structurally similar growth substrates benzoate and \( p \)-hydroxybenzoate.

Catabolic funnelling in Sagittula stellata E-37. Before addressing the primary question of mixed substrate growth, it was first useful to have a broader understanding of aromatic compound catabolism in E-37. Experimental studies in roseobacters are largely restricted to the protocatechuate (pca) branch of the \( \beta \)-ketoadipate pathway. Activity of the ring-cleaving enzyme, protocatechuate 3,4-dioxygenase (PcaHG) has been previously shown to be inducible by growth on POB in E-37 and other roseobacters (39). Furthermore, \( pobA \), the gene encoding a hydroxylase that mediates the conversion of POB to protocatechuate, appears to be coordinately
expressed with the pca genes in E-37 and other roseobacters (38). To address which additional aromatic substrates capable of supporting growth are processed via the pca pathway, PcaHG enzyme assays were performed on extracts of E-37 grown on different substrates, only one of which (p-coumarate) is predicted to generate POB as a catabolic intermediate. Basal level activity (<0.04 µmol pca min⁻¹ µg protein⁻¹) was observed for cells grown on acetate, benzoate, and phenylacetate suggesting that the pca pathway is not induced when provided these growth substrates. Conversely, specific activity of PcaHG was detected in cells grown on ferulate, p-coumarate, p-hydroxybenzoate, protocatechuate, quinate, and vanillate (>0.50 µmol pca min⁻¹ µg protein⁻¹), suggesting that all of these compounds are catabolized via the pca pathway (Fig. 1). In some cases, these data are supported by the presence of gene homologs to known catabolic enzymes from soil bacteria (Table 1 and Fig. 1). It is not yet clear what, if any, compounds other than benzoate are degraded through the box pathway. Transcriptional assays of pcaH were also performed on E-37 grown solely on acetate, benzoate, or POB. pcaH transcripts were significantly higher (p≤0.01) in POB-grown cells compared to those grown on either acetate or benzoate. These findings corroborate the PcaHG enzyme assay data (Fig. 2 and Table 1).

Growth on benzoate induces boxA expression in Sagittula stellata E-37. Like many alphaproteobacteria, roseobacters lack the catechol (cat) branch of the β-ketoadipate pathway that is a well-described route for benzoate degradation in other taxa. Instead E-37 contains the complete complement of box genes encoding for enzymes that convert benzoate to the TCA cycle intermediates acetyl-CoA and succinyl-CoA (12). Growth on benzoate as a sole carbon source has been demonstrated previously in E-37 (32), but experimental observations linking the box pathway to this physiology have not yet been performed. Here, we observed increased
abundance of boxA transcripts when the strain was grown on benzoate relative to cells grown solely on acetate or POB, indicating that E-37 uses the benzoyl-CoA pathway for benzoate catabolism (Fig. 2). These results also suggest the box pathway is strongly inducible and therefore likely subject to transcriptional regulation. The approximate 5-fold difference in transcript abundance between boxA and pcaH when grown on benzoate and POB, respectively, may be the result of a combination of factors, including differences in promoter regulation and strength, as well as transcript stability. Given these differences, transcript abundance for the mixed substrate experiments described below is expressed relative to those obtained for cells grown on each substrate alone.

Simultaneous catabolism of benzoate and p-hydroxybenzoate. In order to assess whether there was a preferential use of either benzoate or POB in non-limiting carbon conditions, E-37 was provided a mixture of both compounds at equal concentrations (1 mM each). Cell density, transcript abundance, PcaHG enzyme activity, and substrate concentration were monitored throughout the growth cycle for each of three biological replicates (Fig. 3). Collectively, these data provided no evidence for substrate preference. Replicate growth curves were monophasic, and while both boxA and pcaH transcript abundance are on average 58-95% of that found in cultures grown on either substrate alone, the normalized relative quantities (NRQ) of both transcript abundances were significantly higher than basal levels and remained high even as substrate concentrations fell. Furthermore, PcaHG specific activities remained unchanged over time and were equivalent to that found in POB-grown cells. Over the course of the experiment, both benzoate and POB were removed from the growth medium at approximately the same rate (Fig. S1). However, substrate concentrations in early logarithmic phase cultures indicate that...
POB is removed from the medium at a slightly faster rate than benzoate during the initial growth phase. The rates of disappearance for benzoate and for POB during simultaneous catabolism were not significantly different from disappearance rates when E-37 was grown on equimolar carbon concentrations of each substrate individually (data not shown).

Simultaneous aromatic catabolism confers enhanced growth rates. Growth kinetics were monitored to compare the physiology of cells simultaneously catabolizing benzoate and POB to cells grown on each substrate individually at the same carbon concentrations. The growth rate for E-37 grown on both compounds simultaneously ($\mu=0.129$) was significantly higher ($p<0.001$) than cells grown on benzoate ($\mu=0.048$) and POB ($\mu=0.048$) alone (Fig. 4). Interestingly, there was a difference in cell yield when this strain was grown on equimolar concentrations of substrates that differ by a single hydroxyl group. Early stationary phase biomass yields of 100 ml cultures of E-37 grown on POB (69.13±2.28 mg C) were significantly lower ($p<0.015$) than benzoate-grown cells (79.57±4.90 mg C), while the mixed substrate grown cells yielded an intermediary value (74.27±4.89 mg C). One explanation for the observed differences in biomass is there is a difference in the ATP yield for the substrates. In fact, theoretical energy calculations suggest more ATP is made per molecule of benzoate proceeding through box than ATP per molecule of POB proceeding through pob-pca (Table S2).

Genome analysis and growth assays with other strains. To ascertain whether a diagnostic genetic signature might be evident in bacteria demonstrating the simultaneous catabolism phenotype, a bioinformatics analysis of bacterial genomes containing the box and pca pathways
was undertaken. Searches indicate that all putative box operons contain a gene, typically designated boxR, with high homology to a XRE-type transcriptional regulator, suggesting functional similarity among the bacteria harboring this pathway (data not shown). Conversely, gene synteny and genetic regulators for POB catabolism are much less conserved. A focused analysis of bacterial genomes that contain both the box and pca pathways (as of Jan 2013) revealed significant variation in the organization of genes for POB catabolism (pobA and the pca genes) and their transcriptional regulators (Fig. S2). To date, co-localization of pobA with the pca genes is unique to roseobacters, and pobA transcription appears to be coordinate with the pca genes and under control of a LysR-type regulator, denoted PcaQ (38). Outside of the Roseobacter clade, gene organization indicates pobA transcription is mediated by the activity of an adjacently located regulatory protein-encoding pobR, belonging to either the AraC or IclR family (40-42). Of the strains analyzed, simultaneous catabolism studies have only been performed on Cupriavidus necator JMP 134, which possesses an AraC-type PobR protein and exhibits a substrate preference for benzoate over POB (19).

Given the unique pobA gene organization identified in roseobacters, we hypothesized that the absence of a PobR homolog facilitates the growth phenotypes described here. As a first step in exploring this hypothesis, we performed additional mixed substrate growth experiments with the bacterium Variovorax paradoxus EPS that contains both the box and pca pathways and has an IclR-type pobR, as well as another roseobacter, Ruegeria pomeroyi DSS-3, that lacks a pobR homolog (Fig. S2). It was first confirmed that both organisms could grow on the two substrates individually (data not shown). In mixed substrate experiments, simultaneous catabolism of
benzoate and POB was observed in DSS-3, however, preferential consumption of POB was observed in EPS (Fig. S3).

Discussion

When a bacterium is provided a mixture of growth supporting compounds, the utilization profile is generally substrate concentration dependent. Under non-limiting (replete) carbon concentrations, sequential utilization of substrates by bacteria is typically observed, with the substrate supporting the highest growth rate receiving preference (43). This response has been well studied for bacteria provided a mixture of two sugars (43), but has also been demonstrated with substrate mixtures from other compound classes, including organic acids and aromatic compounds (e.g., (44, 45)). Control mechanisms that are responsible for substrate utilization hierarchies include substrate transport into the cell (46, 47), transcriptional regulation (48, 49), or post-translational modification of enzyme(s) (50). However, these regulatory controls are often relieved in substrate limiting concentrations, when mixed substrate use is essential for growth (e.g., (51, 52)). In fact, as oligotrophic conditions dominate the microbial landscape (53), it is possible that substrate preferences are the exception rather than the rule in most natural environments (54). However, we demonstrate here simultaneous catabolism of two aromatic compounds processed through separate ring-cleaving pathways in roseobacter representatives occurs under carbon replete conditions (C:N:P ratio of >10,000:59:1). Furthermore, we show that this metabolic versatility leads to an increased growth rate, which may contribute to the competitiveness of these organisms in natural systems, particularly in coastal salt marshes, in which aromatic compounds are a significant component of the dissolved organic carbon pool (55). Consistent with this hypothesis is the observation that roseobacters are one of the most
abundant groups of bacteria associated with decaying *Spartina alterniflora* (56), a primary source of lignin-derived aromatic compounds to these coastal systems (57).

The cross-regulatory mechanisms resulting in substrate preference when strains are provided mixtures of POB and benzoate are complex and difficult to predict from genome sequences alone. Variations in the regulatory proteins and gene organization of each pathway can result in differences in substrate preferences. For example, PobR proteins belong to one of two families (AraC and IclR) and activate *pobA* transcription in response to the inducer POB (19, 40). Yet the regulatory mechanisms that dictate repression of *pobA* transcription in the presence of benzoate vary among the organisms possessing different PobR representatives. For example, in the soil bacterium *Cupriavidus necator* JMP134 benzoate serves as structurally similar anti-inducer to prevent AraC-type PobR-mediated expression of *pobA* (19). Conversely, in the soil bacterium *Acinetobacter baylyi* ADP1 benzoate does not directly modulate the activity of the IclR-type PobR protein found in this strain (40). Instead, repression of the *pca* catabolic genes, whose products are necessary for complete degradation of POB, appears to be primarily mediated by the activities of regulatory proteins of benzoate and catechol catabolic gene loci (BenM and CatM) that upon binding an intermediate of catechol catabolism (*cis, cis* muconate) repress transcription of the *pca* operon, which includes a gene encoding for a POB permease (18, 58).

Our observation that *P. paradoxus* EPS preferentially catabolizes POB over benzoate in mixed substrate experiments is consistent with the notion that the activities of IclR-type PobR proteins are not directly and negatively influenced by benzoate. However, additional studies are needed to confirm this model and to better understand the underlying regulatory mechanisms that lead to the novel POB > benzoate phenotype evidenced in this strain. The unique *pobA-pca* gene...
organization found in roseobacters is suggestive of a more simplified regulatory scheme for
pobA, which may be manifested in the simultaneous catabolism phenotype described here and
may indicate that POB serves as an important substrate in the environmental niches that
roseobacters occupy. Furthermore, most roseobacter genomes contain multiple, potentially
competing ring-cleaving pathways for the degradation of aromatic compounds (59) and raising
the possibility that the simultaneous catabolism phenotype demonstrated here is representative of
group members’ utilization of a broader class of aromatic compounds.

While the specific mechanism(s) that facilitate enhanced growth on mixed substrates are not
clear, the answer may lie at the cell membrane. Prior studies have suggested distinct and
dedicated transport mechanisms contribute to enhanced growth during mixed-substrate use of
glucose and sucrose (60-62). In these previous studies, catabolic enzyme activities were
unchanged in mixed substrate relative to single-substrate experiments. The PcaHG activities
reported for cells in different phases of the growth curve are in accordance with this conclusion
(Fig. 3), although the specific transporters of POB and benzoate in E-37 are not yet known. In
other taxa, PcaK has been shown to transport POB (63), and BenK or BenP transport benzoate
(64, 65). However, homologs to these genes are absent in the E-37 genome (GenBank Accession,
AAYA00000000), as well as those of other roseobacters containing the box and pca pathways.
This suggests an alternative transport system for these substrates exists in lineage members. No
putative transport genes are found in the local vicinity (10 kb) of the pobA-pca operon in E-37.
However, a candidate system for benzoate transport is found directly adjacent to the box operon
(SSE37_24379 and SSE37_24389), which putatively encodes a dicarboxylate TRAP transporter.
A thorough investigation of the POB and benzoate transport systems is needed to confirm that
enhanced growth by strains simultaneously utilizing these compounds is due to the presence and activities of separate, non-competing transport systems for these substrates. An alternative explanation for the observed physiology is downhill diffusion as a result of metabolism.

In the coastal salt marshes in which roseobacters are abundant, the dissolved organic carbon pool is highly aromatic and heterogeneous in structure and in distribution (55). A successful ecological strategy for bacteria in such environments is metabolic versatility, a strategy exemplified by cultivated roseobacter representatives (e.g. (28)). The simultaneous catabolism of growth substrates demonstrated here further illustrates the flexible metabolic characteristic of this abundant group of marine bacteria.
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Table 1. Phenotypic and genetic evidence for aromatic compound catabolism in *S. stellata* E-37.

<table>
<thead>
<tr>
<th>Carbon Substrate</th>
<th>Concentration [mM]</th>
<th>Growth</th>
<th>Specific Activity</th>
<th>Catabolic Gene(s) in E-37</th>
<th>Locus Tag(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>3.5, 7, and 25</td>
<td>+</td>
<td>0.017±0.008</td>
<td>tricarboxylic acid cycle (gltA, acnA, icd, sucABCD, shdABC, funcG, &amp; mult)</td>
<td>SSE37_04079, SSE37_21138, SSE37_04374, SSE37_11134, SSE37_11130, SSE37_11136, SSE37_11132, SSE37_11139, SSE37_11109, SSE37_11107, SSE37_11103, SSE37_11101</td>
</tr>
<tr>
<td>Benzoate</td>
<td>0.5, 1, and 2</td>
<td>+</td>
<td>0.030±0.014</td>
<td>bcl-boxDCBAE</td>
<td>SSE37_24494, SSE37_24490, SSE37_24415</td>
</tr>
<tr>
<td>Caffeate</td>
<td>1.2, and 3</td>
<td>-</td>
<td>n.d.</td>
<td>hecABC</td>
<td>SSE37_01049, SSE37_01045, SSE37_11324</td>
</tr>
<tr>
<td>Chlorophenol</td>
<td>1.2, and 3</td>
<td>-</td>
<td>n.d.</td>
<td>xylE</td>
<td>SSE37_01049</td>
</tr>
<tr>
<td>Ferulate</td>
<td>2</td>
<td>+</td>
<td>(+)</td>
<td>wall &amp; vanAB</td>
<td>SSE37_00305, SSE37_00241</td>
</tr>
<tr>
<td>Gentisate</td>
<td>1.2, and 3</td>
<td>-</td>
<td>n.d.</td>
<td>nagILK</td>
<td>SSE37_01795, SSE37_02720, SSE37_01575</td>
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<tr>
<td>Homogentisate</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>hmgAB &amp; hmgC</td>
<td>SSE37_24444, SSE37_24440, SSE37_14734</td>
</tr>
<tr>
<td>Homoprotocatechuate</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>lpaABC &amp; lpaDEF</td>
<td>SSE37_00633, SSE37_00629, SSE37_23379, SSE37_11379, SSE37_11350</td>
</tr>
<tr>
<td>p-Coumarate</td>
<td>2</td>
<td>+</td>
<td>1.2±0.7</td>
<td>hecABC &amp; pheabcd</td>
<td>SSE37_11325, SSE37_11324</td>
</tr>
<tr>
<td>p-Hydroxybenzoate</td>
<td>0.5, 1, and 2</td>
<td>+</td>
<td>0.4±0.3</td>
<td>pheabcd</td>
<td>SSE37_1837</td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>2</td>
<td>+</td>
<td>0.003±0.017</td>
<td>paaABCDEZ &amp; paaJK</td>
<td>SSE37_00350, SSE37_00356, SSE37_00352, SSE37_00346, SSE37_1753, SSE37_1753, SSE37_1749</td>
</tr>
<tr>
<td>Pheacetate</td>
<td>2</td>
<td>+</td>
<td>0.4±0.4</td>
<td>pcaCHG, pcaDb, pcaI, &amp; pcaF</td>
<td>SSE37_15042, SSE37_15048, SSE37_15052, SSE37_21440, SSE37_21446, SSE37_21442, SSE37_21440, SSE37_23014, SSE37_23019, SSE37_1453</td>
</tr>
<tr>
<td>Quinate</td>
<td>2</td>
<td>+</td>
<td>0.4±0.4</td>
<td>quinBCD</td>
<td>SSE37_03885, SSE37_03947, SSE37_03905</td>
</tr>
<tr>
<td>Salicylate</td>
<td>1.2, and 3</td>
<td>-</td>
<td>n.d.</td>
<td>salA</td>
<td>SSE37_01053</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>2</td>
<td>+</td>
<td>(+)</td>
<td>vanAB</td>
<td>SSE37_01255, SSE37_01247</td>
</tr>
</tbody>
</table>

a. n.d. = not determined.
b. Growth was assessed by monitoring the OD540 of cultures in MBM.
c. PcaHG activity was scored as “-” or “+” if the specific activity was <0.04 or >0.50 µmol pca min⁻¹ µg protein⁻¹, respectively.

d. (+) indicates specific activity was not quantified but levels were >0.50 µmol pca min⁻¹ µg protein⁻¹.

e. unk = Gene(s) involved in the pathway are unknown.

f. Locus tags unique to the E-37 genome are listed in respective order of the catabolic genes.

g. A disparately located nagI duplicate exists (SSE37_02425).
Figure Legends

Fig. 1. Substrates proceeding through the protocatechuate branch of the β-ketoadipate and the aerobic benzoyl CoA pathway in *Sagittula stellata* E-37. Gene designations are based on sequence homology to characterized proteins. Question marks denote uncharacterized genes or substrates. TCA, tricarboxylic acid.

Fig. 2. *boxA* and *pcaH* transcript abundance for E-37 grown solely on POB (2 mM) or benzoate (2 mM). cDNA copy numbers were relativized to those from cells grown on an equimolar C concentration of acetate (7 mM). The horizontal dashed line represents unchanged expression. Error bars represent the standard error of the mean of biological triplicates.

Fig. 3. Simultaneous catabolism of benzoate (1 mM) and POB (1 mM) by E-37. Panels A-C represent three biological replicates. Data for all three biological replicates are shown on separate panels (A-C) due to slight variation in growth phase and sample time points among parallel cultures. The *boxA* and *pcaH* transcript abundances are expressed as a percentage of those when E-37 was grown solely on benzoate or POB, respectively. Error bars represent the standard error of the mean (n = 3 per gene). PcaHG specific activity is also expressed as the percentage of activity obtained from POB-grown cells; error bars represent standard deviation of at least 3 replicates. Standard deviation of the technical variation (n = 3) for benzoate and POB concentrations are smaller than the symbol. NRQ, Normalized Relative Quantity. O.D., Optical Density.
Fig. 4. Growth responses to benzoate (1 mM), POB (1 mM), and a mixture of benzoate (0.5 mM) and POB (0.5 mM) by *S. stellata* E-37. Error bars for O.D. represent the standard deviation of biological replicates (n = 3) for each. O.D., Optical Density.
References


Fig. 2
Fig. 4

- 0.5 mM Benzoate + 0.5 mM POB
- 1 mM Benzoate
- 1 mM POB

O.D. 540 nm vs. Time [h]

0.001 0.01 0.1 1

80 60 40 20 0