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Diverse Organization of Genes of the β-Ketoadipate Pathway in Members of the Marine *Roseobacter* Lineage

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Members of the *Roseobacter* lineage, an ecologically important marine clade within the class α-*Proteobacteria*, harbor genes for the protocatechuate branch of the β-ketoadipate pathway, a major catabolic route for lignin-related aromatic compounds. The genes of this pathway are typically clustered, although gene order varies among organisms. Here we characterize genes linked to *pcaH* and *-G*, which encode protocatechuate 3,4-dioxygenase, in eight closely related members of the *Roseobacter* lineage (pairwise 16S rRNA gene sequence identities, 92 to 99%). Sequence analysis of genomic fragments revealed five unique *pca* gene arrangements. Identical gene organization was found for isolates demonstrating species-level identity (i.e., >99% 16S rRNA gene similarity). In one isolate, six functionally related genes were clustered: *pcaQ*, *pobA*, *pcaD*, *pcaC*, *pcaH*, and *pcaG*. The remaining seven isolates lacked at least one of these genes in their clusters, although the relative order of the remaining genes was preserved. Three genes (*pcaC*, *-H*, and *-G*) were physically linked in all isolates. A highly conserved open reading frame (ORF) was found immediately downstream of *pcaG* in all eight isolates. Reverse transcription-PCR analysis of RNA from one isolate, *Silicibacter pomeroyi* DSS-3, provides evidence that this ORF is coexpressed with upstream *pca* genes. The absence of this ORF in similar bacterial *pca* gene clusters from diverse microbes suggests a niche-specific role for its protein product in *Roseobacter* group members. Collectively, these comparisons of bacterial *pca* gene organization illuminate a complex evolutionary history and underscore the widespread ecological importance of the encoded β-ketoadipate pathway.

The β-ketoadipate pathway provides a model system for studying mechanisms of evolution in an ecologically important catabolic pathway. This widely distributed, typically chromosomally encoded pathway plays a central role in the degradation of naturally occurring aromatic compounds that arise during the decay of lignin and other vascular plant components, as well as in that of environmental pollutants (24). The pathway is biochemically conserved, and the structural genes encoding enzymes of the pathway are similar among the phylogenetically diverse organisms that possess it (24). Despite this mechanistic conservation, studies of a limited number of soil bacteria demonstrate a remarkable diversity of this pathway in terms of gene organization, regulation, inducing metabolites, and transport systems (reviewed in reference 35). This diversification may reflect the distinctive selection pressures faced by the organisms maintaining the pathway and thus may reveal characteristic features that are specific to the bacterial group in which the pathway resides (24, 34).

The degradation of aromatic compounds typically proceeds by conversion of the ring structure to one of several di- or trihydroxylated intermediates in preparation for enzymatic ring cleavage. In natural systems, two of the most prevalent intermediates are protocatechuate and catechol. The separate branches of the β-ketoadipate pathway are initiated as protocatechuate and catechol are cleaved between their two hydroxyl groups by protocatechuate 3,4-dioxygenase (P3,4DO) or catechol 1,2-dioxygenase, respectively. Ring fission is followed by five additional reactions leading to the generation of substrates that feed into the tricarboxylic acid cycle (Fig. 1). In organisms possessing both branches, the pathway converges to form several common intermediates including β-ketoadipate, for which the pathway is named.

The genes encoding the enzymes, transport proteins, and regulatory proteins of the β-ketoadipate pathway are usually present in chromosomal supraoperonic clusters (34). A remarkable example of such an assemblage is demonstrated by the protocatechuate pathway branch in *Acinetobacter* sp. strain ADP1, where more than 40 genes involved in the catabolism of plant-related compounds form a 56-kbp chromosomal cluster (36). However, the extent of gene clustering varies among the phylogenetically diverse microbes possessing the pathway. *Pseudomonas putida* demonstrates a contrasting scenario, as the genes required for the catabolism of protocatechuate are dispersed within the genome in three distinct gene clusters (34). Furthermore, gene order does not appear to be maintained within the operons except in cases where genes may coevolve because they encode subunits of a single enzyme (e.g., *pcaHG* and *pcaIJ* [see Fig. 1]) (24).

The marine *Roseobacter* lineage provides an ideal system for genetic studies of aromatic compound catabolism. Representatives of this lineage are abundant in coastal seawater, aerobic sediments, and decaying plant material from coastal salt marshes (9, 19, 28, 45). The presence of the protocatechuate branch of the β-ketoadipate pathway has been demonstrated in several isolates, and roseobacters have widespread capabilities for the transformation of lignin-related aromatic monomers, including *p*-hydroxybenzoate, vanillate, and ferulate (4,

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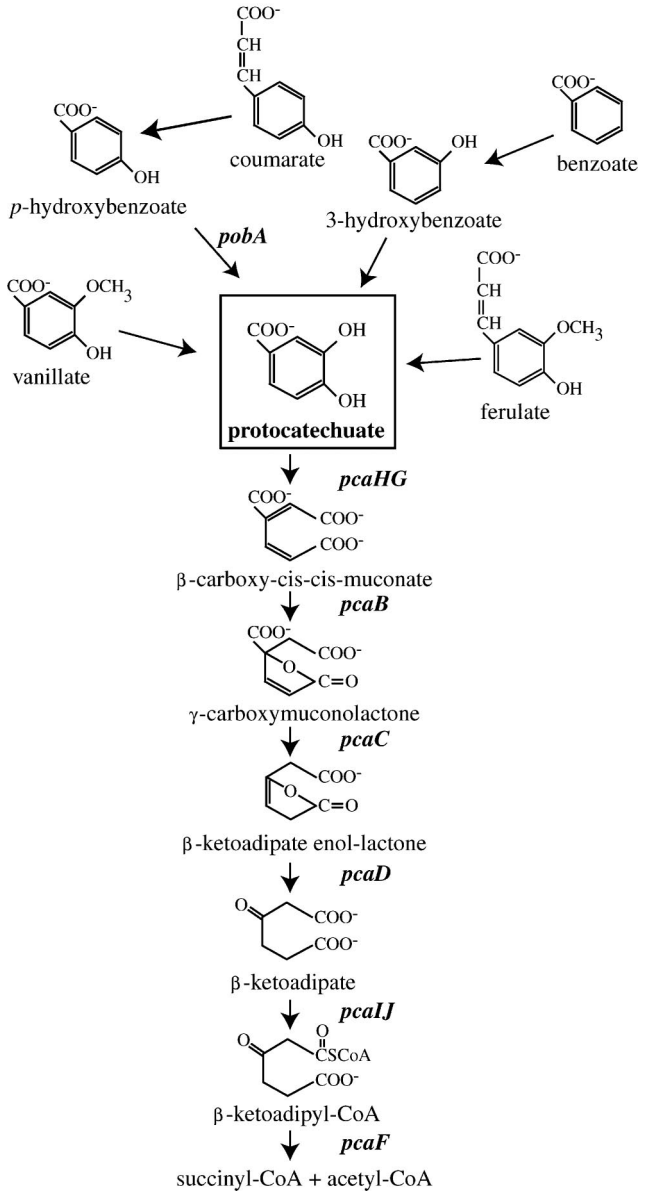

FIG. 1. Schematic of the protocatechuate branch of the β-ketoadipate pathway. Gene designations are italicized. CoA, coenzyme A.

5). Furthermore, more than half of the *pcaH* genes retrieved by PCR amplification from salt marsh bacterial communities could be traced to members of the *Roseobacter* lineage (5). To gain a better perspective on the conservation of sequence and gene arrangement in closely related organisms, we analyzed the protocatechuate branch of the β-ketoadipate pathway in eight *Roseobacter* group isolates.

MATERIALS AND METHODS

Bacterial isolation, growth conditions, and 16S rRNA gene analysis. Most isolates examined in this study were cultured from seawater, sediments, or decaying salt marsh grass collected in the estuaries and coastal waters of the southeastern United States. As previously described, aromatic substrates were used in the enrichment cultures from which *Sagittula stellata* E-37, *Sulfitobacter* sp. strain EE-36, Y3F, and Y4I were isolated. For the first two strains, the high-molecular-weight (>1,000) fraction of pulp mill effluent was used (18). For the latter two isolates, a lignin-rich pulp mill by-product (Indulin) was used (4). SE45 was cultured from decaying *Spartina alterniflora* (smooth cord grass) by using nonselective, low-nutrient seawater plates (5). *Silicibacter pomeroyi* DSS-3 was derived from a marine dimethylsulfoniopropionate enrichment culture (16, 17). Finally, two isolates that were not obtained from the southeastern U.S. coast were examined: *Sulfitobacter pontiacus* ChLG 10, which was cultured from the Black Sea (43), and *Roseovarius nubinhibens* ISM, which was cultured from the Caribbean Sea (14, 16).

Growth capabilities were tested by using aromatic compounds, each provided at a 2 mM concentration, as the sole substrate in marine basal medium (MBM) with vitamins as previously described (18). Plates were incubated at 28 to 30°C in the dark.

16SrRNA sequences have been reported previously for all isolates (accession numbers are given in parentheses): *S. pomeroyi* DSS-3 (AF098491), *Sulfitobacter* sp. strain EE-36 (AF007254), *R. nubinhibens* ISM (AF098495), Y3F (AF253467), Y4I (AF388307), SE45 (AF388308), *S. stellata* E-37 (U58356), and *S. pontiacus* ChLG 10 (Y13155). Species-level similarity was observed between Y4I and Y3F and between *Sulfitobacter* sp. strain EE-36 and *S. pontiacus* ChLG 10. These pairs, respectively, have 100 or 99.8% identity in regions corresponding to positions 48 to 1484 or 50 to 1506 of the 16S rRNA gene in *Escherichia coli.*

Detection and isolation of catabolic genes from roseobacters. The catabolic gene clusters were initially identified in isolates by targeting a region of *pcaH*. A degenerate PCR primer pair designed from conserved PcaH regions (5) was used to amplify a 212-bp product from all isolates. This product was gel excised, labeled with digoxigenin (DIG) by a random priming reaction (Genius system; Roche Molecular Biochemicals, Indianapolis, Ind.), and used in Southern hybridizations with isolate genomic digestions. DNA fragments of the sizes corresponding to bands giving positive hybridization signals in Southern blot analysis were gel excised and ligated into the pZERO vector (Invitrogen Corp., Carlsbad, Calif.). Colony hybridization of genomic libraries with DIG-labeled DNA probes identified positive clones. In cases where the initial fragment did not contain all functionally related genes in the proximity of *pcaHG* (*S. pomeroyi* DSS-3, *Sulfitobacter* sp. strain EE-36, and SE45), adjacent fragments were identified by Southern hybridization analysis using DIG-labeled probes generated from distal ends of the primary fragment (see Fig. 2).

Sequence determination of the genomic fragments was facilitated by using the GeneJumper Primer Insertion Kit for Sequencing (Invitrogen Corp.), which randomly inserted a minimal version of the *Mu* transposon containing two primer binding sites and a selectable marker into the target DNA.

Expression of *Roseobacter* group isolate DNA in *E. coli*. To express the *pcaHG* genes from isolates SE45 and *R. nubinhibens* ISM under the control of the *lac* promoter in *E. coli*, p4NK and p6NB were constructed. PCR primers were used to introduce restriction sites into amplified *pcaHG* genes for optimal positioning in the pCYB1 expression vector (New England Biolabs, Beverly, Mass.). An *Nde*I cleavage site was introduced just before the ATG start codon of the *pcaH* gene, and either a *Kpn*I (SE45) or a *Bam*HI (*R. nubinhibens* ISM) cleavage site was introduced downstream of the *pcaG* stop codon by PCR amplification using the high-fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). The 1.3-kbp fragments were then ligated into the corresponding sites on the pCYB1 vector. The sequences of the resultant recombination plasmids were confirmed to be correct. Luria-Bertani broth cultures (100 ml) of *E. coli* Top10F′ cells (Invitrogen Corp.) carrying either p4NK or p6NB were grown at 30 or 25°C for 12 h. At the time of inoculation, 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures. The induced cells were harvested by centrifugation, washed once with sterile Tris buffer (50 mM; pH 7.5), and stored at −20°C. Reported specific activities are averages from three independently grown and assayed cultures. No activity was detected in *E. coli* with the cloning vector or with recombinant plasmids in the absence of IPTG.

P3,4DO enzyme assays. Cell pellets were suspended in 200 μl of breaking buffer [50 mM Tris-HCl, 10% glycerol, 5 mM $(NH_4)_2SO_4$, 2.5 mM EDTA, 1 mM dithiothreitol (pH 7.5)]. Cell extracts were prepared as described previously (42), and P3,4DO activity was determined spectrophotometrically by measuring the decrease in absorbance at 290 nm (44). Protein concentrations were determined by the method of Bradford (2).

mRNA isolation and RT-PCR. Total RNA was harvested from early-log-phase cultures of *S. pomeroyi* DSS-3 grown with *p*-hydroxybenzoate (3 mM) or succinate (10 mM) as the sole carbon source by using the RNAqueous kit (Ambion, Inc., Austin, Tex.). RNA preparations were treated with DNase (DNA-Free; Ambion, Inc.) to remove contaminating DNA. Reverse transcription reactions were carried out with a primer binding to a region within the conserved open reading frame (ORF) (primer DSS.CHP.rev, 5′ TTG ACG GTC AGT TGA TTG 3′) by using Omniscript RT (Qiagen, Valencia, Calif.) according to the manufacturer's protocols. cDNA generated in the reverse transcription step was

TABLE 1. Growth of *Roseobacter* group isolates on aromatic compounds

Roseobacter group isolate	Growth[a] of isolate on the following aromatic compound[b]:							
	Benzoate	Chlorogenate	4-CB	Coumarate	Ferulate	POB	PCA	Vanillate
R. nubinhibens ISM	−	−	−	+/−	+	+	+	−
SE45	+	+	+	+	+	+	+	+
S. stellata E-37	+	+	−	+	+	+	−	+
S. pomeroyi DSS-3	+	−	+	+	+	+	+	−
Y3F	+	+	+	+	−	+	+	+
Y4I	+/−	+	−	+/−	−	+	−	+
S. pontiacus ChLG 10	−	+/−	−	+/−	−	+	+	−
Sulfitobacter sp. strain EE-36	+/−	+/−	−	+	+	+	+	−

[a] Growth was determined on MBM plates containing 2 mM growth substrate plus 0.1% vitamins. +, growth within 4 days; +/−, growth within 14 days; −, no growth within 14 days.
[b] 4-CB, 4-chlorobenzoate; POB, *p*-hydroxybenzoate; PCA, protocatechuate.

used in subsequent PCR amplifications using primers pcaG.400.for (5′ GAG TYC TGG CAG GCC 3′) and DSS.CHP.rev. Because primer pcaG.400 is complementary to a region within *pcaG*, the pcaG.400–DSS.CHP.rev primer set was used to determine if the ORF and *pcaG* were cotranscribed. PCR thermal cycling conditions consisted of 30 cycles of 95°C for 45 s and 55°C for 45 s, followed by 72°C for 1 min. Control samples with equivalent concentrations of RNA, but lacking reverse transcriptase, were included in all reverse transcription-PCRs (RT-PCRs).

Sequence determination and analysis. DNA sequences were determined with double-stranded templates and primers that recognized the cloning vector or the GeneJumper transposon. When necessary, new oligonucleotide primers were made based on previously sequenced regions. Either an ABI3700 or an ABI310 automated DNA sequencer (Applied Biosystems, Foster City, Calif.) was used.

Homology searches (BLAST) were carried out at the network server of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Sequences were analyzed using the Wisconsin Package 10.1 (Accelrys, Burlington, Mass.). Phylogenetic trees were constructed for sequences with the PHYLIP package (13) by using evolutionary distances (Jukes Cantor or Kimura) and tree-building algorithms (Neighbor or Fitch) or a parsimony method (ProtPars). The *pcaC*, *-H*, and *-G* gene sequences were tested for intragenic recombination by using Partial Likelihoods Assessed through Optimisation (PLATO; available at http://evolve.zoo.ox.ac.uk/program). The conserved hypothetical proteins were analyzed for the presence of signature sequence motifs by using the PROSITE (http://ca.expasy.ch/prosite/), PRINTS (http://www.bioinf.man.ac.uk/dbbrowser/PRINTS/), and BLOCKS (www.blocks.fhcrc.org/) programs. The conserved hypothetical protein was also searched for putative membrane-spanning regions by using the MEMSAT2 program (http://bioinf.cs.ucl.ac.uk/psipred).

Nucleotide sequence accession numbers. Sequences reported here were submitted to the GenBank database under the following accession numbers: AF253465, AF253466, and AY457916 to AY457921.

RESULTS

Choice of *Roseobacter* group isolates. Studies of salt marsh bacterial communities indicate that the ability to cleave protocatechuate is a common and environmentally significant trait of the *Roseobacter* lineage (5). In agreement with this conclusion, *pcaH* sequences were obtained from 16 of 19 *Roseobacter* group members screened by PCR with a degenerate primer set (5). To characterize the genetic regions associated with this trait, isolates were selected from a roseobacter collection of salt marsh and coastal ocean isolates (5, 19, 20). Five strains (*S. stellata* E-37, *S. pontiacus* ChLG 10, *R. nubinhibens* ISM, and isolates Y3F and SE45) were chosen for this study because their *pcaH* sequences were identical to a 159-bp region of *pcaH* retrieved from bacterial communities associated with decaying plant material (5). Two additional isolates, Y4I and *Sulfitobacter* sp. strain EE-36, were selected because 16S rRNA analysis demonstrated species-level similarity to other chosen isolates, Y3F and *S. pontiacus* ChLG 10, respectively (see Materials and Methods). Finally, *S. pomeroyi* DSS-3 was selected because it is a well-characterized representative of a large, distinct subgroup within the *Roseobacter* lineage (16). The marine *Roseobacter* lineage forms a monophyletic clade within the α-3 subclass of the *Proteobacteria* (19). Pairwise sequence identities of 16S rRNA genes among the eight roseobacters of this study ranged from 92 to 100%.

Growth on aromatic compounds. To ensure that the genes under investigation have physiological relevance, growth was tested on aromatic compounds degraded via protocatechuate by other bacteria (Table 1). Although only SE45 grew on all eight substrates, every *Roseobacter* isolate grew on at least four of these compounds. Furthermore, all grew well on *p*-hydroxybenzoate, a feature common to organisms possessing the protocatechuate branch of the β-ketoadipate pathway (24). Interestingly, the two isolate pairs with species-level similarity (Y3F and Y4I; *Sulfitobacter* sp. strain EE-36 and *S. pontiacus* ChLG 10) had different substrate utilization profiles (Table 1). Collectively, these growth capabilities support the involvement of the *pca* genes in protocatechuate catabolism by these bacteria.

Identification of catabolic genes in roseobacters. Genomic fragments hybridizing to *pcaH* were identified and isolated from *S. pontiacus* ChLG 10, *Sulfitobacter* sp. strain EE-36, *S. pomeroyi* DSS-3, *R. nubinhibens* ISM, Y4I, and SE45. DNA sequence and homology analysis indicated that these fragments carried multiple genes of the protocatechuate branch of the β-ketoadipate pathway. Genes predicted to encode γ-carboxymuconolactone decarboxylase (PcaC), and P3,4DO (PcaHG) (Fig. 1) were identified in these six roseobacters as well as in two previously characterized *Roseobacter* group members, *S. stellata* E-37 and isolate Y3F (4). A comparison of the deduced genomic arrangements in all eight strains is shown in Fig. 2. In some isolates, neighboring sequences were predicted to encode an enol-lactone hydrolase (PcaD), *p*-hydroxybenzoate hydroxylase (PobA), and/or a LysR-type transcriptional regulator (PcaQ). In strains carrying *pcaQ*, its upstream and divergent location relative to the adjacent *pca* gene cluster was consistent with the predicted regulatory function (41). No ORFs showing similarity to transcriptional regulators were evident in the immediate vicinity of the *pca* genes in either *Sulfitobacter* strain. The orientation of the *pca* genes and their proximity to one another suggest that they are transcribed as a single unit (Fig. 2), as has been demonstrated previously for some soil bacteria (8).

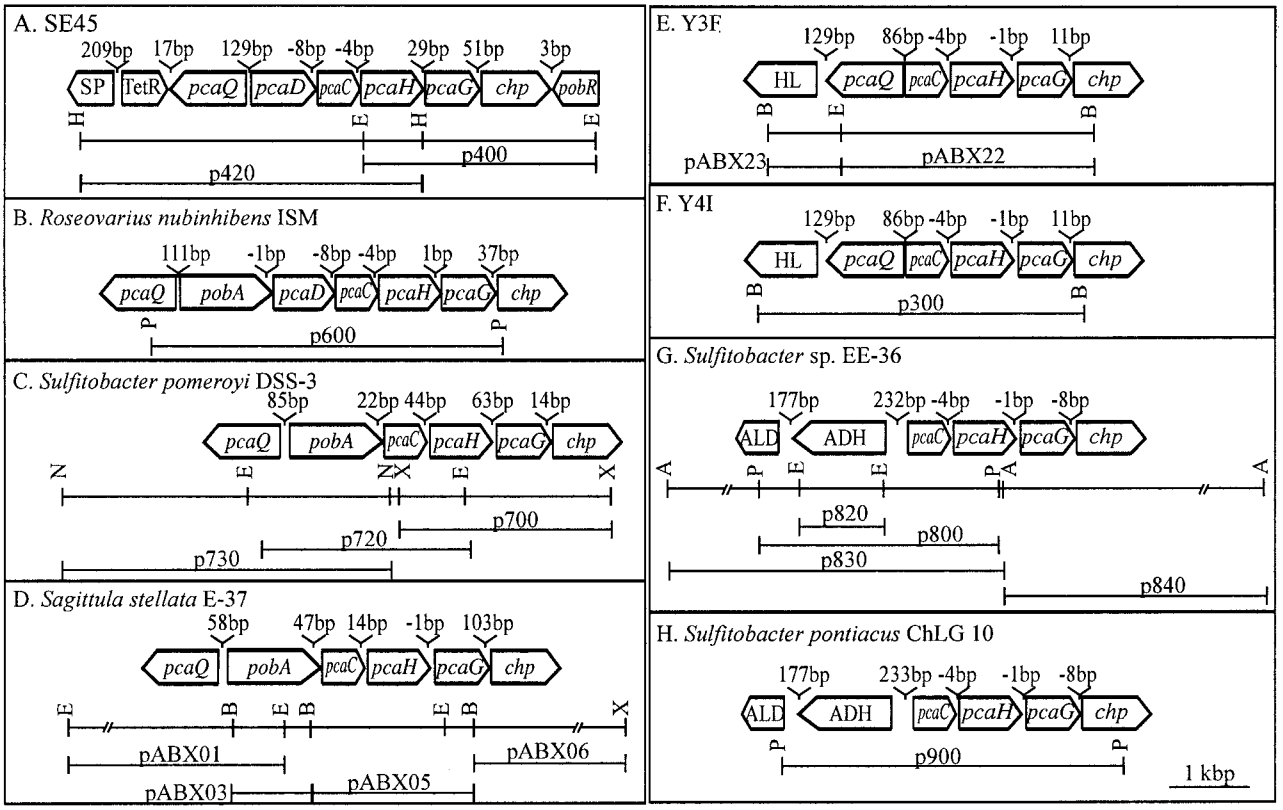

FIG. 2. Restriction maps of genomic fragments from isolate SE45 (A), *R. nubinhibens* ISM (B), *S. pomeroyi* DSS-3 (C), *S. stellata* E-37(D), isolate Y3F (E), isolate Y4I (F), *Sulfitobacter* sp. strain EE-36 (G), and *S. pontiacus* ChLG 10 (H). The locations of the genes and their transcriptional directions are shown relative to selected restriction endonuclease recognition sites. A, *Apa*I; B, *Bam*HI; E, *Eco*RI; N, *Nsi*I; P, *Pst*I; X, *Xho*I. Horizontal lines indicate DNA regions contained on recombinant plasmids (designations given above the lines). Genes are characterized in Table 2 and in the text.

G+C contents have been determined to be 65.0, 66.8, and 66.0% for isolates *S. stellata* E-37, *S. pomeroyi* DSS-3, and *R. nubinhibens* ISM, respectively (16, 20). The G+C contents of the *pca* genes (Table 2) are consistent with those of the genomes of these organisms. The lengths of the *pobA* and *pca* genes as well as the molecular masses, calculated from the deduced amino acids (Table 2), are consistent with those found for their soil counterparts. Additional ORFs located in the vicinity of the *pca* genes are described in Table 3.

Sequence analysis of the *pcaC*, *-H*, and *-G* genes from roseobacters. The three genes present in all eight isolates encode two distinct enzymes catalyzing reactions in the degradation of protocatechuate (*pcaC*, *pcaH*, and *pcaG* [Fig. 1]). Among the *Roseobacter* group isolates, pairwise comparisons of the deduced amino acid sequences for each comparable protein (PcaC, PcaH, and PcaG) ranged from approximately 55 to 76% identity. Comparisons were also carried out with the corresponding sequences from the soil bacteria *Acinetobacter* sp. strain ADP1, *Pseudomonas aeruginosa* PAO1, *P. putida* ATCC 23975, *Sinorhizobium meliloti* 1021, *Mesorhizobium loti* MAFF303099, *Caulobacter crescentus* CB15, *Agrobacterium tumefaciens* C58, and *Rhodococcous opacus* 1CP. The identities of the *P. aeruginosa* PAO1, *S. meliloti* 1021, *M. loti* MAFF303099, and *C. crescentus* CB15 genes were inferred from genomic sequence data. In these comparisons, the identities between corresponding deduced protein sequences ranged from approximately 28 to 39%.

Residues known to be important for catalytic function were well conserved within all of the *Roseobacter* group PcaHG sequences. These included residues involved in coordinating the nonheme Fe^{3+} (Tyr408, Tyr447, His460, and His462) as well as critical active-site residues (e.g., Pro15, Arg133, Trp400, Trp449, and Ile491) (30, 48). In all, 85 of the ~450 residues were completely conserved in the 16 PcaHG sequences examined. Sequence analysis revealed a 72-bp insertion in SE45 *pcaG* that was confirmed by PCR amplification and sequencing of the region from genomic DNA preparations (data not shown). The position of the insertion in SE45 corresponds to the region near residue 125 of PcaG from *Acinetobacter* sp strain ADP1. Based on the crystal structures of PcaHG from *P. putida* ATCC 23975 and *Acinetobacter* sp. strain ADP1 (30, 48), the insertion in SE45 is likely to reside in a loop on the outside face of the enzyme.

The *Roseobacter* group PcaHG sequences do not form a cohesive lineage, as indicated by phylogenetic trees (Fig. 3A). The overall tree topology was identical for trees constructed for individual and combined PcaG and PcaH subunits. Sequences from *S. stellata* E-37 and the two *Sulfitobacter* strains cluster with α-proteobacterial sequences but not with the other *Roseobacter* group isolates (Fig. 3A). However, the deep

TABLE 2. Characterization of β-ketoadipate pathway genes identified in *Roseobacter* group isolates

Roseobacter group isolate	*pcaQ*			*pobA*			*pcaD*			*pcaC*			*pcaH*			*pcaG*		
	% G+C	Length (bp)	Stop codon[a]	MM[b] (kDa)	% G+C	Length (bp)	Stop codon	MM (kDa)	% G+C	Length (bp)	Stop codon	MM (kDa)	% G+C	Length (bp)	Stop codon	MM (kDa)	% G+C	Length (bp)	Stop codon	MM (kDa)
R. nubinhibens ISM	62.1	227[c]			63.2	1,170	TGA	43.4	71.9	789	TGA	27.8	66.7	411	TGA	15	62.7	729	TGA	27.3	65.5	621	TAG	22.6
SE45	68.5	930	TGA	33.7					68.7	789	TGA	27.6	66.7	387	TGA	14.1	63.5	726	TGA	27.1	66.8	693	TGA	25.3
S. stellata E-37	65.7	960	TAA	34.4	63.7	1,181	TGA	44.1					64.6	393	TGA	14.4	66.7	723	TGA	26.8	65.1	602	TGA	21.9
S. pomeroyi DSS-3	66.8	918	TGA	33.1	63.6	1,170	TAG	43.8					64.8	378	TGA	13.8	63.0	729	TGA	27.3	66.0	621	TGA	22.6
Y3F	69.1	929	TAG	32.9									67.3	395	TGA	14.3	63.7	738	TGA	27.7	64.2	618	TGA	22.9
Y4I	69.2	932	TAG	33.1									69.1	396	TGA	14.3	64.8	738	TGA	27.7	64.4	621	TGA	22.8
S. pontiacus ChLG 10													62.7	381	TGA	13.7	60.1	732	TGA	27.1	59.4	606	TGA	22.1
Sulfitobacter sp. strain EE-36													61.9	381	TGA	13.7	60.7	732	TGA	27	58.9	606	TGA	22.2

[a] Stop codon for deduced gene product.
[b] Molecular mass of deduced gene product.
[c] Partial gene sequence was obtained.

branches are difficult to resolve, and thus the phylogenetic relationship of these proteins within the context of other α-proteobacteria is difficult to interpret with the sequences on hand. In contrast, the phylogenetic relationships among the PcaC proteins are similar to those determined from 16S rRNA gene analysis (Fig. 3B and 4). Branch order was maintained when PcaC and PcaHG trees were constructed by using alternative tree-building algorithms (Fitch) and a parsimony method (ProtPars) (data not shown).

Incongruent topologies of the PcaC and PcaHG phylogenetic trees could be the result of recombination (inter- and/or intragenic) or differences in rates of evolutionary change among the corresponding genes. This was tested by using the PLATO program (21). We found no evidence of intragenic recombination within the sets of 16 *pcaC*, *pcaH*, or *pcaG* genes analyzed (8 from roseobacters and 8 from phylogenetically diverse soil bacteria). Furthermore, the relative rates of evolution were assessed by the method outlined by Dykhuizen and Green (11). The similarity in the percentages of divergence of *pcaC*, *-H*, and *-G* among the 16 genes analyzed (32.2, 34.2, and 32.7%, respectively) suggested that the genes are evolving at approximately the same rate. Significant differences were evident for all three genes relative to the 16S rRNA gene, which had significantly lower sequence divergence as determined by analysis of variance (9.6%; $P < 0.001$). A regression of pairwise distances of each of the PcaC, PcaH, and PcaG proteins against pairwise distances of the 16S rRNA gene provided slopes that were not significantly different from one another, likewise suggesting similar rates of evolution of the *pca* genes. Therefore, the different topologies seen for the PcaC and PcaHG protein trees might simply be attributed to the relatively small data set available for analysis.

Expression of the *pcaHG* genes in *E. coli*. To investigate function, the *pcaHG* genes of two roseobacters, SE45 and *R. nubinhibens* ISM, were transformed into *E. coli*, a bacterium that does not encode P3,4DO. The roseobacter *pcaHG* genes resulted in IPTG-inducible P3,4DO activity in cell extracts of the plasmid-bearing *E. coli* strains (258 ± 0.053 or 379 ± 103 nmol/min/mg for the SE45 or *R. nubinhibens* ISM genes, respectively). Surprisingly, for the *R. nubinhibens* ISM genes, activity was observed when plasmid-bearing *E. coli* strains were grown at 25°C but not at 30°C. Although the reason for the temperature sensitivity is not evident, difficulties have been encountered previously with heterologous production of P3,4DO in *E. coli* (38). Possible toxicity associated with expression of *pcaHG* could result in a decreased plasmid copy number (10) or the sequestration of the protein in inclusion bodies. Such problems could be more pronounced when the host is grown at 30°C. Nevertheless, the activity of the PcaHG enzyme from *R. nubinhibens* ISM is indicative of a functional protocatechuate pathway. The broader functional significance of this pathway within the lineage is further supported by a previous report of P3,4DO activity in enzymes from two additional roseobacters, *S. stellata* E-37 and isolate Y3F (4).

Sequence analysis of additional *pca*-related genes. The *pobA* gene, identified in *S. stellata* E-37, *S. pomeroyi* DSS-3, and *R. nubinhibens* ISM (Fig. 2), should encode a hydroxylase for the conversion of *p*-hydroxybenzoate to protocatechuate (Fig. 1). The deduced amino acid sequences of PobA from the roseobacters were >64% identical to comparable sequences

TABLE 3. Additional ORFs in the vicinity of the *pca* genes

Strain(s) and gene designation[a]	Putative function or role	Most similar gene and/or bacterium		
		Designation	% Amino acid similarity (identity)	SwissProt accession no.
SE45				
SP	Shikimate dehydrogenase	*aroE2*, *Ralstonia solanacearum*	65 (57)	NP_522957
TetR	TetR-like transcriptional regulator	*Agrobacterium tumefaciens*	59 (50)	NP_356128
pobR	AraC-like transcriptional regulator	*pobR*, *Rhizobium leguminosarum*	42 (32)	AAA83008
Y3F and Y4I, HL	Zn-dependent hydrolase	*soxG*, *Paracoccus denitrificans*	60 (51)	X79242
Sufitobacter strains				
ALD	Aldehyde dehydrogenase, subunit III	*Mesorhizobium loti*	70 (58)	NP_104855
ADH	Glutathione-dependent alcohol dehydrogenase	*adhI*, *Rhodobacter sphaeroides*	87 (80)	P72324

[a] As shown in Fig. 2.

from soil bacteria. The regions associated with flavin adenine dinucleotide and substrate binding (46) were highly conserved among all the sequences.

Pairwise comparisons of deduced amino acid sequences of *Roseobacter* group PcaD proteins ranged from 60 to 70%. The deduced amino acid sequence also suggests homology to the CatD protein, which catalyzes the analogous reaction in the catechol branch of the β-ketoadipate pathway (23). The conserved active-site cysteine demonstrated to be critical to hydrolysis in PcaD from *Pseudomonas* sp. strain B13 (37) is present in the sequences of both *Roseobacter* group isolates. The amino acids surrounding this residue are also fairly well conserved (GYXXXCXA).

The *Roseobacter* group PcaQ proteins, putative LysR-type regulators, were found to be 39 to 76% identical to PcaQ proteins from other α-proteobacteria (*A. tumefaciens* C58, *S. meliloti* 1021, and *M. loti* MAFF303099). Sequence similarity to several LysR-type regulators of the catechol branch of the β-ketoadipate pathway and of the modified *ortho*-cleavage chlorocatechol pathway was also observed (Fig. 3C). Pairwise PcaQ comparisons showed 45.0 to 98.7% deduced amino acid similarity among the roseobacters. The region of highest similarity among the PcaQ proteins was in the amino terminus, an area presumed to comprise a helix-turn-helix motif for DNA binding (41). A 55-residue stretch of amino acids in the central region of the protein proposed to be involved in inducer binding (7) appears more highly conserved among the PcaQ proteins than among the other LysR-type proteins. By analogy to PcaQ of *A. tumefaciens* A348 and other LysR-type regulators, the *Roseobacter* group PcaQ proteins might be expected to negatively autoregulate their own synthesis and also to activate the expression of genes downstream of, and divergently transcribed from, *pcaQ* (33).

A conserved hypothetical protein present in all roseobacters. A highly conserved ORF, designated a conserved hypothetical protein gene (*chp*), was found immediately downstream of, and oriented in the same direction as, *pcaG* in all eight roseobacters (Fig. 2). The complete gene was retrieved from *S. stellata* E-37 and isolate SE45 and was found to be 828 or 837 bp long, respectively. The two genes share 73.9% identity at the nucleotide level. Partial sequences of the *chp* gene were obtained for the remaining six roseobacters. Pairwise comparisons of *S. stellata* E-37, SE45, *S. pontiacus* ChLG 10, and *S. pomeroyi* DSS-3 show ≥71.8% nucleotide identity for the *chp* gene.

The location of the *chp* gene suggests that it may be cotranscribed with the adjacent *pca* genes (Fig. 2). This suggestion was supported by RT-PCR analysis of RNA isolated from cultures of *S. pomeroyi* DSS-3 grown on succinate (uninduced) or *p*-hydroxybenzoate (induced), a metabolic precursor of protocatechuate (Fig. 5). Amplification of cDNA containing *pcaG* and *chp* was achieved in induced cultures only. However, the CHP function remains unknown. Protein motifs or signature sequences that might reveal catalytic or functional properties of this hypothetical protein (see Materials and Methods) were not apparent. The absence of transmembrane helices suggests that the putative protein is cytoplasmic. Sequence similarity places the CHP within the Pfam protein family DUF849 (www.sanger.ac.uk/Software/Pfam). Not including the *Roseobacter* group CHP sequences described here, the family currently consists of 24 prokaryotic sequences. All are hypothetical proteins with no known function. With the exception of the *Roseobacter* group *chp* genes, none of the genes encoding Pfam DUF849 members are clustered with genes for aromatic compound catabolism.

Species-level comparisons. Sequence similarity in *pca* gene-containing regions was determined for the two pairs of isolates demonstrating species-level similarity of the 16S rRNA gene. Y3F and Y4I share 98.2% sequence identity over 3,354 bp, and *Sulfitobacter* sp. strain EE-36 and *S. pontaicus* ChLG 10 share 97.6% identity over 3,597 bp.

DISCUSSION

The diverse nature of the organization of genes involved in the catabolism of protocatechuate among closely related organisms is evident from the sequence analysis of eight roseobacters. Five unique gene arrangements were identified, with identical gene organization in each of the two species pairs (Fig. 2). Identical *pca* gene organization was also evident

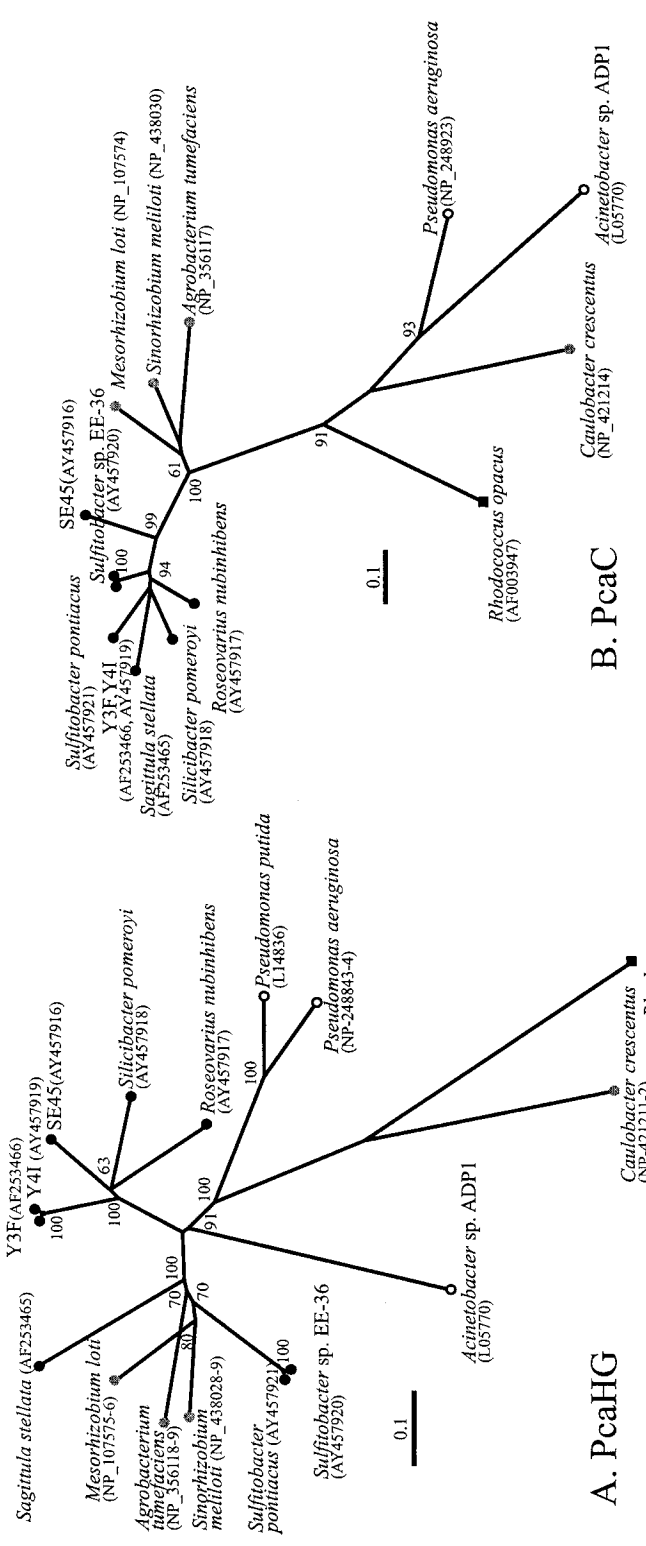

FIG. 3. (A) Phylogenetic tree of PcaHG protein sequences. The tree is based on the deduced amino acid sequence encoded by the *pcaHG* genes and is unrooted, with PcaHG from *R. opacus* 1CP as the outgroup. (B) Phylogenetic tree of PcaC protein sequences. The tree is based on the deduced amino acid sequence encoded by the *pcaC* genes and is unrooted. In *R. opacus* 1CP and *C. crescentus* CB15, *pcaC* and *pcaD* have fused into a single gene (*pcaL*) encoding a protein demonstrating both PcaC and PcaD activities (12); the PcaC-like segment of PcaL was used in this analysis. The protein sequence from *R. opacus* 1CP served as the outgroup. (C) Phylogenetic tree of LysR-type protein sequences. The tree is based on deduced amino acids encoded by the *pcaQ*, *tcbR* (47), *clcR* (6), *tfdR* (27), *benM*, and *catM* (7) genes and is unrooted, with CatR (40) as the outgroup. Bootstrap values of ≥50% are given at branch nodes. Bars indicate Kimura distances. Solid circles, *Roseobacter* group members; shaded circles, non-*Roseobacter* α-proteobacteria; open circles, γ-proteobacteria; solid squares, gram-positive bacteria. GenBank accession numbers are provided in parentheses.

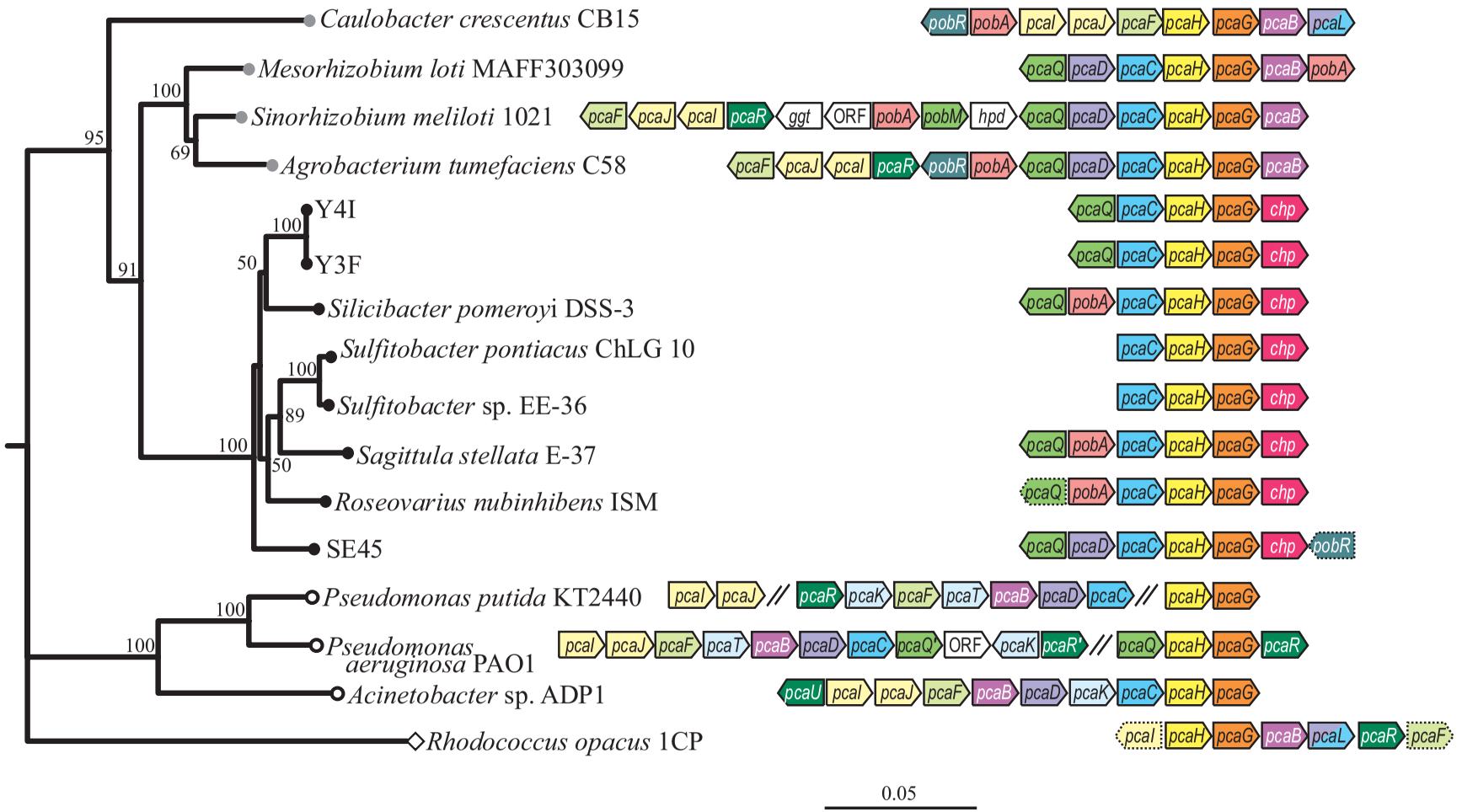

FIG. 4. Phylogenetic tree of 16S rRNA gene sequences. The tree is based on 1,300 nucleotides (positions 111 to 1411; *E. coli* numbering system), with the gene from *R. opacus* 1CP (AB032565) as the outgroup. Bootstrap values of ≥50% are given at branch nodes. Bar indicates Jukes-Cantor distances. The *pca* gene organization of each of the following strains is shown: *C. crescentus* CB15 (SwissProt accession no. NP_421206 to -14), *M. loti* MAFF303099 (NP_107572 to -8), *S. meliloti* 1021 (NP_438027 to -41), *A. tumefaciens* C58 (NP_356109 to -20), isolate Y4I (GenBank accession no. AY457919), isolate Y3F (AF253466), *S. pomeroyi* DSS-3 (AY457918), *S. pontiacus* ChLG 10 (AY457921), *Sulfitobacter* sp. strain EE-36 (AY457920), *S. stellata* E-37 (AF253465), *R. nubinhibens* ISM (AY457917), isolate SE45 (AY457916), *P. putida* KT2440 (AAN69545 to -6, AAN66998 to -67004, AAN70228 to -9), *P. aeruginosa* PAO1 (NP_248917 to -27, NP_248842 to -5), *Acinetobacter* sp. strain ADP1 (L05770), and *R. opacus* 1CP (AF003947). *pcaB*, β-carboxy-*cis*,*cis*-muconate lactonizing enzyme; *pcaC*, γ-carboxy-muconolactone decarboxylase; *pcaD*, β-ketoadipate enol-lactone hydrolase; *pcaHG*, P3,4DO; *pcaF*, β-ketoadipyl coenzyme A thiolase; *pcaIJ*, β-ketoadipate succinyl coenzyme A transferase; *pcaK* and *pcaT*, transport proteins; *pcaL*, *pcaDC* gene fusion; *pobA*, *p*-hydroxybenzoate hydroxylase; *hpd*, *p*-hydroxyphenylpyruvate dioxygenase; *ggt*, γ-glutamyltranspeptidase; *pcaQ*, *pcaQ′*, *pobR*, *pcaR′*, *pobM*, *pcaU*, and *pcaR*, transcriptional regulators. Dark blue, AraC-type transcriptional regulator; dark green, IclR-type transcriptional regulator; light green, LysR-type transcriptional regulator. Double diagonal lines indicate that >10 kbp separates the transcriptional units. Dotted outlines indicate that only partial sequences have been obtained for these genes.

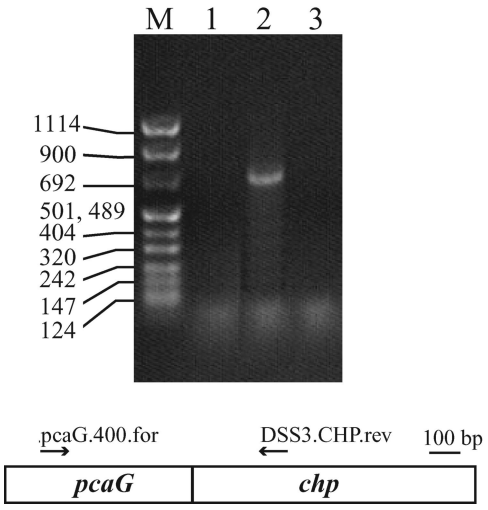

FIG. 5. RT-PCR analysis of *chp* in *S. pomeroyi* DSS-3 cultures. RNAs extracted from *S. pomeroyi* DSS-3 cultures grown on *p*-hydroxybenzoate (lane 2) or succinate (lane 3) served as the template in RT-PCRs. The orientations and locations of the primers used in amplification reactions are shown beneath the gel. Lane 1, a reaction lacking template; lane M, DNA molecular weight markers. Sizes of the standard (in base pairs) are shown to the left of the gel.

for *S. stellata* E-37 and *S. pomeroyi* DSS-3, which share 93.1% sequence identity of the 16S rRNA gene and 64.1% sequence similarity over a 4.97-kbp genomic region containing the *pobA* and *pca* genes.

In the *Sulfitobacter* isolates, the absence of a regulatory gene in the immediate vicinity of *pcaCHG* differs from the genetic arrangement in the other *Roseobacter* group isolates. Since it is common for transcriptional regulators to control the expression of distal genes, this arrangement does not imply anything significant about whether *pca* gene expression is constitutive or regulated in the *Sulfitobacter* strains. For example, the PcaR regulator in *P. putida* is encoded in a *pca* region that is distant from the *pcaHG* genes that it also regulates (32) (Fig. 4). However, these variations in genetic arrangement necessarily affect which genes will be coordinately transcribed within operons and regulons. Thus, the eight roseobacters in this study appear to represent at least five alternative regulatory schemes for the catabolism of *p*-hydroxybenzoate and/or protocatechuate (Fig. 2).

The presence of the *chp* homologs immediately downstream of *pcaG* in all eight isolates is intriguing. The proximity and orientation of this putative gene suggest that it is cotranscribed with the *pca* genes, as demonstrated for one of the roseobacters (Fig. 5). Therefore, it might be expected to play a role in the degradation of protocatechuate or related aromatic compounds. Since this gene is uniquely associated with the *pca* genes of *Roseobacter* group isolates, its function may be related to a substrate or environmental feature that characterizes the ecological niche of this bacterial group. Further genetic and biochemical investigations are necessary to elucidate the function of CHP.

Six enzymatic steps encoded by eight distinct genes (*pcaGHBCDIJF*) complete the conversion of protocatechuate to tricarboxylic acid cycle intermediates (see Fig. 1). Clustering of all eight genes within a single operon can be found in some bacteria. However, it appears that distribution of these functionally related genes in two or more distinct genetic loci is the norm (Fig. 4). A preliminary genome sequence has recently become available for the *Roseobacter* group member *S. pomeroyi* DSS-3 (www.tigr.org); it indicates that the *pca* genes are found in four distinct regions of the genome, grouped as *pcaCHG*, *pcaBD*, *pcaIJ*, and *pcaF*. A defining characteristic of the *Roseobacter* group *pcaCHG* clusters appears to be the absence of the gene encoding the enzyme mediating the step immediately following ring cleavage, *pcaB*. In other organisms examined to date, *pcaB* is physically linked with the genes encoding the two subsequent reactions (*pcaCD* or *pcaL*) (Fig. 4), in agreement with the notion that coordinated expression of genes is an economical strategy for maintaining the fluidity of sequential enzymatic steps. This coordination may be particularly relevant for the gene encoding PcaB, because its substrate, β-carboxymuconate, is known to be a toxic intermediary metabolite (22). Examination of the regulatory strategies invoked by members of the *Roseobacter* lineage may reveal unique mechanisms to ensure stringent coordination of genes involved in the formation and degradation of β-carboxymuconate.

Whether or not both branches of the β-ketoadipate pathway are present within a single bacterium may be pivotal in dictating the genetic organization of genes in the pathway. Both the catechol and protocatechuate branches of the pathway have been found in phylogenetically diverse soil microorganisms including *Acinetobacter* sp. strain ADP1, *P. putida* KT2440, *P. aeruginosa* PAO1, and *R. opacus* 1CP. In contrast, there is no evidence of the catechol branch of the pathway in the *Roseobacter* group isolates (4; www.tigr.org) or in other *pca*-containing α-proteobacteria, including *A. tumefaciens* C58, *S. meliloti* 1021, *M. loti* MAFF303099, or *C. crescentus* CB15, based on genome sequence analyses. Organisms possessing both branches of the pathway may require additional regulatory mechanisms for dictating a preferential hierarchy in substrate uptake when presented with the mixture of aromatic compounds found in natural environments (1, 15, 29). This cross-regulation between the two branches of the pathway might serve to prevent mismatched interactions between the structurally similar enzymes and substrates of the pathway (3). In addition, the presence of both branches of the pathway may lead to a greater selective pressure to maintain the genes of each branch within a minimum number of transcriptional units.

The importance of transport proteins in defining the biological individuality of organisms harboring the β-ketoadipate pathway is becoming increasingly evident (35, 49). *Acinetobacter* sp. strain ADP1, *P. putida* KT2440, and *P. aeruginosa* PAO1, members of the γ-*Proteobacteria*, have catabolic *pca* regions that include *pcaK*, a gene that encodes a transport protein in the major facilitator superfamily (31). Similar genetic arrangements have also been identified in some α-proteobacteria. For example, *C. crescentus* CB15 has a *pcaK*-like gene immediately downstream of *pcaL* (NP_421214). However, in some α-proteobacteria the *pca* regions include genes that may encode proteins of the ABC transport family. Examples of this type of arrangement are seen in *A. tumefaciens* C58 (NP_356121) and *M. loti* MAFF303099 (NP_107571). In other

cases, such as *S. meliloti* 1021, no genes encoding transport proteins are evident in the immediate vicinity of the *pca* genes. As depicted in Fig. 2, the *pcaHG* regions from the *Roseobacter* isolates did not include transport genes. In these isolates, transport genes could be associated with *pca* genes, such as *pcaB*, that are not linked to the regions characterized. In fact, *S. pomeroyi* DSS=3 has a gene adjacent to *pcaBD* that may encode a major facilitator-type transporter (www.tigr.org). The location of transport genes may further reflect the specific compound(s) that serves as a natural substrate(s) and inducer(s) of the pathway. Roseobacters can degrade diverse compounds via the protocatechuate branch of the pathway, yet regulation remains to be investigated in depth (4, 5).

Although the organization of genes within operons provides coordinated and potentially economic genetic regulation, operon organization does not tend to be conserved. Operon rearrangement appears to be selectively neutral in long-term evolution (25). However, despite extensive changes in individual operons (Fig. 4), it is remarkable that multiple transcriptional units with related function remain clustered in diverse and distantly related bacteria. Various factors have been proposed to account for the selective advantage conferred by this type of supraoperonic clustering during the course of evolution. This genetic arrangement facilitates the horizontal gene transfer of complete functional units (26) and provides the potential for multiple functionally related genes to be coamplified (39). A related topic that has received far less attention because it is difficult to address experimentally is whether the subtle variations in genetic organization are ecologically significant. By characterizing the specific arrangements of functionally related *pca* genes in different bacteria, these studies provide a first step in assessing the broader ecological implications of genetic variation for niche partitioning.

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