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Characterization of the β-Ketoadipate Pathway in *Sinorhizobium meliloti*

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Aromatic compounds represent an important source of energy for soil-dwelling organisms. The β-ketoadipate pathway is a key metabolic pathway involved in the catabolism of the aromatic compounds protocatechuate and catechol, and here we show through enzymatic analysis and mutant analysis that genes required for growth and catabolism of protocatechuate in the soil-dwelling bacterium *Sinorhizobium meliloti* are organized on the pSymB megaplasmid in two transcriptional units designated *pcaDCHGB* and *pcaIJF*. The *pcaD* promoter was mapped by primer extension, and expression from this promoter is demonstrated to be regulated by the LysR-type protein PcaQ. β-Ketoadipate succinyl-coenzyme A (CoA) transferase activity in *S. meliloti* was shown to be encoded by SMb20587 and SMb20588, and these genes have been renamed *pcaI* and *pcaJ*, respectively. These genes are organized in an operon with a putative β-ketoadipyl-CoA thiolase gene (*pcaF*), and expression of the *pcaIJF* operon is shown to be regulated by an IclR-type transcriptional regulator, SMb20586, which we have named *pcaR*. We show that *pcaR* transcription is negatively autoregulated and that PcaR is a positive regulator of *pcaIJF* expression and is required for growth of *S. meliloti* on protocatechuate as the carbon source. The characterization of the protocatechuate catabolic pathway in *S. meliloti* offers an opportunity for comparison with related species, including *Agrobacterium tumefaciens*. Differences observed between *S. meliloti* and *A. tumefaciens pcaIJ* offer the first evidence of *pca* genes that may have been acquired after speciation in these closely related species.

Aromatic acids constitute an important source of carbon and energy for soil-dwelling microorganisms and accumulate primarily as the result of the degradation of plant-derived molecules, including lignin. Many aromatic compounds may be converted to one of two common intermediates, protocatechuate or catechol, which are metabolized to tricarboxylic acid intermediates via the β-ketoadipate pathway (Fig. 1A) (18). In *Agrobacterium tumefaciens*, genes encoding enzymes involved in protocatechuate catabolism are organized into two distinct operons (36). Expression of the *pcaDCHGB* operon is induced by pathway metabolites β-carboxy-*cis*,*cis*-muconate and γ-carboxymuconolactone via the LysR-type transcriptional regulator protein PcaQ (35, 37). Genes in this operon are involved in the conversion of protocatechuate to the pathway intermediate β-ketoadipate. Expression of the *pcaIJF* operon is induced in the presence of β-ketoadipate (36), and these genes mediate the conversion of β-ketoadipate to the end products succinate and acetyl-coenzyme A (CoA). The transcriptional regulator involved in modulating expression of the *pcaIJF* operon in *A. tumefaciens* is an adjacent IclR-type regulator encoded by *pcaR* (39).

The gram-negative bacterium *Sinorhizobium meliloti* forms a symbiotic relationship with alfalfa through the establishment of root nodules. The β-ketoadipate pathway is present in many members of *Rhizobiaceae* examined to date, emphasizing the importance of aromatic acid catabolism in this family (41, 42). The publication of the *S. meliloti* genome has facilitated the identification and characterization of many metabolic pathways (10), and here we report the characterization of the protocatechuate branch of the β-ketoadipate pathway in *S. meliloti*. Except for *pcaIJ* orthologues, we demonstrate that the *pca* genes are organized, function, and are regulated in *S. meliloti* in a manner similar to that previously established for *A. tumefaciens*. Unexpectedly, the *S. meliloti* genes SMb20587 and SMb20588 were found to encode proteins with low sequence similarity to the two protein subunits of β-ketoadipate succinyl-CoA transferase (PcaI and PcaJ) in *A. tumefaciens*, *Acinetobacter baylyi* strain ADP1, and *Pseudomonas putida*. Through overexpression of SMb20587 and SMb20588 in *S. meliloti* followed by purification of β-ketoadipate succinyl-CoA transferase activity, these two genes are demonstrated to encode β-ketoadipate succinyl-CoA transferase activity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used throughout this study are described in Table 1. *Escherichia coli* was grown at 37°C in LB broth. *Sinorhizobium meliloti* was grown at 30°C in M9 minimal medium (Difco) or LB broth supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LBmc). M9 minimal medium was supplemented with 1.0 mM $MgSO_4$, 0.25 mM $CaCl_2$, 1 μg/ml D-biotin, and 10 ng/ml $CoCl_2$. Unless otherwise specified, carbon sources were added to M9 minimal medium as follows: 0.5% (vol/vol) glycerol, 15 mM arabinose, 30 mM adipate (Sigma-Aldrich), or 5 mM protocatechuate (Sigma-Aldrich). For *S. meliloti*, antibiotics were used at the following concentrations (in μg/ml): streptomycin, 200; neomycin, 200; gentamicin, 60; spectinomycin, 200; tetracycline, 5.

Transposon mutagenesis. Tn*5* mutagenesis of *S. meliloti* was performed by mating the suicide plasmid pRK602 into wild-type derivative strain Rm1021. Neomycin-resistant colonies were patched onto M9 minimal media with protocatechuate or glucose as the sole carbon source. Mutants unable to grow on protocatechuate (Pca^-) were examined for ability to grow with succinate as a sole carbon and energy source to eliminate mutants deficient in succinate metabolism.

The pLAFR1 clone bank of *S. meliloti* Rm1021 DNA (12) was screened to isolate clones capable of complementing Pca^- strains. Spot matings were per-

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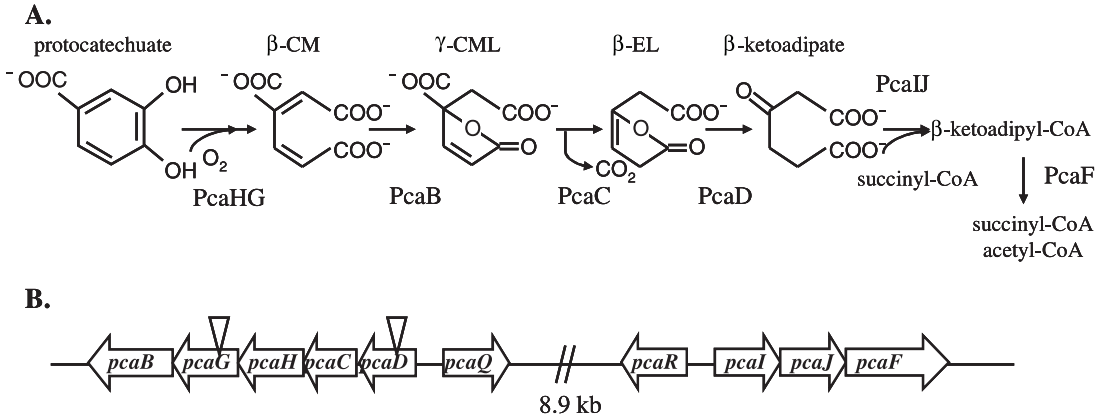


FIG. 1. Protocatechuate catabolism in *S. meliloti*. (A) The protocatechuate branch of the β-ketoadipate pathway is involved in the catabolism of protocatechuate to intermediates which are funneled into the tricarboxylic acid cycle. β-CM, β-carboxy-*cis*,*cis*-muconate; γ-CML, γ-carboxymuconolactone; β-EL, β-ketoadipate enol-lactone. (B) Schematic depiction of the *pca* genes on the pSymB megaplasmid. Inverted triangles indicate the locations of transposon insertions in *S. meliloti* strains RmG879 (left triangle) and RmG867 (right triangle).

formed with Pca$^-$ mutants, using the clone bank, and strains carrying the complementing cosmids were selected on M9 minimal media with protocatechuate as a sole carbon source. DNA sequencing was provided by Mobix (McMaster University, Hamilton, Ontario, Canada).

Rothera test. An overnight LBmc culture was centrifuged and washed with M9 minimal medium. Cells were subcultured into M9 minimal medium supplemented with 0.1% arabinose and 5 mM protocatechuate for overnight incubation at 30°C. Cells were centrifuged and resuspended in 0.02 M Tris-HCl, pH 8.0, to an optical density (OD) of 1.0. Toluene (0.5 ml) was added to 2 ml resuspended cells, which was incubated at 30°C with shaking for 1 h. $(NH_4)_2SO_4$ (1 g) was added, and the mixture was vortexed. One drop of a fresh aqueous sodium nitroprusside (1%) solution was added, followed by the addition of 1 drop of concentrated NH_3 (29%), and the mixture was vortexed. Development of a purple color within 5 min following the addition of NH_3 was considered a positive test for the presence of β-ketoadipate (20).

Protocatechuate 3,4-dioxygenase activity assays. Overnight cultures grown in LBmc were washed and subcultured into M9 minimal medium supplemented with 0.1% arabinose and 5 mM protocatechuate. Tetracycline was included in the growth medium for strains carrying pTH178. Upon harvest, cells were centrifuged, washed, and resuspended into 4 ml buffer (20 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.8) per gram cells. Aliquots of cells were frozen at −80°C until used in the assay.

Prior to use, 10 μl of 0.1 M dithiothreitol was added (per ml) as the aliquots thawed on ice. Cells were disrupted via sonication, and extracts were centrifuged to remove intact cells and cellular debris. The dioxygenase assay was performed as previously described (8), with the following modifications. The temperature of the assay was maintained at 30°C, and the reaction was monitored by following the reduction in absorbance at 293 nm using a Contron Uvikon 930 double-beam spectrophotometer. Protein concentrations were determined using Bio-Rad protein assay reagent, with bovine serum albumin as the standard.

Construction of an *S. meliloti pcaQ*::Ω strain. An Ω cassette encoding gentamicin resistance from pHP45Ω*aac* (3) was introduced into a PstI site located 35 bp downstream of the predicted *pcaQ* translational start site as follows. An 893-bp fragment centered upon the PstI site was PCR amplified using *S. meliloti* Rm1021 genomic DNA as a template. This fragment was cloned into the suicide vector pJQ200 uc1 (44) via NotI to create plasmid pTH1577. The Ω cassette was PCR amplified and cloned into pTH1577 via PstI, yielding pTH1592. The NotI fragment, encompassing *pcaQ*::Ω, was subcloned from pTH1592 into a derivative of pVO155 (30) to create pTH1882. This pVO155 derivative (pTH1883) lacks the *gusA* reporter gene present in the parental vector and was selected for use because it is unable to replicate in *S. meliloti* and carries a gene specifying neomycin resistance. pTH1882 was mated into *S. meliloti lac* strain RmG212, and recombinants were selected for by plating onto LB agar supplemented with streptomycin plus gentamicin. Colonies were patched onto LB agar plus neomycin to identify recombinants in which the suicide vector had recombined out of the genome, leaving the Ω cassette behind. Southern hybridization was performed on DNA extracted from Gm^r Nm^s recombinants to confirm the location of the antibiotic cassette and absence of the suicide vector. In this case, an EcoRV digest of genomic DNA isolated from putative RmG212 *pcaQ*::Ω mutants and RmG212 was hybridized with a labeled (random primed DNA labeling kit; Roche) probe encompassing the *pcaQ* PstI site. A shift corresponding to an ~2-kb increase (compared to the wild type) was noted in the putative *pcaQ* mutants, consistent with the incorporation of the 1.8-kb Gm^r cassette into the PstI site and the subsequent excision of the integrating vector. As well, hybridization of the EcoRV genomic DNA digest with labeled probe corresponding to the (Gm^r) Ω cassette indicated the presence of the antibiotic cassette within the shifted bands observed in the putative mutants. The Ω probe failed to hybridize with the EcoRV-digested RmG212 genomic DNA.

Construction of an *S. meliloti pcaR*::Ω strain. An Ω cassette encoding streptomycin/spectinomycin resistance from pHP45Ω (43) was introduced into a SacII site located 76 bp downstream of the predicted *pcaR* translational start site. A 963-bp fragment encompassing the SacII site within *pcaR* was PCR amplified using *S. meliloti* Rm1021 DNA as a template and Vent DNA polymerase (New England Biolabs). This blunt-ended fragment was cloned into pUC119 at a SmaI site to create plasmid pTH1338. The Ω cassette was PCR amplified and cloned into the SacII site in pTH1338 to produce pTH1340. A NotI fragment, encompassing *pcaR*::Ω, was subcloned from pTH1340 into the suicide vector pJQ200 to yield pTH1351. This plasmid was mated into *S. meliloti* Rm1021 and recombinants were selected for by plating onto LB agar supplemented with streptomycin plus gentamicin. A single Gm^r colony was inoculated into LBmc and grown in the absence of antibiotic selection. The overnight culture was plated onto LB agar supplemented with 5% sucrose and spectinomycin. Sp^r colonies were patched onto LB agar plus gentamicin to confirm excision of the suicide plasmid. Southern hybridization was performed on DNA extracted from Sp^r Gm^s sucrose-resistant colonies to confirm the location of the Ω cassette and verify the loss of the integrating plasmid. Briefly, separate XhoI and SalI digests of genomic DNA isolated from putative Rm1021 *pcaR*::Ω mutants and Rm1021 were hybridized with a labeled (random primed DNA labeling kit; Roche) probe encompassing the *pcaR* SacII site. In each case, a shift corresponding to an ~2-kb increase (compared to the wild type) was noted in the putative *pcaR* mutants, consistent with the incorporation of the 2.1-kb antibiotic cassette into the SacII site and the excision of the integrating vector. As well, hybridization of separate XhoI and SalI genomic DNA digests with labeled probe corresponding to the (Sm^r Sp^r) Ω cassette indicated the presence of the antibiotic cassette within the shifted bands observed in the putative mutants. The Ω probe failed to hybridize with either SalI- or XhoI-digested Rm1021 genomic DNA.

Construction of an *S. meliloti pcaF*::*gusA* strain. A transcriptional fusion between the annotated *pcaF* gene on the pSymB megaplasmid and a promoterless *gusA* gene was created in *S. meliloti* wild-type derivative Rm1021 and PcaR$^-$ strain RmK1014. The fusion was designed such that *pcaF* was not disrupted, and this was verified by growth with protocatechuate as a sole carbon source. A 431-bp fragment spanning the 3′ end of *pcaF* was PCR amplified and cloned into the pVO155 (30) derivative pTH1360 to create pTH1559. In pTH1360, the original *gusA* reporter gene present in pVO155 has been replaced by the *gusA* gene present in pFus1, which has a superior ribosome binding site and is expressed more efficiently than its pVO155 counterpart (R. Zaheer and T. M. Finan, unpublished data). pTH1559 was mated into Rm1021 and RmK1014, and

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
S. meliloti		
Rm1021	Sm^r derivative of wild-type strain SU47	28
Rm5004	Rm1021 *recA*::Tn*5* Sm^r Nm^r	Strain collection
RmG212	Rm1021 *lac* Sm^r	Strain collection
RmG867	Rm1021 *pcaD*::Tn*5* Sm^r Nm^r	This study
RmG879	Rm1021 *pcaG*::Tn*5* Sm^r Nm^r	This study
RmK927	Rm5004 (pTH1459) Sm^r Tc^r	This study
RmK948	RmG212 *pcaG*::Tn*5* Sm^r Nm^r	This study
RmK1014	Rm1021 *pcaR*::Ω Sm^r Sp^r	This study
RmK1015	Rm1021 *pcaF*::*gusA* Sm^r Nm^r	This study
RmK1016	Rm1021 *pcaF*::*gusA pcaR*::Ω Sm^r Nm^r Sp^r	This study
RmP134	RmG212 *pcaQ*::Ω Sm^r Gm^r	This study
RmP135	RmG212 (pTH468) Sm^r Tc^r	This study
RmP136	RmP134 (pTH468) Sm^r Gm^r Tc^r	This study
RmP892	Rm1021 (pTH1335) Sm^r Tc^r	This study
RmP893	RmK1014 (pTH1335) Sm^r Tc^r	This study
RmP894	RmK948 (pTH468) Sm^r Nm^r Tc^r	This study
E. coli		
DH5α	F^- ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44* λ^- *thi-1 gyrA96 relA1*	Bethesda Research Laboratories, Inc.
BL21	*E. coli* B F^- *ompT hsdS*$_B$(r_B^- m_B^-) *dcm* Tet^r *gal* λ(DE3) *endA* Hte [*argU proL* Cam^r]	Stratagene
Plasmids		
pLAFR1	IncP cosmid cloning vector; Tc^r	12
pFus1	Broad-host-range *gusA* transcriptional reporter plasmid; Tc^r	45
pMP220	Broad-host-range *lacZ* transcriptional reporter plasmid; Tc^r	48
pHP45Ω	pBR322 derivative with Ω element; Ap^r Sp^r Sm^r	43
pHP45Ω*aac*	pBR322 derivative with Ω element; Ap^r Gm^r	3
pRK602	pRK600Ω::Tn*5*, suicide vector used in transposon mutagenesis of Rm1021; Cm^r Nm^r	9
pJQ200 uc1	Suicide vector with *sacB* to select for plasmid excision; Gm^r	44
pVO155	Suicide vector with promoterless *gusA*; Ap^r Nm^r	30
pTH178	pLAFR1 derivative complementing Pca^- phenotype of RmG867 and RmG879; Tc^r	This study
pTH468	434-bp EcoRI-XbaI PCR product encompassing *pcaD-pcaQ* intergenic region in pMP220 (*pcaD*::*lacZ*); Tc^r	This study
pTH1227	Broad-host-range derivative of pFus1 with P_{tac} promoter inserted upstream of *gusA*; Tc^r	J. Cheng and T. M. Finan, unpublished data
pTH1335	153-bp EcoRI-PstI PCR product encompassing *pcaR-pcaI* intergenic region in pFus1 (*pcaR*::*gusA*); Tc^r	This study
pTH1338	963-bp blunt-ended PCR product encompassing SacII site within *pcaR*, cloned into pUC119 via SmaI; Ap^r	This study
pTH1340	ΩSm/Sp^r from pHP45Ω into pTH1338 via SacII; Ap^r Sm^r Sp^r	This study
pTH1351	*pcaR*::Ω from pTH1340 into pJQ200 uc1 via NotI; Sm^r Sp^r Gm^r	This study
pTH1360	pVO155 derivative with *gusA* from pFus1; Ap^r Nm^r	R. Zaheer and T. M. Finan, unpublished data
pTH1459	1,701-bp EcoRI-PstI PCR product encompassing *pcaIJ* in expression vector pTH1227; Tc^r	This study
pTH1559	431-bp SpeI-XbaI PCR product encompassing 3′ end of *pcaF* into pTH1360 (*pcaF*::*gusA*); Ap^r	This study
pTH1577	893-bp NotI PCR product encompassing PstI site within *pcaQ* cloned into pJQ200 uc1; Gm^r	This study
pTH1592	ΩGm^r from pHP45Ω into pTH1577 via PstI site; Gm^r	This study
pTH1882	*pcaQ*::Ω from pTH1592 into pTH1883 via NotI; Ap^r Nm^r Gm^r	This study
pTH1883	pTH1360 with *gusA* removed via NotI digest; Ap^r Nm^r	This study

recombination of the vector into the *S. meliloti* genome was selected by plating cells onto LB agar supplemented with streptomycin plus neomycin.

Purification of β-ketoadipate succinyl-CoA transferase activity in *S. meliloti*. β-Ketoadipate succinyl-CoA transferase was purified according to the method of Kaschabek et al. (24), with some modifications. An overnight culture of RmK927 was subcultured into 4 liters LBmc supplemented with tetracycline, and cells were grown with shaking at 30°C. Expression of genes SMb20587 and SMb20588 was induced at an OD of 0.3 to 0.4 with the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 5 mM protocatechuate. After 4 h of induction, cells were harvested in the late exponential growth phase (OD of 1.2 to 1.4). The pellet was resuspended in 30 ml buffer (100 mM Tris-HCl, 0.5 mM dithiothreitol, pH 7.0) and then lysed via five passages through a French pressure cell at 110 MPa. The cell extract was cleared by centrifugation at 100,000 × *g* for 60 min. Solid $(NH_4)_2SO_4$ was added to give 75% saturation, and the precipitate was collected by centrifugation at 8,000 × *g* for 20 min. The pellet was redissolved in buffer B1 [50 mM Tris-HCl, 1 M $(NH_4)_2SO_4$, 1 mM EDTA, pH 7.0], and the extract was cleared by centrifugation. The supernatant was loaded onto a phenyl Sepharose CL-4B (Amersham Biosciences) column preequilibrated with buffer B1, and protein was eluted in a linear gradient of $(NH_4)_2SO_4$ from 1 to 0 M at a flow rate of 0.3 ml/min. Nine 2-ml fractions with β-ketoadipate succinyl-CoA transferase activity were pooled and dialyzed into buffer A1 (50 mM Tris-HCl, 0.5 mM EDTA, pH 7.0).

The dialyzed fractions were loaded onto a Source 30Q (Amersham Biosciences) column preequilibrated with buffer A1, and protein was eluted in a

linear gradient of NaCl from 0 to 1 M at a flow rate of 0.3 ml/min. Twelve 0.5-ml fractions with β-ketoadipate succinyl-CoA transferase activity were pooled and dialyzed into 5 mM potassium phosphate, pH 7.0.

The fractions were loaded onto a CHT ceramic hydroxyapatite (Bio-Rad) column preequilibrated with 5 mM potassium phosphate, pH 7.0. Elution occurred in a linear gradient of potassium phosphate from 10 to 400 mM at a flow rate of 0.3 ml/min. Twelve 1-ml fractions with β-ketoadipate succinyl-CoA transferase activity were collected and pooled. An attempt was made to concentrate the enzyme within the pooled extract by using Nanosep microconcentrators; however, the majority of the purified enzyme was lost at this step, as revealed by subsequent β-ketoadipate succinyl-CoA transferase assays and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie brilliant blue.

β-Ketoadipate succinyl-CoA transferase assays. β-Ketoadipate succinyl-CoA transferase assays were performed as previously described (54), with the following modifications. Briefly, the enzyme assays were performed with UV-Star (flat-bottomed) 96-well microtiter plates (Greiner Bio-One) and a Safire microplate reader (Tecan). To start the reaction, protein samples were added to a buffered reaction mixture (200 mM Tris-HCl, 40 mM $MgCl_2$, 10 mM β-ketoadipate [Sigma-Aldrich], 0.4 mM succinyl-CoA, pH 8.0) to a final volume of 0.2 ml (path length, 0.52 cm). The formation of β-ketoadipyl-CoA:Mg^{2+} was monitored at 305 nm over a temperature range of 22 to 23°C. An extinction coefficient of 16,300 M^{-1} cm^{-1} was used to calculate the formation of the β-ketoadipyl-CoA:Mg^{2+} complex (24). One unit of activity is defined as the amount of enzyme required to convert 1 μmol of substrate to product in 1 min under the conditions of the assay.

Protein identification via mass spectrometry. Mass spectrometry analyses were provided by the McMaster Regional Centre for Mass Spectrometry (McMaster University, Hamilton, Ontario, Canada). The following peptides were used in the identification of the proteins: with a molecular mass of 37.6 kDa, peptides R.NGNVLIEGIVGVQK.E, R.MTPDILYDQLIGVGAAR.G, and R.IMSLAEAVEENVR.D; with a molecular mass of 35.4 kDa, peptides R.NGNVLIEGIVGVQK.E and R.MTPDILYDQLIGVGAAR.G; and with a molecular mass of 28.5 kDa, peptides R.FANLNTTVVGPYDHPK.V, K.FAETVIETPAPTETELVVLR.D, and R.ITTGFLGGAQIDR.F.

Isolation of total RNA from *S. meliloti*. Cultures were grown with shaking at 30°C in LBmc ± 5 mM protocatechuate to an OD of 0.6 to 0.7. Total RNA was isolated from *S. meliloti* Rm1021 by use of a hot phenol method as previously described (27).

Primer extension. Primer extension reactions were performed using 50 μg of total *S. meliloti* RNA as previously described (27). The following primers were used for extension reactions: for the identification of the *pcaD* start site, 5′ GAAATCCGTGCCGAGCGAGTTGATGAAGAC 3′, and for the identification of the *pcaI* start site, 5′ GAGAGACATTATCCGCGCCATCG 3′ and 5′ CGTCTCTGACATTCTCCTCTACCG 3′. Sequencing reactions were performed using a Sequenase version 2.0 DNA sequencing kit (USB). The same primer was used in sequencing and primer extension reactions.

β-Galactosidase enzyme assays. *S. meliloti* cultures were grown overnight at 30°C in LBmc and washed with 0.85% saline prior to subculture. Cells were subcultured into M9 minimal media supplemented with a carbon source as indicated and grown with shaking at 30°C for 4 h. Aliquots (50 to 200 μl) of cells were added directly to Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 40 mM, β-mercaptoethanol, pH 7.0) with 5% chloroform and 0.0025% SDS. The reaction was started with the addition of 200 μl 2-nitrophenyl β-D-galactopyranoside (ONPG) (4 mg/ml) and stopped upon addition of 500 μl 1 M Na_2CO_3. β-Galactosidase activities were calculated according to the method of Miller (29).

Enzyme assays were performed using derivatives of RmG212 (Rm1021 *lac*) to reduce background LacZ enzyme activity. RmK948 was created through the transduction of *pcaG*::Tn*5* into RmG212, using RmG879 as a donor strain.

β-Glucuronidase enzyme assays. Overnight LBmc cultures of *S. meliloti* were grown and washed with 0.85% saline. Cells were subcultured into M9 minimal media supplemented with a carbon source as indicated and grown with shaking at 30°C for 4 h. Cultures were centrifuged and resuspended into a buffer consisting of 50 mM sodium phosphate, 50 mM dithiothreitol, and 1 mM EDTA, pH 7.0. Enzyme assays were performed according to the method of Reeve et al. (45), with the exception that assays were performed at room temperature (20 to 22°C).

RESULTS AND DISCUSSION

Isolation and characterization of *pca* Rm1021 mutants. To directly identify genes involved in protocatechuate catabolism in *S. meliloti*, we screened a transposon Tn*5* insertion library for mutants able to grow with succinate but unable to grow with protocatechuate (Pca^-) as a sole carbon source. The precise insertion sites of the transposon in the *S. meliloti* genome were determined by DNA sequencing (see Materials and Methods). Strains RmG867 and RmG879 were found to carry Tn*5* within genes annotated to encode β-ketoadipate enol-lactone hydrolase (PcaD) and protocatechuate 3,4-dioxygenase alpha-subunit (PcaG), respectively. Analysis of the *S. meliloti* genome sequence suggests that *pcaD* and *pcaG* are organized in an operon with a γ-carboxymuconolactone decarboxylase (*pcaC*), protocatechuate 3,4-dioxygenase β-subunit (*pcaH*), and β-carboxy-*cis*,*cis*-muconate cycloisomerase (*pcaB*) (Fig. 1B) (10).

To determine whether β-ketoadipate accumulated from protocatechuate metabolism in either the *pcaD* or the *pcaG* mutant, we performed the Rothera test, which detects the presence of β-ketoadipate and thus indicates whether protocatechuate has been metabolized to this pathway intermediate. The wild-type strain Rm1021 exhibited a Rothera-positive phenotype, whereas the mutants RmG867(*pcaD*) and RmG879(*pcaG*) were Rothera negative, indicating that these strains failed to metabolize protocatechuate to β-ketoadipate (Fig. 1A). Protocatechuate 3,4-dioxygenase catalyzes the first step in the degradation of protocatechuate, the conversion of protocatechuate to β-carboxy-*cis*,*cis*-muconate (Fig. 1A) (31). Examination of *S. meliloti* extracts from cultures grown in minimal medium containing arabinose and protocatechuate revealed that protocatechuate 3,4-dioxygenase activity was readily detected in the parent strain, Rm1021, whereas no activity was detected in RmG867(*pcaD*) and RmG879(*pcaG*) (data not shown). Since the dioxygenase consists of protein subunits encoded by two genes (*pcaHG*), disruption of either of these genes (as in RmG879) would result in a corresponding loss of protocatechuate 3,4-dioxygenase activity. The enzyme β-ketoadipate enol-lactone hydrolase (PcaD) mediates the conversion of β-ketoadipate enol-lactone to β-ketoadipate (31), and a mutation in *pcaD* (as in RmG867) would block this step of the pathway, preventing the production of β-ketoadipate (Fig. 1A). Organization of the *pcaDCHGB* operon ensures that insertion of a transposon within *pcaD* would also disrupt expression of downstream *pca* genes, including *pcaHG* (Fig. 1B). The loss of protocatechuate 3,4-dioxygenase activity in RmG867(*pcaD*) presumably results from the polar nature of the mutation in this strain and supports the assumption that the genes in question are organized as a single transcriptional unit.

An *S. meliloti* Rm1021 pLAFR1 clone (12), pTH178, was isolated on the basis of its ability to complement the Pca^- phenotype of the RmG867 and RmG879 mutants. The presence of the pTH178 plasmid in strains RmG867 and RmG879 restored a Rothera-positive phenotype and protocatechuate 3,4-dioxygenase activity to the RmG867 and RmG879 mutant strains (data not shown). Consistent with its ability to complement *pcaD* and *pcaG* mutant phenotypes, DNA sequencing of pTH178 revealed that this cosmid carries the *pcaDCHGB* region in its entirety.

Regulation of expression of the *pcaDCHGB* operon. In *S. meliloti*, the *pcaDCHGB* operon is located adjacent to, and transcribed divergently from, a gene encoding a product with

TABLE 2. Expression of *pcaD-lacZ* fusion in *S. meliloti* strains

Strain	Relevant genotype	Growth condition	β-Galactosidase activity[a] (Miller units [SD])
RmP135	Rm1021 *lac* (pTH468)	Glycerol	590 (18.5)
		Glycerol plus PCA[b]	8,210 (288.6)
RmP136	Rm1021 *lac pcaQ*::Ω (pTH468)	Glycerol	530 (39.6)
		Glycerol plus PCA	311 (8.6)
RmP894	Rm1021 *lac pcaG*::Tn*5* (pTH468)	Glycerol	468 (36.3)
		Glycerol plus PCA	550 (20.2)

[a] Shown are averages of values obtained from three independent cultures grown overnight in LBmc and subcultured into M9 minimal medium with 0.5% glycerol ± 5 mM protocatechuate at 30°C for 4 h.
[b] PCA, protocatechuate.

similarity to the *A. tumefaciens* LysR-type transcriptional regulator PcaQ. To examine the possible involvement of PcaQ in regulating *pcaDCHGB* expression, an *S. meliloti* interposon knockout mutant of *pcaQ* was constructed. The *pcaQ* mutant strain was unable to utilize protocatechuate as a sole carbon source; however, growth of the mutant strain was comparable to that of the wild type in media containing glucose or glycerol as the carbon source (data not shown).

To monitor expression from the *pcaD* promoter, the *pcaD-pcaQ* intergenic region was cloned into pMP220 (48), with the *pcaD* promoter in the same orientation as a promoterless *lacZ* reporter gene. The resulting replicating plasmid, pTH468, was conjugated into RmG212 (*lac* mutant Rm1021 derivative), RmP134 (*pcaQ*::Ω derivative of RmG212), and RmK948 (*pcaG*::Tn*5* derivative of RmG212), a strain that is incapable of metabolizing protocatechuate to β-carboxy-*cis*,*cis*-muconate. Expression of *pcaD* (as measured by β-galactosidase activity) was examined following growth in minimal medium containing glycerol as a carbon source and in medium containing glycerol plus protocatechuate. In the wild-type background, expression of *pcaD* was induced greater than 10-fold in the presence of protocatechuate, while *pcaD* expression did not increase under similar growth conditions in RmP134(*pcaQ*) (Table 2). This suggests that a product encoded by *pcaQ* is required for induction of *pcaD* expression. The low level of expression of *pcaD* in both the uninduced wild-type and *pcaQ* mutant backgrounds suggests that PcaQ does not act as a repressor of *pcaD* transcription. Expression of *pcaD* was also not induced in the *pcaG* mutant background (RmK948), and this suggests that protocatechuate itself does not act as an inducing agent. Presumably, an intermediate in the β-ketoadipate pathway is required to induce *pcaDCHGB*. Given the similarity that exists in genetic organization and regulation of the β-ketoadipate pathways in *S. meliloti* and *A. tumefaciens* (38), it is likely that β-carboxy-*cis*,*cis*-muconate and γ-carboxymuconolactone serve as coinducers in the PcaQ-regulated expression of the *pcaDCHGB* operon in *S. meliloti*; however, this matter was not examined further.

Identification of *pcaDCHGB* transcriptional start sites. Primer extension analysis was performed on RNA isolated from *S. meliloti* strain Rm1021 grown in LBmc in the presence and absence of protocatechuate prior to RNA isolation. A 30-mer oligonucleotide complementary to the 5′ *pcaD* coding region was used to prime the extension reaction. Two major extension products were obtained only with template RNA isolated from cells grown in the presence of protocatechuate, indicating transcriptional start sites at G and C residues located 14 and 15 nucleotides upstream of the predicted *pcaD* translational start site, respectively (Fig. 2A).

The sequence upstream of the *pcaD* transcriptional start sites has AT-rich sequences centered at −10 and −35 hexanucleotide regions (Fig. 2B). Alignment of the sequences upstream of the *pcaD* genes from *S. meliloti* and *A. tumefaciens* with the region upstream of the annotated *Mesorhizobium loti pcaD* gene indicates that these regions are conserved among the three species (Fig. 2B). However, the *M. loti* sequence that corresponds to the inferred *S. meliloti* −10 hexanucleotide includes the annotated *M. loti pcaD* translational start site. Alignment of the PcaD amino acid sequences from *S. meliloti*, *A. tumefaciens*, and *M. loti* reveals that the *M. loti* protein consists of an additional 8 amino acids at the N terminus that are absent in the other two species (Fig. 2C). These results suggest that the correct start codon for *pcaD* in *M. loti* is located 24 bp downstream of the annotated site. Interestingly, this would mean that the *M. loti* gene employs a GTG translational start codon, as is predicted for *pcaD* in *S. meliloti*.

Examination of the *pcaD* promoter region also reveals potential PcaQ binding sites, several of which have also been conserved among *S. meliloti*, *A. tumefaciens*, and *M. loti*. As a member of the LysR family of transcriptional regulators, PcaQ may be expected to recognize and bind elements established upon a (T-N_{11}-A) consensus binding motif (16, 47). Examination of the *pcaD-pcaQ* intergenic region from *S. meliloti* revealed seven T-N_{11}-A motifs. Of these, two T-N-A-N_9-A motifs span the −35 region in *S. meliloti* and are conserved in *A. tumefaciens* and *M. loti* (Fig. 2B). As *pcaD* and *pcaQ* are separated by a 94-bp intergenic region, it is possible that binding of PcaQ to a given site(s) may simultaneously exert positive and negative effects with respect to the expression of *pcaD* and *pcaQ*, respectively, as has been shown for the *crgA-mdaB* genes in *Neisseria meningitidis* (6, 21). PcaQ-mediated autoregulation has been described previously for *A. tumefaciens* (37), and we similarly have evidence of autoregulation in *S. meliloti* (data not shown). This work is being pursued using purified PcaQ to facilitate the identification of PcaQ binding sites.

Identification and purification of PcaIJ. Genes encoding the β-ketoadipate succinyl-CoA transferase proteins (PcaIJ) have not been identified in the *S. meliloti* genome (13). Two genes (SMb20587 and SMb20588) are annotated as encoding subunits of a CoA transferase, and these are located approximately 10 kb from the *pcaDCHGB* operon. The SMb20587 and SMb20588 genes lie upstream of the *pcaF* gene, annotated to encode β-ketoadipyl-CoA thiolase. The low amino acid sequence similarity of SMb20587 and SMb20588 with other PcaIJ proteins prevented their annotation as PcaIJ orthologues.

To establish whether SMb20587 and SMb20588 encode β-ketoadipate succinyl-CoA transferase activity, these genes were cloned into pTH1227, an IPTG-inducible expression vector carrying the *tac* promoter, to give pTH1459. *S. meliloti* (pTH1459) cells were induced with both protocatechuate and

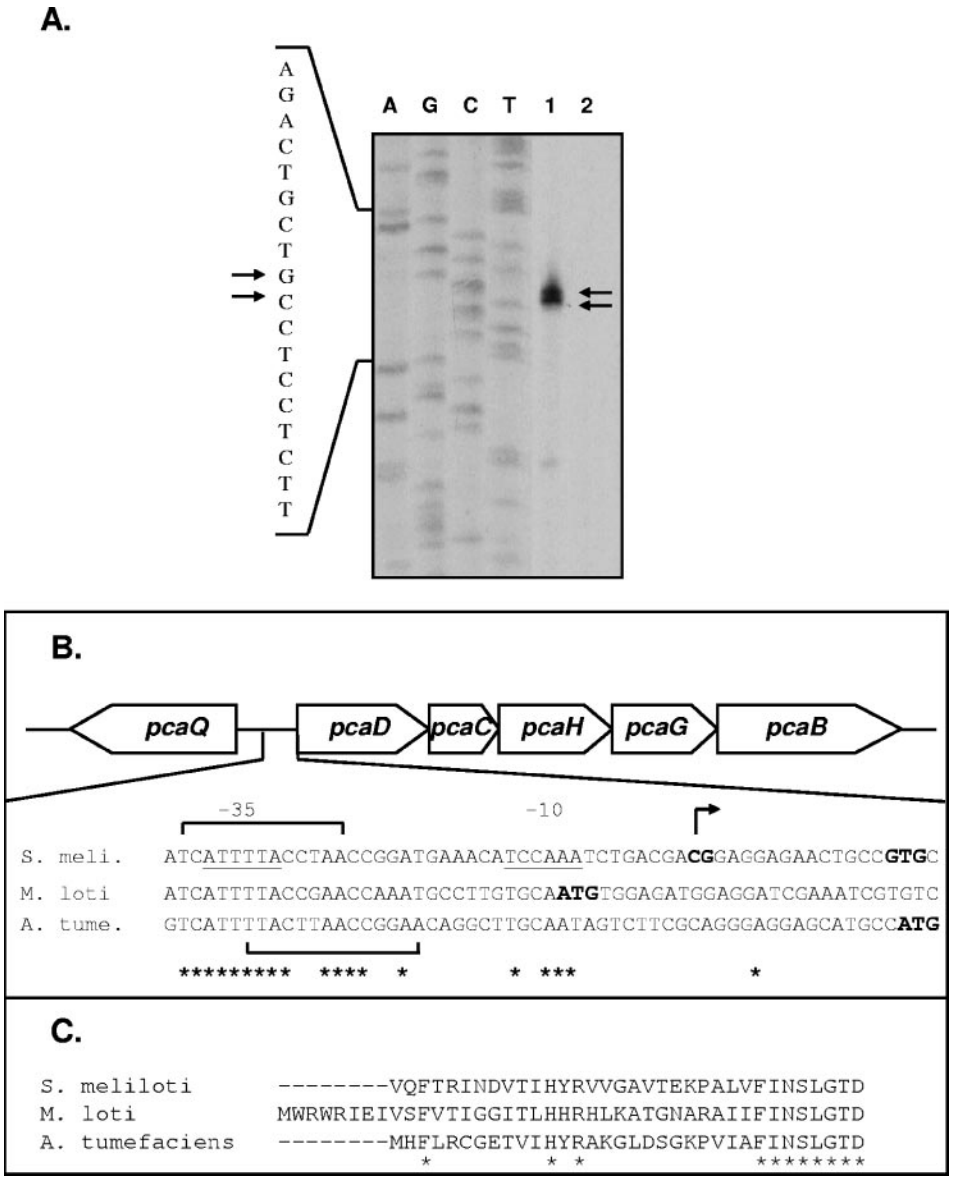


FIG. 2. Analysis of the *pcaD* promoter in *S. meliloti*. (A) Primer extension reactions were performed using RNA isolated from *S. meliloti* strain Rm1021 grown in the presence (lane 1) and absence (lane 2) of 5 mM protocatechuate. Sequencing reactions were performed with the same primer used in primer extension reactions, and results are shown to the left of the extension products. The arrows indicate the extension products (right) and identify the corresponding nucleotides (left). (B) Schematic depiction of the *pcaQ-pcaDCHGB* genes, including an alignment of the *S. meliloti* (*S. meli*.) *pcaDp* region with the sequence upstream of *pcaD* in *M. loti* and *A. tumefaciens* (*A. tume*.) (GenBank accession numbers NC_003078, NC_002678, and NC_003305, respectively). The two *S. meliloti pcaD* transcriptional start sites are identified in bold, and the inferred −10 and −35 regions are underlined. Nucleotides conserved among all three species are indicated by an asterisk, and predicted translational start sites are indicated (bold, enlarged font). Two putative PcaQ binding sites present in *S. meliloti*, *A. tumefaciens*, and *M. loti* are indicated by brackets. (C) Alignment of PcaD amino acid sequences (N terminus), as annotated in *S. meliloti*, *M. loti*, and *A. tumefaciens* genome sequences. Invariant residues are indicated by an asterisk. Sequences were aligned using CLUSTAL W (50).

IPTG. Whole-cell lysate obtained from induced cultures exhibited β-ketoadipate succinyl-CoA transferase activity as detected spectrophotometrically by the increase in absorbance at 305 nm that accompanies formation of the β-ketoadipyl-CoA: Mg^{2+} complex (see Materials and Methods). This activity was sequentially purified to near homogeneity by use of a combination of ammonium sulfate precipitation and chromatography with columns containing phenyl Sepharose CL-4B, Source 30Q, and CHT ceramic hydroxyapatite (see Materials and Methods for details) (Fig. 3). SDS-PAGE analysis demonstrated the presence of a 28-kDa band that was consistent with the predicted size (27.7 kDa) of the β-subunit encoded by SMb20588. A second, higher-molecular-mass protein was present as a doublet of 36- and 38-kDa bands, which we thought could be an anomalously migrating species of the alpha-subunit (predicted size, 31.2 kDa) encoded by SMb20587. To positively identify these polypeptide species, all three bands were subjected to a trypsin digest and tandem

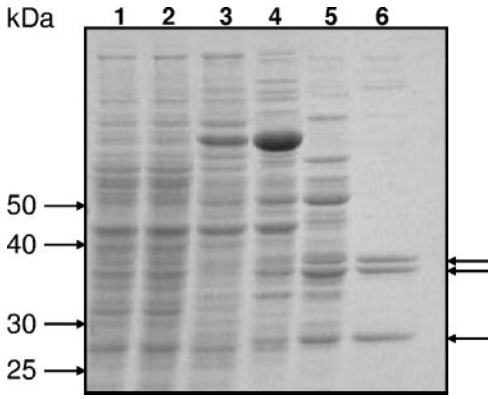


FIG. 3. Purification of β-ketoadipate succinyl-CoA transferase in *S. meliloti*. *S. meliloti* Rm5004 carrying pTH1459 was grown in M9 minimal medium with 5 mM protocatechuate and 1 mM IPTG. Protein samples were subjected to SDS-PAGE in a 10% gel followed by staining with Coomassie brilliant blue. Lane 1, crude cell lysate derived from uninduced culture; lane 2, crude cell lysate obtained from cells induced by 1 mM IPTG and 5 mM protocatechuate; lane 3, ammonium sulfate precipitate of induced cell lysate; lane 4, pooled eluate from phenyl Sepharose column; lane 5, pooled eluate from Source 30Q (anion-exchange) column; lane 6, pooled eluate from hydroxyapatite column. Molecular masses are shown on the left. Arrows (right) indicate the proteins identified by mass spectrometry. The 36- and 38-kDa proteins were identified as the alpha-subunits of a CoA transferase encoded by gene SMb20587; the 28-kDa protein was identified as the β-subunit of a CoA transferase encoded by gene SMb20588.

mass spectrometry. The 28-kDa protein was confirmed as the β-subunit of a CoA transferase encoded by gene SMB20588. Both the 36- and 38-kDa proteins were identified as the alpha-subunits of a CoA transferase encoded by gene SMB20587. We have not explored why the alpha-subunit is expressed as a doublet, but it may be because expression was induced both from the pSymB megaplasmid (using protocatechuate as an inducer) and from an IPTG-inducible expression vector. Possibly, an alternative start codon was used in translation from the expression vector. Examination of the nucleotide sequence encoding the alpha-subunit revealed two potential in-frame start codons downstream of the annotated start site. Translation initiation from these alternative start sites would generate proteins differing by either 4 or 22 amino acids relative to the full-length protein. The difference in molecular mass between the doublet proteins as estimated from SDS-PAGE is ~2 kDa (or approximately 20 amino acids), and this difference might therefore be explained by the use of two distinct translational start sites. In any case, polypeptides encoded by SMb20587 and SMb20588 are enriched to near homogeneity in a protein sample purified solely on the basis of β-ketoadipate succinyl-CoA transferase activity.

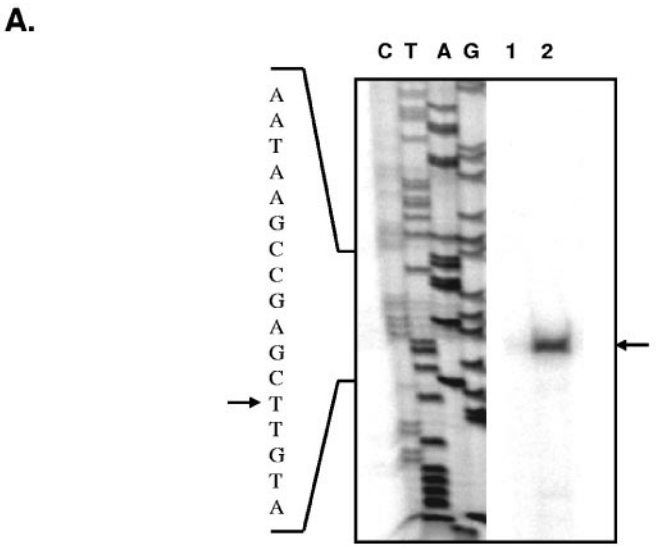
The transfer of CoA to β-ketoadipate has been documented previously as resulting from the nonspecific activity of an adipate succinyl-CoA transferase (5, 19). To eliminate the possibility that an adipate succinyl-CoA transferase had been purified inadvertently, we performed enzyme assays with β-ketoadipate and with increasing concentrations of adipate to examine substrate specificity (Table 3). Addition of equimolar amounts of adipate did not result in a significant decrease in enzyme activity (7% decrease in activity); however, a fivefold-greater concentration of adipate (relative to β-ketoadipate) in a reaction reduced activity by 56%. These results indicate that although adipate might compete with β-ketoadipate as a substrate when present in a greater concentration, β-ketoadipate is the preferred substrate of this enzyme. Enzyme activity was also dependent upon the presence of β-ketoadipate, succinyl-CoA, and Mg^{2+}, and omission of any one of these reagents from the reaction mixture abolished activity (<0.01 μmol/min/mg) (data not shown). We therefore concluded that SMb20587 and SMb20588 encode subunits of a β-ketoadipate succinyl-CoA transferase, and these genes were named *pcaI* and *pcaJ*, respectively.

The β-ketoadipate pathway is composed of protocatechuate and catechol branches, and either *pca* or *cat* genes (or both) may be present within a given species. Accordingly, β-ketoadipate succinyl-CoA transferase activity may be encoded by either *pca* and/or *cat* genes (*pcaIJ* and *catIJ*, respectively), and these may share sequence similarity (15, 25). Amino acid sequence identity between the *S. meliloti* PcaIJ and that of *A. baylyi* (25) (PcaI, 21%; PcaJ, 20%), *P. putida* (33) (PcaI, 21%; PcaJ, 23%), *Bradyrhizobium japonicum* (23) (PcaI, 17%; PcaJ, 20%), and even *A. tumefaciens* (53) (PcaI, 19%; PcaJ, 21%) is limited. In contrast, the sequence identity that exists between the *S. meliloti* PcaIJ and that of *M. loti* (22) (PcaI, 63%; PcaJ, 71%) and *Pseudomonas aeruginosa* PAO1 (49) (PcaI, 70%; PcaJ, 60%) and *Pseudomonas* sp. strain B13 CatIJ (15) (CatI, 68%; CatJ, 60%) is extensive. Likewise, signature sequences typically present in PcaI and PcaJ of many species are absent or modified in their *S. meliloti* counterparts, a situation comparable to that previously described by Göbel et al. (15). For PcaI, an N-terminal glycine cluster ([DN]-[GN]-X[2]-[LIVMFA][3]-G-G-F-X[3]-G-X-P) (52) present in *A. tumefaciens*, *B. japonicum*, *A. baylyi*, and *P. putida* proteins has been modified in *S. meliloti* by the deletion of one glycine residue and the replacement of another with glutamic acid. This modification has been reported previously for CatI of *Pseudomonas* sp. strain B13 (15) and may also be observed to occur in *P. aeruginosa* and *M. loti pcaIJ*. For PcaJ, an N-terminal signature sequence ([LF]-[HQ]-S-E-N-G-[LIVF][2]-[GA]) (33) present in *B. japonicum*, *A. baylyi*, and *P. putida* is absent in *S. meliloti*. The E-S-G motif reported for CatJ of *Pseudomonas* sp. strain B13 (15) and present in *P. aeruginosa*, *M. loti*, and a glutaconate-CoA transferase of *Acidaminococcus fermentans* is also conserved in *S. meliloti*. Thus, the differences observed between β-ketoadipate succinyl-CoA transferases of one group of species (*A. baylyi*, *P. putida*, *A. tumefaciens*, and *B. japonicum*) and another (*Pseudomonas* sp. strain B13, *P. aeruginosa*, *M. loti*, and *S. meliloti*) are striking, especially since closely

TABLE 3. β-Ketoadipate succinyl-CoA transferase activity in the presence of adipate[a]

Assay condition	Ratio of adipate to β-ketoadipate	Transferase activity (mU/mg protein [SD])	% Transferase activity (SD)
0 mM adipate		539 (12.6)	100 (2.3)
2 mM adipate	1:5	562 (24.4)	104 (4.5)
10 mM adipate	1:1	503 (24.2)	93 (4.5)
50 mM adipate	5:1	236 (24.0)	44 (4.5)

[a] Enzyme assays were performed using purified enzyme. One unit of enzyme is the amount required to convert 1 μmol of β-ketoadipate to β-ketoadipyl-CoA in 1 min under assay conditions.



B.

-35 -10 +1

TAAATTGACGCTTGCGAAGAACAGGCGCTTATTCGGCTCGAACATCACCGGAGGAAAACGATG

C.

Promoter sequences		Gene	Reference
-35	-10		
CTTGAC	ACTGATT-CGCGGAAGTG GGATTC	*incA1*	(27)
CTTGTC	TTGGGTC-AGCCTTGCCG GTATGT	*pckA*	(32)
CTTGAC	CAAATTC-CAGTAATAAG CAATTT	*ntrA*	(1)
CTTGAC	TTCGATC-GATGTTCGGG AGAATG	*hemA* P2	(26)
GTTGAC	CACTGAT-CGCTTTGAAG GAAGAA	*hemA* P1	(26)
CTTGAT	TCCATTAACTTCAGGGTT CTCTAA	*nodD1*	(11)
CTTGCG	CGCCAGC-GCAAGCCGCG CTAACA	*trpE*	(2)
ATTTTA	CCTAACCGGATGAAACAT CCAAAT	*pcaD*	This work
ATTGAC	GCTTGCG-AAGAACAGGC GCTTAT	*pcaI*	This work

FIG. 4. Analysis of the *pcaI* promoter in *S. meliloti*. (A) RNA isolated from *S. meliloti* wild-type derivative strain Rm1021 was used in primer extension reactions. Lane 1, no extension products were observed using RNA obtained from cells grown in the absence of protocatechuate; lane 2, a single extension product was observed using RNA derived from cells grown in the presence of protocatechuate. Extension reactions were performed using two different primers; however, only results obtained with one of the two primers are shown (reactions with both primers identified the same start site). Sequencing reactions were performed using the same primer used in extension reactions, and results are shown to the left of the extension reaction products. The arrows indicate the extension product (right) and identify the corresponding nucleotide (left). (B) *pcaI* promoter region, including the inferred −10 and −35 regions (underlined). The transcriptional start site is identified (in bold, enlarged font), and the start codon is also indicated (enlarged font at far right). (C) Comparison of the *pcaI* promoter with other previously determined promoters in *S. meliloti*.

related organisms, such as *A. tumefaciens* and *S. meliloti*, carry different forms of the enzyme.

Identification of *pcaI* transcriptional start site. To facilitate our analysis of transcriptional regulation of *pcaIJF*, we determined the transcriptional start site of the operon by using primer extension analysis. Two different primers were used to map the start site, and each yielded a single extension product only with RNA derived from cells grown in the presence of protocatechuate (Fig. 4A). The transcriptional start site of the *pcaIJF* operon mapped to an A residue located 20 nucleotides upstream of the predicted PcaI start codon. Figure 4B shows the sequence of the *pcaI* promoter, including the inferred −10 and −35 hexanucleotide regions. Examination of the promoter region revealed the presence of a 9-bp palindromic sequence (5′ GCTTATTCG 3′) coincident with the predicted −10 region. Comparison of *pcaIp* with other *S. meliloti* promoters reveals some sequence similarity, particularly with respect to the inferred −35 regions (1, 2, 11, 26, 27, 32).

Regulation of the *pcaIJF* operon requires an IclR-type regulator encoded by SMb20586. The regulatory systems associated with the protocatechuate branch of the β-ketoadipate pathway have been described for a few species. In *P. putida*, expression of *pca* genes (with the exception of *pcaHG*) is regulated by the IclR-type transcriptional regulator PcaR, with

TABLE 4. Expression of *pcaF-gusA* fusion in *S. meliloti* wild-type and PcaR$^-$ backgrounds

Strain	Relevant genotype	Growth condition	β-Glucuronidase activity[a] (Miller units [SD])
RmK1015	Rm1021 *pcaF*::*gusA*	Glycerol	27 (2.7)
		Glycerol plus adipate	131 (11.7)
		Glycerol plus PCA[b]	259 (18.6)
RmK1016	Rm1021 *pcaF*::*gusA* *pcaR*::Ω	Glycerol	52 (3.0)
		Glycerol plus adipate	48 (4.1)
		Glycerol plus PCA	66 (6.8)

[a] Shown are averages of values obtained from three independent cultures grown overnight in LBmc and subcultured into M9 minimal medium with 0.5% glycerol ± 5 mM protocatechuate or 30 mM adipate at 30°C for 4 h.
[b] PCA, protocatechuate.

TABLE 5. Expression of *pcaR-gusA* fusion in *S. meliloti* wild-type and PcaR$^-$ backgrounds

Strain	Relevant genotype	Growth condition	β-Glucuronidase activity[a] (Miller units [SD])
RmP892	Rm1021 (pTH1335)	Glycerol	62 (2.2)
		Glycerol plus PCA[b]	42 (2.6)
RmP893	Rm1021 *pcaR*::Ω (pTH1335)	Glycerol	291 (8.2)
		Glycerol plus PCA	261 (3.1)

[a] Shown are averages of values obtained from three independent cultures grown overnight in LBmc and subcultured into M9 minimal medium with 0.5% glycerol ± 5 mM protocatechuate at 30°C for 4 h.
[b] PCA, protocatechuate.

β-ketoadipate serving as an inducing metabolite (17, 34). In *A. baylyi*, expression of the *pcaIJFBDKCHG* operon is regulated by the IclR-type protein PcaU (14), which acts as both a repressor and an activator of the operon, depending upon the presence of the inducer protocatechuate (51). In *A. tumefaciens* and *Rhizobium leguminosarum*, β-ketoadipate succinyl-CoA transferase activity is likewise induced by β-ketoadipate, and in *A. tumefaciens*, expression of *pcaIJ* is subject to regulation by an IclR-type protein (36, 39).

In *S. meliloti*, the *pcaIJF* operon is located beside a gene (SMb20586) encoding a putative IclR-type protein. To determine whether this putative regulator is involved in the regulation of *pcaIJF* gene expression, a streptomycin/spectinomycin antibiotic cassette was used to inactivate SMb20586, generating strain RmK1014 (as verified by Southern hybridization). RmK1014 was unable to grow with protocatechuate but grew like the wild type with glycerol or glucose as the sole carbon source, showing that SMb20586 is essential for protocatechuate metabolism (data not shown).

A promoterless *gusA* reporter gene was inserted at the 3′ end of the *pcaIJF* operon in the genome in order to monitor expression of this operon. The fusion was designed such that *pcaF* was not disrupted, as was verified by growth of the fusion strain with protocatechuate as a sole carbon source. In the wild-type Rm1021 background, expression of *pcaF*::*gusA* increased 10-fold and 5-fold following growth in the presence of protocatechuate and adipate, respectively (Table 4). In this instance, adipate was used as an analogue of β-ketoadipate. In contrast, expression of *pcaF*::*gusA* in the SMb20586 mutant background was minimal regardless of whether protocatechuate or adipate was in the growth media. This demonstrated that a product encoded by SMb20586 was required for *pcaIJF* expression.

The regulator encoded by SMb20586 from *S. meliloti* shares 59% amino acid identity with PcaR of *A. tumefaciens* (53) and 41% identity with PcaR from *P. putida* (46). In *P. putida* and *A. tumefaciens*, adipate is utilized as an inducer analogue of β-ketoadipate (36, 40), and based on the inducing activity of adipate as revealed in Table 4, we conclude that β-ketoadipate is the in vivo metabolite responsible for *pcaIJF* expression in *S. meliloti*. Based upon its amino acid sequence similarity and role in the regulation of *pcaIJF* expression, we have renamed SMb20586 *pcaR*.

Transcriptional regulator PcaR participates in autoregulation. To examine whether PcaR could also autoregulate its own synthesis, the region upstream of the *pcaR* translational start site was amplified and cloned into the *gusA* reporter vector pFus1 (45) to create pTH1335. This plasmid was then conjugated into *S. meliloti* strains Rm1021 and RmK1014 (a *pcaR*::Ω derivative), and promoter activity was monitored via β-glucuronidase enzyme assays (Table 5). In both strains, reporter enzyme activities are comparable in cells grown with and without protocatechuate, indicating that *pcaR* expression is not influenced by the presence of this compound. Expression of *pcaR*::*gusA* in the low-copy-number plasmid pTH1335 is relatively low (comparable to expression of the *pcaF*::*gusA* fusion in uninduced cells), as expected of a regulatory gene. However, expression from the *pcaR* promoter was increased fivefold in the *pcaR* mutant compared to that in Rm1021 (Table 5). These results demonstrate that PcaR expression is negatively autoregulated.

Analysis of the β-ketoadipate pathways in *S. meliloti* and *A. tumefaciens*. Many aspects regarding the organization and regulation of genes encoding enzymes involved in the upper portion of the β-ketoadipate pathway (metabolism of protocatechuate to β-ketoadipate) are conserved between *S. meliloti* and *A. tumefaciens*. In addition to amino acid sequence similarities between homologues of the two species (which range from 60 to 77% identity), the organizations of genes into a single operon (*pcaDCHGB*) are identical. Likewise, regulation of the operon in both species is mediated by a LysR-type transcriptional regulator. Identification of the *S. meliloti pcaD* transcriptional start site and comparison with *M. loti* and *A. tumefaciens* sequences reveal that the *pcaD* promoter is likely conserved among these species. Similarly, previous work utilizing an *A. tumefaciens pcaD*::*lacZ* fusion indicates that the PcaQ binding sites of this species are recognized by the *S. meliloti* homologue (38). Although the evidence is limited, it is also quite likely that β-carboxy-*cis*,*cis*-muconate and γ-carboxymuconolactone serve as coinducing metabolites required for the PcaQ-regulated expression of the *pcaDCHGB* operon in *S. meliloti*, as has been shown for *A. tumefaciens*.

With respect to genes whose products participate in the lower portion of the pathway (conversion of β-ketoadipate to

succinate and acetyl-CoA), certain similarities between *S. meliloti* and *A. tumefaciens* may also be observed. In both species, genes encoding subunits of a β-ketoadipate succinyl-CoA transferase (*pcaI* and *pcaJ*) are organized into an operon whose expression is regulated by an IclR-type transcriptional regulator (PcaR), with β-ketoadipate serving as a coeffector. On the other hand, amino acid sequence identity between PcaIJ of *S. meliloti* and *A. tumefaciens* is quite low and signature sequences present in the *A. tumefaciens* PcaIJ are absent or modified in the *S. meliloti* protein.

Comparison of the β-ketoadipate pathways in *S. meliloti* and *A. tumefaciens* can be extended to include PobA (4-hydroxybenzoate hydroxylase), an enzyme involved in the catalysis of 4-hydroxybenzoate to protocatechuate. In *S. meliloti*, a gene annotated as *pobA* is situated between the two *pca* operons. Despite the presence of this gene, *S. meliloti* strain Rm1021 is unable to utilize 4-hydroxybenzoate as a sole carbon source and we were unable to isolate an Rm1021 mutant that acquired this capability (data not shown). Likewise, an auxanographic study of *Rhizobiaceae* reported *S. meliloti* incapable of growing upon this compound (41). Moreover, using two independent transcriptional fusions to the *S. meliloti pobA* gene (utilizing *gfp* and *gusA* as reporters), we detected only basal *pobA* expression which did not increase upon addition of 4-hydroxybenzoate or protocatechuate to the growth medium (data not shown). In contrast, *A. tumefaciens* is able to grow at the expense of 4-hydroxybenzoate (41), and *pobA* expression in this species is induced by 4-hydroxybenzoate via PobR (39). In *A. tumefaciens* (and *R. leguminosarum*), an AraC family transcriptional regulator (PobR) regulates *pobA* expression (39), but there are no *araC* homologues located nearby in the *S. meliloti* genome. A putative LysR-type regulator (SMb20582) is located directly downstream of *pobA*, and although members of this family of regulators are typically transcribed divergently from a target gene, it is possible that this gene encodes a *pobA* regulator. In *A. baylyi*, an IclR-type regulator positively regulates expression of the *pobA* gene (7); however, the only close IclR gene in *S. meliloti* has been identified as *pcaR* (this work) because it regulates *pcaIJF* expression. Expression levels of *pobA* (as determined by reporter enzyme assays) in wild-type and *pcaR* mutant backgrounds are comparable, and we have concluded that PcaR is not involved in the regulation of *pobA* gene expression. Consequently, the identity of a regulator of *pobA* expression in *S. meliloti*, if one exists, remains obscure.

The supraoperonic organization of genes whose products participate in the catabolism of protocatechuate and related compounds has been well documented (4, 18, 39). In *A. tumefaciens* and *S. meliloti*, the two *pca* operons are clustered in close proximity, flanking the putative *pobA* gene. It has been proposed that this supraoperonic organization in *A. tumefaciens* arose as the result of the acquisition of these genes as a unit and that the protocatechuate pathway evolved prior to the divergence of *Agrobacterium* and *Rhizobium* species (39). Although much of the genetic organization and regulation in these systems has been conserved, the differences observed (particularly) with respect to PcaIJ of *S. meliloti* and *A. tumefaciens* are inconsistent with a shared history. It may be that although the protocatechuate catabolic pathway was established prior to *Agrobacterium* and *Rhizobium* speciation, the *pcaIJ* genes present today were acquired at some point afterwards independently in one or both genera. Possibly, this punctuated assembly of the two *pca* operons resulted in the loss of the *pobA* regulator, leaving *S. meliloti* unable to efficiently metabolize 4-hydroxybenzoate.

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