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Conservation of PcaQ, a Transcriptional Activator of *pca* Genes for Catabolism of Phenolic Compounds, in *Agrobacterium tumefaciens* and *Rhizobium* Species

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In *Agrobacterium tumefaciens* A348, control of five genes for catabolism of the phenolic compound protocatechuate to β-ketoadipate is exerted by the gene *pcaQ*. The product of *pcaQ* is a transcriptional activator which is distinct from regulators of the β-ketoadipate pathway characterized in other bacterial groups. An investigation of whether *pcaQ* is present and conserved in related *Rhizobium* species employed Southern hybridization and an agrobacterial *pcaD*::*lacZ* promoter probe plasmid. These studies revealed that homologs of the activator are widespread among members of the family *Rhizobiaceae*, being present in *Rhizobium leguminosarum*, *Rhizobium fredii*, *Rhizobium meliloti*, *Rhizobium etli*, and *Rhizobium tropici*.

In *Agrobacterium tumefaciens*, the phenolic compound *p*-hydroxybenzoate and the hydroaromatic compounds quinate and shikimate are catabolized to tricarboxylic acid cycle intermediates via the protocatechuate branch of the β-ketoadipate pathway. The *pca* genes for the protocatechuate pathway are clustered in a supraoperon in *A. tumefaciens* A348 (Fig. 1A and B) (13). A novelty of *A. tumefaciens pca* gene regulation is that control of the *pcaDCHGB* operon is mediated by two unstable metabolites, β-carboxy-*cis*,*cis*-muconate and γ-carboxymuconolactone, in concert with the product of a regulatory gene, *pcaQ*. Characterization of *pcaQ* has revealed that its product is a member of the LysR family of bacterial regulators, and it resembles other members of this family in many respects (14).

Surveys of aromatic compound utilization have shown that protocatechuate appears to be a universal growth substrate for *Rhizobium* species as well as for the more distantly related *Bradyrhizobium* species (2, 16, 25). Previous studies indicated that the unusual pattern of regulation of protocatechuate catabolic enzymes in *Rhizobium leguminosarum* bv. trifolii (18) is similar to that found in *A. tumefaciens* (13). In *R. leguminosarum*, enzymes for at least two initial steps are induced by β-carboxy-*cis*,*cis*-muconate, and the β-ketoadipate succinyl-coenzyme A transferase is induced by β-ketoadipate. It was not known whether the common features of *pca* gene regulation in *A. tumefaciens* and *R. leguminosarum* reflect a common origin for the regulatory molecules governing the *pca* structural genes. In addition, it was unknown whether other members of the *Rhizobiaceae* share these unique elements of regulation with *A. tumefaciens* and *R. leguminosarum*. This study was designed to investigate whether homologs of *A. tumefaciens pcaQ* exist in rhizobia and whether functional PcaQ polypeptides are distributed widely in this group of bacteria.

Bacterial strains and conjugations, LacZ assays, and Southern hybridizations. Bacterial strains and plasmids used in this study are listed in Table 1. The broad-host-range plasmid pARO80 contains an operon fusion between the agrobacterial *pcaDCHGB* promoter and the promoterless *lacZ*-Km^r cassette of pKOK6 (6). The first 33 nucleotides of *pcaQ*, 95 bp of the *pcaQ-pcaD* intergenic region, and 5 bp presumed to be the 5′ end of *pcaD* lie upstream of the promoterless *lacZ*-Km^r cassette, and 117 bp of a truncated *pcaC* are downstream of the cassette in pARO80 (14, 15). Plasmids were maintained in *Escherichia coli* cells with 12.5 μg of tetracycline ml^{-1} and 30 μg of kanamycin ml^{-1}.

Conjugations between *E. coli* S17-1 cells carrying pARO158 or pARO80 and different recipient strains were carried out at 30°C overnight as previously described (14). *Rhizobium meliloti* and *Pseudomonas putida* were mated on Luria-Bertani medium (22). Other rhizobial strains were mated on peptone-yeast extract medium (9). Selection and purification of transconjugants employed minimal medium (16) with the following supplements: 10 mM glucose, 0.5 μg of biotin ml^{-1}, 2 μg of calcium pantothenate ml^{-1}, and 40 μg of nicotinate ml^{-1} for *Rhizobium etli* and *Rhizobium tropici*; 10 mM glucose and 0.5 μg of biotin ml^{-1} for *R. meliloti*; 10 mM arabinose, 0.5 μg of biotin ml^{-1}, 2 μg of calcium pantothenate ml^{-1}, and 1 μg of thiamine-HCl ml^{-1} for *R. leguminosarum*; 10 mM arabinose, 0.5 μg of biotin ml^{-1}, and 1 μg of thiamine-HCl ml^{-1} for *Rhizobium fredii*; 15 mM glycerol for *P. putida*; and 10 mM arabinose for *A. tumefaciens*. A level of 1 μg of tetracycline ml^{-1} was used to select for the pRK415-based plasmids in *Rhizobium* and *Agrobacterium* strains, and a level of 12.5 μg of tetracycline ml^{-1} was used for *P. putida.*

For β-galactosidase (LacZ) assays, turbid cultures were diluted 1:50 into fresh minimal medium and grown in a rotary shaker at 30°C in the presence of inducing or noninducing carbon sources. Vitamin and antibiotic supplementation of minimal medium was as described above for conjugations. Inducing carbon sources, added to the above-described media at a concentration of 5 mM, were protocatechuate for *R. meliloti*, *R. etli*, and *R. tropici* and quinate for the other strains. An exception was *R. fredii*, for which inducing conditions were quinate alone. Strains carrying plasmids were harvested at the following optical densities at 620 nm: 0.5 to 0.7 for *A. tumefaciens*, 0.8 for *R. leguminosarum* bv. trifolii, 1.3 for *R. fredii*, 1.0 for *P. putida*, and 0.9 for *R. meliloti*, *R. tropici*, and *R. etli*. Cells were harvested and permeabilized as previously described (14). The method of Miller (8) was followed to assay for LacZ activity. Since *A. tumefaciens*(pARO158) and *R. meliloti*(pARO158) had negligible LacZ backgrounds, this plasmid was not included in further tests with rhizobial strains.

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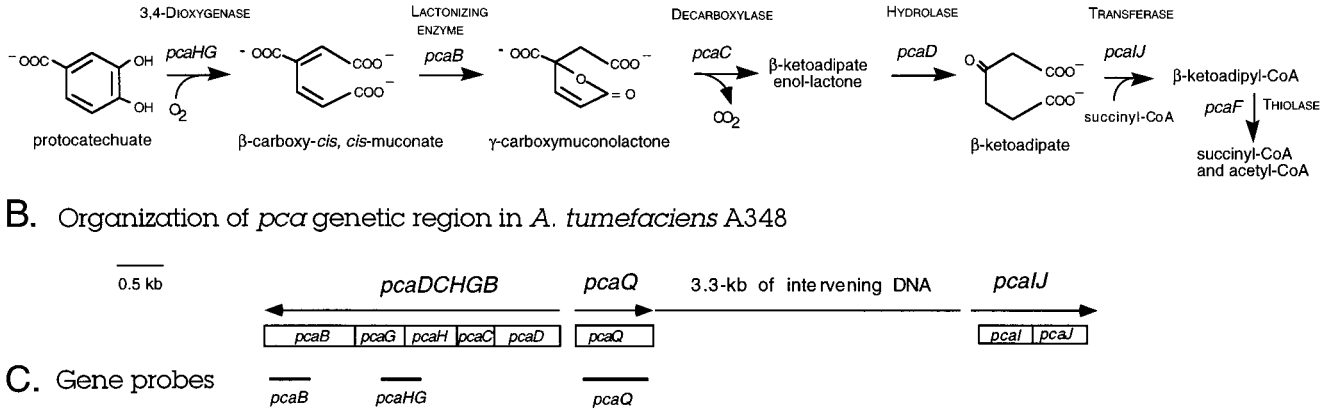

FIG. 1. Protocatechuate branch of the β-ketoadipate pathway. (A) Enzymatic steps in the catabolism of protocatechuate to tricarboxylic acid cycle intermediates. CoA, coenzyme A. Genes encoding each enzyme are shown next to the arrows for the reaction. (B) Organization of *pca* genes in *A. tumefaciens* A348 (13). (C) Gene fragments used as probes aligned beneath their corresponding sequences in the *pca* gene cluster of *A. tumefaciens.*

Standard techniques of molecular biology were used in plasmid and gene manipulations (20) and isolation of chromosomal DNA (1). Blotting was carried out by published procedures (20). Random primed labeling of probes with digoxigenin-dUTP, Southern hybridization, blocking of filters, and detection with Lumi-Phos 530 were based on the protocol for the Genius system of the Boehringer Mannheim Corporation (Indianapolis, Ind.). Probes for *pcaQ*, *pcaHG*, and *pcaB* were derived from plasmids pARO472, pARO162, and pARO62, respectively (Table 1; Fig. 1C).

Identification of *pcaQ* homologs in three rhizobial species by Southern hybridization. Previous characterization of the *pca* genetic region in *A. tumefaciens* A348 identified *pcaQ* on 7.4-kb *Eco*RI, 2.5-kb *Pst*I, and 13.5-kb *Sal*I fragments of DNA (13). Southern hybridization with the *A. tumefaciens pcaQ* probe revealed that a *pcaQ* homolog is present in *R. leguminosarum* bv. trifolii on an approximately 18-kb *Bam*HI fragment and a 10.5-kb *Pst*I fragment (Fig. 2). This correlates with the use of β-carboxy-*cis*,*cis*-muconate as a coinducer of at least two enzymes of protocatechuate catabolism in this strain. Two species uncharacterized with respect to *pca* gene regulation, *R. fredii* and *R. meliloti*, also carry homologs of *pcaQ*. *R. fredii* PRC201 possesses a 2.4-kb *Eco*RI fragment which hybridized strongly with the *pcaQ* probe under conditions of reduced stringency. Southern hybridization of the *pcaQ* probe with *Sal*I-digested chromosomal DNA of *R. meliloti* FA1021 revealed two bands at about 6.3 kb and 1.75 kb. *R. meliloti* 102F28 showed a pattern of bands identical to those of strain FA1021 after different enzymatic treatments (*Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I).

Evidence that *pcaQ* is linked to other genes of protocatechuate metabolism in rhizobial strains. In *R. leguminosarum* bv. trifolii MNF9000, hybridization signal bands for *pcaQ* coincided with those for *pcaHG* in digestions with four different restriction enzymes. Even in the absence of a *pcaQ* mutant strain of *R. leguminosarum*, there is enough evidence from this study and previous research (18) to conclude that PcaQ activates expression of at least *pcaC* and *pcaD*. In *R. fredii* PRC201, digestions with three different restriction enzymes showed identical hybridization signal patterns with the *pcaQ* and the *pcaB* probes. The data for *R. meliloti* FA1021 and 102F28 indicated linkage of *pcaQ* with *pcaHG* and *pcaB* in several different restriction enzyme digestions. Although no conclusion can be drawn as to what particular *pca* genes are under *pcaQ* control, its apparent linkage to other *pca* genes gives credence to the hypothesis that *pcaQ* has evolved in concert with them and that it functions in regulating at least some *pca* genes in rhizobial species.

PcaQ homologs in diverse rhizobial species activate transcription of an *A. tumefaciens pcaD*::*lacZ* fusion. In order to study whether the sequences homologous to *pcaQ* encode functional PcaQ proteins, a *pcaD*::*lacZ* promoter-probe plasmid, pARO80, was introduced into rhizobial strains. Expression of LacZ in rhizobial strains relied on the ability of heterologous PcaQ polypeptides to act in *trans* on the agrobacterial *pcaD* promoter. Figure 3 shows *pcaD*::*lacZ* expression in different strains under conditions in which coinducer was present or absent. *A. tumefaciens* provided both positive and negative controls in a homologous, defined background (14). Under inducing conditions, *A. tumefaciens* ADO2044(pARO80), which lacks PcaQ, expressed LacZ levels that were similar to those of the uninduced A348(pARO80) parental strain (Fig. 3A). The lack of *trans* activation of *pcaD*::*lacZ* transcription in ADO2044(pARO80) was in accord with the protocatechuate-negative phenotype of the strain and indicated that no "cross-talk" from homologs of PcaQ occurred in *A. tumefaciens*. Expression of LacZ from the pARO80 plasmid was tested in another bacterium that was not expected to possess PcaQ. In *P. putida* PRS2000(pARO80), no activation of the agrobacterial *pcaD*::*lacZ* promoter was observed (Fig. 3A).

R. leguminosarum bv. trifolii MNF9000(pARO80), the only rhizobial strain documented to have β-carboxy-*cis*,*cis*-muconate as a coinducer (18), showed evidence of PcaQ activation of *pcaD*::*lacZ* transcription only under inducing conditions (Fig. 3B). The two other species shown to have *pcaQ* homologs by Southern hybridization, *R. fredii* PRC201 (Fig. 3A) and *R. meliloti* FA1021 (Fig. 3B), similarly exhibited LacZ activity under inducing conditions when they carried pARO80. The ability of *R. etli* and *R. tropici* to activate *pcaD*::*lacZ* transcription under inducing conditions (Fig. 3B) indicates that they possess PcaQ and catabolize protocatechuate via the β-ketoadipate pathway. These findings underscore the apparent universality of protocatechuate as a growth substrate for rhizobia

TABLE 1. Bacteria and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
A. tumefaciens		
A348	Cm^r Nal^r Rif^r Sm^r; derived from C-58, containing the cryptic plasmid pAtC58 and the octopine Ti plasmid pTiA6	5
ADO2044	Spc^r; *pcaQ1*::Ω mutant of A348	12
P. putida PRS2000	Wild-type PRS1 (ATCC 12633) derivative	10, 24
Rhizobium spp.		
R. etli bv. phaseoli CFN42		21
R. fredii PRC201		USDA Nitrogen Fixation Laboratory, Beltsville, Md.
R. leguminosarum bv. trifolii MNF9000		18
R. meliloti FA1021		16
R. meliloti 102F28		16
R. tropici CFN299		7
E. coli		
DH5α	F$^-$ ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^-)*supE44* λ$^-$ *thi-1 gyrA96 relA1*	Gibco-BRL[b]
S17-1	*recA pro hsdR*, RP4-Tc::Mu-Km::Tn*7* integrated into the chromosome	23
Plasmids		
pARO190	Ap^r; mobilizable derivative of pUC19	11
pKOK6	Ap^r Km^r Tc^r; contains 4.6-kb promoterless *lacZ*-Km^r cassette for contructing operon fusions	6
pRK415	Tc^r; broad host range; *lac* p/o	4
pUC18	Ap^r; *lac* p/o	28
Plasmids with insertions of agrobacterial DNA		
pARO46	Ap^r Km^r; 13-kb *Eco*RI fragment containing *pca* genes and *pcaI*1::Tn*5* in pUC18	13
pARO56	Ap^r Km^r; 8.3-kb *Sal*I fragment containing part of *pcaD*1::Tn*5* in pUC18	13
pARO62	Ap^r; 2.5-kb *Hin*dIII-*Sph*I subclone of pARO56 in pARO190; spans *pcaHG* and most of *pcaB*; *Nru*I-*Sph*I digestion yields a 0.4-kb probe specific for *pcaB*	This study
pARO80	Km^r Tc^r; *pcaD3*::*lacZ*-Km^r; pARO158 with *lacZ* cassette of pKOK6 replacing the 1.0-kb *Nsi*I fragment of *pcaDC*	14
pARO144	Ap^r; 2.4-kb *Pst*I-*Eco*RI fragment of pARO46 in pUC19	12
pARO158	Tc^r; 1.24-kb *Pst*I-*Hin*dIII fragment of pARO144 containing the first 33 nucleotides of *pcaQ*, the *pcaD-pcaQ* intergenic region, *pcaD*, and a truncated *pcaC* oriented in pRK415 so that the direction of transcription of *pcaD* is opposite to that from the *lac* promoter of the vector	14
pARO162	Ap^r; pARO144 with a 1.3-kb *Pst*I-*Hin*dIII deletion; retains all of *pcaG* and part of *pcaH*; *Eco*RI-*Eco*RV digestion yields a 0.45-kb fragment with the 3′ end of *pca*H and the 5′ half of *pcaG*	This study
pARO462	Ap^r; 2.5-kb *Pst*I insertion of pARO46 in pUC18	This study
pARO472	Ap^r; pARO462 with an exonuclease III-generated 1.75-kb deletion at the insertion end distal to *pcaQ*; the 0.75-kb insertion extends from 30 bp within the 5′ end of *pcaQ* to 150 bp short of the 3′ end of the gene	This study

[a] Cm, chloramphenicol; Nal, nalidixic acid; Rif, rifampin; Sm, streptomycin; Spc, spectinomycin; Ap, ampicillin; Tc, tetracycline; p/o, promoter/operator.
[b] Competent *E. coli* DH5α cells were purchased from Bethesda Research Laboratories, Inc. (Gibco-BRL), Gaithersburg, Md.

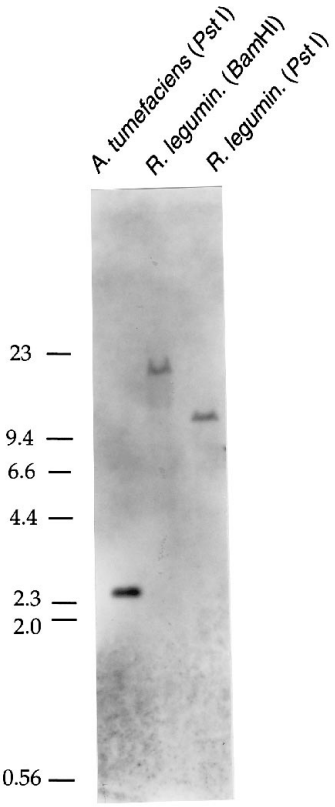

FIG. 2. Southern hybridization of an agrobacterial *pcaQ* probe with *A. tumefaciens* and *R. leguminosarum* bv. trifolii (*R. legumin.*) chromosomal DNA digests. The enzyme used for digestion is next to the strain label at the top of the figure. Size markers to the left are in kilobases. The amount of agrobacterial chromosomal DNA digest applied was about 20% of the amount of rhizobial DNA.

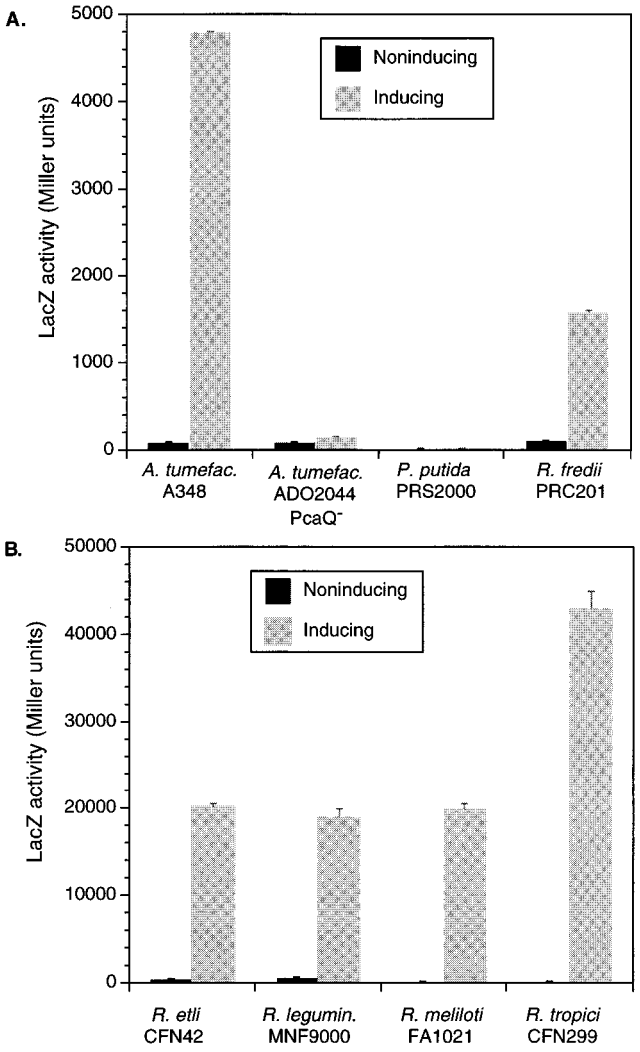

FIG. 3. Ability of strains to activate transcription of agrobacterial *pcaD*::*lacZ* in *trans* in the presence and absence of coinducers. All strains carried pARO80 (*pcaD*::*lacZ*-Km^r), and *pcaD*::*lacZ* expression was measured by β-galactosidase activity (Miller units). Cells grown under the respective inducing conditions but not carrying pARO80 had LacZ activities of 1 U or less, except for *R. etli*, *R. leguminosarum*, and *R. tropici*, which had 8 to 10 U. The *A. tumefaciens* results were reported previously (14). Abbreviations: *A. tumefac.*, *A. tumefaciens*; *R. legumin.*, *R. leguminosarum* bv. trifolii. Standard deviation is of measurements from three independent transconjugants, except for *P. putida* (two replicates).

and provide further evidence that the *pca* genes are widely distributed in this group.

In general, the heterologous PcaQ proteins were very effective in activating transcription from the agrobacterial *pcaD* promoter. For most of the strains, levels of induction of LacZ from pARO80 in the homologous and heterologous genomic backgrounds corresponded to previously published levels of induction measured for enzymes of protocatechuate catabolism (12, 17, 18), i.e., roughly 15- to 40-fold.

According to 16S rRNA nucleotide sequence comparisons among members of the alpha subdivision of purple bacteria, *A. tumefaciens* has a separate lineage from the *Rhizobium* species used in this study, and the rhizobia fall into three subgroups: (i) *R. etli*, (ii), *R. leguminosarum* and *R. tropici*, and (iii) *R. fredii* and *R. meliloti* (3, 21, 26, 27). The widespread conservation of the PcaQ regulator in members of the family *Rhizobiaceae* suggests that its absence would likely be the exception rather than the rule. The simplest explanation for the conservation of PcaQ proteins is that the protocatechuate pathway, with the controls characteristic of those found in *A. tumefaciens*, was established prior to the divergence of bacteria in the family *Rhizobiaceae*. This conclusion is consistent with the hypothesis that regulatory genes governing established pathways of phenolic catabolism were recruited to regulate pathogenic or symbiotic associations (19). Given the complex genomes of bacteria in this family, it is also conceivable that genetic elements of the pathway have been exchanged through horizontal evolution. This fluidity would result in apparent discontinuities in evolutionary distances relative to presumedly stable markers such as 16S rRNA sequences. As more data on the organization and nucleotide sequences of *pca* genes in rhizobial species become available, it should be possible to gauge the contribution of lateral evolution.

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