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Organization and Control of Genes Encoding Catabolic Enzymes in *Rhizobiaceae*

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The family *Rhizobiaceae* is a diverse bacterial group comprising rhizobia and agrobacteria. In a symbiotic partnership with plants, rhizobia form nitrogen-fixing nodules on plant roots; agrobacteria are plant pathogens. Studies of the molecular mechanisms of these bacteria-plant interactions have revealed that phenolic compounds produced by plants serve as inducers of rhizobial nodulation genes and agrobacterial virulence genes. Underlying the ability of agrobacteria and rhizobia to respond to specific phenolics as signals for nodulation or virulence is their capacity to utilize numerous aromatics, including phenolics, as a source of carbon and energy. It is known that, in many microbes, the aerobic degradation of numerous aromatic compounds to tricarboxylic acid cycle intermediates is achieved by the β-ketoadipate pathway. The universal distribution of the protocatechuate catabolic pathway, one branch of the β-ketoadipate pathway, in members of the *Rhizobiaceae* as well as its qualities of being a peripheral, multi-step sequence of reactions, well-characterized in other microbes, makes it a model system for studying the organization and control of catabolic enzymes in this group of organisms.

Initial studies have focused on the organization and regulation of the β-ketoadipate pathway in *Agrobacterium tumefaciens*. A significant achievement of this research has been the cloning, identification and characterization of a novel regulatory gene that modulates expression of an adjacent *pca (protocatechuate)* structural gene, *pcaD*. Regulation of *pcaD* is mediated by the regulatory gene, termed *pcaQ*, in concert with the intermediate β-carboxy-*cis,cis*-muconate. β-carboxy-*cis,cis*-muconate is an unstable chemical, not marketed commercially, and it is unlikely to permeate *Escherichia coli* cells if supplied in media. Because of these factors, characterization of *pcaQ* in *E. coli* required an *in vivo* delivery system for β-carboxy-*cis,cis*-muconate. This was accomplished by designing an *E. coli* strain that expressed an *Acinetobacter calcoaceticus pcaA* gene for conversion of protocatechuate to β-carboxy-*cis,cis*-muconate. The commercially marketed and affordable chemical, protocatechuate, was supplied in the growth medium, and *E. coli* cells accumulated β-carboxy-*cis,cis*-muconate intracellularly.

Studies of *pcaQ* in this strain of *E. coli* led to the conclusion that β-carboxy-*cis,cis*-muconate is an inducer and allowed localization of *pcaQ* next to *pcaD*. An antibiotic-marked omega transcription-terminator element was used to mutate *pcaQ*. Introduction of the mutation into wild type *A. tumefaciens* allowed characterization of the effect of the mutation on gene expression and of the ability of the *pcaQ* gene supplied *in trans* to influence such expression. The latter studies indicated that *pcaQ* encodes a transcriptional activator that complements the mutated *pcaQ in trans*. A preprint reporting this work, "Positive Regulation of Phenolic Catabolism in *Agrobacterium tumefaciens* by the *pcaQ* gene in response to β-carboxy-*cis,cis*-muconate", is included. It has been submitted to the Journal of Bacteriology for publication and was just returned to the editor, having been revised in accordance with reviewers' comments.

The utilization of β-carboxy-*cis,cis*-muconate as an inducer of *pca* genes appears to be an unusual trait, as evaluated by its characterization in diverse bacteria. Work carried out in collaboration with the laboratory of Andrew Glenn of Murdoch University in Australia employed Tn*5* mutants of *Rhizobium leguminosarum* biovar *trifolii* to elucidate the regulation of the β-ketoadipate pathway. This study revealed that at least two genes were induced by β-carboxy-*cis,cis*-muconate, and the *pcaE* gene was induced by β-ketoadipate. These findings were published early in the first year of this grant; a reprint is included with the progress report.

In concert with the studies of *pcaQ*, agrobacterial *pca* structural genes have been cloned. The effects of transposon Tn*5* mutagenesis and site directed mutagenesis on gene expression have revealed that four *pca* genes are organized in an operon under unified transcriptional control. Studies of gene expression of sub-clones has revealed that the order of transcription is *pcaDCAB*. Thus, the *pcaQ* gene product governs the entire *pcaDCAB* operon. Approximately 4-kb from this operon is the *pcaE* genetic unit, transcribed in the opposite direction. The *pcaE* gene encodes a CoA transferase for the second to last enzymatic step of the pathway. The transferase is induced by the pathway metabolite β-ketoadipate, and its structual gene is regulated separately from the *pcaDCAB* operon. Two other bacterial species distant from *A. tumefaciens* have been characterized with respect to *pca* gene organization. In *Pseudomonas putida*, the genes *pcaBDC* form an operon induced by β-ketoadipate; in *A. calcoaceticus* the genes *pcaEFBDCA* are in an operon induced by protocatechuate. A novel induction mechanism and gene arrangement accompanied evolution of the pathway in *Agrobacterium*. This work on *pca* gene organization and regulation has been written up and should be submitted for publication soon.

Although gene rearrangements have occurred, DNA sequence comparisons show conservation of primary sequences within the rearranged genes. The *pca* genes from *P. putida* and *A. calcoaceticus*, closely related species of the gamma branch of proteobacteria, have been cloned and sequenced by other workers. In the course of sequencing *pcaQ* from *Agrobacterium*, the genes adjacent to *pcaQ* are being sequenced. *Agrobacterium* has been placed in the alpha branch of proteobacteria, and comparison of translated sequences has revealed varying degrees of homology with the corresponding genes from the distantly related bacteria of the gamma branch.

Another gene of phenolic catabolism, *pobA*, has been found to be in a supraoperonic cluster between the *pca* genes in *Agrobacterium* and contiguous to *pcaQ*. The *pobA* gene encodes *p*-hydroxybenzoate hydroxylase, a nicotinamide-flavoprotein monooxygenase that converts *p*-hydroxybenzoate to protocatechuate. The translated *pobA* gene product has a high degree of homology with the amino acid sequences of two *p*-hydroxybenzoate hydroxylases from the gamma bacterial group. It shares a common ancestry with other flavin monooxygenases such as salicylate hydroxylase, 2,4-dichlorophenol hydroxylase, and phenol hydroxylase The addition of the more divergent *Agrobacterium p*-hydroxybenzoate hydroxylase sequence for comparison with the other two *pobA* gene products should help to clarify the role of certain amino acid sequences in the function of this enzyme.

Research on aromatic catabolism in bacteria of the family *Rhizobiaceae* led to the discovery that the chemical *p*-toluidine serves as an extremely sensitive chromogenic indicator for diphenolic intermediates of aromatic catabolism. The compound forms a colored precipitate with protocatechuate and catechol, as well as substituted catechols. Formation of the colored complex was found to be dependent on the presence of iron in medium. The *p*-toluidine indicator has been used in our research to study protocatechuate accumulation by mutant strains and to characterize the products of genes cloned in *Escherichia coli* for particular enzymatic activities. For example, the use of *p*-toluidine to detect the accumulation of protocatechuate when *p*-hydroxybenzoate was included in medium contributed to the discovery that the *pobA* gene was on a sub-cloned DNA fragment adjacent to *pcaQ*.

The discovery of a chromophore for diphenolics should have widespread applications. It is relevant to studies of diverse pathways of aromatic catabolism, including those of toxic environmental pollutants which frequently are degraded *via* substituted catechols. It is applicable to the study of structural or regulatory mutants altered in the expression of diverse enzymes that give rise to protocatechuate or catechols and of the oxygenases that act upon protocatechuate or catechols. In

addition, the detection system opens up the possibility of creating vectors in which the insertion of cloned DNA into an aromatic catabolic gene can be screened chromogenically. A reprint on this research, "Application of *p*-Toluidine in Chromogenic Detection of Catechol and Protocatechuate, Diphenolic Intermediates in Catabolism of Aromatic Compounds" is included.

Research in Progress

The similarity of an unusual induction pattern in *Agrobacterium* and *Rhizobium leguminosarum* raises the possibility of a *pcaQ* homolog in the latter bacterium and related *Rhizobium* species. It has been hypothesized that phenolic-responding genes that regulate nodulation and virulence genes evolved from regulatory genes that govern catabolism of phenolics. The *pcaQ* gene is being sequenced, and it is already clear that its product belongs to the family of DNA binding proteins which includes *nodD*. Sequencing has defined the location of *pcaQ* and allowed the isolation of a probe specific for *pcaQ*. Southern hybridizations are being performed. If they show that the agrobacterial *pcaQ* gene indeed has a homolog in *Rhizobium*, this will be strong evidence that the pathways for phenolic catabolism evolved before *Agrobacterium* and *Rhizobium* diverged. Fusions of the *pcaQ* gene with *lacZ* and of the *pcaD* gene with *lacZ* have been made in order to further study the inducer specificity of *pcaQ* and the regulation of *pcaQ* itself.

In addition to the isolation of a probe of *pcaQ*, other probes for specific agrobacterial *pca* genes and for a conserved region of the *pobA* gene have been created. They are being used to see if a similar supraoperonic clustering of genes for phenolic catabolism occurs in *Rhizobium* species related to *Agrobacterium*. This first step is necessary in order to optimize hybridization conditions with heterologous probes. The first structural gene probe that has been tested, *pcaA* from *Agrobacterium*, hybridizes very nicely, under conditions of reduced stringency, with a single band of *R. leguminosarum* DNA which has been digested with *Eco*RI or *Sal*I. An extension of the Southern hybridization work will be to look at *pca* mRNA transcripts in *Agrobacterium* and *Rhizobium*. In addition, evidence has led us to believe that the *pca* genes are located on megaplasmids in *Rhizobium*. The Southern hybridization work is aimed at localizing the *pca* genes to the chromosome or a megaplasmid in *Agrobacterium* and *Rhizobium*. If the genes are indeed located on a megaplasmid in *Rhizobium*, this work should help in understanding the nature of megaplsmids.

Our research has focused upon *Agrobacterium* and its relatives because that work has been so productive, but research on *Bradyrhizobium japonicum* is continuing as well. Southern blots of *pca* probes with DNA from *Rhizobium* are being carried out with DNA from *Bradyrhizobium* at the same time. Positive results using *pcaA* to probe *Bradyrhizobium japonicum* DNA lead us to expect that two genes known to be even more highly conserved, *pcaE* and *pobA*, will likewise prove to be effective probes.

PUBLICATIONS

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