JOURNAL OF BACTERIOLOGY, Jan. 1986, p. 288–292
0021-9193/86/010288-05$02.00/0
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Vol. 165, No. 1

Enzymes of the β-Ketoadipate Pathway Are Inducible in *Rhizobium* and *Agrobacterium* spp. and Constitutive in *Bradyrhizobium* spp.

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Received 2 July 1985/Accepted 16 October 1985

Protocatechuate is a universal growth substrate for members of the family *Rhizobiaceae*, and these bacteria utilize the aromatic compound via the β-ketoadipate pathway. This report describes transcriptional controls exercised by different subgroups of the *Rhizobiaceae* over five enzymes that catalyze consecutive reactions in the pathway: protocatechuate oxygenase (EC 1.13.11.3), β-carboxy-*cis*,*cis*-muconate lactonizing enzyme (EC 5.5.1.2), γ-carboxymuconolactone decarboxylase (EC 4.1.1.44), β-ketoadipate enol-lactone hydrolase (EC 3.1.1.24), and β-ketoadipate succinyl-coenzyme A transferase (EC 2.8.3.6). All five enzymes were inducible in the fast-growing strains *Agrobacterium rhizogenes*, *Agrobacterium tumefaciens*, *Rhizobium fredii*, *Rhizobium meliloti*, *Rhizobium leguminosarum*, and *Rhizobium trifolii*. Specific activities in induced cells ranged from 5- to 100-fold greater than those found in uninduced cells. In contrast to the fast-growing strains and members of every other microbial taxon examined to date, the slow-growing *Bradyrhizobium japonicum* and cowpea *Bradyrhizobium* spp. constitutively expressed four of the five enzymes; protocatechuate oxygenase was the only inducible enzyme in this group. The slow-growing strains included different DNA homology groups, so it appears likely that constitutive expression of the four enzymes is a common trait in the bradyrhizobia. This property points to the importance of aromatic compounds and aromatic catabolites in the nutrition of these organisms.

Members of the family *Rhizobiaceae* form intimate associations with plants: *Rhizobium* and *Bradyrhizobium* species fix nitrogen in symbiosis with legumes, and *Agrobacterium* species produce galls and other malformations on many types of plants. Diverse aromatic and hydroaromatic compounds are produced by plants, and many of these chemicals are used as growth substrates by members of the family *Rhizobiaceae* (4, 9, 12, 16, 18). Rhizobial species vary in their nutritional capabilities. For example, the relatively slow-growing bradyrhizobia were shown to be more nutritionally versatile than representatives of faster-growing rhizobia and agrobacteria when presented with aromatic and hydroaromatic substrates (18). A universal trait, shared by all of the *Rhizobiaceae* examined, was the ability to grow at the expense of protocatechuate (4, 12, 16, 18).

Members of the family *Rhizobiaceae* utilize protocatechuate via the β-ketoadipate pathway (Fig. 1). In all of the bacteria and fungi studied to date, transcription of genes for enzymes of the β-ketoadipate pathway is regulated by induction (2, 3, 6, 24). Several studies of rhizobia and bradyrhizobia have suggested that the pathway is inducible in these organisms (4, 9, 21, 22). Investigation of substrate-dependent oxygen consumption revealed that a cowpea *Rhizobium* strain, unusual in being fast growing, demonstrated inducible systems for the oxidation of *p*-hydroxybenzoate and other compounds (22). In another study of substrate-dependent oxygen consumption, a strain of *Rhizobium leguminosarum* exhibited constitutive systems for the catabolism of a number of tricarboxylic acid cycle intermediates and sugars, but the dissimilation of *p*-hydroxybenzoate and several other compounds occurred inducibly (9). Moreover, in *R. leguminosarum* and *Rhizobium trifolii*, the initial degradation of *p*-hydroxybenzoate is mediated by an inducible uptake system and an inducible hydroxylase (4).

Bradyrhizobium japonicum I-110 constitutively expresses a positive chemotactic response to β-ketoadipate (19). This trait suggested to us that some enzymes involved in the dissimilation of aromatic compounds may also be expressed constitutively in *Bradyrhizobium* species. To test this possibility, we determined the specific activities of five enzymes associated with the dissimilation of protocatechuate after growth of representatives of the *Rhizobiaceae* on inducing and noninducing substrates.

MATERIALS AND METHODS

Media and growth conditions. Bacterial strains used in this study are listed in Table 1. Fast-growing strains are defined as those which have doubling times of 2 to 4 h on yeast extract-mannitol medium, whereas slow-growing strains are those with doubling times of 6 h or more (26).

Bacterial strains were maintained on plates containing yeast extract-mannitol medium (27). Cells from a single colony were preadapted to growth on a particular substrate in a tube containing 5 ml of mineral medium. The 5-ml inoculum was then transferred to a 500-ml baffled Erlenmeyer flask containing 150 ml of defined mineral medium (18). The medium for growth of *B. japonicum* 110 on quinate was supplemented with 0.01% yeast extract. Carbon sources were prepared as 0.5 M concentrated solutions and filter sterilized. They were added aseptically to the culture media to a final concentration of 15 mM for succinate and 10 mM for other substrates. Unlike many aromatic compounds, the hydroaromatic substrates quinate and shikimate are not toxic to rhizobial species at 10 mM (18). Liquid cultures were incubated at 30°C on a New Brunswick environmental Gyrotory shaker at 240 rpm. Contamination was routinely checked, and strains were tested for their ability to nodulate appropriate legume hosts as described previously (18). Cultures were harvested in late exponential or early stationary phase, corresponding to an optical density of 0.8 to 1.1 at 600 nm, by centrifugation at 4°C. *R. leguminosarum* 2368 and cowpea *Bradyrhizobium* sp. strain 3179 grown on succinate

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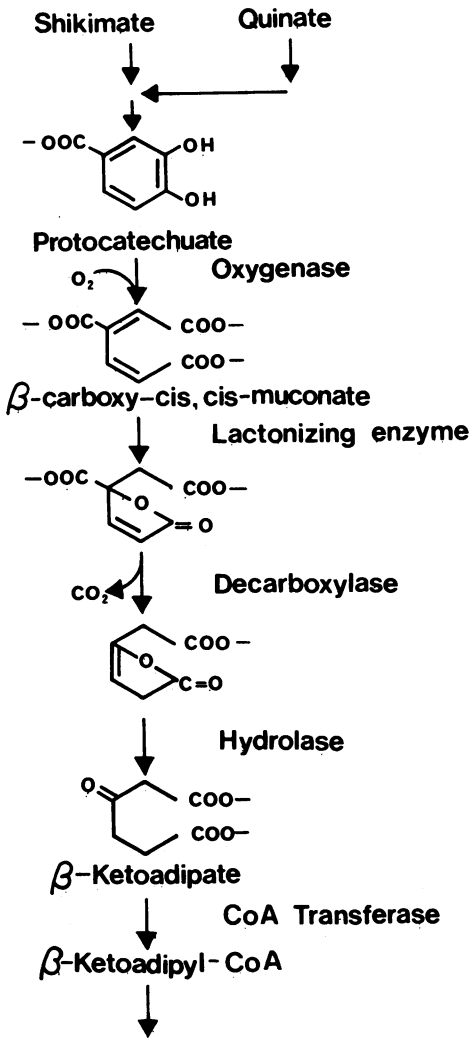

FIG. 1. The protocatechuate branch of the β-ketoadipate pathway for the conversion of hydroaromatic compounds and protocatechuate to succinyl-CoA and acetyl-CoA. Each arrow represents a specific enzymatic step.

were harvested at lower cell concentrations, as was cowpea *Bradyrhizobium* sp. strain 32H1 grown on shikimate and on succinate. Cells were washed twice with 20 mM Tris hydrochloride buffer at pH 8.0 containing 25 μM dithiothreitol. They were resuspended in 0.6 to 1.0 ml of buffer and stored at −20°C before use.

Preparation of cell extracts. Thawed cells at 4°C were broken open by three 20-s bursts using a Braun-Sonic 2000 apparatus and centrifuged at 4°C to remove cell debris and unbroken cells. The supernatant provided the crude extract used in enzyme assays. Assays were performed on two independent extracts from cells grown on a given carbon source. Extracts of cells grown under inducing and noninducing conditions were assayed on the same day with the same assay mixes. *Pseudomonas putida* PRS2000 grown on quinate or *p*-hydroxybenzoate served as a positive control in all assays.

Enzyme assays. The following enzymes were assayed according to published procedures: protocatechuate-3,4-dioxygenase (EC 1.13.11.3 [7]), β-carboxy-*cis*,*cis*-muconate lactonizing enzyme (EC 5.5.1.2 [17]), γ-carboxymuconolactone decarboxylase (EC 4.1.1.44 [17]), β-ketoadipate enol-lactone hydrolase (EC 3.1.1.24 [17]), β-ketoadipate succinyl-coenzyme A (CoA) transferase (EC 2.8.3.6 [17]). A unit of enzyme activity is defined as the amount that removes 1.0 μmol of substrate per min. Mixtures of extracts of induced and uninduced cells revealed no inhibitors of enzyme activity. The protein concentrations of extracts were determined by the method of Lowry et al. (15) with bovine serum albumin as the standard.

RESULTS

Patterns of induction for the five enzymes that convert protocatechuate to β-ketoadipyl-CoA in six fast-growing rhizobial and agrobacterial strains are presented in Table 2. Table 3 presents patterns of induction for four bradyrhizobial strains that can grow with the inducing substrates quinate or shikimate. Slow-growing *B. japonicum* 61A76 does not grow with these compounds; Table 3 shows specific activities in extracts of this strain after growth with the noninducing substrate succinate.

Of the examined rhizobial strains, *R. fredii* exhibited the most rapid growth with quinate; doubling times of 5 h were observed. For other rhizobia, doubling times with quinate or shikimate ranged between 7 and 20 h, and within this range there was no consistent pattern that drew distinctions between the bradyrhizobia and the other organisms. Induction patterns for *P. putida* PRS2000, a strain that grows on the hydroaromatic compounds with a doubling time of 75 min, is included in the table to allow comparison of enzyme levels of the *Rhizobiaceae* with those in an organism that metabolizes the compounds far more rapidly.

Regulation of the protocatechuate enzymes in fast-growing organisms. Enzymes that mediate catabolism of protocatechuate are inducible in agrobacteria and in the fast-growing rhizobia (Table 2). Quinate, an inducing growth substrate that is metabolized via protocatechuate, elicited synthesis of

TABLE 1. Bacterial strains

Strains	Source[a]
A. rhizogenes A4	D. Tepfer, CNRA, Versailles, France
A. tumefaciens B6	S. R. Long, Stanford University, Stanford, CA 94305
R. fredii 192	H. H. Keyser, USDA, Nitrogen Fixation Lab, Beltsville, MD 20705
R. meliloti 102F28	R. S. Smith, The Nitragin Co., Inc., Milwaukee, WI 53209
R. leguminosarum 2368	H. H. Keyser, USDA
R. trifolii 2066	H. H. Keyser, USDA
P. putida PRS2000	This laboratory
B. japonicum 110	H. H. Keyser, USDA
B. japonicum 61A76	R. S. Smith, The Nitragin Co.
Cowpea *Bradyrhizobium* sp. strain 32H1	R. S. Smith, The Nitragin Co.
Cowpea *Bradyrhizobium* sp. strain 3241	R. S. Smith, The Nitragin Co.
Cowpea *Bradyrhizobium* sp. strain 3179	H. H. Keyser, USDA

[a] Abbreviations: CNRA, Centre National de la Recherche, Agronomique; USDA, U. S. Department of Agriculture.

TABLE 2. Expression of five enzymes of the β-ketoadipate pathway in strains of fast-growing rhizobia and agrobacteria

Strain	Growth conditions[a]	Sp act (U/mg of protein)				
		Oxygenase	Lactonizing enzyme	Decarboxylase	Hydrolase	Transferase
A. rhizogenes A4	Quinate	0.08	0.29	0.29	0.06	0.08
	Succinate	0.004	0.02	0.01	0.008	0.001
A. tumefaciens B6	Quinate	0.33	1.11	0.37	0.22	0.10
	Succinate	0.02	0.04	0.005	0.01	0.001
R. fredii 192	Quinate	0.48	2.13	0.72	0.54	0.04
	Mannitol	0.01	0.12	0.02	0.02	0.001
R. meliloti 102F28	Quinate	0.33	1.25	0.43	0.14	0.04
	Succinate	0.01	0.08	0.003	0.02	0.0002
R. leguminosarum 2368	Quinate	0.24	0.31	0.32	0.19	0.09
	Succinate	0.026	0.05	0.015	0.02	0.001
R. trifolii 2066	Quinate	0.34	0.28	0.50	0.11	0.14
	Arabinose	0.01	0.005	0.01	0.001	0.001
P. putida PRS2000	Quinate	2.1	0.67	1.63	1.13	0.02
	Succinate	0.02	0.02	0.03	0.03	0.0001

[a] It was necessary to use different noninducing carbon sources since some of the strains grew poorly on succinate.

each enzyme at a specific activity exceeding by 6- to 200-fold that found in uninduced cells. Regulation was most lax in *R. leguminosarum*, in which induction increased the specific activities of the four enzymes that convert protocatechuate to β-ketoadipate by 6- to 21-fold. Induction of the β-ketoadipyl-CoA transferase in this strain was controlled relatively tightly. Induction of the transferase in all of the agrobacteria and rhizobia examined resulted in increases in specific activity ranging from 40- to 200-fold; fully induced levels of this enzyme were severalfold higher than those found in induced cultures of the rapidly growing *P. putida* (Table 2). Induced levels of the other agrobacterial and rhizobial enzymes, with the exception of β-carboxy-*cis,cis*-muconate lactonizing enzyme, generally were lower than those found in extracts derived from induced cells of *P. putida*. Expressed as a fraction of the specific activity of the corresponding induced enzyme in *P. putida*, the average specific activities for the agrobacterial and rhizobial enzymes were as follows: protocatechuate oxygenase, 14%; β-carboxy-*cis,cis*-muconate lactonizing enzyme, 134%; γ-carboxymuconolactone decarboxylase, 27%; enol-lactone hydrolase, 19%.

R. fredii 192 is similar to *B. japonicum* in its ability to nodulate soybeans, although the former strain is ineffective in fixing nitrogen on most improved soybean cultivars. Like other fast-growing strains and in contrast to the bradyrhizobia, *R. fredii* inducibly forms enzymes for catabolism of protocatechuate (Table 2).

Regulation of the protocatechuate enzymes in bradyrhizobia. Enzymes that catalyze the dissimilation of protocatechuate are, for the most part, independent of transcriptional controls in the bradyrhizobia. The sole exception to this pattern, protocatechuate oxygenase, demonstrated an activity in induced cells that exceeded levels in uninduced cells by 5- to 15-fold (Table 3). The specific activities of the other four enzymes do not appear to be strongly influenced by growth substrate. The lactonizing enzyme, decarboxylase, and hydrolase are produced constitutively at low levels, corresponding to roughly 5 to 20% of the specific activities found in induced cultures of the rapidly growing *P. putida* (Table 3). Constitutive expression of the CoA transferase that acts on β-ketoadipate is at a much higher level in the bradyrhizobia. As illustrated in Fig. 2, growth of the organisms on inducing carbon sources does not influence the expression of the transferase to any significant extent. Furthermore, levels of the CoA transferase in the bradyrhizobia are comparable to those found in induced cultures of *P. putida* (Fig. 2).

TABLE 3. Expression of five enzymes of the β-ketoadipate pathway in strains of slow-growing bradyrhizobia

Strain	Growth conditions	Sp act (U/mg of protein)				
		Oxygenase	Lactonizing enzyme	Decarboxylase	Hydrolase	Transferase
B. japonicum 110	Quinate	0.16	0.10	0.22	0.16	0.02
	Succinate	0.02	0.09	0.20	0.13	0.015
B. japonicum 61A76	Succinate	0.016	0.06	0.10	0.10	0.02
Cowpea *Bradyrhizobium* sp. strain 32H1	Shikimate	0.23	0.09	0.26	0.22	0.02
	Succinate	0.05	0.10	0.22	0.28	0.02
Cowpea *Bradyrhizobium* sp. strain 3241	Shikimate	0.15	0.03	0.20	0.16	0.03
	Succinate	0.01	0.03	0.22	0.10	0.03
Cowpea *Bradyrhizobium* sp. strain 3179	Quinate	0.06	0.04	0.09	0.06	0.01
	Succinate	0.01	0.04	0.05	0.04	0.01
P. putida PRS2000	Quinate	2.1	0.67	1.63	1.13	0.02
	Succinate	0.02	0.02	0.03	0.03	0.0001

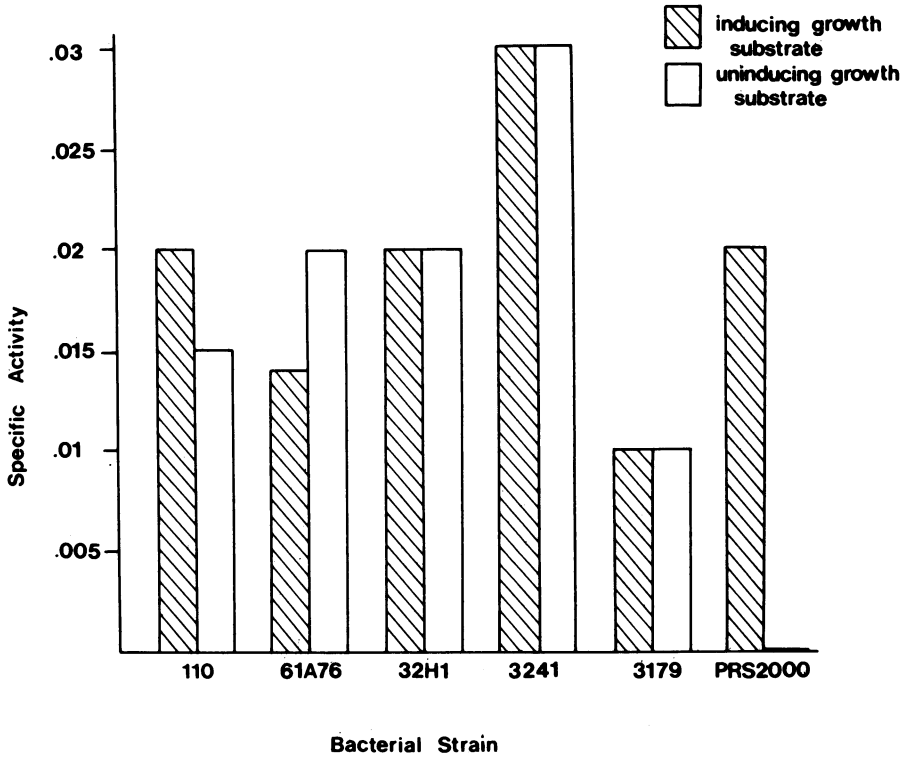

FIG. 2. Constitutive expression of the β-ketoadipate succinyl-CoA transferase in five bradyrhizobial strains. Strains 110 and 61A76 are *B. japonicum* strains; 32H1, 3241, and 3179 are cowpea *Bradyrhizobium* sp. strains; and PRS2000 is a *P. putida* reference strain. Under inducing conditions, cells were grown at the expense of quinate or shikimate. Strain 61A76 was grown at the expense of mandelate since it does not grow with quinate or shikimate (18). The noninducing growth substrate was succinate. Specific activity is expressed as micromoles per minute per milligram of protein.

Some of the bradyrhizobium strains represented in Fig. 2 are known to fall into different DNA homology groups. The bradyrhizobium strains 61A76 and 110 (Fig. 2) fall into two different DNA homology groups (11) and are highly divergent in their symbiotic gene sequences (25). The cowpea strain 3179 may bear a subspecies relationship to strain 110, but it is less closely related to the cowpea strain 32H1 (Fig. 2) (11). Therefore it seems likely that constitutive expression of the β-ketoadipate succinyl-CoA transferase will prove to be a universal characteristic of the bradyrhizobia. The consistency of regulatory patterns shown in Fig. 2 and Table 3 suggests that constitutive expression of the lactonizing enzyme, the decarboxylase, the hydrolase, and the transferase is a characteristic shared by the bradyrhizobia and by no other microbial group that has been examined for this property.

DISCUSSION

Inducible regulation of protocatechuate catabolism in fast-growing organisms. Studies of rhizobia have revealed some degree of constitutivity in both utilization of (9, 22) and chemotaxis to (1, 8, 10) carbohydrates. Therefore it is somewhat remarkable that the wide range of agrobacteria and rhizobia included in this study invariably demonstrate an inducible response in the synthesis of enzymes for the catabolism of protocatechuate. Clearly it is premature to generalize about the regulation of catabolic pathways in these organisms. Our observations with agrobacteria and rhizobia reinforce the generalization, based on numerous investigations with diverse organisms, that enzymes for the β-ketoadipate pathway are regulated by induction. This generalization would be without exception if it were not for the properties of the bradyrhizobia, discussed below.

Constitutive synthesis of enzymes in bradyrhizobia. Oligotrophic bacteria have been defined as organisms that grow in media containing low concentrations of organic matter, ranging from 1 to 15 mg of organic carbon per liter (20). With their ability to survive and even grow, under certain conditions, in distilled water (5), bradyrhizobia certainly can be classified as oligotrophic. Severe limitation of growth substrate is likely to restrict the amount of carbon and energy that oligotrophs can dedicate to protein synthesis. Selection for or against transcriptional control mechanisms must be determined in part by the interplay between the effectiveness of the controls in preventing synthesis of unnecessary proteins and the biosynthetic demands imposed by synthesis of the control mechanisms themselves.

The constitutive levels for the three enzymes that convert β-carboxy-*cis*,*cis*-muconate to β-ketoadipate are quite low in the bradyrhizobia. Perhaps, in this case, the physiological expense of transcriptional control does not counterbalance the relatively low biosynthetic expenditure required for constitutive synthesis of the enzymes. It would be of interest to know whether a high degree of unregulated catabolic enzyme synthesis characterized by low specific activities is commonly found in microorganisms adapted to an oligotrophic environment.

The bradyrhizobia constitutively form β-ketoadipate succinyl-CoA transferase at levels comparable to those found in fully induced cultures of *P. putida*. The specific activities of the pure enzyme from widely divergent bacterial sources are roughly the same (28; Ka-Leung Ngai, unpublished obser-

vations). Thus it is reasonable to propose that the amount of transferase constitutively synthesized in bradyrhizobia is about the same as that found in induced *P. putida* cultures, approximately 0.4% of the cell protein. The fact that constitutive expression of the CoA transferase, a trait unique to bradyrhizobia among organisms that have been characterized in this regard, has been conserved against a background of substantial evolutionary divergence suggests that the trait has been selected in the niche of the organisms. It should be noted that six free-living representatives of bradyrhizobia examined grew at the expense of β-ketoadipate (18). In further support of the inference that β-ketoadipate plays an important role in the ecology of bradyrhizobia is the fact that chemotaxis to β-ketoadipate is a constitutive trait in *B. japonicum* I-110 (19).

The biological source of the β-ketoadipate is unknown. The compound can be excreted during the microbial dissimilation of aromatic compounds (14) and is formed quantitatively from aromatic precursors in extracts of appropriately induced cells (23). The most likely source of aromatic precursors is plant cells or litter. This raises the possibility that the bradyrhizobia utilize β-ketoadipate as members of consortia that may include other microorganisms or plant cells.

ACKNOWLEDGMENTS

We thank Ka-Leung Ngai for providing purified enzymes for use in coupled enzyme assays, Anne Harrison for preparation of the manuscript, and Andrew Glenn for his critical reading of the manuscript.

This research was funded by the Celanese Research Company.

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