Nutritional Diversity of *Rhizobiaceae* Revealed by Auxanography

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Many aromatic compounds are toxic when supplied at concentrations employed in most growth media. This effect was demonstrated when rhizobia and agrobacteria were grown in auxanographic plates in which cells were seeded in agar and exposed to a gentle gradient of substrate concentration. An auxanographic nutritional survey with representative strains revealed that *Rhizobium japonicum* and cowpea *Rhizobium* sp. could utilize a relatively large proportion of the aromatic and hydroaromatic compounds tested; *Rhizobium leguminosarum*, *Rhizobium trifolii* and *Agrobacterium* species displayed intermediate nutritional versatility; *Rhizobium meliloti* was relatively fastidious. The hydroaromatics quinate and shikimate were not toxic. Most of the strains examined grew at the expense of one or both of these substrates. Quinate was metabolized via protocatechuate and 3-oxoadipate. All of the strains examined were able to grow at the expense of protocatechuate and therefore must contain six structural genes for the enzymes required to convert this aromatic compound to common intermediary metabolites. Conservation of pathways for aromatic catabolism against a background of wide evolutionary divergence among the *Rhizobiaceae* suggests that pressures for selection of the traits were exerted throughout the evolutionary history of the organisms. A probable selective pressure is competition for nutrients in the soil. In addition, the ability of agrobacteria and rhizobia to respond to aromatic compounds may have selective value in bacterial–plant interactions.

INTRODUCTION

Nutritional surveys contributed to the classification of rhizobia (Graham, 1964*a*; Graham & Parker, 1964; Moffett & Colwell, 1968) and agrobacteria (Keane *et al.*, 1970; White, 1972; Kersters *et al.*, 1973). Patterns of carbohydrate utilization suggested that relatively fast growing rhizobia and agrobacteria are nutritionally diverse whereas slow growing rhizobia appear to be nutritionally fastidious. It is clear from the work of Parker *et al.* (1977) and Glenn & Dilworth (1981) that some rhizobial strains grow at the expense of aromatic compounds, yet most aromatic growth substrates have been overlooked in previous nutritional surveys. This investigation was undertaken to see if genes for the catabolism of aromatic compounds are distributed widely among members of the *Rhizobiaceae*.

Early in our work it became apparent that most aromatic compounds are toxic to members of the *Rhizobiaceae*. In order to explore the full genetic potential of strains, the compounds were supplied in low concentration by auxanography in a defined minimal medium. A similar method was employed by Graham (1964*b*) to observe growth supplementation of rhizobia and agrobacteria by carbohydrates and Krebs cycle intermediates in an undefined medium. The work reported here, the first systematic survey of the *Rhizobiaceae* employing aromatic, hydroaromatic and related compounds, demonstrates substantial catabolic potential within the biological group and indicates that the slow growing strains may be more nutritionally versatile than previously recognized.

Abbreviations: MM, defined minimal medium; YM, yeast extract/mannitol medium.

Table 1. *Bacterial strains*

Species	Strain	Source
Fast growing strains		
<i>Rhizobium meliloti</i>	102F28	J. C. Burton & R. S. Smith, The Nitragen Co. Inc., Milwaukee, Wisconsin 53209, USA
<i>R. meliloti</i>	FA1021	F. M. Ausubel, Harvard University
<i>Rhizobium leguminosarum</i>	2368	H. H. Keyser, USDA, Nitrogen Fixation Lab., Beltsville, MD 20705, USA
<i>R. leguminosarum</i>	2376	
<i>Rhizobium trifolii</i>	2066	
<i>R. trifolii</i>	162S33	J. C. Burton & R. S. Smith, The Nitragen Co. Inc.
<i>R. trifolii</i>	BP100	<i>Trifolium repens</i> L. nodules; Hamden, Ct., USA
<i>R. trifolii</i>	HM100	<i>Trifolium pratense</i> L. nodules; Hamden, Ct., USA
<i>Rhizobium japonicum</i>	192	H. H. Keyser, USDA, Nitrogen Fixation Lab.
<i>Agrobacterium tumefaciens</i>	B6	S. R. Long, Stanford University
<i>Agrobacterium rhizogenes</i>	A4	D. Tepfer, CNRA, Versailles, France
Slow growing strains		
<i>Rhizobium japonicum</i>	61A76	J. C. Burton & R. S. Smith, The Nitragen Co. Inc.
<i>R. japonicum</i>	110	H. H. Keyser, USDA, Nitrogen Fixation Lab.
<i>R. japonicum</i>	123	
Cowpea <i>Rhizobium</i> sp.	3179	
Cowpea <i>Rhizobium</i> sp.	3241	R. S. Smith, The Nitragen Co. Inc.
Cowpea <i>Rhizobium</i> sp.	32H1	

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 generation times of 2 to 4 h on yeast extract/mannitol medium, whereas slow growing strains are those with generation times of 6 h or more (Vincent, 1977). The organisms were maintained on plates containing yeast extract/mannitol medium (YM; Wacek & Brill, 1976). Colonies were picked from YM plates and placed in a defined liquid minimal medium (MM; Ornston & Stanier, 1966) containing Hutner's 'Metals 44' (Cohen-Bazire *et al.*, 1957) and biotin at $0.5 \mu\text{g ml}^{-1}$; the medium was modified in that it contained 0.0125 M each of KH_2PO_4 and Na_2HPO_4 . The minimal medium for strains of *R. leguminosarum* and *R. trifolii* contained additional supplements of thiamin.HCl at $1 \mu\text{g ml}^{-1}$ and calcium pantothenate at $2 \mu\text{g ml}^{-1}$. Carbon sources were prepared as 0.5 M or 1.0 M concentrated solutions, neutralized with NaOH, and filter sterilized. Catechol and sodium benzoate were obtained from MCB Manufacturing Chemists Inc., Cincinnati, Ohio 45212, USA. *cis,cis*-Muconate was synthesized chemically (Riegel & Lilienfeld, 1945), and all other carbon sources were purchased from Sigma. Liquid cultures were incubated at 30°C on a gyrotory New Brunswick Environmental shaker at 240 r.p.m. Growth was measured by monitoring optical density at 600 nm.

Auxanography (Lederberg, 1946) was used to screen strains for their ability to utilize aromatic compounds as a source of carbon and energy. Cells were grown in 5 ml growth tubes to early stationary phase, corresponding to an optical density of 0.7 at 600 nm, using 10 mM-succinate as a growth substrate. *Rhizobium japonicum* strain 192, *R. leguminosarum* and *R. trifolii* were exceptions in that they were grown on media containing 10 mM-glucose, arabinose or mannitol. Cells were rinsed with an equal volume of MM lacking carbon source. Resuspended cells were then mixed with MM containing 1.5% (w/v) purified Noble agar (Difco) at a temperature below 50°C , and 20 ml of the media containing the cells was poured into each $15 \times 100 \text{ mm}$ Petri dish. Each Petri dish received approximately 2×10^9 cells. The plates were left overnight to solidify before carbon sources were added. Up to three different carbon sources were spotted onto the edge of a plate using 15 to $30 \mu\text{mol}$ of each substrate; each carbon source was tested on at least three plates. Plates were incubated at 30°C for up to 4 d. All of the above operations were carried out under aseptic conditions.

Contamination was routinely checked by microscopic examination of cultures and by streaking cells onto a YM plate containing 0.0025% Congo red. Most of the strains used in this study were distinct in morphology and growth rates. In addition, strains were tested for their ability to nodulate appropriate host plants.

Nodulation tests. Rhizobial strains were examined for symbiotic properties by inoculation onto seedlings. Seeds were surface sterilized by brief immersion in 70% (v/v) ethanol, rinsing with sterile distilled water, treatment with 50% (v/v) Chlorox for 10 or 15 min, and rinsing six more times with sterile distilled water. Seeds of 'Siratro' and vetch were pretreated with concentrated H_2SO_4 for 10 min. They were rinsed with sterile distilled water nine times prior to the Chlorox treatment. Seeds were germinated on sterile agar slants in tubes supplied with Jensen's medium (Vincent, 1970). Plants were grown for 4 to 6 weeks in a growth chamber at 24°C , 18000 lx, and with a 16 h photoperiod. Rhizobia were grown in liquid YM medium, rinsed, and resuspended in distilled water. Week old plants were inoculated with approximately 10^7 bacteria in 0.5 ml.

The host plant nodulated by all of the *Rhizobium japonicum* and cowpea *Rhizobium* sp. strains was 'Siratro' (*Macroptilium atropurpureum*, PI no. 316463). Vetch, *Vicia sativa* cv. 'Woodford', formed nodules when inoculated with the *Rhizobium leguminosarum* strains, and *Trifolium pratense* var. 'Pennscoot' was nodulated by all strains of *Rhizobium trifolii* except strain 162S33. *Medicago sativa* var. 'Iroquois' was the host of *Rhizobium meliloti* strains. 'Siratro' and vetch seeds were kindly provided by Gill Lovell of the USDA Regional Plant Introduction Station, Experiment, Ga. 30212, USA. Other seeds were obtained from Agway Inc.

RESULTS

Genetic potential revealed by auxanography. Auxanography revealed the genetic potential of strains to grow with compounds that were toxic at high concentrations. Anthranilate, benzoate, cinnamate, catechol, *p*-coumarate, gallate, gentisate, mandelate, *p*-hydroxybenzoate, syringate and vanillate supported growth at low substrate concentration, revealed by an arc of growth away from the origin of substrate application to the plate (Fig. 1). The toxicity of the compounds was reflected in the distance of the arc from the origin, and it varied from strain to strain. In some cases growth was limited to a narrow band and, in others, a broad band (Fig. 1).

Auxanography also distinguished between mutation or variation in a minority of cells and the slow growth of an entire population, a distinction which might have been overlooked in liquid cultures. For example, all of the *R. trifolii*, *R. leguminosarum* and *Agrobacterium tumefaciens* strains formed mutant or variant colonies readily when exposed to shikimate (Table 2), and thus the wild type cells possessed all the genes required for efficient utilization of this substrate.

Protocatechuate, a universal growth substrate for rhizobia. Protocatechuate supported the growth of all the strains included in this survey. The compound is relatively nontoxic to many strains, but its chemical instability makes it undesirable as a growth substrate for metabolic studies. On the other hand, quinate or shikimate, stable nontoxic growth substrates which are utilized via protocatechuate (Fig. 2), supported growth of almost all the examined strains. A representative of each species that utilized quinate was shown to convert it to 3-oxoadipate, determined as material giving a positive Rothera reaction (Evans, 1947; Stanier *et al.*, 1966). This evidence indicated that quinate and protocatechuate were metabolized via the pathway outlined in Fig. 2.

Growth substrates that distinguish between fast growing and slow growing strains. The survey revealed that the slow growing strains examined grew at the expense of adipate whereas fast growing strains did not. Adipate, a nonmetabolizable analogue of the inducer 3-oxoadipate in some *Pseudomonas* species (Ornston & Parke, 1976), was nontoxic and thus might be used to facilitate isolation of slow growing rhizobia from the soil without interference from fast growing rhizobia or agrobacteria.

Fig. 1. Growth patterns on auxanographic plates. (a) Growth of *A. tumefaciens* with quinate, a non-toxic substrate. (b) Arcs observed after growth of *R. leguminosarum* strain 2376 with moderately toxic substrates benzoate and *p*-hydroxybenzoate. (c) Narrow arc observed after growth of *R. meliloti* strain 102F28 with the extremely toxic substrate anthranilate.

Table 2. Growth substrates utilized by fast growing strains of *Rhizobiaceae*

Substrate	<i>R. meliloti</i>			<i>R. japonicum</i> 192	<i>R. leguminosarum</i>			<i>R. trifolii</i>				<i>A. rhizogenes</i> A4	<i>A. tumefaciens</i> B6
	FA1021	102F28			2376	2368	2066	162S33	BP100	HM100			
Anthranilate	—*	+		—	—	—	—	—	—	—	—	—	
Benzoate	—	—		+	+	+	(+)	—	+	—	—	—*	
Catechol	—	—		+	+	+	+	—	+	—	—	—*	
<i>p</i> -Coumarate	—	—		—	+	+	+	+	+	+	—	—	
Ferulate	—	—		—	+	(+)	(+)	(+)	+	+	—	—	
Gallate†	—	—		—	—	—	—	—	—	—	+	—	
3-Oxo adipate	—	—		—	—	—	—	—	—	—	—*	—*	
<i>p</i> -Hydroxybenzoate	—	—		+	+	+	+	+	+	+	+	+	
Protocatechuate	+	+		+	+	+	+	+	(+)	+	+	+	
Quinate	—*	+		+	+	+	+	+	+	+	+	+	
Shikimate	—	—		—	—*	—*	—*	—*	—*	—*	+	—*	

+, Growth; —, no growth; (+), very faint growth. In addition to the compounds listed, fast growing strains did not utilize adipate, cinnamate, gentisate, muconate, mandelate, salicylate, syringate or vanillate.

* Mutant or variant colonies appeared in agar after more than 72 h at 30 °C.

† Gallic acid was applied as a solid; oxidation of the compound to a black substance made the scoring of plates difficult with the fast growing strains.

Fig. 2. Pathways for the catabolism of aromatic and hydroaromatic compounds via 3-oxoadipate in prokaryotes. Each arrow represents a specific enzymic step. As shown in this paper, quinate is degraded to 3-oxoadipate by agrobacteria and rhizobia. Pathways used by the organisms for catabolism of most of the other compounds included in this study remain to be determined.

All of the slow growing strains except possibly *Rhizobium* sp. strain 32H1 grew at the expense of vanillate whereas the fast growing strains did not. It should be noted that other workers have reported a fast growing rhizobial strain which was able to utilize a wide range of aromatics including vanillate (Glenn & Dilworth, 1981).

Growth patterns of fast growing strains. The fast growers could be distinguished among themselves by their responses to ten aromatic and hydroaromatic compounds (Table 2). The two *R. meliloti* strains were most fastidious with respect to these compounds, demonstrating the genetic potential to utilize only three of them (Table 2). Protocatechuate was the sole tested compound that supported the growth of *R. meliloti* strain FA1021, but the organism readily acquired the ability to grow with either anthranilate or quinate. Thus the nutritional potential of this strain was identical to that of the independent *R. meliloti* isolate, strain 102F28 (Table 2).

Two *R. leguminosarum* strains and four *R. trifolii* strains shared almost the same catabolic pattern, utilizing seven of the ten compounds. The only difference between the two species was failure of two of the *R. trifolii* strains to share a reproducibly positive growth response with benzoate (Table 2). *R. japonicum* 192, a fast growing isolate from China (Keyser *et al.*, 1982) was

Table 3. *Growth substrates utilized by slow growing rhizobial strains*

Substrate	<i>R. japonicum</i> 61A76	<i>R. japonicum</i> 110	<i>R. japonicum</i> 123	<i>Rhizobium</i> sp. 3179	<i>Rhizobium</i> sp. 3241	<i>Rhizobium</i> sp. 32H1
Adipate	+	+	+	+	+	+
Anthranilate	+	+	(+)	(+)	(+)	(+)
Benzoate	+	+	(+)	—	—	(+)
Cinnamate	+	(+)	(+)	—	—	(+)
Catechol	+	—	—	—	—	—
<i>p</i> -Coumarate	+	+	+	+	+	+
Ferulate	+	+	+	+	+	(+)
Gallate*	+	+	+	+	+	+
Gentisate	+	+	+	+	+	+
3-Oxo adipate	+	+	+	+	+	+
Mandelate	+	(+)	(+)	—	(+)	(+)
<i>cis,cis</i> -Muconate	+	—	—	—	—	—
<i>p</i> -Hydroxybenzoate	+	+	(+)	+	+	(+)
Protocatechuate	+	+	+	+	+	+
Quinate	—	+	+	+	+	+
Shikimate	—	+	+	+	+	+
Syringate	—	+	+	(+)	—	—
Vanillate	+	+	+	+	+	(+)

+, Growth; —, no growth; (+), very faint growth. Salicylate did not serve as a growth substrate for any of the above strains.

* Gallic acid was applied as a solid.

similar to the *R. leguminosarum* strains except that it did not mutate to growth at the expense of shikimate and did not grow with *p*-coumarate or ferulate (Table 2).

To test whether strains had lost the ability to utilize any of the tested compounds during long-term storage, two *R. trifolii* strains, BP100 and HM100, were isolated from nodules of native white and red clover plants, respectively, and subjected to auxanography after being sub-cultured once on YM. Their growth patterns were similar to those of *R. leguminosarum* and *R. trifolii* strains from culture collections.

Representatives of the genus *Agrobacterium* have been classified into two major biotypes on the basis of biochemical tests and their differential ability to utilize several growth substrates (Elkan, 1981). A representative of biotype 1, *A. tumefaciens* B6, and one of biotype 2, *A. rhizogenes* A4, were included in this survey. The two *Agrobacterium* strains were distinguishable from the *leguminosarum*-*trifolii* group by their failure to utilize *p*-coumarate and ferulate and by the ability of the *A. rhizogenes* strain to catabolize gallate. With respect to other compounds tested, the two *agrobacteria* appeared to be more closely related to the *leguminosarum*-*trifolii* strains than they were to the *R. meliloti* strains (Table 2). This contrasts with the conclusions of White (1972) and Graham (1964*a*) who found that, on the basis of numerous criteria, both *Agrobacterium* biotypes were more closely related to *R. meliloti* than to *R. leguminosarum*.

Growth patterns of slow growing strains. Whereas fast growing strains demonstrated the ability to utilize from one to seven of the proffered substrates, slow growing strains showed a relatively full growth yield at the expense of from eight to fifteen of the compounds (Table 3). The three *R. japonicum* strains included in the survey fall into different DNA homology groups within this species (Hollis *et al.*, 1981). The cowpea *Rhizobium* sp. strain 3179, also known as 3G4b4, may bear a subspecies relationship to *R. japonicum* strains 110 and 123, whereas *Rhizobium* sp. strain 32H1 falls outside this group (Hollis *et al.*, 1981). Thus, across a broad spectrum of DNA homology groups, slow growing rhizobia demonstrated the capacity to utilize aromatic compounds and showed different patterns of utilization.

A number of aromatic compounds produced low growth yields with the slow growing rhizobial strains (Table 3); even after prolonged incubation, the growth yields did not increase in these cases. Quinate and shikimate, in addition to being non-toxic, allowed growth to a full yield.

In liquid MM, shikimate supported a doubling time of 11 h for *Rhizobium* sp. 3241. The same strain had a doubling time of 20 h with quinate and 6 h with succinate. Cells grown in the presence of quinate or shikimate were usually small and relatively uniform in appearance, even those of strains like *R. trifolii* strain 2066 which assumed enlarged, pleomorphic shapes in MM supplemented with succinate as sole carbon source.

DISCUSSION

Auxanographic analysis of diverse rhizobial species revealed considerable metabolic potential overlooked in earlier nutritional surveys of these organisms. Slow growing rhizobia, generally considered to be nutritionally fastidious, were able to use eight to fifteen of the nineteen substrates presented to them as sole sources of carbon and energy; fast growers were able to utilize seven or fewer of the nineteen substrates.

The results suggest that some of the tested compounds may prove useful in elucidating taxonomic relationships among the *Rhizobiaceae* and in isolating particular groups from the soil. For example, the distinction between fast and slow growers in the ability to utilize adipate warrants further study. Many additional aromatic compounds, existing in free form or covalently bound to other aromatics or sugars, may occur in or around the roots of plants. Growth studies with these compounds may reveal additional nutritional versatility among members of the *Rhizobiaceae*.

Protocatechuate served as sole source of carbon and energy for the seventeen representatives of the *Rhizobiaceae* examined. Quinate and shikimate, metabolized via protocatechuate, were commonly used growth substrates. At least six structural genes encode the enzymes that degrade protocatechuate via the 3-oxoadipate pathway (Fig. 2). Conservation of these genes across a broad biological spectrum indicates that there have been selective pressures to maintain them. Possible functions of the genes encoding enzymes of aromatic catabolism are in enabling rhizobia and agrobacteria to survive and to compete effectively in the soil, an environment containing low levels of aromatics derived from the breakdown of lignin and other plant products.

In addition, genes for aromatic catabolism may be involved in bacterial-plant interactions. Most of the aromatic acids used in this survey are widely distributed in plants (Ribereau-Gayon, 1972). Aromatic acids released from plants (Harborne, 1980) may serve as chemo-attractants for rhizobia and agrobacteria. The possibility that aromatic compounds contribute to the metabolism of bacteroids in root nodules is raised by the observation that the total phenolic concentration in alfalfa root nodules exceeds the concentration in roots by more than twofold (Vance, 1978). The hypothesis that aromatics play a role in rhizobial-plant interactions is being tested by obtaining rhizobial strains defective in aromatic catabolism and comparing the properties of wild type and mutant strains.

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