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Invalidity of the Concept of Slow Growth and Alkali Production in Cowpea Rhizobia

BLANCA S. HERNANDEZ[1] AND DENNIS D. FOCHT[2]*

Department of Soil and Environmental Sciences, University of California, Riverside, Riverside, California 92521,[1,2] and Universidad de Panama, Facultad de Ciencias Naturales y Farmacia, Panama City, Panama[1]

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A total of 103 rhizobial strains representing the cowpea miscellany and *Rhizobium japonicum* were studied with regard to growth rate, glucose metabolic pathways, and pH change in culture medium. Doubling times ranged from 1.4 ± 0.04 to 44.1 ± 5.2 h; although two populations of "fast-growing" and "slow-growing" rhizobia were noted, they overlapped and were not distinctly separated. Twenty-four strains which had doubling times of less than 8 h all showed NADP-linked 6-phosphogluconate dehydrogenase (6-PGD) activity, whereas only one slow-growing strain (doubling time, 10.8 ± 0.9 h) of all those tested showed 6-PGD activity. Doubling times among fast growers could not be explained solely by the presence or absence of 6-PGD activity (r^2 = 0.14) because the tricarboxylic acid cycle and the Emden-Meyerhoff-Parnas pathway were operative in both 6-PGD-positive and 6-PGD-negative strains. Growth rate and pH change were unrelated to each other. Fast- or slow-growing strains were not associated with any particular legume species or group of species from which they were originally isolated, with the exception of *Stylosanthes* spp., all nine isolates of which were slow growers. We conclude that 6-PGD activity is a more distinctive characteristic among physiologically different groups of rhizobia than doubling times and that characterization of the cowpea rhizobia as slow-growing alkali producers is an invalid concept.

Cowpea rhizobia were defined by Norris (22) as the ancestral type that arose in the tropics. They were characterized as typically slow growing and producing alkali in the medium; the latter characteristic was interpreted to signify that alkali was produced in response to acidic soil conditions common to the tropics (23). In contrast, fast growth and acid production have been generally associated with rhizobia which nodulate temperate legumes, with the exception of *Rhizobium japonicum*, which is considered to be more closely related to the cowpea miscellany (10, 22). Recent studies show, however, that pH change in culture is dependent upon the carbon source or the amount of phosphate buffer (24, 30). In fact, the concept that the cowpea rhizobia-*R. japonicum* group are slow growers may be inaccurate in light of recent reports on fast-growing strains (6, 9, 17, 27, 30).

The classification of rhizobia in *Bergey's Manual*, 8th ed. (4), is still based primarily upon the traditional cross-inoculation groups developed by Baldwin and Fred (1) rather than upon the physiology of the bacteria. Consequently, the isolation and diagnosis of a rhizobial endophyte from a cowpea cultivar (e.g., *Vigan unguiculata*) would be designated as a cowpea miscellany, whereas one isolated from soybeans (*Glycine max*) would be designated as *R. japonicum* despite the fact that the two are generally indistinguishable physiologically and frequently cross-inoculate the two cultivars. Graham (10) has recommended that all species of cowpea rhizobia and *R. japonicum* be reclassified as *Phytomyxa*. This recommendation was essentially carried out by Jordan (13), except for the nomenclatural designation of the generic name *Bradyrhizobium* (Greek bradus: slow) to distinguish the "slow-growing, alkali-producing" rhizobia from the "fast-growing, acid-producing" strains, the latter which retain the generic name *Rhizobium*. Some of the pertinent criteria used for this delineation are as follows. "Moderate turbidity is produced only after 3 to 5 days or longer in agitated broth. Produce an alkaline reaction in mineral salts-mannitol medium after 28 days at 27°C" (13). A recent study by Kennedy and Greenwood (16), however, has shown that slow growth and alkali production were not mutually inclusive characteristics. Moreover, the concept of Norris (22) which associates slow growth with alkali production and fast growth with acid production is based upon neither a sufficiently large sample size nor any quantitative relationship of growth rates and pH changes.

The physiological mechanism of slow versus fast growth was advanced by Elkan (7) and verified by Martinez-de Drets and Arias (19). Slow-growing rhizobia lack NADP-linked 6-phosphogluconate dehydrogenase (6-PGD; EC 1.1.1.43), the key enzyme of the hexose monophosphate pathway, and instead use the Entner-Doudoroff pathway. Hexoses can also be metabolized by rhizobia through the Emden-Meyerhoff-Parnas pathway, although Martinez-de Drets and Arias (19) have reported that fructose-diphosphate aldolase (FDPA; EC 44.1.2.3), the key enzyme of the Emden-Meyerhoff-Parnas pathway, is present in low quantities. Others (14, 21, 25) have found that it occurs in both fast- and slow-growing rhizobia.

In light of the recent expansion of rhizobial technology to agricultural development in developing countries of the tropics and to subtropical areas of developed countries (e.g., the United States and Australia), basic physiological characteristics of these bacteria must be understood to provide a working framework for microbiologists. Many uncertainties and contradictions regarding physiological traits of cowpea rhizobia and *R. japonicum* exist because all studies, to our knowledge, have encompassed a relatively small sample of strains that may not have been representative of a naturally occurring population. The following study, therefore, was undertaken with 103 isolates representing a diverse source of isolation with respect to geography and cultivar to determine whether (i) growth rate and pH change are mutually

* Corresponding author.

exclusive properties, (ii) cowpea rhizobia and *R. japonicum* are clearly delineated into fast- and slow-growing groups, (iii) glucose catabolic pathways are related to growth rate, and (iv) certain plants are specifically associated only with fast- or slow-growing strains, rather than both.

MATERIALS AND METHODS

***Rhizobium* strains.** A total of 105 strains were used in the study. All P strains were isolated at the University of Panama, Panama City; 3389 and all IHP strains were obtained from P. Dart, ICRISAT, Patancheru, India. TAL strains were obtained from NifTAL, Paia, Hawaii. Strains 136, 726, 727, 807, 891, 1460, 1504, 1505, 1943, and 1944 were obtained from CIAT, Cali, Columbia. Strains 8A55, 24A17, 24Z1, 32H1, 42B1, 43B3, 94A5, 94A8, 61-A101, 101B1, 105A1, 145A10, 145Z2, 150B1, 176A22, 176A26, 176A27, and 176A28 were obtained from J. C. Burton, Nitragin Co., Madison, Wis. Strains 110, 191, 192, 193, and 194, the fast-growing strains from the People's Republic of China, were obtained from H. Keyser, U.S. Department of Agriculture, Beltsville, Md. Strains L1, L7, and L9 were obtained from J. S. LaFavre, University of California, Riverside. Strain 406 was obtained from M. Alexander, Cornell University, Ithaca, N.Y. Strain ATCC 10324, the proposed type species *Bradyrhizobium japonicum* (13), was obtained from the American Type Culture Collection, Rockville, Md. When all strains used in this investigation were inoculated axenically to the legumes from which they were isolated, all formed nodules and thus satisfied Koch's postulates.

TABLE 1. Doubling times of rhizobia and source of isolation (host plant)

Host plant	Doubling time (h)	No. of strains	Strain designations
Glycine max	3.2–22.5	11	P218, P220, 191, 192, 193, 194, ATCC 10324, USDA 110, TAL102, TAL379, 61-A-101
Vigna unguiculata	1.7–26.6	9	176A22, 176A26, 176A27, 176A28, TAL411, P132, 32H1, 3389, 406
Leucaena leucocephala	1.7–26.6	6	1460, 1943, 1944, 94A5, 94A8, CB81
Cajanus cajan	1.4–44.1	47	IHP1, IHP24, IHP71, IHP147, IHP194, IHP195, IHP229, P130, P131, P133, P136, P137, P138, P139, P140, P141, P142, P144, P146, P147, P148, P149, P150, P151, P152, P153, P155, P156, P157, P158, P162, P163, P164, P165, P167, P170, P171, P179, P180, P183, P184, P185, P193, P194, P195, P338, P350
Stylosanthes spp.	12.0–29.3	9	150B1, 807, P119, P121, P123, P124, P126, P128, P134
Desmodium spp.	3.6–24.3	5	136, 726, 727, 891, 1504
Prosopis spp.	1.9–11.1	3	L1, L7, L9
Sesbania sp.	3.6–15.0	2	145A10, 145Z2
Mimosa spp.	2.9–7.8	2	101B1, 105A1
Cassia spp.	14.1–26.7	2	24A17, 24Z1
Lablab spp.	23.1–35.8	2	42B1, 42B3
Calopogodium spp.	23.0	1	1505
Medicago spp.	2.9	1	TAL380
Centrosema spp.	31.0	1	P122
Arachis spp.	12.5	1	8A55
Phaseolus vulgaris	31.3	1	P174

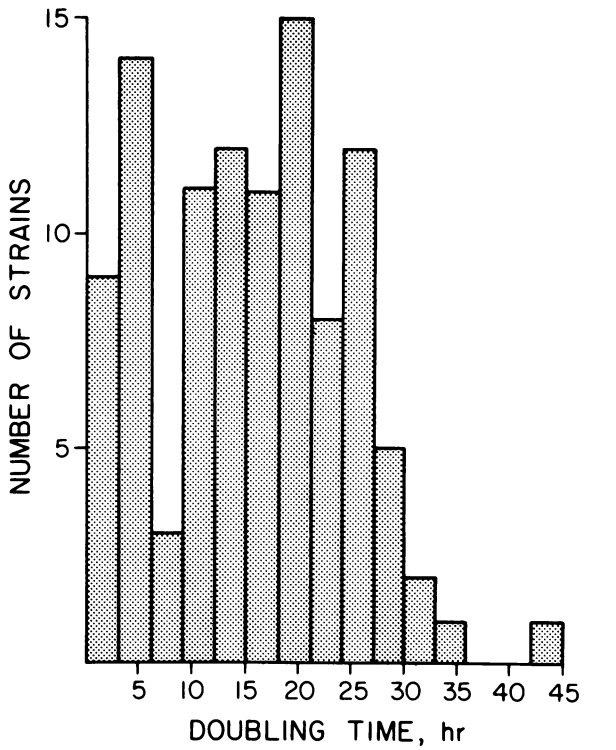

FIG. 1. Histogram of doubling time for 103 rhizobial strains (minimum, 1.4 h; maximum, 44.1 h).

Culture media. Cultures were grown in yeast extract broth (28) containing 1% glucose as a carbohydrate source. The medium was adjusted to pH 6.8 before being autoclaved (5 min at 20 lb/in^2). *Rhizobium* strains were grown in side-arm Erlenmeyer flasks (125 ml) which contained 30 ml of yeast extract-glucose broth. At late exponential growth (optical density, 0.6 to 0.8), 0.5 ml was inoculated into side-arm flasks containing 50 ml of yeast extract-glucose broth. Each strain was grown in sets of three replicates on a rotary platform shaker (125 rpm) at 26°C. Optical density readings were determined at 525 nm with a Bausch & Lomb Spectronic 20 spectrophotometer at intervals of 1, 2, 3, 6, or 12 h, depending on the growth rate of the strain. Doubling times

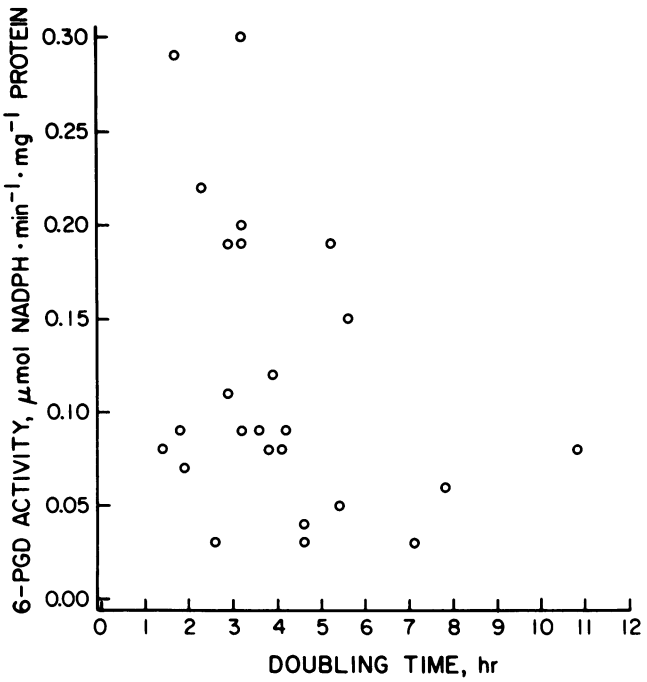

FIG. 2. Relationship of 6-PGD activity and doubling time for 25 rhizobial strains (r^2 = 0.13, F = 5.13, and α = 0.01 for linear regression; r^2 = 0.15, F = 5.61, and α = 0.01 for hyperbolic regression).

were calculated from the exponential growth phase. The final pH was measured at an optical density of 1.0.

Preparation of crude cell-free extracts. Rhizobial cultures were grown in batches of 100 ml of yeast extract-glucose broth, harvested at the late exponential phase of growth by centrifugation at 10,000 × g for 10 min in a Sorvall RC-5 refrigerated centrifuge, and washed twice with 0.05 M sodium phosphate buffer (pH 6.9). Cells were resuspended by adding just enough buffer (1 to 2 ml) to cover the pellet. The cells were disrupted with a Branson sonicator in an ice bath (three 1-min bursts separated by 5-s intervals). Cell-free extracts were obtained by centrifugation at 37,000 × g for 20 min. The extracts were kept in an ice bath at all times and used within 4 h from the time of preparation. Protein content was determined by the method of Lowry et al. (18).

Chemicals. 6-Phosphogluconic acid (sodium salt), isocitric acid (trisodium salt), ATP (sodium salt), D-fructose-6-phosphate (disodium salt), aldolase calibration solution, glycylglycine, malic acid (disodium salt), sodium potassium tartrate (crystalline tetrahydrate), and Folin & Ciocalteu's phenol reagent were obtained from Sigma Chemical Co., St. Louis, Mo. Tris-hydrochloride buffer, albumin (bovine factor V), and NADP were obtained from Matheson, Coleman and Bell, Norwood, Ohio, and hydrazine sulfate was obtained from Merck & Co., Inc., Rahway, N.J.

Enzyme assays. Dehydrogenases were assayed by the method described by Keele et al. (15). The reaction mixture for 6-PGD (EC 1.1.1.43 or EC 1.1.1.44) contained 250 μmol of glycylglycine buffer (pH 8.5), 1.5 μmol of 6-phosphogluconic acid, 1 μmol of NADP, 30 μmol of $MgSO_4$, and 0.1 ml of cell-free extract (the protein concentration ranged from 0.1 to 0.5 mg in the cuvette). The final volume of the mixture was 3.0 ml. The reaction mixture for NADP-linked isocitrate dehydrogenase (EC 1.1.1.42) contained 250 μmol of glycylglycine buffer, 30 μmol of $MgSO_4$, 1 mg of NADP, 3 μmol of isocitrate trisodium salt (or 6 μmol of malic acid disodium salt), and 0.1 ml of cell extract (0.5 to 1.0 mg of protein). The final volume was 3.0 ml. Upon the addition of NADP, change in absorbance was measured spectrophotometrically (340 nm). Specific activities were calculated by the method of Bergmeyer (2) and expressed as micromoles of NADPH per minute per milligram of protein.

The presence of FDPA (EC 4.1.2.13) was determined by the method of Brisou (3), which is based on the colorimetric measurement of 2,4-dinitrophenylhydrazone at 540 nm. The reaction mixture containing 0.5 ml of fructose-1,6-diphosphate (in 0.01 M Tris-hydrochloride buffer, pH 7.2), 1.0 ml of Tris-hydrochloride buffer, 0.1 ml of hydrazine sulfate (0.056 M), and 0.3 ml of crude cell-free extract (0.5 to 1.0 mg of protein) was placed in a water bath at 25°C for 60 min. After the addition of 1 ml of trichloroacetic acid (8%), the reaction mixture was centrifuged at 10,000 × g for 10 min, and 0.5 ml of supernatant was mixed with 0.5 ml of NaOH (0.7 M) and 0.5 ml of 2,4-dinitrophenylhydrazone. This mixture was placed in a water bath at 38°C for 10 min to develop the hydrazone for colorimetric determination. Specific activities were expressed as micromoles of product formed per milligram of protein.

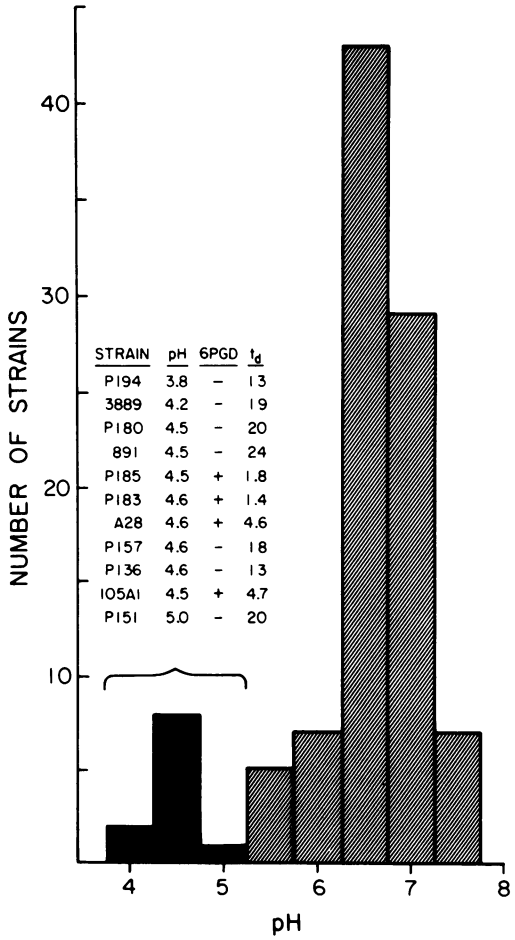

FIG. 3. Histogram of final pH change in culture medium for 103 rhizobial strains. Acid-producing strains (■) are listed above with corresponding final pH, presence (+) or absence (−) of 6-PGD activity, and doubling time (t_d) to illustrate the lack of correlation between acid production and rapid growth.

RESULTS

The doubling times of the rhizobial strains (Table 1) did not correlate with host plant (i.e., source of isolate). Strains which were positive and negative in relation to 6-PGD activity were isolated from *V. unguiculata*, *Cajanus cajan*, *G. max*, *Leucaena leucocephala*, and *Desmodium*, *Sesbania*, and *Prosopis* spp. Although the assay which we and others (16, 19) have used does not distinguish between 6-PGD which forms 6-phospho-2-keto-D-gluconate (EC 1.1.1.43) and that which forms D-ribulose 5-phosphate (EC 1.1.1.44), we are concerned only with the presence or absence of any 6-PGD activity, since strains that lack activity can not use the hexose monophosphate pathway, which is supposedly the reason for slow growth (7). Strains which had less than the detectable limit (0.005 μmol of NADPH per mg of protein per min) of 6-PGD activity were listed as negative. The range for 6-PGD-positive strains was 0.030 to 0.290 (Fig. 1). Isocitrate dehydrogenase activity ranged from 0.03 to 0.92 μmol of NADPH per mg of protein per min, and FDPA activity ranged from 0.04 to 27.0 μg of product per mg of protein per min in all strains tested.

Although doubling times for 103 rhizobial strains appear to be distributed into two populations, they overlapped and were not divided into two exclusive populations of fast and slow growers (Fig. 1). Nevertheless, rhizobia were separated into two groups at a doubling time of 7.8 ± 0.5 h on the basis of 6-PGD activity. Only one strain (P130) which had a doubling time of greater than 7.8 h (10.8 ± 0.9) had 6-PGD activity. However, faster growth did not necessarily signify higher specific activities of 6-PGD, as noted (Fig. 2) by the poor linear correlation ($r^2 = 0.13$) or curvilinear correlation ($r^2 = 0.15$). (The curvilinear relationship was considered since this would be expected from Monod kinetics if the doubling time had been based on a single enzyme.) To determine whether growth rates among fast growers could be explained by use of alternative pathways, namely the Emden-Meyerhoff-Parnas pathway and the tricarboxylic acid cycle, we measured FDPA and isocitrate dehydrogenase activities, respectively, in all 6-PGD-positive strains and among seven randomly chosen strains that were 6-PGD negative. Isocitrate dehydrogenase and FDPA were detected in strains that were both 6-PGD positive and 6-PGD negative, and correlation with respect to growth rate was noted ($r^2 = 0.01$).

There was no correlation between doubling time and acid or alkali production, nor were the acid-producing strains confined solely to fast growers (Fig. 3).

DISCUSSION

Consistent with previous reports (19, 21), our results show that the presence of 6-PGD is associated with fast growth in rhizobia. Martinez-de Drets and Arias (19) reported doubling times which ranged from 3.4 to 8.3 and 14.3 to 33.3 h for fast- and slow-growing strains, respectively. Kennedy and Greenwood (16) noted that strains considered a priori as typically fast growing had doubling times of 5.5 h or less, whereas the slow growers had doubling times of 8.5 h or more. However, two of their strains had intermediate doubling times of 7.0 and 8.0 h, yet the one with the shorter doubling time had no 6-PGD activity, whereas the other one did. Although the lower (1.4 h) and upper (44.1 h) limits for doubling times in our study were similar to those obtained by these authors, we did not obtain separation of rhizobia into two distinct groups on the basis of growth rates. In the light of our larger sample size (103 as opposed to 38 strains used by Kennedy and Greenwood), we would expect to find more intermediate strains. On the basis of the histogram for doubling times (Fig. 1), three strains are intermediate.

The tendency to associate fast- or slow-growing rhizobia with a particular host legume or cross-inoculation group originates from the earlier works of Fred et al. (8). Moreover, utilization of cross-inoculation groups as a classification criterion for rhizobia has already been rejected by several investigators (5, 10, 11, 12, 20, 26). The results from our study indicate that strains isolated from *V. unguiculata*, *C. cajan*, *G. max*, *L. leucocephala*, and *Desmodium*, *Sesbania*, and *Prosopis* spp. can be either 6-PGD positive or 6-PGD negative, whereas only 6-PGD-negative strains were isolated from *Stylosanthes* spp. Since only two or fewer strains were isolated from the seven other plants represented in this study, no conclusion can be made regarding growth rate and inoculation specificity. Nevertheless, our data negate the relationship between inoculation group and growth rate put forth recently by Gibson et al. (9) for some of these plants. Others have also reported that rhizobial growth is not associated with the ability to nodulate legume species or genera of *Acacia* (6), *G. max* (17), or *Lotus*, *Lupine*, *Phaseolus*, or *Macroptillium* spp. (29).

The detection of significant levels of isocitrate dehydrogenase in both 6-PGD-positive and -negative strains indicates that the tricarboxylic acid cycle is widely operative in these bacteria. The occurrence of the tricarboxylic acid cycle in glucose metabolism of rhizobia is well established (15, 16, 19, 25). The Emden-Meyerhoff-Parnas pathway and the tricarboxylic acid cycle occurred in both 6-PGD-positive and 6-PGD-negative strains. Thus, the poor quantitative correlation between 6-PGD activity and doubling time among fast-growing strains may be due to the simultaneous operation of other metabolic pathways.

The lack of correlation between acid or alkali production and doubling times in our study invalidates the hypothesis of Norris (23) regarding pH change in rhizobia. Consistent with these results was the statement of Kennedy and Greenwood (16) that acid and alkali production are not useful criteria. Furthermore, it is evident that some cowpea strains and *R. japonicum* are physiologically indistinct from other rhizobia, since they possess 6-PGD and metabolize glucose via more than one metabolic pathway. The latter is consistent with previous genetic studies (5) which revealed the similarities among cowpea rhizobia, *R. japonicum*, and other rhizobia on the basis of overlapping DNA base ratios. Finally, it is clear from our study that a precise doubling time for what constitutes a slow versus a fast grower is slightly ambiguous. Rather than argue whether the doubling time for fast growers is less than 6 h (if the three intermediate strains in Fig. 1 are excluded), 8 h (as suggested by Kennedy and Greenwood [16]), or 11 h (in accordance with the relationship to 6-PGD activity), we suggest that the presence or absence of 6-PGD would resolve the few cases where doubling time (8 to 11 h) might be ambiguous and would thus be a distinguishing physiological criterion in rhizobia.

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