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Enzymatic Basis for Differentiation of *Rhizobium* into Fast- and Slow-Growing Groups

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Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and other enzymes related to carbohydrate metabolism were studied in rhizobia. A nicotinamide adenine dinucleotide phosphate-6-phosphogluconate dehydrogenase was detected in strains of the fast-growing group of *Rhizobium* but not in strains of the slow-growing group. An enzymatic differentiation of rhizobia was established.

Previous investigators (3, 4) reported differences in the utilization of carbohydrates by different strains of rhizobia. Their findings allowed them to subdivide rhizobia into two groups: fast-growing strains and slow-growing strains. The enzymatic basis for this division is not known. Katznelson and Zagallo (5) found two enzymes of the pentose phosphate pathway in three strains of rhizobia: a nicotinamide adenine dinucleotide phosphate (NADP)-linked glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and an NADP-linked 6-phosphogluconate dehydrogenase (6PGD) (EC 1.1.1.43). However, Keele et al. (6, 7) reported the absence of NADP-6PGD in *Rhizobium japonicum*. This would suggest that enzymatic differences exist in rhizobia which could be detected by a comparative study of G6PD, 6PGD, and other enzymes related to carbohydrate metabolism.

The organisms (Table 1) were grown in a yeast extract-glucose medium (6) at 30 C in an oscillatory shaker (100 rev/min) for 72 hr and washed in 0.05 M sodium phosphate buffer (*p*H 7.2). Cell-free extracts were prepared as reported earlier (9) or by sonic exposure (ten 1-min treatments with a Branson sonifier) and were assayed no more than 3 hr later. G6PD and 6PGD were determined by following the reduction of NADP. The incubation mixture (1.0 ml) contained: sodium glucose-6-phosphate (Sigma Chemical Co., St. Louis, Mo.) or sodium-6-phosphogluconate (Sigma), 1 μmole; glycylglycine buffer (*p*H 8.0), 80 μmoles; NADP (Sigma), 0.3 μmole; MgSO4, 10 μmoles; and cell-free extract. A higher amount of sodium glucose-6-phosphate and sodium-6-phosphogluconate (5 μmoles) in the above incubation mixture did not result in an increase in activities of the enzymes. The following determinations were made by procedures described previously: isocitrate dehydrogenase (EC 1.1.1.42; reference 8), malate dehydrogenase (EC 1.1.1.37; reference 8), fructosediphosphate aldolase (EC 4.1.2.13; reference 10); formation of pyruvate from 6-phosphogluconate (6); and protein (2).

Consistent with previous findings (5, 6), NADP-G6PD was detected in all the strains of rhizobia studied (Table 2). Fourteen of these strains were of the fast-growing group with specific growth rates (R) between 0.12 and 0.29 doublings per hr (Table 1), and twenty strains were of the slow-growing group with specific growth rates between 0.03 and 0.07 doublings per hr. The specific activity of this enzyme was several times higher in cell-free extracts from fast-growing strains than in cell-free extracts from slow-growing strains. G6PD was found not to be specific for NADP and the ratio of NADP-G6PD to NAD-G6PD ranged from 2 to 6.5 in the different cell-free extracts of rhizobia studied.

Activity of NADP-6PGD was detected in the cell-free extracts from 14 fast-growing strains but was not detected in the cell-free extracts from any of slow-growing strains. Reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) was not oxidized by any of the cell-free extracts studied, and mixing experiments showed that the absence of enzyme activity in the slow-growing strains did not result from an inhibitor capable of inhibiting enzyme from the fast-growing strains. There was no induction of NADP-6PGD by growing the slow-growing strains in media described by

TABLE 1. *Rhizobia strains employed and their growth rates*

Organism	Source[a]	Specific growth rate[b]
Rhizobium japonicum QA888	Department of Agriculture, Queensland, Australia	0.04
R. japonicum D151	Institut de Recherche et Production Vegetale, Prague, Czechoslovakia	0.04
R. japonicum E2	Instituto de Investigación y Fomento Agrícola-Ganadero, Santa Fe, Argentina	0.03
R. japonicum E10	Department of Bacteriology, Corvallis, Ore.	0.04
R. japonicum 10324	ATCC	0.04
R. japonicum 5000	IPEACS	0.05
R. japonicum 5006	IPEACS	0.06
R. japonicum 532	University of Wisconsin, Madison, Wis.	0.05
R. lupini G7	IMIA	0.03
R. lupini SV623	Department of Bacteriology, University of Uppsala, Uppsala, Sweden	0.04
R. lupini 3G1a1	USDA	0.04
R. lupini 3C2b1	USDA	0.05
R. lupini D48	Institut de Recherche et Production Vegetale, Prague, Czechoslovakia	0.07
Rhizobium sp. (for cowpea) QA549	Department of Agriculture, Queensland, Australia	0.16
Rhizobium sp. (for cowpea) 614-J	National Institute of Agricultural Sciences, Tokyo, Japan	0.05
Rhizobium sp. (for cowpea) C14	IMIA	0.05
Rhizobium sp. (for cowpea) 316U6	USDA	0.04
Rhizobium sp. (for cowpea) CB33	CSIRO	0.05
Rhizobium sp. (for cowpea) CB756	CSIRO	0.06
Rhizobium sp. (for cowpea) 9931	ATCC	0.03
Rhizobium sp. (for desmodium) CB627	CSIRO	0.03
R. meliloti SU277	Sydney University, Sydney, Australia	0.25
R. meliloti U45	Laboratorio de Microbiología de Suelos, Ministerio de Ganadería y Agricultura, Montevideo, Uruguay	0.22
R. meliloti CC2079	CSIRO	0.21
R. meliloti SU47	Sydney University, Sydney, Australia	0.22
R. meliloti 9930	ATCC	0.20
R. trifolii TA1	Department of Agriculture, Tasmania, Australia	0.27
R. trifolii U28	Laboratorio de Microbiología de Suelos, Ministerio de Ganadería y Agricultura, Montevideo, Uruguay	0.29
R. trifolii WA67	Department of Agriculture, Western Australia, Australia	0.22
R. trifolii WU290	Western Australia University, Western Australia, Australia	0.24
R. trifolii NZ29	Department of Agriculture, New Zealand	0.15
R. leguminosarum 10004	ATCC	0.16
Rhizobium sp. (for phaseolus vulgaris) F306	IPEACS	0.12
Rhizobium sp. (for lotus corniculatus) B816	Department of Microbiology, Kensington, Australia	0.14

[a] ATCC, American Type Culture Collection, Rockville, Md.; CSIRO, Commonwealth Scientific and Industrial Research Organization, Brisbane, Australia; IMIA, Instituto de Microbiología e Industrias Agropecuárias, Castelar, Argentina; IPEACS, Instituto de Pesquisas e Experimentação Agropecuárias do Centro-Sul, Rio de Janeiro, Brazil; USDA, United States Department of Agriculture, Washington, D.C.

[b] Growth was measured (1) by turbidimetry method, which was calibrated by counting the cells in a Petroff-Hauser chamber. Specific growth rate is expressed in generations per hour in exponential phase.

TABLE 2. *Enzyme activities in cell-free extracts of rhizobia*[a]

Strain	Glucose-6-phosphate dehydrogenase[b]	6-Phosphogluconate dehydrogenase[b]	Isocitrate dehydrogenase[c]	Malate dehydrogenase[c]	Fructose 1-6P aldolase[d]	ED (6-phosphogluconate) pyruvate[e]
Slow-growing						
Rhizobium japonicum						
QA888	17	<0.5	132	1,150	10	0.8
E2	22	<0.5	200	1,390	<0.5	3.5
E10	26	<0.5	430	2,080	<0.5	4.5
D151	29	<0.5	220	870	3	1.8
532	18	<0.5	280	920	5	ND[f]
5000	12	<0.5	180	520	<0.5	0.7
5006	45	<0.5	204	880	<0.5	4.3
10324	30	1.1	285	870	7	4.2
R. lupinus						
G7	51	<0.5	290	1,520	8	2.3
D48	82	<0.5	390	1,900	12	0.7
SV623	24	<0.5	150	890	<0.5	1.1
3Gla1	60	<0.5	380	970	11	3.2
3G2b1	35	<0.5	375	1,990	<0.5	1.4
Rhizobium sp. (for cowpea)						
614-J	55	<0.5	380	1,860	10	5.0
C14	20	<0.5	260	1,520	ND	1.2
316U6	25	<0.5	395	1,260	10	0.5
CB-33	60	<0.5	202	1,500	10	2.2
9931	34	3	142	790	13	2.0
Rhizobium sp. (for desmodium)						
CB-627	23	0.5	112	620	<0.5	1.1
Fast-growing						
R. meliloti						
SU-47	280	132	358	565	15	27
SU-277	310	160	300	1,450	8	42
U45	167	100	354	595	11	20
CC2079	156	75	235	540	13	ND
9930	290	85	434	790	4	20
R. trifolii						
TA1	276	167	500	2,030	<0.5	2.6
U28	340	115	385	2,250	11	25
NZ29	755	237	430	1,252	11	14
WA67	232	145	755	2,600	9	14
WU290	101	101	416	1,320	5	23
R. leguminosarum						
10004	290	88	384	330	8	28
Rhizobium sp. (for phaseolus vulgaris)						
F-306	160	25	217	554	ND	10
Rhizobium sp. (for lotus corniculatus)						
E-816	256	100	485	356	16	19
Rhizobium sp. (for cowpea)						
QA549	101	53	175	1,260	3	10

[a] Enzyme activities were determined at 25 C, and a control without substrate was run with each assay.

[b] Expressed as nanomoles of nicotinamide adenine dinucleotide phosphate reduced per minute per milligram of protein.

[c] Expressed as nanomoles of nicotinamide adenine dinucleotide phosphate reduced per minute per milligram of protein.

[d] Expressed as nanomoles of reduced nicotinamide adenine dinucleotide oxidized per minute per milligram of protein.

[e] Expressed as nanomoles of pyruvate formed per minute per milligram of protein.

[f] Not determined.

Katznelson et al. (5) or Keele et al. (6) for 48, 72, or 96 hr.

Concurrent studies were conducted to detect the presence of certain enzymes involved in the tricarboxylic acid cycle, the Entner-Doudoroff or the glycolytic pathway in the cell-free extracts of the 34 strains of rhizobia studied (Table 2). No significant differences were detected either in malate dehydrogenase or in NADP-specific isocitrate dehydrogenase. The activity of fructosediphosphate aldolase was very low in most of the strains, and it was not detected at all in several of them. This would suggest that EMP pathway does not operate to any extent in rhizobia.

As reported earlier for four strains (5, 6), the enzymes of the Entner-Doudoroff pathway appeared in all the cell-free extracts, but the specific activity was several times higher in the fast-growing strains. Only low levels of pyruvate accumulated since it was also metabolized. Addition of sodium arsenite and hydrazine to the incubation mixture inhibited the transformation of 6-phosphogluconate to pyruvate by several cell-free extracts. On the basis of their 6PGD activity, two subgroups may be established. One group has NADP-6PGD activity, and the strains in this subgroup have the highest specific growth rates. A second group has no NADP-6PGD and coincidently includes slow-growing strains. This subdivision based on enzymatic determinations is consistent with the classification of Vincent (11) for these strains. Therefore, the enzymatic characteristics of rhizobia may be useful for distinguishing between the two groups of *Rhizobium* species.

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