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Biochemical Characterization of Fast-and Slow-Growing *Rhizobium japonicum*

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Fast-growing과 Slow-growing *Rhizobium japonicum*의 생화학적 특성

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Rhizobium japonicum isolates from all around Korea could be classified into two groups, i.e., acid producing fast-growers with 2.4 hour mean generation time and non-acid producing slow-growers in yeast extract-mannitol medium with 13.1 hour mean generation time. Tested fast-growers were higher in 6-phosphogluconate dehydrogenase activity than slow-growers were and used sucrose as carbon source whereas slow-growers did not. Fast-grower R4, R257, R278, showed tolerance even in 0.5M NaCl or above and the growth of all the strains tested were inhibited at below pH 4.5. Relative symbiotic activities of nitrogen fixation for these isolated with *Glycine max* cv. Jangyeobkong (commercial soybean cultivar mostly cultivated in Korea) ranged 0.1 to 2.0 comparing to that of *R. japonicum* L-259 (NRRL), without regard to their growth rate.

The species of *Rhizobium* genus have been divided into two groups on the basis of growth rate and acid production in yeast extract-mannitol medium (8).

R. japonicum causes formation of root nodules on soybean and has been typically classified as slow-growing species with doubling time 6 to 13 hours.

Recently, the presence of fast-growing *R. japonicum* in China was reported (5). Thereafter, their biochemical characterization were conducted and came to the result that these strains shared more characteristics in common with the other fast-growing group than the slow-growing group of rhizobia.

There is a report that a salt-tolerant *R. japonicum* formed effective symbiosis on American soybean cultivars (18).

In parrallel, it was also elucidated that *nif* genes appeared mostly on the plasmids of fast-growing *R. japonicum* strains (13).

About 600 *Rhizobium* species were isolated from various areas of Korea last 3 years, and fast-and slow-growing *R. japonicum* among them were characterized.

Materials and Methods

Media and Strains

R. japonicum strains were grown in yeast extract-mannitol media containing yeast extract 1.0g, mannitol 10g, K_2HPO_4 0.5g, NaCl 0.2g, $MgSO_4.7H_2O$ 0.2g, $FeCl_3$ 4.88mg per liter distilled water, and plant nutrient solution used in plant growth was prepared as previously described (1). R series *R. japonicum* strains used were isolated from the root nodules of legume cultivars in various areas of Korea (place names of the origin were not described here), *R. japonicum* L-259 was from NRRL (Northern Region Research Center), *R. trifolii* 14480, *R. leguminosarum* 10004 were from ATCC and

R. phaseoli 124K14 was kindly provided by M.J. Cho (Kyeang Sang National Univ., Korea). Various legume seeds used in this research were provided by National Crop Experiment Station (Suwon, Korea).

Determination of Generation Times and Acid Production by *R. japonicum*

50 ml yeast extract-mannitol media were inoculated at 2% with precultured mid-log phase rhizobia and shaken reciprocally at 120 strokes/min., 28°C. Samples for determination of mean generation times were taken during exponential growth phase and absorbances at 600nm were measured in parallel with cell counting. Samples for determination of pH changes were taken after 3 days at above growth condition.

Plant Growth Conditions

Seeds were surface sterilized in 70% ethanol and 0.1% $HgCl_2$, washed exhaustively in sterile water, and pregerminated. Three day-old seedlings were selected and planted in sterile vermiculite moistened with nitrogen-free plant nutrient solution in glass tubes (28x180mm). They were inoculated with desired strain (approximately 10^9 cells/plant) after 5 days later and controls consisted of uninoculated plants. Plants were grown in Shimadzu model SGA-213S controlled environment growth chamber at 25±1°C, 70±5% RH with a light intensity 750 μE/cm^2 per s and a photoperiod of 15 hr.

Sucrose Utilization Test

Cultures were grown in Keele's medium (10), containing sucrose or glucose (final concentration 1%) and no carbon source. Growth determinations were conducted with 500ml sidearm flasks with shaking at 30°C. The turbidity of the cultures was determined spectrophotometrically at 600nm and generation time was calculated from semi-log plot of time vs. absorbance.

Determination of Symbiotic Nitrogen Fixing Activity

Plants were harvested four weeks after the inoculation and the nodulated roots were transferred into rubber stoppered bottle (Wheaton scientific, 25 ml) and acetylene reducing activities of them were assayed according to previous method (17).

Measurement of Growth in the Presence of Salts and Acid

Mid-log phase seed cultures of rhizobia were inoculated at 0.2% into each yeast extract-mannitol broth of various NaCl concentration (0 to 0.8 M) and of different pH (2 to 7) and were incubated at 28°C with reciprocal shaking. The cell densities of fast-growers were determined with absorbance at 600nm after 48 hrs and those of slow-growers were measured

Table 1. Generation times and acid-production of fast- and slow-growing *R. japonicum*

Strains [a]	Generation time (hrs)	Final pH [b]	Strains	Generation time (hrs)	Final pH
Fast-growing *Rhizobium japonicum*			Slow-growing *Rhizobium japonicum*		
R4	2.5	6.39	R13	8.3	7.14
R234	1.6	3.70	R67	19.1	7.08
R257	2.9	6.29	R97	10.3	6.94
R271	1.7	5.35	R158	17.8	8.64
R278	2.5	4.32	R168	13.7	7.09
R289	3.0	3.98	R214	11.9	6.90
Mean	2.4	5.01	R224	11.9	6.98
			L-259	10.6	7.19
			Mean	13.1	7.22
Other fast-growing Rhizobia					
R. leguminosarium ATCC 10004	3.1				
R. phaseoli 124K14	5.3				
R. trifolii ATCC 14480	5.3				

a Tested strains were roughly selected on the basis of nodule condition, when isolated in the field.
b The initial pH of yeast-extract mannitol media was adjusted to 6.8.

after 96 hrs.

6-Phosphogluconate Dehydrogenase Activity

Late-log phase cells were harvested and crude cell extracts were prepared through a French pressure as previously described (15). 6-phosphogluconate dehydrogenase (EC 1.1.1.43) activities were determined according to the method of Martinez De-Drets & Arias (12). Protein was quantitated by the method of Schacterle & Pollack (16) and specific activities were expressed as nanomoles of reduced NADP formed per minute per milligram of protein.

Results

Growth Rate and Acid Production of Fast- and Slow-Growing *R. japonicum*

About 24% among tested Korean *R. japonicum* isolates produced acid in yeast extract-mannitol medium and acid production was associated with fast growth rate and these results were consistent with previous report (5). The generation time of fast-growers were slightly shorter than those of other fast-growing rhizobia (Table 1). Acid production of fast-growers was not affected by variation of initial pH and the optimal pH for growth was 6.0 to 7.0 (unpublished data).

6-Phosphogluconate Dehydrogenase and Sucrose Utilization

Several fast- and slow-growers of *R. japonicum* isolates were compared in 6-phosphogluconate dehydrogenase activities and sucrose utilization. Fast-growers were higher in 6-phosphogluconate dehydrogenase activity than slow-growers (Table 2). As a while, the growth rates of fast-growers did not decrease when sucrose was substituted for glucose as a carbon source but slow-growers did not grow in sucrose (Table 2). Conclusively, the fast-growers were positive in both above two tests and seemed to be much similar to the other typical fast-growing rhizobia.

Salt and Acid Tolerance

The growth of fast-growing *R. japonicum* R4, R257, R278, were not inhibited in the prescence of 0.5M NaCl in the least and other fast-growers R271, R247 in 0.4M NaCl also showed around 50% growth of that without NaCl. In contrast, *R. phaseolus* 124K14 and *R. leguminosarum* ATCC 10004 did not show any growth in above 0.2M NaCl (Table 3). All the *R. japonicum* tested did not grow at below pH 5.0 (Table 3) showing transient growth response between pH 4 and pH 5, and maximum growth between pH 6.0, and pH 7.0, which is known to be the optimal pH for rhizobial growth (8).

Symbiotic Nitrogen Fixing Activities of *R. japonicum*

Glycine max cv. Jangyeobkong were inoculated with several fast- and slow- growers of *R. japonicum* isolates. Acetylene reducing activities of nodulated roots by these

Table 2. 6-phosphogluconate activities and sucrose utilization of *R. japonicum*

	6-phosphogulconate[a] dehydrogenase	Sucrose utilization
Fast-grower		
R4	45	+
R257	ND[b]	+
R271	ND	+
R275	46	ND
R278	ND	+
R289	69	ND
Slow-grower		
R67	ND	−
R138	ND	−
R214	3	−
R240	2.9	−

a Activities are expressed as nmoles of NADPH produced per minute per mg protein

b Not determined

Table 3. Salt and acid tolerance of fast- and slow- growing *R. japonicum*

	Salt tolerance[a]	Sensitivity to pH 4.5
Fast-grower		
R4	0.6	+
R247	0.2	+
R257	0.5	+
R271	0.2	+
R278	0.6	+
R289	0.1	+
R. phaseolus 124K14	0.1	+
R. leguminosarum ATCC 10004	0.1	+
Slow-grower		
R138	0.1	+
R158	0.3	+
R214	0.1	+
L-259	0.1	+

a Tolerance was defined as NaCl concentration (M) in which cells showed normal growth rate.

Table 4. Acetylene reducing activity of *R. japonicum* strains[a]

R. japonicum strain[b]	Activity (μmole C_2H_4/root. hr)
Fast-grower	
R4	2.49
R247	2.48
R271	1.40
R278	2.88
R289	2.88
Slow-grower	
R13	0.17
R67	3.08
R97	2.28
R138	3.08
R168	2.42
R214	3.15
R221	1.57
R224	0.87
R228	2.14
R240	2.76
R256	0.24
R264	0.66
L-259	1.67

a Six replicates were used per treatment and results are expressed as mean.
b All unioculated controls were devoid of nodules.

Table 5. Inoculation test of *R. japonicum* into various legumes[a]

Effective Nodulation	
Glycine max	cv. Jangyeobkong
Glycine max	cv. Kwangkyo
Glycine max	cv. Hill
Glycine max	cv. Heuktaejaerae 58
Glycine max	cv. Williams
Glycine max	cv. Clark
Glycine max	cv. Crawford
Phaseolus angularis	cv. Chungwonpat
Ineffective Nodulation[b]	
Phaseolus radiatus	
Phaseolus multiformis	
No Nodulation	
Pisum sativum	
Trifolium repens	

a Three to four replicates were used per treatment and all uninoculated controls were devoid of nodules.
b Ineffective nodulation means that *Rhizobium* could form nodules in legumes but didn't express nitrogen fixing activity.

isolates were around that of *R. japonicum* L-259 (NRRL), known to have relatively high nitrogen fixing activity (9) and their activities were independent upon the growth rate (Table 4). Although the results were obtained from the symbiosis with soybean of Korean cultivar, many fast- and slow-growing *R. japonicum* showed relatively high symbiotic nitrogen fixing activities and especially, some fast- growers attract much interest as excellent legume inoculants.

Host range of *R. japonicum*

All the tested fast- and slow-growers of *R. japonicum* caused formation of root nodules effectively on *Phaseolus angularis* as well as some commercial soybean cultivars (Table 5). They also caused formation of root nodules on *Phaseolus radiatus* and *Phaseolus multiformis* ineffectively. The result remains to be elucidated in detail whether it is due to the host or the strain itself, however, it is noteworthy to consider that fast-growing *R. japonicum* formed ineffective symbiosis on most commercial soybean cultivars and one strain, *R. japonicum* USDA 191 formed effective symbiosis on several commercial soybean cultivars (5,19).

Discussion

A fast-growing *R. japonicum* group isolated in Korea usually produces acid in yeast extract-mannitol medium, whereas a slow-growing *R. japonicum* group is associated with alkaline reaction. This suggested the prescence of great biochemical differences between two groups: thereafter many investigators reported on that (4,11,12,15). From this point of view, experiments on 6-phosphogluconate and sucrose utilization were performed as diagnostic tests differentiating two groups and the results were consistent with previous report (15).

Salt and acid tolerance, symbiotic nitrogen fixing activity and host range specificity were determined expecting that fast-growing *R. japonicum* would make promising materials as legume inoculants. Recently, Yelton et. al (18), reported salt tolerant *R. japonicum* which effectively nodulated American soybean cultivars. However, this strain grew at decreased rate in 0.4M NaCl, whereas some fast-growing *R. japonicum* here did grow at the same rate even in 0.6M NaCl

and showed some growth in 0.8M NaCl. But all the tested strain had an usual optimum pH for rhizobial growth and showed sensitivity to below pH 4.5.

Typically, *Rhizobium* species have been designated in terms of host plant cross-inoculation groups (8). However, it is generally recognized that this criterion is inadequate since cross-inoculation groups are not mutually exclusive (3), and host range is determined by plasmid (2,7). Some fast- and slow-growing *R. japonicum* here commonly nodulated some beans which have not been cross-inoculation group, as well as soybean and reisolates from each root nodules formed effective symbiosis with counterpart legumes again (unpublished data).

Additionally, several fast-growing *R. japonicum* assumed unique pigments on yeast extract-mannitol plate, which are not common character of *R. japonicum*, except to *R. phaseolus* (2,7) and a few *R. japonicum* (14).

The significance of this is to be estimated but its usefulness as genetic marker and relatedness to other function of symbiosis deserves to be exploited. Conclusively, some unusual characteristics of fast-growing *R. japonicum* described above suggest the possibility that these stains could be developed as excellent legume inoculants.

요 약

국내에서 분리된 대두근류균을 생육속도에 따라서 2.4시간의 평균세대시간을 가지는 fast-grower와 13.1시간의 평균세대시간을 가지는 slow-grower로 나눌 수 있었다. Fast-grower는 6-phosogluconate dehydrogenase 효소역가가 slow-grower 보다 높았으며 sucrose를 탄소원으로 이용하였다.

Fast-grower 중에서 특히 R4, R257, R278은 NaCl 0.5M 농도에서도 생육하였으며 시험한 대두근류균은 모두 pH 4.5 이하에서 생육이 저지되었다. 또한 식물배양기 조건하에서 접종실험을 한 결과 시험된 모든 대두품종에 양호하게 근류를 형성하였으며 L-259 (NRRL) 균주에 비해서 0.1-2 배의 질소고정력을 나타내었다.

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