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Growth of *Rhizobium japonicum* Strains at Temperatures Above 27°C†

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A study was conducted to examine the growth responses of different *Rhizobium japonicum* strains to increasing temperatures, determine the degree of variability among strains in those responses, and identify temperature-related growth characteristics that could be used to select temperature-tolerant strains. Each of 42 strains was grown in liquid culture for 96 h at 19 incubation temperatures ranging from 27.4 to 54.1°C in a temperature gradient apparatus. Growth was estimated by measuring the change in optical density over time. Strains differed in their responses to increasing temperatures. Three characteristic temperatures were determined for each strain: the temperature giving the maximum optical density at 96 h (optimum temperature), the maximum temperature allowing a continuous increase in optical density during the 96-h period (maximum permissive temperature), and the maximum temperature allowing growth of the cultures after they were transferred to a uniform incubation temperature of 28°C (maximum survival temperature). The three characteristic temperatures varied among strains and had the following ranges: optimum temperature, from 27.4 to 35.2°C; maximum permissive temperature, from 29.8 to 38.0°C; and maximum survival temperature, from 33.7 to 48.7°C. Significant positive correlations were found between maximum permissive temperature and optimum temperature and between maximum permissive temperature and maximum survival temperature. Eight strains which had the highest maximum permissive temperature, optimum temperature, and maximum survival temperature were considered tolerant of high temperatures and were able to grow at temperatures higher than those previously reported for the most tolerant *R. japonicum* strains. The strains were of diverse geographical origin, but the response to high temperatures was not related to their origin. Evaluation of the temperature responses in pure culture may be useful in the search for *R. japonicum* strains better suited to environments in which high soil temperature is a limiting factor.

Temperature is one of the most important factors influencing bacterial growth and survival in natural environments, including soils (7). Surface soil temperatures between 40 and 60°C have been reported for tropical and subtropical areas (3, 6, 15). This is also true for the soils of the southeastern United States (16). Temperature records for soils planted with soybeans (*Glycine max* (L.) Merr.) in North Carolina show that the top 1 cm of the soil profile may reach 48°C and that at least the top 3.8 cm of the soil profile is frequently exposed to temperatures above 38°C during most of June and July (unpublished data).

It has been suggested that the growth and survival of rhizobia in soils and their symbiotic association with leguminous plants are adversely affected by high soil temperatures (4, 11-14). Survival of rhizobia in inoculant materials has also been found to be severely diminished by high storage temperatures (2).

Differences in tolerance of high temperatures among species and strains of *Rhizobium* have been recognized (1, 8-11, 13, 19), and selection of strains for temperature tolerance has been suggested as a means of overcoming temperature stress (1, 9, 11, 13, 14, 20). The relative abundance of different *Rhizobium japonicum* (Kirchner) Buchanan serogroups in soybean nodules has also been found to be affected by soil temperature (20).

Bowen and Kennedy (1) and Ishizawa (9) tested several strains belonging to different cross-inoculation groups of *Rhizobium* and

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found that only a few would grow at 42.5°C; the *R. japonicum* strains did not grow above 35.6°C. Six *R. japonicum* strains tested by Graham and Parker (8) were unable to grow at 39°C. Based on these studies, few generalizations about the temperature tolerance of *R. japonicum* can be made. Therefore, a study was conducted to evaluate temperature relationships among strains of *R. japonicum* from various geographical origins. The objectives of the study were to determine the variability among strains in growth response to temperatures between 27.4 and 54.1°C, to identify temperature-related growth characteristics which may be used to select high-temperature-tolerant strains, and to identify high-temperature-tolerant strains.

MATERIALS AND METHODS

Organisms. A total of 42 strains of *R. japonicum* were studied (Table 1). Stock cultures were maintained on agar slants of HEPES-MES-gluconate medium containing the following (in grams per liter): yeast extract, 0.25; potassium gluconate, 5.0; L-arabinose, 0.5; Na_2HPO_4, 0.125; Na_2SO_4, 0.25; NH_4Cl, 0.32; $MgSO_4 \cdot 7H_2O$, 0.18; $FeCl_3$, 0.004; $CaCl_2 \cdot 2H_2O$), 0.013; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma Chemical Co., St. Louis, Mo.), 1.3; and 2-(*N*-morpholino) ethanesulfonic acid (MES; Sigma), 1.1 (17). The medium was adjusted to pH 6.6 with 1 N NaOH before autoclaving. All strains were found to form nodules on Lee soybeans growing in pots with vermiculite as the rooting medium.

Temperature gradient study. (i) Growth at different temperatures. A total of 19 temperature treatments were imposed with a Poly-Temp apparatus (temperature gradient bar; Lab-Line Instruments, Inc., Melrose Park, Ill.). The temperatures used were (in °C) 27.4, 28.9, 29.8, 31.1, 32.5, 33.7, 35.2, 36.7, 38.0, 39.1, 40.5, 42.3, 43.7, 45.5, 47.2, 48.7, 50.5, 52.2, and 54.1. These temperatures were maintained within ±0.2°C. The bacteria were grown in test tubes (18 by 150 mm or 8 by 100 mm) containing 10 or 4 ml of HEPES-MES-gluconate broth, respectively. The test tubes were previously matched to make them suitable for optical density (OD) readings. A starter culture of each strain was grown to the early stationary phase in 250-ml Erlenmeyer flasks containing 50 ml of HEPES-MES-gluconate broth. Then, fresh broth was inoculated with a volume of starter culture equivalent to 1% of the volume of fresh broth. After thorough mixing, aliquots were taken to fill the sterile test tubes for the growth studies. Sets of three strains or three replicates of one strain were assayed at a time. Bacterial growth was monitored by measuring the OD of the cultures at 600 nm with a Spectronic 100 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). The OD was measured at the following incubation times (h): 0, 12, 24, 48, 72, and 96. At the end of 96 h, the mean OD at the optimum temperature (OT) for all strains was 0.750, with a standard deviation of ±0.020. At this time, there were ca. 10^9 cells ml of medium^{-1}. Preliminary observations involving 18 strains indicated that interpretations based on a 96-h incubation period were not different from interpretations based on a 216-h incubation period.

TABLE 1. *R. japonicum strains used in this study*

Strain	Source[a]	Origin
509	1	Iowa
527, 532c, 543, 572	1	Brazil
586	1	Maryland
587	1	Brazil (Rio Grande do Sul)
588	1	Brazil (Rio de Janeiro)
596	1	United States
599	1	Unknown
1a11	2	Brazil
5019	2	Brazil (Rio de Janeiro)
NC 1001, NC 1004, NC 1005, NC 1010	3	North Carolina
NC 1016, NC 1017, NC 1028, NC 1029	3	North Carolina
NC 1030, NC 1031, NC 1032, NC 1033	3	North Carolina
NC 1034	3	North Carolina
TAL 102	4	Florida
TAL 183	4	Mississippi
TAL 184, TAL 185	4	Wisconsin
TAL 299	4	Unknown
TAL 649	4	Malaysia
USDA 24, USDA 31	5	Wisconsin
USDA 38	5	Japan
USDA 62, USDA 94	5	North Carolina
USDA 76	5	California
USDA 110	5	Florida
USDA 122, USDA 124, USDA 138	5	Mississippi
USDA 123	5	Iowa

[a] The sources of bacterial cultures were: (1) R. H. Miller, Department of Agronomy, The Ohio State University, Columbus, Ohio; (2) C. Vidor, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; (3) A. G. Wollum, Department of Soil Science, North Carolina State University, Raleigh, N.C.; (4) P. Somasegaran, University of Hawaii NifTAL Project, Paia, Hawaii; and (5) D. F. Weber, Cell Culture and Nitrogen Fixation Laboratory, Plant Physiology Institute, U.S. Department of Agriculture, Beltsville, Md.

The initial OD of the cultures varied by no more than 0.002 U within each strain. The number of viable cells at the beginning of the assay was estimated to be about 10^7 per ml of culture, based on plate dilution counts (18). In an attempt to minimize O_2 stress, we vigorously shook the contents of each tube in a Vortex mixer for about 5 s every 3 h during the day and twice at night, for a total of six shakings in 24 h. The reliability of OD measurements in estimating bacterial growth at different temperatures was assessed by comparing the number of viable cells estimated by plate dilution counts (18) and the OD of shake cultures of strains USDA 110, 587, and TAL 184 at 28 and 36°C (data not shown). A linear relationship was found between OD and viable cell counts to an OD of 0.66 ($r_2 = 0.87$; $P = 0.0001$). The incubation temperature had no effect on this relationship. Before the study, test tubes containing sterile growth medium were incubated in the temperature gradient bar for 132 h to determine the effect of temperature on the OD of the

medium itself, but no changes in OD were found.

The reproducibility of temperature-related growth characteristics was determined for four strains, from the standpoints of within an experiment and between experiments. From two to four replicates of each strain were used. The standard deviation of the characteristic temperatures was ±0.81°C. There were no significant differences among replicates of strains either within an experiment or among different experiments.

(ii) Survival tests. At the end of the incubation period in the temperature gradient bar, the cultures were transferred to an incubator maintained at 28 ± 1.5°C to determine the capability of the cultures to grow after removal of the temperature treatments. The OD of the cultures was determined as previously indicated at 1, 2, 4, and 6 days after initiation of the survival tests. The cultures were shaken in a Vortex mixer every 12 h as previously indicated. A bacterial culture was considered to have survived the temperature treatment when its OD increased at any time during the 6-day period after removal from the Poly-Temp apparatus. A 6-day incubation period was selected for this test because preliminary experiments in which growth was monitored for 10 days indicated that the number of cultures exhibiting increases in OD did not increase after day 6.

Shake cultures. Growth curves were obtained by growing bacterial cultures simultaneously in two reciprocating shaker water baths maintained at 28 and 37.5°C. The cultures were grown in 50-ml Erlenmeyer flasks with a side arm for inoculation and sampling and a tube for OD measurements. A 13-ml amount of inoculated growth medium was dispensed into each flask. The shaker baths were operated at a frequency of about 150 strokes per min. The OD determinations were done as previously described. A standard deviation of ±0.021 U was found for the OD determinations in preliminary studies.

RESULTS

Responses to increasing temperatures. Great diversity was observed among strains in response to increasing temperature. For each strain, there was a temperature range within which growth continuously increased during the incubation period. The lower temperature limit of this range was 27.4°C, and the upper limit varied with the strain from 29.8 to 38.0°C. The upper limit of this temperature range for each strain was referred to as the maximum permissive temperature (MPT). This response is shown in Fig. 1A by the growth curves of strain TAL 649 at 27.4 and 38.0°C and strains USDA 110 and USDA 122 at 27.4°C.

Within the temperature range that allowed a continuous increase in OD, a specific temperature value gave the highest OD for each strain at the end of the incubation period. That value was considered as the OT for growth of the respective organism under the conditions of the test. For most strains, the OD readings were proportionally less as temperatures decreased or increased from the OT. OT may be overestimated for the strains which grew best at 27.4°C because temperatures lower than 27.4°C were not included.

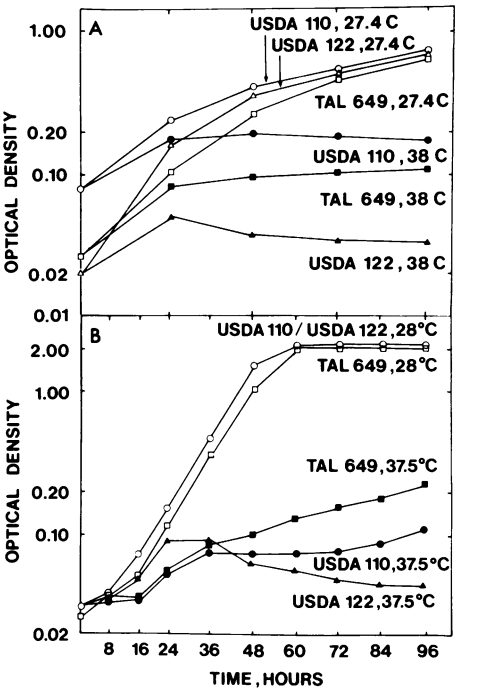

FIG. 1. *Growth curves of R. japonicum strains representing different degrees of tolerance of high temperatures. (A) Growth in the Poly-Temp apparatus. (B) Growth in shake cultures. Values for strains USDA 110 and USDA 122 at 28°C in (B) were almost identical and are represented by a single curve.*

A second growth response which was temperature and time dependent was found. Above the MPT there was generally an initial increase in the OD of the cultures, followed by a decrease in OD over time. It was established that the decrease in OD at high temperatures was correlated with the initiation of cell lysis. The time at which the OD of a given strain began to decrease varied with the temperature level, generally being earlier at the higher temperatures. The time of the initial decrease in OD was dependent on both strain and temperature.

Survival tests. The maximum temperature that allowed growth of a given strain during the period after transfer from the Poly-Temp apparatus to a uniform incubation temperature of 28°C was referred to as the maximum survival temperature (MST) of the strain. All the strains tested survived the temperature treatments up to 33.7°C, but the proportion of strains surviving higher temperatures declined progressively (Fig. 2). The decline in the number of surviving strains was especially noticeable between 38.0 and 42.3°C. A total of 76% of the strains survived

the former temperature, whereas only 19% survived the latter. No strain tested survived temperatures above 48.7°C. The MST was higher than the MPT for all strains, with the exception of TAL 102, USDA 24, and USDA 122, for which the MST was equal to the MPT.

Distribution of the strains by response to temperature. To better evaluate the performance of the strains at high temperatures, we categorized the strains according to their OT, MPT, and MST (Fig. 3). The OT varied from 27.4 to 35.2°C. Fourteen strains, representing 33.3% of the total, attained the highest OD at the lowest temperature studied. Only 7.1% of the strains had an OT of 35.2°C. The remainder of the strains had an OT between 28.9 and 33.7°C. The MPT varied from 29.8 to 38.0°C. Strains having an MPT of either 35.2 or 36.7°C constituted 71.4% of the total. Only 9.5% of the strains had an MPT of 38.0°C.

Shake culture studies. Growth curves of selected strains with shake culture conditions at two incubation temperatures were obtained to confirm the results of the studies conducted in the Poly-Temp apparatus. The data in Fig. 1B, which includes three representative strains, show a close relationship with the results found with the Poly-Temp apparatus (Fig. 1A). Other studies (data not shown) in which shake cultures were grown at 28 and 36°C or 28 and 40°C also confirmed the results of the temperature gradient studies. Although at a given temperature growth rates were lower in the temperature gradient studies than in the shake culture studies, the relative temperature effects were comparable between the two systems; therefore, strain comparisons based on the temperature gradient studies were considered valid. In addition, the Poly-Temp apparatus permits the simultaneous evaluation of growth at 19 different temperatures, a condition which would be difficult to duplicate with a shaker water bath system.

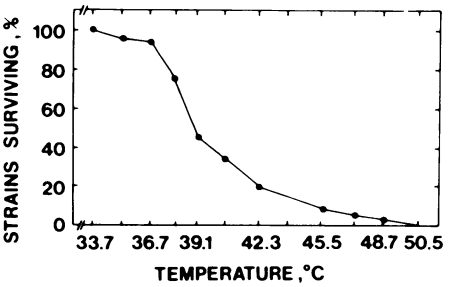

FIG. 2. *Relative number of strains surviving different incubation temperatures for 96 h.*

DISCUSSION

Strains of *R. japonicum* differ in response to incubation temperatures above 27.4°C. Temperature changes as small as 1.5°C had a noticeable effect on the different strains, indicating the advisability of using a large number of temperature levels when studying temperature effects on the growth of rhizobia. The observed responses to temperature indicate that diversity exists among *R. japonicum* strains and suggest that searching for strains tolerant of high temperatures can be successful.

The criteria used in this investigation to char-

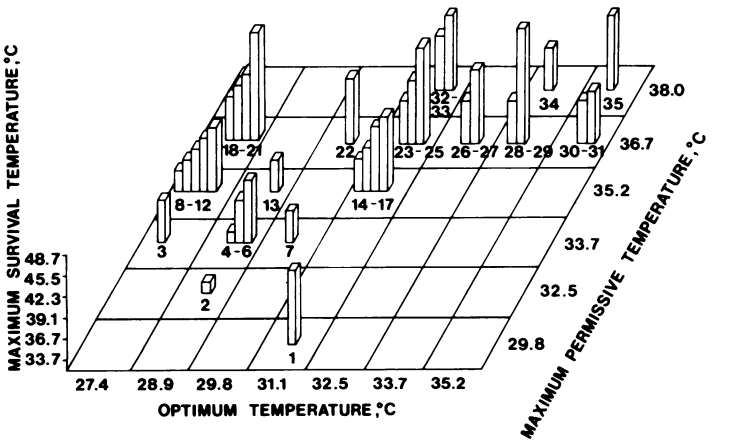

FIG. 3. *Distribution of the strains according to their OT, MPT, and MST. Each column represents one or more of the following strains: 1, NC 1031; 2, USDA 123; 3, USDA 76, NC 1028; 4, USDA 24; 5, 572; 6, 543; 7, 527; 8, USDA 122; 9, 596; 10, TAL 299, USDA 124, NC 1016; 11, NC 1004; 12, NC 1017; 13, 509, 532, 599, NC 1001; 14, USDA 38; 15, NC 1034; 16, USDA 94; 17, NC 1032; 18, USDA 62; 19, USDA 138, 1all; 20, 586; 21, TAL 185; 22, USDA 110; 23, TAL 183; 24, NC 1029; 25, 5019; 26, 588; 27, USDA 31; 28, NC 1033; 29, TAL 184; 30, NC 1005; 31, NC 1030; 32, NC 1010; 33, TAL 649; 34, TAL 102; 35, 587. The MST of the strains may be obtained with the vertical scale.*

acterize the behavior of rhizobial strains at increasing temperatures may have ecological significance. For instance, a strain having a low OT might be exposed for long periods of time in the soil environment to temperatures too high to allow for adequate growth. However, a strain with a high MPT or MST may have the capacity to withstand high soil temperatures without its growth being completely suppressed. Strains 587, TAL 102, NC 1030, NC 1005, TAL 184, NC 1033, TAL 649, and NC 1010 appear to be tolerant of high temperatures. Additional studies are needed to verify whether these strains have a competitive advantage in the soil and as symbionts for soybean plants under high root temperature environments, as compared with more susceptible strains, such as USDA 123, NC 1031, USDA 76, NC 1028, USDA 24, 572, and 543.

The tolerant strains were able to grow at temperatures higher than those previously reported for the most tolerant *R. japonicum* strains (1, 9). No clear relationship was found between the geographical origin of the strains and their relative tolerance of high temperatures, despite reports by other investigators (5, 21). Among the most tolerant strains, some were from Brazil (strain 587), others were from Wisconsin (TAL 102 and TAL 184), and some were from North Carolina (NC 1005, NC 1010, NC 1030, and NC 1033). The strains isolated from North Carolina soils showed a broad range of temperature tolerance. Several strains of subtropical origin had a relatively low temperature tolerance. Strains 588 and 5019 displayed only moderate tolerance, in spite of having a tropical origin.

Based on pure culture studies, it is possible to identify *R. japonicum* strains which have a distinctive tolerance of high temperatures. Whether these same strains will perform adequately as symbionts of soybean plants when grown under soil-root temperature stress remains to be evaluated. If there is a significant correlation between strain performance under temperature stress in pure culture and strain behavior in a temperature-stressed symbiotic system, pure culture evaluation may be a useful tool in the search for *R. japonicum* strains better suited for soil environments where high temperatures constitute a limitation for symbiotic and saprophytic competence of rhizobia.

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