APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1994, p. 1206–1212
0099-2240/94/$04.00+0

Vol. 60, No. 4

Effects of Temperature Stress on Bean-Nodulating *Rhizobium* Strains

JAN MICHIELS, CHRISTEL VERRETH, AND JOS VANDERLEYDEN*

F. A. Janssens Laboratory of Genetics, Catholic University of Leuven, B-3001 Heverlee, Belgium

Received 26 July 1993/Accepted 31 January 1994

High soil temperatures in tropical areas limit nodulation and dinitrogen fixation by strains of *Rhizobium*. Several heat-tolerant bean-nodulating *Rhizobium* strains have been isolated previously. However, the basis of their resistance to heat remains unknown. In this study, we compared the effects of heat on symbiotic nitrogen fixation, cell survival, amino acid uptake, and protein synthesis in a heat-tolerant (CIAT899) and a heat-sensitive (CNPAF512) bean-nodulating *Rhizobium* strain. Acetylene reduction activity of nodulated roots excised from unstressed plants was strongly diminished at 35 or 40°C when plants were nodulated either by CIAT899 or by CNPAF512. When these strains were tested under free-living conditions, survival at 40°C as well as the kinetics of L-[^{35}S]methionine uptake and protein synthesis at 35 and 40°C indicated the higher tolerance of CIAT899 than of CNPAF512 to thermal stress. The synthesis of heat shock proteins was detected in both strains, although at different temperatures. Increased synthesis of 14 heat shock proteins in CNPAF512 and of 6 heat shock proteins in CIAT899 was observed at 40 and 45°C, respectively. A heat shock protein of approximately 21 kDa, of which the synthesis was strongest in both *Rhizobium* strains upon a temperature shift up, was also conserved in several other bean-nodulating rhizobia. Acquired thermotolerance in CIAT899 was shown to depend on protein synthesis.

High soil temperatures in tropical areas are a major problem for biological nitrogen fixation by legume crops. Common beans (*Phaseolus vulgaris* L.) are considered to be particularly sensitive. High root temperatures have been shown to strongly affect bacterial infection and N_2 fixation in several legume species, including soybean (30), clover (38), pea (8), guar (2), peanut (19), cowpea (36, 39), and beans (17, 37). Critical temperatures for N_2 fixation are 30°C for clover and pea and range between 35 and 40°C for soybean, guar, peanut, and cowpea. Nodule functioning in common beans is optimal between 25 and 30°C and is hampered by root temperatures between 30 and 33°C (15, 35, 37). Temperature dependence of nodulation and symbiotic nitrogen fixation has been shown to depend on, in addition to the plant cultivar, the nodulating strain (2, 22, 30). Several bean-nodulating rhizobia that are able to nodulate beans and fix atmospheric nitrogen on a 40/23°C (day/night) regime have been described recently (18).

Although a considerable amount of work has been invested in the study of the effect of elevated temperature on nodule development and functioning, no such an analysis has been conducted at the level of the microsymbiont. In order to gain better insight into the adaptation of bacteria to high temperatures, we studied the effects of heat on the rate of symbiotic nitrogen fixation, bacterial survival, amino acid uptake, and protein synthesis in a heat-tolerant (CIAT899) and a heat-sensitive (CNPAF512) bean-nodulating *Rhizobium* strain.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are *Rhizobium leguminosarum* biovar phaseoli CNPAF512 (29); *Rhizobium tropici* CIAT899 (13, 27), CFN299 (26, 27), and F98.5 (28a); *Rhizobium etli* CFN 42 (41); and *Rhizobium fredii* BR814 and *Rhizobium* sp. BR816 (18).

* Corresponding author. Phone: (32-16)220921. Fax: (32-16)200720. Electronic mail address: Janssens%faj%agr@CC3.KULEUVEN.AC.BE.

Media and growth conditions. *Rhizobium* strains were grown in TY (0.5% tryptone, 0.3% yeast extract, 7 mM $CaCl_2$) or yeast extract-mannitol medium (47) at 30°C. Cultures containing approximately 5×10^8 cells per ml were used in all experiments.

Viable cell counts. Cells were grown overnight at 30°C. Portions (1.5 ml) of each culture were incubated in a water bath at 40°C. Samples (0.1 ml) were removed at various times. Serial dilutions of each sample were spread onto TY solid medium and incubated at 30°C. Colonies were counted after 2 to 3 days.

To determine the induction of thermotolerance, 1.5-ml samples of an overnight culture were incubated for 1 h at 40°C or for 10 min at 45°C and subsequently shifted to 50°C. At intervals, 0.1-ml samples were removed. Controls were kept at 30°C prior to a shift to 50°C. Viable cell counts were performed as described. To inhibit protein synthesis during the 1-h preincubation period at 40°C, tetracycline was added to a concentration of 4 μg/ml. Such a concentration inhibits cell growth at 30°C but does not affect cell viability as judged from the number of viable cells after a 1-h incubation period at 40°C in the presence of tetracycline at 4 μg/ml.

***In vivo* labeling of proteins with L-[^{35}S]methionine and gel electrophoresis.** Cultures in exponential phase were divided into 1.5-ml aliquots and preheated at the appropriate temperature for 5 min or otherwise as indicated. *Rhizobium* cells were pulse-labeled for 5 min with L-[^{35}S]methionine (Amersham) added at 15 μCi/ml. The labeling was stopped by transfer of the samples to ice. Samples were centrifuged for 5 min at room temperature and washed twice in phosphate-buffered saline (pH 7.2) containing K_2HPO_4 (1.24 g/liter), KH_2PO_4 (0.39 g/liter), and NaCl (8.8 g/liter).

For one-dimensional (1D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the cell pellet was solubilized in 200 μl of SDS-lysis buffer consisting of 25 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 100 mM dithiothreitol, and 0.002% bromophenol blue. Samples were heated at 95°C and centrifuged for 5 min. The lysed cell samples were

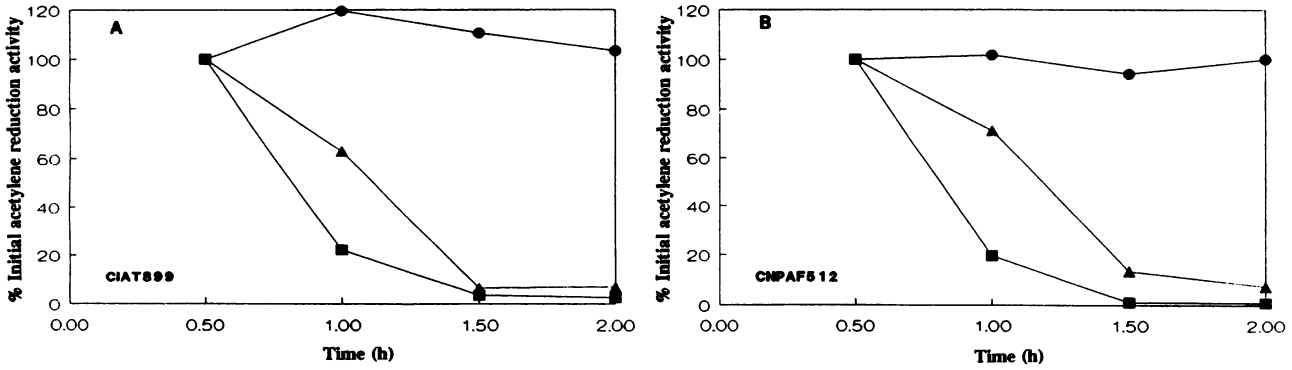

FIG. 1. ARAs of roots nodulated by *R. tropici* CIAT899 (A) or *R. leguminosarum* bv. phaseoli CNPAF512 (B) at elevated temperatures. Nodulated roots were preincubated at 28°C for 30 min prior to transfer to 28°C (●), 35°C (▲), or 40°C (■). Plants were assayed for ARA at 30-min intervals. The amount of ethylene produced during the 30-min preincubation period at 28°C was set to 100%. Nitrogen fixation activity is expressed as a percentage of the initial ARA. Data are the means for six independent plant cultures.

loaded onto slab gels (14.5 by 17 by 0.15 cm, 5% stacking gel, 15% separating gel, 0.1% SDS). Electrophoresis was carried out according to the method of Laemmli (21) at 150 V for 4 h. Following electrophoresis, gels were stained with Coomassie brilliant blue R-250, destained, and dried prior to autoradiography.

For 2D gel electrophoresis, cell pellets were suspended in 0.75 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, and 40 mM dithiothreitol). Preparation of proteins and 2D gel electrophoresis were performed as previously described (6, 33). Immobiline DryStrip (Pharmacia) was used when a narrow pH gradient was required.

L-[^{35}S]methionine uptake and incorporation. Cells were grown in yeast extract-mannitol medium containing 0.1 g of yeast extract per liter instead of 0.4 g/liter. Cultures were labeled as described above, and protein samples were prepared as for SDS-PAGE. To determine the amount of L-[^{35}S]methionine accumulated in the cells, 5-μl aliquots of the lysed cell samples were spotted on Whatman 3MM filter paper and allowed to dry for 5 min. For counting radioactivity, filters were put into scintillation vials, mixed with 3.5 ml of liquid scintillation cocktail (Instagel II; Packard), and analyzed in a Pharmacia Wallac 1410 liquid scintillation counter. To assay the amount of L-[^{35}S]methionine incorporated into proteins, 5-μl aliquots of each lysed cell sample were spotted on Whatman 3MM filter paper, dried for 5 min, and washed in 10% (wt/vol) trichloroacetic acid, 5% trichloroacetic acid, and 100% ethanol, consecutively. Radioactivity was counted as described above. Protein concentration was determined by the bicinchoninic acid assay (42) with bovine serum albumin as a standard.

Plant culture and acetylene reduction assay. *Phaseolus vulgaris* cv. Negro Argel seeds were surface sterilized by consecutive immersion in concentrated H_2SO_4 for 2 min, in sterile distilled H_2O several times, in 100% ethanol for 1 min, and in 0.2% $HgCl_2$ (wt/vol) for 2 min and washed 10 times in sterile distilled H_2O. The treated seeds were germinated for 2 days in the dark on 1.5% water agar plates. The seedlings were transferred aseptically to sterile test tubes (15 by 200 mm) containing a 1:1 mixture of quartz sand and vermiculite and 20 ml of Jensen medium (47). The seedlings were inoculated with approximately 10^8 *Rhizobium* cells resuspended in 10 mM $MgSO_4$. Plants were grown in the growth chamber (12-h day, 28°C during the day, 24°C during the night) and harvested 5 weeks after inoculation.

Nitrogen fixation was assayed by the acetylene reduction method. The roots of the plants were excised and placed in test tubes (15 by 200 mm). The tubes were tightly stoppered with rubber caps, and acetylene was injected to a final concentration of 10%. To determine the initial acetylene reduction activity (ARA) of each sample, the tubes were incubated for 30 min in the water bath at 28°C prior to the incubation at the appropriate temperature (28, 35, or 40°C). Ethylene production was assayed at 30-min intervals. Ethylene production was quantified on a Hewlett Packard 5890A gas chromatograph equipped with a PLOT fused silica column. Propane was used as an internal standard. At 28°C, ARAs of 20 plants nodulated by CNPAF512 and CIAT899 were 1.9 ± 0.7 (mean ± standard deviation) and 2.7 ± 1.5 μmol of C_2H_2 plant^{-1} h^{-1}, respectively.

RESULTS

Effect of temperature on nitrogen fixation. To determine the effect of elevated temperature on nitrogen fixation activity, the ARAs of roots excised from unstressed plants nodulated either by CIAT899 or by CNPAF512 were measured at 28, 35, and 40°C. The kinetics of temperature repression of nitrogenase activity were determined as described for Fig. 1. When plants were shifted to 35 or 40°C, ARA was dramatically reduced. Repression of nitrogen fixation activity was most dramatic at 40°C. Similar decreases in ARA on plants nodulated either by CNPAF512 or by CIAT899 were observed.

Survival and protein synthesis at high temperature. Cell viability of both strains at 40°C was determined. A clear difference in survival between CIAT899 and CNPAF512 was observed at this temperature (Fig. 2). In contrast to CIAT899, the number of viable cells of CNPAF512 starts to decline immediately after the temperature shift. Likewise, a comparison of cell viabilities of both strains at 45 and 50°C indicated the higher resistance of CIAT899 to thermal stress (results not shown). These results are in agreement with the data obtained by Martínez-Romero et al. (27) describing *R. tropici* as a relatively temperature-tolerant strain compared with *R. leguminosarum* bv. phaseoli.

The effect of temperature on the rate of protein synthesis was monitored. In order to take into account possible artifacts due to differences in amino acid transport at various temperatures, the amount of L-[^{35}S]methionine incorporated in proteins precipitable with 10% trichloroacetic acid was normal-

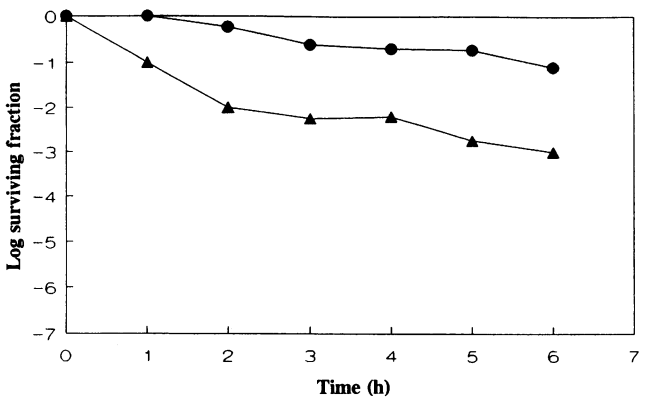

FIG. 2. Survival of *R. leguminosarum* bv. phaseoli CNPAF512 (▲) and *R. tropici* CIAT899 (●) at high temperature. Cells grown at 30°C were shifted at time zero to 40°C and bacterial survival was determined at the indicated times.

ized by the amount of L-[^{35}S]methionine accumulated in the cells as determined in whole-cell extracts. The results are shown in Fig. 3.

During incubation at 30, 40, or 45°C, no clear increase or decrease of the rate of accumulation of L-[^{35}S]methionine in CIAT899 cells was observed (Fig. 3C). Accumulation rates varied between 0.68 and 1.00 times the initial rate at 30°C. At 30°C, the accumulation rates in CNPAF512 varied between 0.65 and 1.00 times the initial rate at 30°C (Fig. 3A). At 40 and 45°C, CNPAF512 cells accumulated L-[^{35}S]methionine at a constant rate during the first 60 and 20 min, respectively; thereafter, accumulation rates rapidly declined. These data indicate that, as opposed to CIAT899, substrate transport in CNPAF512 cells was hampered at 40 and 45°C after 60 and 20 min, respectively.

In Fig. 3B and D, protein synthesis is expressed as the percentage of incorporated L-[^{35}S]methionine (cpm of L-[^{35}S]methionine incorporated [min · μg of cells]$^{-1}$)/(cpm of L-[^{35}S]methionine accumulated [min · μg of cells]$^{-1}$). Clear differences in protein synthesis were observed between both strains upon increase of the incubation temperature. Firstly, in contrast to CNPAF512, an increased protein synthesis in CIAT899 was observed at 40°C compared with synthesis at 30°C. Secondly, whereas protein synthesis in CIAT899 at 40°C was reduced by one-fifth after 5 h of incubation, a reduction of one-half was observed in CNPAF512 after 2 h. Similarly, at 45°C protein synthesis in CIAT899 decreased by a factor of 2 after 50 min of incubation; the same reduction of protein synthesis in CNPAF512 was reached in less than 20 min. These results were confirmed by analysis of the 1D protein profiles of the L-[^{35}S]methionine-labeled cells (see Fig. 5A and B).

Protein analysis by 1D and 2D PAGE. Synthesis of specific proteins in CIAT899 and CNPAF512 was investigated by pulse-labeling with L-[^{35}S]methionine at 30, 40, and 45°C and analysis by 1D or 2D gel electrophoresis. Following a shift from 30 to 40°C, the intensity of most proteins in CNPAF512 remained unaffected or decreased (Fig. 4A and B). The synthesis of at least 14 proteins increased after shift up. The sizes of these heat shock proteins (hsps) were about 12_2, 17_2,

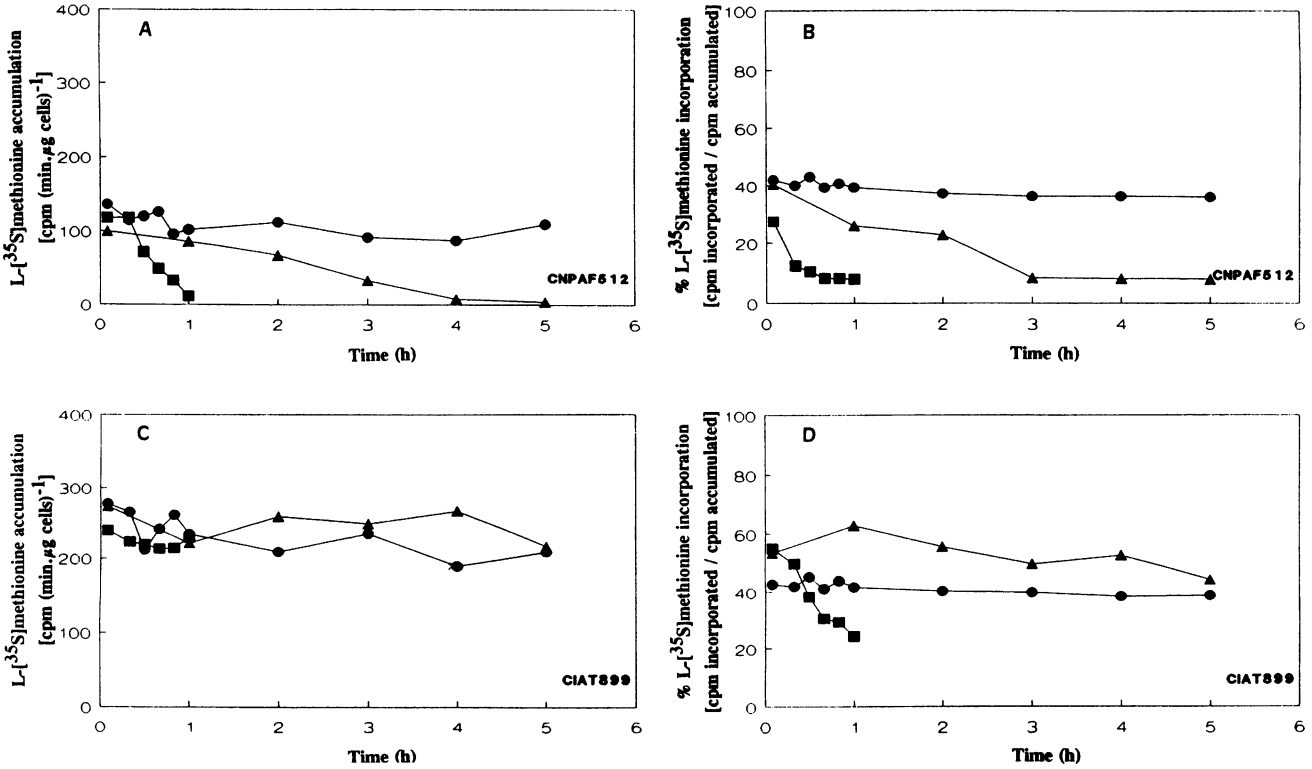

FIG. 3. Effects of temperature on the accumulation (A and C) and incorporation (B and D) of L-[^{35}S]methionine in *R. leguminosarum* bv. phaseoli CNPAF512 (A and B) and *R. tropici* CIAT899 (C and D) cells. Cells grown at 30°C were shifted at time zero to 30°C (●), 40°C (▲), or 45°C (■) and pulse-labeled for 5 min with L-[^{35}S]methionine at the indicated times. The results are the means of two independent experiments.

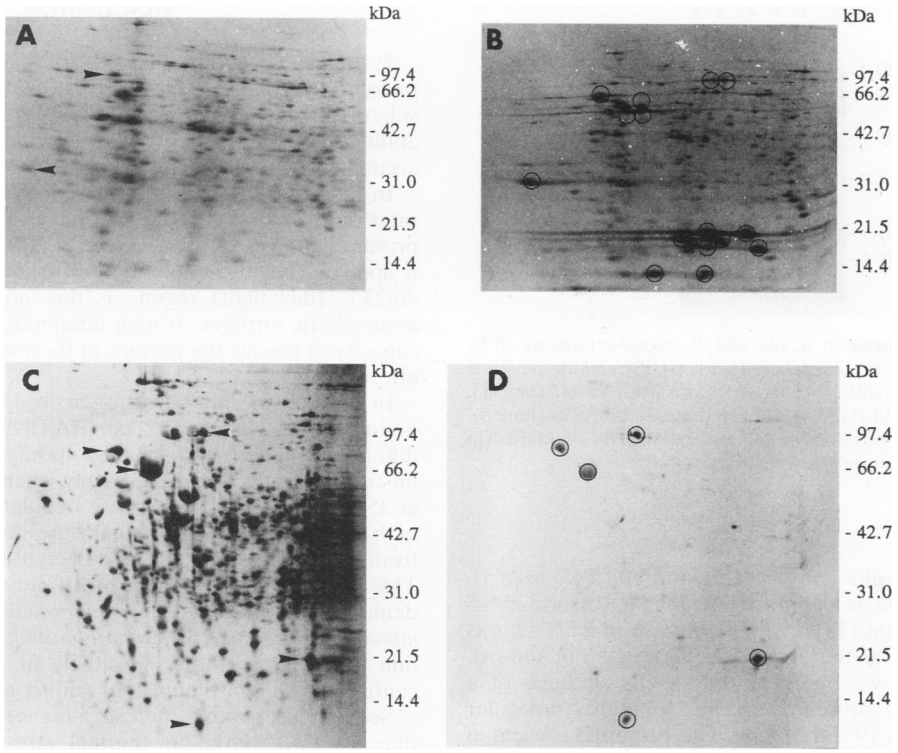

FIG. 4. 2D resolution of L-[^{35}S]methionine-labeled proteins from *R. leguminosarum* bv. phaseoli CNPAF512 (A and B) and *R. tropici* CIAT899 (C and D). Proteins were separated in the first dimension by isoelectric focusing (pH range, acid [left] to basic [right]) and then resolved in the second dimension by SDS-PAGE. Cells were pulse-labeled for 10 min, 20 min after transfer to the appropriate incubation temperature. The incubation temperature was 30°C (A), 40°C (B and C), or 45°C (D). hsps of CNPAF512 (B) and CIAT899 (D) are circled. The corresponding proteins in CNPAF512 at 30°C (A) or in CIAT899 at 40°C (C) are indicated by arrowheads. The molecular mass standards (in kilodaltons) are indicated on the right.

19_2, 21_2, 33, 58_2, 61, 68, and 97 kDa (the numbers of proteins with the same molecular mass but differing in isoelectric points, possibly isoforms, are indicated as subscripts). Synthesis of the 12-, 17-, 19-, and 21-kDa proteins was not observed at 30°C. Similar patterns of hsp synthesis were observed 5, 20, 40, and 60 min after the temperature shift up to 40°C. Protein synthesis was completely stopped after 3 h postshift (data not shown). The synthesis of these hsps was also observed following a temperature shift up from 30 to 45°C (Fig. 5A). Synthesis of the 21-kDa band was particularly strong compared with that of the other hsps. While protein synthesis in CNPAF512 decreased as a function of time, the highest intensity of the 21-kDa protein band was obtained after 20 min of incubation at 45°C. Protein synthesis had completely ceased after 60 min of incubation at this temperature.

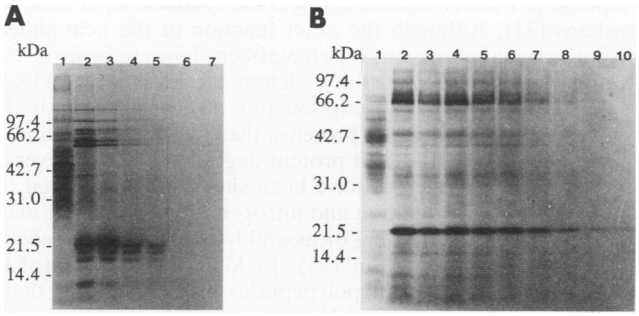

FIG. 5. hsp synthesis in *R. leguminosarum* bv. phaseoli CNPAF512 (A) and *R. tropici* CIAT899 (B). Cells grown at 30°C were pulse-labeled with L-[^{35}S]methionine for 5 min either at 30°C (lanes 1) or 45°C (lanes 2 to 10). At 45°C, cells were labeled 5 (lanes 2), 20 (lanes 3), 30 (lanes 4), 40 (lanes 5), 50 (lanes 6), 60 (lanes 7), 80 (lane 8), 100 (lane 9), and 120 (lane 10) min after transfer. The molecular mass standards (in kilodaltons) are indicated on the left.

In CIAT899, the specific synthesis of hsps was observed at 45 but not at 40°C (Fig. 4C and D). At 40°C, the synthesis of all proteins was increased from the levels at 30°C and was maintained for at least 5 h (data not shown). At 45°C, only five major protein spots were observed. The sizes of these hsps were 12, 21, 65_2, 68, and 97 kDa. The 65-kDa protein represents a doublet. This was demonstrated by 2D analysis with isoelectric focusing carried out in a narrow pH-4.5-to-5.4 gradient. At 45°C, incorporation of L-[^{35}S]methionine into hsps decreased gradually as a function of time (Fig. 5B). The synthesis of the 21-kDa protein band was particularly strong compared with that of the other hsps and continued to show label incorporation during the first 2 h of incubation. The synthesis of hsps was also observed 5 min after shift to 46 or 47°C but was strongly reduced when the incubation temperature was shifted to 48°C and was completely inhibited at 50°C (data not shown).

To investigate whether the strongly synthesized 21-kDa protein was also present in other bean-nodulating rhizobia, their heat shock response was determined (Fig. 6). The *Rhizobium* strains used were CFN 42 (*R. etli*), BR814 (*R. fredii*), BR816 (*Rhizobium* spp.), CFN299 (*R. tropici*, formerly

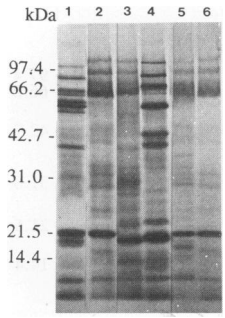

FIG. 6. Protein synthesis in *R. etli* and *R. tropici* strains at 45°C. Cells grown at 30°C were pulse-labeled with L-[^{35}S]methionine for 5 min at 45°C, 5 min after shift. *Rhizobium* strains are CFN-42 (lane 1), CIAT899 (lane 2), BR814 (lane 3), BR816 (lane 4), CFN299 (lane 5), and F98.5 (lane 6). The positions of the molecular mass standards (in kilodaltons) are indicated on the left.

R. leguminosarum bv. phaseoli type IIa), and F98.5 (*R. tropici*). CIAT899 was included as a control. BR814, BR816, and F98.5 are heat-tolerant strains (18). Protein synthesis in CFN 42 is as temperature sensitive as that in CNPAF512 (data not shown). In all strains tested, we observed the strong synthesis of a low-molecular-weight protein with an apparent molecular mass ranging between 19 and 21 kDa. This protein is present in heat-tolerant as well as in heat-sensitive strains. Whether this protein is related to other known hsps remains to be determined.

Acquired thermotolerance. The capacity of adapting to high temperatures by a short pretreatment at a sublethal temperature has been documented for a great variety of cell types. We observed survival after 30 min at 50°C enhanced by as much as 4 orders of magnitude compared with the controls when cells were preincubated at 40°C (Fig. 7). When cells were preheated at 45°C, cell viability after 30 min at 50°C was 100-fold higher than in the control culture. To determine the role of protein synthesis in the acquisition of thermotolerance, tetracycline, an inhibitor of protein synthesis, was added during the preincubation period. Cells incubated at 40°C in the presence of tetracycline (4 μg/ml) were as thermosensitive at 50°C as cells kept at 30°C during the preincubation period.

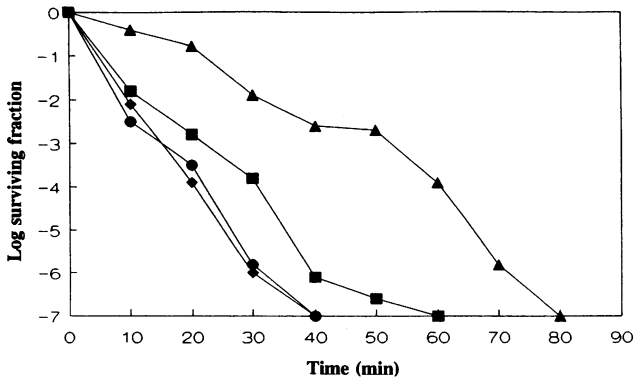

FIG. 7. Survival of *R. tropici* CIAT899 at 50°C following exposure at 30°C for 60 min (●), at 40°C for 60 min (▲), at 45°C for 10 min (■), or at 40°C for 60 min in the presence of 4 μg of tetracycline per ml (◆).

DISCUSSION

R. tropici CIAT899, in contrast to *R. leguminosarum* bv. phaseoli CNPAF512, is able to nodulate beans and fix atmospheric nitrogen on a diurnal 40/23°C (day/night) regime. The ability to fix nitrogen was assessed on the basis of total N accumulation in shoots (18, 28a).

In this study, we have compared the effects of heat on the rates of nitrogen fixation, cell survival, amino acid uptake, and protein synthesis in CIAT899 and CNPAF512. From our data, it appears that the symbiotic performance of CIAT899 on a 40/23°C (day/night) regime is due not to its ability to fix atmospheric nitrogen at high temperature but probably to its capacity to survive the periods of thermal stress and to recover afterwards.

In a first experiment, nitrogen fixation activities in plants nodulated by CNPAF512 or CIAT899 were determined at different temperatures. ARA by nodulated roots excised from unstressed plants declined sharply when roots were incubated at 35 or 40°C, when plants were nodulated either by CIAT899 or by CNPAF512. The temperature dependence of nitrogen fixation may be partly due to a decreased *nif* gene expression. The temperature sensitivity of *nif* gene expression has been demonstrated in *Klebsiella pneumoniae* (49), *Bradyrhizobium japonicum* (1), and *Azospirillum brasilense* (45) and is probably due to the temperature sensitivity of the NifA protein, the central activator protein of *nif* and *fix* genes (5, 23, 49).

Several observations indicate a higher tolerance of CIAT899 than of CNPAF512 to thermal stress. Firstly, survival of CIAT899 cells in liquid medium at elevated temperatures was significantly higher than that of CNPAF512 cells. Secondly, in contrast with CNPAF512, the uptake of L-[^{35}S]methionine in CIAT899 was not affected by a temperature increase. Finally, protein synthesis in CIAT899 was less thermosensitive than in CNPAF512. At 40°C, synthesis of normal proteins in CIAT899 was maintained for at least 5 h whereas this was drastically reduced in CNPAF512 2 h after temperature shift up. The higher tolerance of CIAT899 towards elevated temperatures may allow this strain to survive periods of thermal stress. Under these conditions, nitrogen fixation activity is shut off but may resume when temperatures are lower.

The effect of high temperatures on protein synthesis has been studied in bacteria, plants, and animals. All organisms so far examined respond to a sudden increase in growth temperature by inducing the synthesis of a small number of hsps (24, 32). Some of them have been highly conserved throughout evolution (3, 4, 14, 28). The induction of the heat shock response in *Escherichia coli* involves the synthesis of at least 20 proteins (31). Although the exact function of the heat shock response remains unclear, hsps have been shown to be involved in important cellular functions such as the initiation of DNA replication (34, 40), transcription (43, 48), protein synthesis (46), the correct folding of proteins, the assembly of multiprotein complexes (9, 10), and protein degradation (11). Several hsps or homologs thereof have been shown to be involved in the processes of nodulation and nitrogen fixation. GroEL may assist in the correct folding or assembly of *Rhizobium meliloti* NodD into an active form (25). In *K. pneumoniae*, GroEL interacts with several Nif polypeptides and determines their rates of synthesis and levels of accumulation (12). In *B. japonicum*, the anaerobic expression of a GroEL homolog is dependent on NifA and NtrA (7). The *R. fredii* NolC protein, involved in cultivar-specific nodulation of soybean, is homologous to the *E. coli* hsp DnaJ (20).

An increase in growth temperature of CNPAF512 and CIAT899 cells was accompanied by the specific synthesis of

several hsps. Whether these hsps contribute to the observed differences in heat tolerance between both strains is unclear. The synthesis of two hsps of 12 kDa and approximately 21 kDa after temperature shift up was also observed in several other bean-nodulating *Rhizobium* strains belonging to different species. Such a conservation may be indicative of an important cellular function of these proteins. The establishment of a correlation between thermotolerance and the synthesis of specific proteins would be greatly facilitated by the use of (nearly) isogenic strains differing only in heat tolerance.

The phenomenon whereby cells can adapt to high temperatures when they are first exposed to a sublethal dose of heat has been termed acquired thermotolerance. In several organisms, the appearance of thermotolerance has been correlated with the production of hsps (24). Several hsps, including hsp70, strongly conserved over large taxonomic distances, have been shown to be directly implicated in acquired thermotolerance (16). Enhanced cell survival at 50°C was observed when CIAT899 cells were first exposed to a temperature of 40 or 45°C. The acquired thermotolerance was found to be dependent on protein synthesis. It can be speculated that the appearance of thermotolerance at 50°C after a preincubation at 45°C is related to the synthesis of six major proteins at 45°C, as no additional protein synthesis occurs during incubation at 50°C. A much higher level of thermotolerance was obtained when cells were preheated at 40°C than at 45°C. This difference may be accounted for by quantitative and/or qualitative differences in the synthesis of proteins at both temperatures.

Although resistance to temperature stress may constitute a prerequisite for symbiotic nitrogen fixation in tropical areas, heat may also affect other properties. High temperatures have been shown to affect the stability of the symbiotic properties of *Rhizobium* strains. The loss of *Rhizobium* symbiotic effectiveness at elevated temperature was attributed to deletions or to the curing of the symbiotic plasmid (44, 50). In *R. tropici* strains, the symbiotic plasmid seems to be particularly stable, even after prolonged incubation at high temperature (27). In addition to the resistance of CIAT899 to thermal stress, the stability of its symbiotic properties may constitute a second advantage in tropical and subtropical areas.

ACKNOWLEDGMENTS

J.M. is a recipient of a fellowship from the Instituut voor Wetenschappelijk Onderzoek in de Nijverheid en de Landbouw. Part of this work was supported by an EEC-STDII program [TS2-0199-C(GDF)].

We thank R. De Mot for critically reading the manuscript.

REFERENCES

1. **Alvarez-Morales, A., and H. Hennecke.** 1985. Expression of *Rhizobium japonicum nifH* and *nifDK* operons can be activated by the *Klebsiella pneumoniae nifA* protein but not by the product of *ntrC*. Mol. Gen. Genet. **199:**306–314.
2. **Arayankoon, T., H. H. Schomberg, and R. W. Weaver.** 1990. Nodulation and N_2 fixation of guar at high root temperature. Plant Soil **126:**209–213.
3. **Bardwell, J. C. A., and E. A. Craig.** 1984. Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible *dnaK* gene are homologous. Proc. Natl. Acad. Sci. USA **81:**848–852.
4. **Bardwell, J. C. A., and E. A. Craig.** 1987. Eukaryotic M_r 83,000 heat shock protein has a homologue in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84:**5177–5181.
5. **Brooks, S. J., J. J. Collins, and W. J. Brill.** 1984. Repression of nitrogen fixation in *Klebsiella pneumoniae* at high temperature. J. Bacteriol. **157:**460–464.
6. **De Mot, R., and J. Vanderleyden.** 1989. Application of two-dimensional protein analysis for strain fingerprinting and mutant analysis of *Azospirillum* species. Can. J. Microbiol. **35:**960–967.
7. **Fischer, H. M., M. Babst, T. Kaspar, G. Acuña, F. Arigoni, and H. Hennecke.** 1993. One member of a *groESL*-like chaperonin multigene family in *Bradyrhizobium japonicum* is coregulated with symbiotic nitrogen fixation genes. EMBO J. **12:**2901–2916.
8. **Frings, J. F. J.** 1976. The *Rhizobium*-pea symbiosis as affected by high temperatures. Meded. Landbouwhogesch. Wageningen **76:**1–76.
9. **Gaitanaris, G. A., A. G. Papavassiliou, P. Rubock, S. J. Silverstein, and M. E. Gottesman.** 1990. Renaturation of denatured lambda repressor requires heat shock proteins. Cell **61:**1013–1020.
10. **Goloubinoff, P., A. A. Gatenby, and G. E. Lorimer.** 1989. GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose biphosphate carboxylase oligomers in *Escherichia coli*. Nature (London) **337:**44–47.
11. **Gottesman, S., M. E. Gottesman, J. E. Shaw, and M. L. Pearson.** 1981. Protein degradation in *E. coli*: the *lon* mutation and bacteriophage lambda N and cII protein stability. Cell **24:**225–233.
12. **Govezensky, D., T. Greener, G. Segal, and A. Zamir.** 1991. Involvement of GroEL in *nif* gene regulation and nitrogenase assembly. J. Bacteriol. **173:**6339–6346.
13. **Graham, P. H., S. E. Viteri, F. Mackie, A. A. T. Vargas, and A. Palacios.** 1982. Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. Field Crops Res. **5:**121–128.
14. **Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix, and R. J. Ellis.** 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature (London) **333:**330–334.
15. **Hernandez-Armenta, R., H. C. Wien, and A. R. J. Eaglesham.** 1989. Maximum temperature for nitrogen fixation in common bean. Crop Sci. **29:**1260–1265.
16. **Hightower, L. E.** 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell **66:**191–197.
17. **Hungria, M., and A. A. Franco.** 1993. Effects of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L. Plant Soil **149:**95–102.
18. **Hungria, M., A. A. Franco, and J. I. Sprent.** 1993. New sources of high-temperature tolerant rhizobia for *Phaseolus vulgaris* L. Plant Soil **149:**103–109.
19. **Kishinevsky, B. D., D. Sen, and R. W. Weaver.** 1992. Effect of high root temperature on *Bradyrhizobium*-peanut symbiosis. Plant Soil **143:**275–282.
20. **Krishnan, H. B., and S. G. Pueppke.** 1990. *nolC*, a *Rhizobium fredii* gene involved in cultivar-specific nodulation of soybean, shares homology with a heat-shock gene. Mol. Microbiol. **5:**737–745.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **La Favre, A. K., and A. R. J. Eaglesham.** 1986. The effects of high temperatures on soybean nodulation and growth with different strains of bradyrhizobia. Can. J. Microbiol. **32:**22–27.
23. **Lee, H. S., D. K. Berger, and S. Kustu.** 1993. Activity of purified NifA, a transcriptional activator of nitrogen fixation genes. Proc. Natl. Acad. Sci. USA **90:**2266–2270.
24. **Lindquist, S.** 1986. The heat-shock response. Annu. Rev. Biochem. **55:**1151–1191.
25. **Long, S. R., R. F. Fisher, J. Ogawa, J. Swanson, D. W. Ehrhardt, E. M. Atkinson, and J. S. Schwedock.** 1991. *Rhizobium meliloti* nodulation gene regulation and molecular signals, vol. 1, p. 127–133. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions. Kluwer Academic Publishers, Dordrecht, The Netherlands.
26. **Martínez, E., R. Palacios, and F. Sánchez.** 1987. Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. J. Bacteriol. **169:**2828–2834.
27. **Martínez-Romero, E., L. Segovia, F. M. Mercante, A. A. Franco, P. Graham, and M. A. Pardo.** 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. **41:**417–426.
28. **McMullin, T. W., and R. L. Hallberg.** 1988. A highly evolutionarily conserved mitochondrial protein is structurally related to the protein encoded by the *Escherichia coli groEL* gene. Mol. Cell. Biol. **8:**371–380.

28a. **Mercante, F. M.** 1993. M.Sc. thesis. Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil.

29. **Michiels, J., A. Vande Broek, and J. Vanderleyden.** 1991. Molecular cloning and nucleotide sequence of the *Rhizobium phaseoli recA* gene. Mol. Gen. Genet. **228:**486–490.
30. **Munevar, F., and A. G. Wollum.** 1982. Response of soybean plants to high root temperature as affected by plant cultivar and *Rhizobium* strain. Agron. J. **74:**138–142.
31. **Neidhardt, F. C., and R. A. VanBogelen.** 1987. Heat shock response, p. 1334–1345. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
32. **Neidhardt, F. C., R. A. VanBogelen, and V. Vaughn.** 1984. The genetics and regulation of the heat-shock proteins. Annu. Rev. Genet. **18:**295–329.
33. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
34. **Paek, K.-H., and G. C. Walker.** 1987. *Escherichia coli dnaK* null mutants are inviable at high temperature. J. Bacteriol. **169:**283–290.
35. **Pankhurst, C. E., and J. I. Sprent.** 1976. Effects of temperature and oxygen tension on the nitrogenase and respiratory activities of turgid and water-stressed soybean and French bean root nodules. J. Exp. Bot. **27:**1–9.
36. **Philpotts, H.** 1967. The effect of soil temperature on nodulation of cowpeas (*Vigna sinensis*). Aust. J. Exp. Agric. Anim. Husb. **7:**372–376.
37. **Piha, M. I., and D. N. Munns.** 1987. Sensitivity of the common bean (*Phaseolus vulgaris* L.) symbiosis to high soil temperature. Plant Soil **98:**183–194.
38. **Possingham, J. V., D. V. Moyne, and A. J. Anderson.** 1965. Influence of elevated shoot and root temperature on nitrogen fixation. Plant Physiol. (Bethesda) **39:**561–563.
39. **Rainbird, R. M., C. A. Atkins, and J. S. Pate.** 1983. Effect of temperature on nitrogenase functioning in cowpea nodules. Plant Physiol. (Bethesda) **73:**392–394.
40. **Sakakibara, Y.** 1988. The *dnaK* gene of *Escherichia coli* functions in initiation of chromosome replication. J. Bacteriol. **170:**972–979.
41. **Segovia, L., J. P. W. Young, and E. Martínez-Romero.** 1993. Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. **43:**374–377.
42. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
43. **Taylor, W. E., D. E. Straus, A. D. Grossman, Z. F. Burton, C. A. Gross, and R. Burgess.** 1984. Transcription from a heat-inducible promoter causes heat shock regulation of the sigma subunit of *E. coli* RNA polymerase. Cell **38:**371–381.
44. **Toro, N., and J. Olivares.** 1986. Analysis of *Rhizobium meliloti* Sym mutants obtained by heat treatment. Appl. Environ. Microbiol. **51:**1148–1150.
45. **Tripathi, A. K., and W. Klingmüller.** 1992. Temperature sensitivity of nitrogen fixation in *Azospirillum* spp. Can. J. Microbiol. **38:** 1238–1241.
46. **VanBogelen, R. A., V. Vaughn, and F. C. Neidhardt.** 1983. Gene for heat-inducible lysyl-tRNA synthetase (*lysU*) maps near *cadA* in *Escherichia coli*. J. Bacteriol. **153:**1066–1068.
47. **Vincent, J. M.** 1970. A manual for the practical study of the root-nodule bacteria. IBP Handbook no. 15. Blackwell Scientific Publications, Oxford.
48. **Wada, M., and H. Itikawa.** 1984. Participation of *Escherichia coli* K-12 *groE* gene products in the synthesis of cellular DNA and RNA. J. Bacteriol. **157:**694–696.
49. **Zhu, J., and W. J. Brill.** 1981. Temperature sensitivity of the regulation of nitrogenase synthesis by *Klebsiella pneumoniae*. J. Bacteriol. **145:**1116–1118.
50. **Zurkowski, W.** 1982. Molecular mechanism for loss of nodulation properties of *Rhizobium trifolii*. J. Bacteriol. **150:**999–1007.