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Adaptation to Nutrient Starvation in *Rhizobium leguminosarum* bv. phaseoli: Analysis of Survival, Stress Resistance, and Changes in Macromolecular Synthesis during Entry to and Exit from Stationary Phase

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The nitrogen-fixing bacterium *Rhizobium leguminosarum* bv. phaseoli often has to survive long periods of starvation in the soil, when not in a useful symbiotic relationship with leguminous plants. We report that it can survive carbon, nitrogen, and phosphorus starvation for at least 2 months with little loss of viability. Upon carbon starvation, *R. leguminosarum* cells were found to undergo reductive cell division. During this period, they acquired the potential for long-term starvation-survival, levels of protein, DNA, and RNA synthesis were decreased to base levels, and pool mRNA was stabilized. The starved cells are ready to rapidly restart growth when nutrients become available. Upon addition of fresh nutrients, there is an immediate increase in the levels of macromolecular synthesis, pool mRNA destabilizes, and the cultures enter exponential growth within 5 to 8 h. The starved cells were cross-protected against pH, heat, osmotic, and oxidative shock. These results provide evidence for a general starvation response in *R. leguminosarum* similar to that previously found in other bacteria such as *Escherichia coli* and *Vibrio* sp.

Nongrowth is probably the rule rather than the exception in most natural environments, including soil, with the majority of bacteria spending most of their time in nutrient-limited stationary phase (15, 31). Bacteria have evolved a number of mechanisms that allow them to survive under nutrient starvation conditions and to resume growth once nutrients become available again. Some bacteria, such as bacilli, clostridia, and azospirilli, undergo major differentiation programs leading to the formation of highly stress resistant endospores or cysts (3, 32). However, even without the formation of such elaborately differentiated cells, bacteria enter starvation-induced programs that allow them to survive long periods of nongrowth and to restart growth when nutrients become available again. This often leads to the formation of metabolically less active cells that are more resistant to a wide range of environmental stresses (4, 15, 16, 24, 28, 34). This adaptation to starvation conditions is often accompanied by a change in cell size as well as the induction of genes and the stabilization of proteins that are essential for long-term survival. The best-studied examples of starvation-survival in nondifferentiating bacteria are *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio* sp. strain S14, which show qualitative similarities in their survival responses (4, 16, 27, 29, 39).

There is little known about the starvation-survival of bacteria indigenous to soil (41). Most carbon in soil is present as recalcitrant compounds, such as humic substances and lignins, that may also complex available compounds (25), and so soil can be considered an oligotrophic environment (45). The resulting low amount of available carbon in soil generally precludes bacterial growth, and it is estimated that soil microbes typically receive sufficient energy for just a few cell divisions per year (7, 33). Nutrients may become available locally, for example, in decaying plant and animal material or via plant roots, which are one of the major sites of carbon input into soil. Consequently, the rhizosphere is a soil region with a transiently high availability of carbon and the compounds released will be in a form readily available to soil bacteria (41). Soil bacteria that have evolved in close association with plants, such as rhizobia and pseudomonads, would benefit from being able to quickly escape the starvation state and colonize the plant root. Indeed, the life cycle of rhizosphere bacteria on annual crops can be regarded as a regular feast-and-famine existence (41).

Lutgenberg and coworkers have shown, using *Rhizobium leguminosarum* bv. viciae, that the conditions under which rhizobia are grown are of prime importance to their ability to attach to root hairs. Nutrient limitation coincides with optimal attachment, and the type of limitation determines whether host lectins are involved in the attachment process (14, 36, 37, 38). For example, under carbon limitation, there is induction of a nonspecific attachment mechanism involving neither host plant lectins nor *nod* genes (36).

We are investigating the starvation-survival of *R. leguminosarum* bv. phaseoli to understand how it maintains viability, during the long periods of starvation that are inevitably encountered, in the bulk soil and in the rhizosphere. We are also interested in whether the persistence of bacteroids in the root nodule, in a nongrowing but metabolically active state, and the presence of an undifferentiated subpopulation of vegetative cells which can be recovered as CFU from the nodule (8, 20, 48) relate to the maintenance of cultures of free-living cells under starvation-induced stationary-phase conditions. In this paper, we report on the nature of the starvation-survival response of *R. leguminosarum* bv. phaseoli with respect to the survival kinetics, the development of a long-term survival capability, changes in cell size and shape, macromolecular synthesis, and stability, and the development of a stress-resistant cell.

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MATERIALS AND METHODS

Growth and starvation of bacteria. *R. leguminosarum* bv. phaseoli 4292 was used in this study. It is a rifampin-resistant strain that carries the biovar phaseoli Sym plasmid pRP2JI and nodulates *Phaseolus vulgaris* (18). Cultures were grown in either YEM or minimal medium. YEM medium at pH 7.5 (43) consisted of (per liter) yeast extract (0.4 g; Oxoid), K_2HPO_4 (0.5 g), NaCl (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), and mannitol (10 g). The minimal medium used was a specially designed morpholinepropanesulfonic acid (MOPS) minimal medium based on a medium described by Jordan (12) and adjusted to contain only one carbon, nitrogen, and phosphorus source. This medium contained (per liter) MOPS (8.37 g, pH 7.2), KOH (1.12 g), $CaCl_2$ (1.12 g), $MgSO_4$ (0.48 g), NH_4Cl (1.07 g), K_2HPO_4 (0.21 g), KH_2PO_4 (0.05 g), mannitol (10 g), NaCl (5 mg), H_3BO_3 (1 mg), $ZnSO_4 \cdot 7H_2O$ (1 mg), $CuSO_4$ (0.5 mg), $MnCl_2 \cdot 4H_2O$ (0.5 mg), $NaMoO_4 \cdot 2H_2O$ (1 mg), EDTA (10 mg), thiamine (0.2 mg), pantothenate (0.01 mg), and biotin (0.2 mg). This medium could easily be manipulated to vary the concentrations of carbon source (mannitol), nitrogen source (NH_4Cl), or phosphorus source (K_2HPO_4 and KH_2PO_4). For solid medium, agar was added at 15 $g.l^{-1}$. For routine culture maintenance, growth of starter cultures, and plating for viable counting, rifampin was added at 25 $\mu g\ ml^{-1}$.

Starter cultures were prepared by inoculating a single colony from a plate stock into 5 ml of medium and incubating it overnight at 30°C in a shaking incubator (200 rpm); 0.5 ml of this growing culture was then subcultured into a second 5 ml of medium and again incubated overnight to ensure that cells were fully adapted to exponential growth. About 5 ml of this starter culture was then inoculated into 50 ml of medium in a sterile 250-ml conical flask to give an initial optical density at 600 nm (OD_{600}) of 0.02. This method of inoculation was used throughout this study unless otherwise stated. Mid-exponential-phase cultures used in this work were cultures 24 h after inoculation which had 10^8 CFU ml^{-1} ($OD_{600} = 0.2$).

Growth was measured spectrophotometrically as OD_{600}, by cell counting and by dry weight (biomass) measurements. For viable counting, samples were taken at appropriate times and plated onto solidified minimal medium after appropriate dilution in medium. Total cell counts were made microscopically, using a hemocytometer slide to count the number of cells in a known volume of medium. The length of bacteria was measured by using a calibrated eye piece graticule in bright-field or Nomarski interference contrast microscopy. Dry weight measurements were made by collecting bacteria from appropriate volumes of cultures by filtration through 0.45-μm-pore-size membrane filters. The filters were then dried at 100°C to constant weight.

Carbon starvation was achieved in one of two ways. Firstly, starvation by exhaustion was established by reduction of the concentration of mannitol in the medium to 5 g $liter^{-1}$. Second, starvation by resuspension was achieved by harvesting bacteria from a culture of an appropriate age by centrifugation, followed by resuspension in minimal medium with no carbon source. Starvation for nitrogen was performed by exhaustion of 0.5 g of NH_4Cl $liter^{-1}$, and starvation for phosphorus was performed by exhaustion of phosphate (0.1 g of K_2HPO_4 and 0.025 g of KH_2PO_4 $liter^{-1}$). All experimental cultures were inoculated to the same starting OD_{600}. Nutrient levels were known to be limiting, as cultures grew to lower cell densities than if normal nutrient concentrations were used.

Determination of protein, RNA, and DNA synthesis. For DNA and RNA synthesis determinations, 0.5-ml samples of cultures were pulsed for 10 min with 1 μCi of [^{3}H]thymidine (89 Ci $mmol^{-1}$, for DNA synthesis) or 1 μCi of [^{3}H] uridine (47 Ci $mmol^{-1}$, for RNA synthesis). After the 10-min pulse, samples were quenched with 4 ml of ice-cold 7.5% trichloroacetic acid (TCA) with 12.5 μg of herring sperm DNA ml^{-1} as a carrier. Samples were kept on ice for at least 15 min before collection of cells on 25-mm glass fiber filter discs (pore size, 1 μm; Gelman Science). Filters were washed with 5 ml of 7.5% TCA, and the radioactivity on the filters was determined by liquid scintillation counting in Cocktail T Scintran (BDH Chemicals Ltd.). DNA and RNA synthesis rates were measured as isotope incorporation determined by scintillation counting. For protein synthesis determination, 0.5-ml samples were pulsed for 10 min with 1 μCi of [^{35}S]methionine (1,067 Ci mol^{-1}) and then quenched with 1.0 ml of ice-cold 7.5% TCA with 1 mM methionine and left for at least 60 min. Samples were then heated at 90°C for 30 min, cooled and collected on glass fiber discs, and washed with 10 ml of 7.5% TCA, and the radioactivity counted as before.

Determination of pool mRNA stability. A change in the potential to synthesize proteins posttranscriptionally could be affected by an increase in the rate of translation or more likely a change in mRNA stability. Therefore, mRNA stability was estimated as the loss of potential to incorporate [^{35}S]methionine after inhibition of transcription by rifampin. In these experiments, the rifampin-sensitive strain *R. leguminosarum* 8002, which is isogenic with 4292, was used. These experiments were done essentially as described by Albertson et al. (1). Control experiments were run to ensure that the concentration of rifampin used inhibited the rate of RNA synthesis rapidly and with the same kinetics at each sample time. This was done by measuring the residual RNA synthesis after the addition of rifampin. Samples of 0.5 ml were pulse-labeled for 20 s with 1 μCi of [^{3}H]uridine at time points 0 to 300 s after the addition of 10 μl of 500 μg of fresh rifampin ml^{-1}. Samples were quenched, filtered, and counted as described above. This was repeated for carbon-starved and exponentially growing cultures to ensure that the kinetics of inhibition were similar. To measure the half-life of the mRNA pool, 0.5-ml culture samples were pulse-labeled for 2 min with 1 μCi of [^{35}S]methionine at time points between 0 and 60 min after the addition of 10 μl of 500 μg of rifampin ml^{-1}. Incorporation was determined by measuring the amount of TCA-precipitable radioactivity as described above.

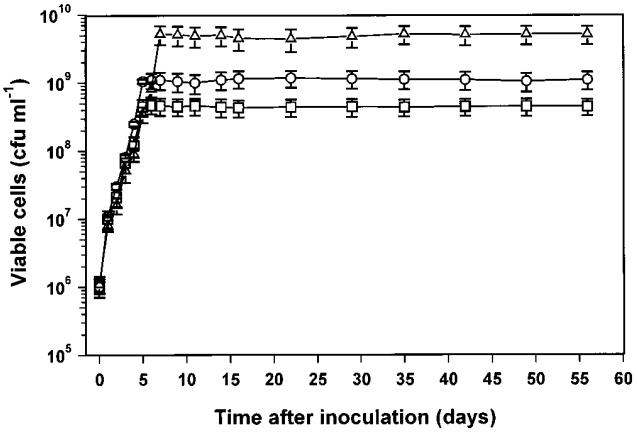

FIG. 1. Long-term survival of *R. leguminosarum* bv. phaseoli following nutrient starvation by exhaustion for either carbon (□), nitrogen (○), or phosphorus (△). Survival was determined by viable counting of samples taken from cultures throughout the experiment.

Stress challenge protocols. Two *R. leguminosarum* 4292 cultures were inoculated into carbon-limiting minimal medium; one was grown to exponential phase (OD_{600} of 0.4), and the other was grown to carbon-starved stationary phase (10 days after inoculation, 6 days into stationary phase). A stress condition was then applied to both cultures, and survival was monitored over a period of 3 h by viable counting. Osmotic shock was applied by addition of a filter-sterilized NaCl solution to a final concentration of 2.5 M. Oxidative stress was applied by the addition of H_2O_2 (to 5 mM), and heat shock was applied by moving the cultures from 28 to 55°C. Acid stress was studied by two methods. First, the internal pH of the cells was lowered by addition of a filter-sterilized sodium acetate solution. Acetate can cross bacterial cell membranes by passive diffusion as the undissociated acid. Once in the cell, it dissociates releasing H^+ ions, thus lowering the internal pH. This dissociation prevents the acetate from diffusing back out of the cell (30). Second, the external pH of the medium was changed. The MOPS buffer used in the minimal medium can buffer down only to a pH of 5.0 and so, to increase this range, was changed in favor of a citrate-phosphate buffer. With this buffer, it was possible to reduce the pH from 7.0 to as low as 3.5 by addition of citric acid, without loss of buffering capacity.

RESULTS

Effect of starvation by nutrient exhaustion on viability and cell size. The long-term survival of *R. leguminosarum* following starvation by nutrient exhaustion for carbon, nitrogen, and phosphorus was monitored by viable counting (Fig. 1). In each case, the levels of viability were maintained close to 100% throughout the 55 days of the experiment.

The kinetics of starvation-induced entry into stationary phase was investigated in more detail by monitoring viable and total cell counts, biomass (dry weight) and OD_{600} (Fig. 2). The times at which the various parameters stabilized differed. During carbon starvation, both the biomass and the OD_{600} stabilized 90 h after inoculation, while cell counts continued to increase at a lower rate and finally stabilized after 120 h (Fig. 2A and B). Between 90 and 120 h after inoculation, the cell numbers increased approximately threefold, from 2×10^8 to 5×10^8 to 6×10^8. During nitrogen starvation, the biomass stabilized at 100 h shortly followed by optical density (Fig. 2D). Cell counts continued increasing but at a reduced rate until 140 h after inoculation, during which time viable cell numbers increased about twofold, from 7×10^8 to 1.5×10^9 CFU ml^{-1} (Fig. 2C). During phosphorus starvation, there was a clear change in the rates of increase of cell numbers, optical density, and biomass from about 100 h after inoculation (Fig. 2E and

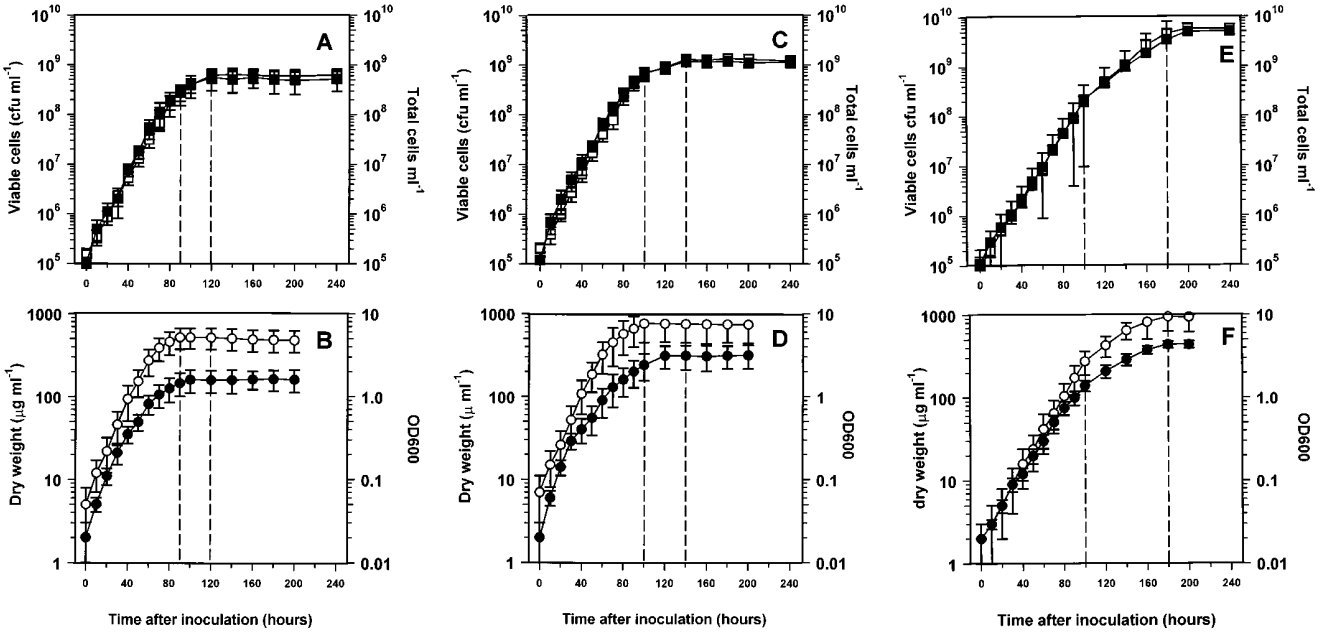

FIG. 2. Changes in total (■) and viable (□) cell numbers, biomass (○), and optical density (●) of *R. leguminosarum* during and following entry into nutrient-starved stationary phases. Cultures were starved by exhaustion for carbon (A and B), nitrogen (C and D), or phosphorus (E and F).

F). This lower rate of increase in these parameters continued until 180 to 200 h. During this period, viable cell numbers increased 24-fold and biomass increased 3.5-fold, a discrepancy that could be due to reductive cell division occurring and/or the utilization of storage compounds to facilitate growth and cell division. Following stabilization of the biomass at 180 h, there was a modest (30%) increase in cell numbers over the next 20 h (Fig. 2E and F). This type of growth pattern, of an extended deceleration phase, has been previously described for phosphorus limitation in, for example, *Klebsiella pneumoniae*, where a biomass increase of sixfold was reported during this phase (23, 26, 44). Carbon starvation was studied in all subsequent experiments, and the time at which OD_{600} stabilizes was taken as the time of entry into stationary phase.

The increase in cell numbers, found following stabilization of biomass levels under all three starvation conditions, suggested that reductive cell division was occurring. This was confirmed by microscopic examination and measurements of cell size during entry into stationary phase (Fig. 3). In carbon-starved cultures, the average cell length changed from 3.20 to 1.75 μm between 100 and 140 h (Fig. 3), and the cells became coccoid (data not shown). Nitrogen-starved cells retained their rod shape but became thinner, and their average cell length fell to 2.1 μm between 100 and 140 h after inoculation (Fig. 3). Phosphate-starved cells also retained their rod shape but were more swollen in appearance and reduced their length from 3.2 to 2.5 μm between 180 and 200 h after inoculation (Fig. 3). Reductive cell division, with concomitant changes in cell shape from rods to cocci, has been reported for many different gram-negative bacteria, including *K. pneumoniae*, *E. coli*, *Vibrio* sp., and *Pseudomonas putida* (6, 16, 19, 22). Reductive cell division increases the probability that some cells of the clonal population will subsequently encounter nutrients and increase the surface area, allowing for more efficient uptake of nutrients.

An alternative way of achieving carbon starvation is to remove growing cells from culture medium by centrifugation and to immediately resuspend them in a medium lacking the carbon source. When exponentially growing *R. leguminosarum* cells were harvested and rapidly transferred to minimal medium without mannitol, their viability dropped rapidly; 8 days after resuspension, there was only 14% viability (Fig. 4). Clearly, *R. leguminosarum* cultures were unable to adapt to the rapid depletion of carbon source. However, a stationary-phase culture resuspended 7 days after inoculation (3 days in stationary phase) still showed 97% survival 8 days later, and it had similar viability after 20 days (Fig. 4). This culture, in which carbon had been depleted by nutrient exhaustion during

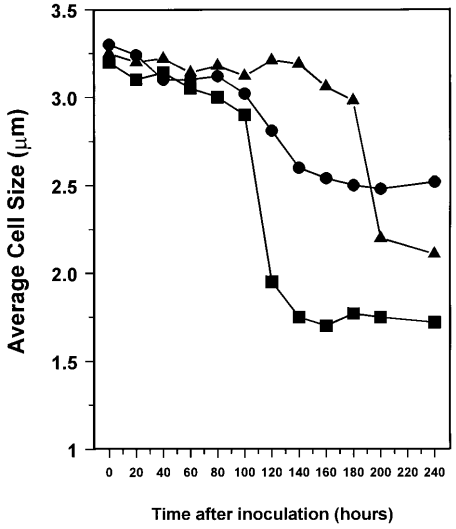

FIG. 3. Changes in the length of *R. leguminosarum* cells during exponential growth and during entry into nutrient-starved stationary phase. Cultures were starved for carbon (■), nitrogen (●), or phosphorus (▲); an average of 100 cells were measured for each data point shown, and the standard deviation was never $>\pm 0.1$ μm.

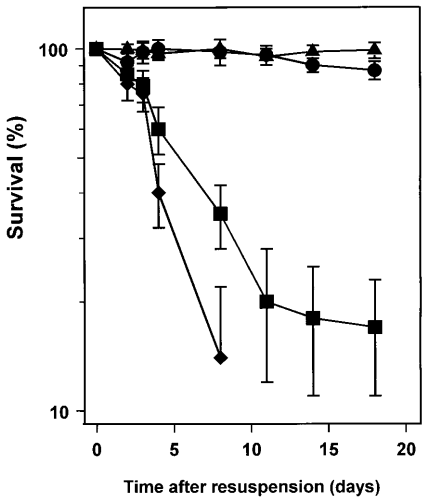

FIG. 4. Survival of *R. leguminosarum* cultures following starvation for carbon by resuspension, and the use of this procedure to investigate the adaptation to stationary-phase survival. Cultures were grown in carbon starvation medium, harvested at various times following inoculation, and then resuspended in minimal medium without a carbon source as described in Materials and Methods. ◆, 1-day-old mid-exponential-phase culture (OD_{600} = 0.4); ■, 4-day-old culture (just entering carbon-starved stationary phase, at the point of biomass stabilization [Fig. 2B]); ●, 5-day-old culture (ca. 30 h in stationary phase); ▲, 7-day-old culture (ca. 72 h in stationary phase). All cultures were resuspended to a density of ca. 5×10^5 CFU ml^{-1}, and survival was determined by sampling and determining viable counts.

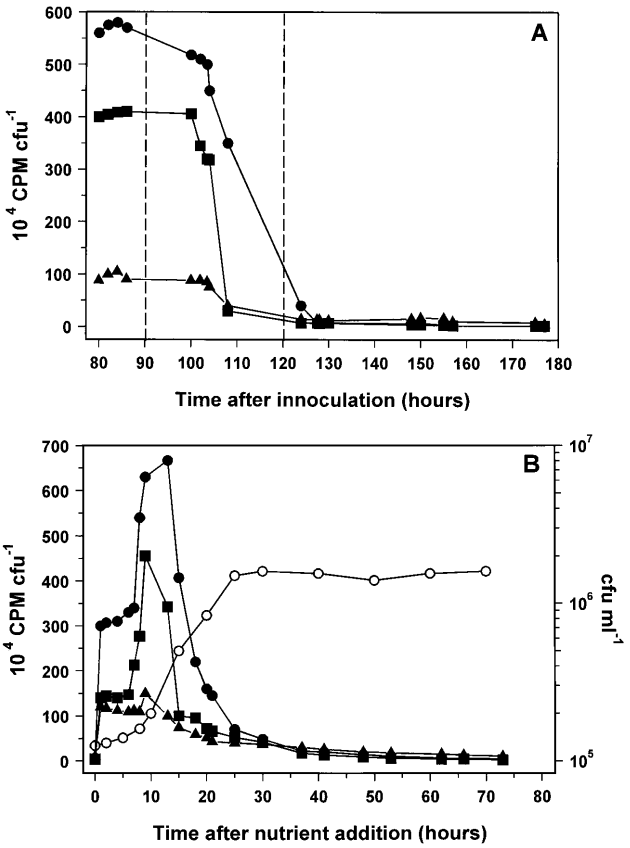

FIG. 5. Synthesis of DNA, RNA, and protein during entry to and recovery from carbon-starved stationary phase. (A) Cultures were grown in minimal medium and allowed to become starved for carbon by exhaustion. Samples were taken throughout the experiment for viable counting and bulk DNA (▲), RNA (■), and protein (●) synthesis determined by measuring incorporation of [^{3}H]thymidine, [^{3}H]uridine, and [^{35}S]methionine, respectively, into TCA-precipitable material. The first dashed vertical line marks the point of entry into stationary phase, where the cell biomass stabilizes and the period of reductive cell division starts. The second dashed vertical line marks the time at which reductive cell division is complete and at which the cells are fully adapted for long-term survival. (B) To monitor macromolecular synthesis during recovery, samples were taken from 10-day-old carbon-starved cultures (6 days in stationary phase) and inoculated into 50 ml of fresh minimal medium to give 1.3×10^5 CFU ml^{-1}. Samples were taken, levels of DNA, RNA, and protein synthesis were determined as described above, and viability was monitored (○).

growth and entry into stationary phase, has become fully adapted for long-term survival. This result indicated that starvation by nutrient exhaustion is the most appropriate way to study starvation survival in *R. leguminosarum*, and it suggested that starvation by resuspension could provide a way of monitoring the temporal adaptation of *R. leguminosarum* to stationary-phase survival. A culture resuspended 4 days (96 h) after inoculation lost viability more slowly that the exponential culture, but 7 days after resuspension, it showed just 35% viability. This was reduced to 20% survival after 11 days but then leveled off, and the remaining cells survived. In contrast, a culture resuspended 5 days (120 h) after inoculation was fully adapted to survival, and viability at 20 days after resuspension was 95%. This finding indicates that the adaptation to stationary-phase survival occurred between 96 and 120 h after inoculation and is coincident with the period of reductive cell division of the carbon-starved culture.

Changes in macromolecular synthesis during entry to and exit from carbon starvation-induced stationary phase. The rate of protein synthesis was measured by pulse-labeling with [^{35}S]methionine during exponential growth and during entry to and maintenance of carbon-starved stationary phase. Incorporation rates were maintained at a steady state during exponential growth. This rate of incorporation declined slowly during the first 10 to 15 h of stationary phase (90 to 105 h after inoculation), corresponding to the first half of the reductive cell division period (Fig. 5A). It then declined more rapidly at a fairly constant rate for the next 25 h, and 130 h into stationary phase it had reached a level approximately 0.2% of the exponential growth levels (Fig. 5A). Therefore, protein synthesis remained high up to the point at which reductive cell division was complete and which coincided with the culture becoming fully adapted for stationary-phase survival (Fig. 4). The kinetics of RNA and DNA synthesis were broadly similar to those for protein synthesis during entry into carbon-starved stationary phase. [^{3}H]thymidine incorporation rates were maintained at close to the exponential growth level for the first 10 to 15 h of stationary phase and then declined rapidly to reach a minimum by about 125 h after inoculation. This reduction in the rate of DNA synthesis occurs in the later stages of the reductive cell division period. The cells probably complete a final round of DNA replication once nutrients become scarce or exhausted. However, the rate of DNA synthesis in stationary phase was relatively high, at about 5% of that in exponential phase. RNA synthesis levels were reduced about 57-fold in stationary phase compared to exponential phase. The reduction in RNA synthesis started part way through the period of reductive cell division, and the time when the minimal level was reached coincided with the end of this period and the full adaptation of the culture to stationary-phase survival.

The kinetics of recovery from carbon-starved stationary phase was followed by subculturing samples from 10-day-old cultures into fresh minimal medium. Viable counts were determined, and samples were taken for pulse-labeling to mon-

itor the rates of protein, RNA, and DNA synthesis during exit from stationary phase. The response of macromolecular synthesis to the readdition of nutrients was multiphasic. The rates of protein, RNA, and DNA synthesis increased 75-, 35-, and 12-fold, respectively, within 40 min of inoculation into fresh medium (Fig. 5B). Synthesis rates remained at these levels for between 6 and 8 h, and viability measurements indicated that this clearly corresponded to the lag phase of the growth cycle (Fig. 5B). After this period, the culture entered exponential growth, concomitant with further two- and threefold increases in protein and RNA synthesis, respectively, and a more modest 30% increase in the rate of DNA synthesis. In this experiment, the culture remained in exponential phase for about four doubling times before becoming carbon starved and reentering stationary phase, with the expected decrease in macromolecular synthesis (Fig. 5B). It is clear from this experiment that a sustained high level of macromolecular synthesis is required before a 10-day-old carbon-starved culture can reinitiate exponential growth. However, the response to the availability to nutrients is immediate and very rapid and results in exponential growth within 5 to 8 h of addition of nutrients.

Changes in pool mRNA stability during entry to and subsequent exit from carbon-starved stationary phase. There are a number of examples where differential control of mRNA stability is used by bacteria to economize on protein synthesis during environmental stress. mRNA turnover is retarded in *E. coli* as a means of maintaining gene expression during anaerobiosis (5) and the entire mRNA pool is stabilized in response to nutrient starvation in *Vibrio* sp. strain S14 (1). Given the very low levels of RNA and protein synthesis in carbon-starved *R. leguminosarum*, which are rapidly restarted upon readdition of nutrients (Fig. 5), we investigated whether there were parallel changes in the pool mRNA stability of *R. leguminosarum*. The pool mRNA stability was determined as the decay rate of the potential to synthesize proteins, determined by the rate of incorporation of [^{35}S]methionine, after complete inhibition of transcriptional initiation by rifampin. In Fig. 6A and B, the rates of [^{35}S]methionine incorporation are shown for mid-exponential- and stationary-phase cultures of *R. leguminosarum* after rifampin addition. The decay was biphasic for exponentially growing cells, with a rapid loss over the first 20 min (half-life = 17 min) followed by a slower second phase (Fig. 6A). It is not known if this slow phase represents a subset of more stable RNA species or if a global defense mechanism has been switched on, stabilizing all of the remaining RNA. Clearly carbon-starved, stationary-phase cells have a markedly lower decay rate of methionine incorporation following rifampin addition, and the kinetics are less obviously biphasic (Fig. 6B). The most likely explanation for this is increased pool mRNA stability in carbon-starved cultures. The half-time of the decay in the carbon-starved culture was approximately 80 min, representing a fivefold stabilization of pool mRNA over exponential cultures. This apparent mRNA stabilization is complete within 120 h of inoculation (data not shown) and coincides with the end of the reductive cell division phase and the drop in the rates of macromolecular synthesis to their minimum levels. However, within 40 min of readdition of nutrients (while growth is in the lag phase and coinciding with the initial increase in the rate of macromolecular synthesis), the decay rate of methionine incorporation had become biphasic and was faster than in mid-exponential cultures (Fig. 6C; half-life for initial phase = 12 min). The decay rate then slowed to the typical exponential rate (Fig. 6D) 10 h after readdition of fresh nutrients. There is a close correlation between the timing of the changes in the rates of protein and RNA synthesis (Fig. 5) and the apparent pool mRNA stability of *R. leguminosarum*

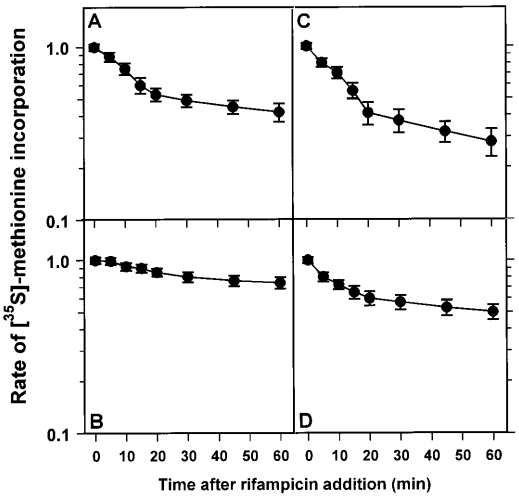

FIG. 6. Residual protein synthesis, after inhibition of mRNA synthesis with rifampin, during exponential growth and at different times following entry into and exit from carbon-starved stationary phase. At time zero, rifampin (0.1 μg ml^{-1}) was added to the culture, and at various times the protein synthesis was determined by monitoring the rate of incorporation of [^{35}S]methionine. Rates are given relative to the rate at time zero. (A) One-day-old culture in mid-exponential phase; (B) 10-day-old carbon-starved, stationary-phase culture (6 days into stationary phase); (C) same culture as in panel B but 40 min after the addition of fresh nutrients; (D) same culture as in panel B but 10 h after the addition of fresh nutrients.

cultures during entry to and exit from stationary phase (Fig. 6). The decrease in protein and RNA synthesis, which is complete at the end of the reductive cell division phase at ~120 h (Fig. 5A), coincides with the maximum stabilization of pool mRNA (Fig. 6B). Similarly, during exit from stationary phase, the immediate increase in macromolecular synthesis (Fig. 5B) is concomitant with a decrease in apparent pool mRNA stability (Fig. 6C).

Development of a general cross-protection to stress in response to nutrient starvation. A widespread feature of nondifferentiating bacteria in which starvation-survival has been studied is the development of generalized stress resistant state upon nutrient starvation. This is characterized by the starved culture having significantly increased resistance to a range of environmental stresses compared to growing cultures (6, 9–11, 16, 28, 34, 42). Therefore, it was clearly something that required investigation in *R. leguminosarum*. We compared the resistance of mid-exponential-phase cultures and carbon-starved stationary-phase cultures (10 days after inoculation) to osmotic, oxidative, and acid stress and to heat shock (Fig. 7). Cultures were osmotically stressed by the addition of NaCl to a final concentration of 2.5 M. It is clear from the results in Fig. 7A that exponential-phase cultures of *R. leguminosarum* are significantly more susceptible to osmotic stress than stationary-phase cultures. By 3 h after the addition of NaCl, 80% of the stationary-phase cells but only 3% of the exponential-phase cells were still viable. Similarly, stationary-phase cells were more resistant to oxidative stress. Following addition of 5 mM H_2O_2, 48% of the stationary-phase cells were viable 3 h after the stress, compared to just 5% of exponential cells (Fig. 7B). When cultures were subjected to a heat shock by being shifted from 28 to 55°C, stationary-phase cultures had increased resistance to heat shock (Fig. 7C). Finally, the resistance of the cultures to two forms of acid stress was tested, first by addition of sodium acetate to lower the internal pH (Fig. 7D) and second by addition of acid to lower the external pH of the

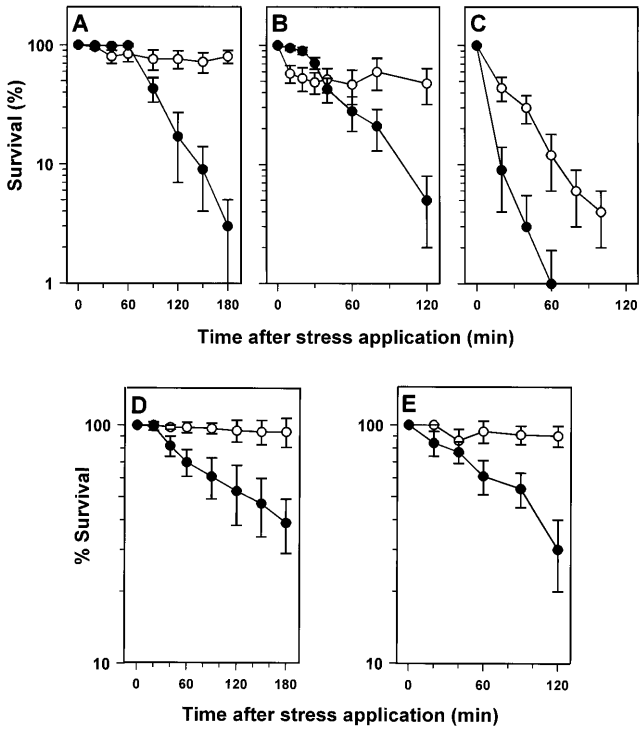

FIG. 7. Stress resistance of exponentially growing (●) and 10-day-old carbon-starved cultures (○) of *R. leguminosarum*. (A) Osmotic stress by addition of NaCl to 2.5 M; (B) oxidative stress by addition of H_2O_2 to 5 mM; (C) heat shock by shifting cultures from 30 to 55°C; (D) acid stress by addition of 25 mM sodium acetate; (E) acid stress by shifting the culture pH from 7.0 to 3.5.

medium (Fig. 7E). In the presence of 25 mM sodium acetate, stationary-phase cells clearly showed a higher survival rate than the exponential-phase culture (Fig. 7D). When the external pH was lowered from pH 7.0 to 3.5, stationary-phase cells survived at pH 3.5 for 2 h with no loss of viability, while over the same time, exponential cells lost 70% viability. Therefore, carbon starvation-induced stationary phase in *R. leguminosarum* was found to cross-protect cells for heat shock, oxidative, osmotic, and acid stress. This indicates that a general stress response, similar to that found in enteric bacteria, is present in stationary-phase *R. leguminosarum.*

DISCUSSION

In this study, we have investigated the starvation-survival response of *R. leguminosarum* bv. phaseoli. *R. leguminosarum* was able to cope with long-term carbon, nitrogen, and phosphorus starvation. Following starvation by exhaustion for these nutrients, *R. leguminosarum* survived for 55 days with little or no loss of viability. The soil bacterium *P. putida* can survive long-term carbon or nitrogen starvation, maintaining full viability for 30 days following starvation and the viability then dropping by 2 orders of magnitude after 6 months (6). However, survival of *P. putida* was severely reduced under phosphorus and sulfate starvation. It is difficult to compare starvation experiments with different bacteria in absolute terms because the ways in which they were starved varied with respect to the concentration of the starving (or other) nutrients and the growth rate prior to starvation. The growth rate of the culture will determine the overall cell composition (21, 44), and so the choice of carbon source for limitation could affect the adaptation to starvation-survival and the long-term survival outcome. In *E. coli*, there is little drop in viability over the first 7 to 10 days in minimal medium following carbon or phosphorus starvation, while nitrogen-starved cultures survive somewhat less well (35). However, upon entry into stationary phase in rich medium, the viability of *E. coli* cultures dropped to 0.1 to 1% after 2 weeks, and these cells exhibited the GASP (growth advantage in stationary phase) phenotype (46, 47). Consequently an important question which follows from these observations is whether the 100% viability, following a long period in stationary phase, precludes the need to accumulate GASP mutations in *R. leguminosarum*. This is something that will be worth investigating in future studies.

We have shown that macromolecular synthesis is carefully controlled following entry into and exit from stationary phase by *R. leguminosarum* and that such changes are concomitant with changes in the apparent pool mRNA stability. In stationary-phase *R. leguminosarum*, the mRNA decay was found to be biphasic, with an initial rapid loss of ability to synthesize mRNA over the first 20 min after inhibition of transcription by rifampin followed by a slower decline. This could be due to the cell sensing a decrease in mRNA levels and so inducing a global protection system or to a particularly stable subset of mRNA molecules. We used an indirect method to measure pool mRNA stability changes. We looked at the loss of the potential to incorporate [^{35}S]methionine after inhibition of transcription by rifampin. There are a number of assumptions behind this method. First, it is assumed that the *R. leguminosarum* RNA polymerase is inhibited by rifampin with similar kinetics during exponential and stationary phases. This was checked in preliminary experiments and found to be the case. Second, it is possible that the loss of methionine incorporation is entirely or in part due to changes in translational efficiency, which cannot be accounted for by the methodology used here. It is known that ribosomal activity is altered in stationary-phase *E. coli* (17), and irreversible changes in translational activation or efficiency cannot be ruled out as contributory factors to our indirect measurements of pool mRNA stability.

Starvation by nutrient exhaustion was used throughout this study for two reasons. First, we considered it to be more likely to resemble starvation in a natural environment. Second, experiments indicated that *R. leguminosarum* cultures resuspended in minimal medium without a carbon source survived very poorly in comparison to carbon-exhausted cultures. We made use of this observation to determine the kinetics of stationary-phase survival-adaptation of *R. leguminosarum* (Fig. 4) and found adaptation to be complete in 5-day-old cultures, that is, 30 h after the onset of reductive cell division and stabilization of the culture biomass. Presumably this period of adaptation is needed for *R. leguminosarum* to marshal its remaining resources prior to complete starvation. Starvation by resuspension may not allow metabolism to shut down in an ordered fashion, leading to unwanted, uncontrolled production of metabolic products that are not conducive to survival. These might include, for example, toxic oxygen species. This contrasts with the situation in other bacteria where starvation by resuspension has been studied over a similar time period. In the soil bacterium *P. putida*, no loss of viability was found 20 days after resuspension of an exponential culture in a medium without carbon (6). Similarly, *Vibrio* sp. strain S14 lost no viability over 10 days when in a similar resuspension experiment it was starved for multiple nutrients or carbon (28). However, in *S. typhimurium* cultures, the viability remained relatively constant for only the first 24 to 48 h following resuspension, and this period of stability was followed by a death phase where culture viability dropped to around 5 to 6% and then remained at this level for many weeks (40).

Using the approaches adopted here, we have been limited to studying starvation-survival at the population level. However, this does not take account of the probability that *R. leguminosarum* cultures are heterogeneous in stationary phase, as has been demonstrated for *E. coli* and *Micrococcus luteus* (13, 35). Our starvation-by-resuspension experiments (Fig. 4) provide evidence for culture heterogeneity. Four-day (96-h)-old cultures had lost 80% viability 11 days after resuspension, while the remaining 20% of population survived (Fig. 4). This could be explained by a heterogeneous culture in which 20% of the culture is able to adapt and survive following resuspension. Use of flow cytometric and cell sorting methods would be an attractive approach to investigate heterogeneity of stationary-phase *R. leguminosarum* in future studies (2).

CFU can be recovered from individual nodule plant cell protoplasts, and these arise from the reproductive growth either of bacteroids or of an undifferentiated subpopulation of bacteria within the nodule, depending on the symbiosis (8, 20, 48). Undifferentiated bacteria must also cease replication and adjust to conditions of the nodule and remain dormant. This might involve adaptation to nutrient starvation-induced stationary phase or a related process. One of our longer-term aims is to understand the relationship between stationary-phase free-living cells, nongrowing differentiated bacteroids, and the undifferentiated subpopulation in the root nodule. While the present study does not directly address these issues, the clear definition of the period of adaptation to stationary-phase survival and some of the physiological changes that occur has allowed us to define the adaptive stages upon which to focus future work. We are currently identifying proteins which are induced at different stages of stationary-phase adaptation and survival. This may help to establish whether stationary-phase-inducible proteins are also important to the survival of bacteroids or undifferentiated rhizobia in root nodules.

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