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Changes in Ester-Linked Phospholipid Fatty Acid Profiles of Subsurface Bacteria during Starvation and Desiccation in a Porous Medium

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Ester-linked phospholipid fatty acid (PLFA) profiles of a *Pseudomonas aureofaciens* strain and an *Arthrobacter protophormiae* strain, each isolated from a subsurface sediment, were quantified in a starvation experiment in a silica sand porous medium under moist and dry conditions. Washed cells were added to sand microcosms and maintained under saturated conditions or subjected to desiccation by slow drying over a period of 16 days to final water potentials of approximately −7.5 MPa for the *P. aureofaciens* and −15 MPa for the *A. protophormiae*. In a third treatment, cells were added to saturated microcosms along with organic nutrients and maintained under saturated conditions. The numbers of culturable cells of both bacterial strains declined to below detection level within 16 days in both the moist and dried nutrient-deprived conditions, while direct counts and total PLFAs remained relatively constant. Both strains of bacteria maintained culturability in the nutrient-amended microcosms. The dried *P. aureofaciens* cells showed changes in PLFA profiles that are typically associated with stressed gram-negative cells, i.e., increased ratios of saturated to unsaturated fatty acids, increased ratios of *trans*- to *cis*-monoenoic fatty acids, and increased ratios of cyclopropyl fatty acids to their monoenoic precursors. *P. aureofaciens* starved under moist conditions showed few changes in PLFA profiles during the 16-day incubation, whereas cells incubated in the presence of nutrients showed decreases in the ratios of both saturated fatty acids to unsaturated fatty acids and cyclopropyl fatty acids to their monoenoic precursors. The PLFA profiles of *A. protophormiae* changed very little in response to either nutrient deprivation or desiccation. Diglyceride fatty acids, which have been proposed to be indicators of dead or lysed cells, remained relatively constant throughout the experiment. Only the *A. protophormiae* desiccated for 16 days showed an increase in the ratio of diglyceride fatty acids to PLFAs. The results of this laboratory experiment can be useful for interpreting PLFA profiles of subsurface communities of microorganisms for the purpose of determining their physiological status.

It has been discovered during the last decade that deep subsurface terrestrial environments can be habitats for diverse microbial communities (2, 4, 6, 7, 10, 13–16, 20, 21, 23, 36). Identification of microbial isolates from these subsurface habitats has revealed that most heterotrophic populations fall into two phylogenetic groups: (i) *Pseudomonas* spp. and related gram-negative, nonfermentative bacilli and (ii) *Arthrobacter* spp. and other GC-rich gram-positive bacteria (2, 5). In situ geochemical conditions suggest that the microbes in these subsurface environments exist under conditions of severe nutrient deprivation, i.e., starvation. Evidence for the starved state of these microbes includes the extremely low concentrations of organic nutrients in uncontaminated subsurface environments and the low adenylate energy charges of subsurface microbial communities (23). Among the tools used for determining the physiological state of nutritionally challenged microorganisms is the analysis of phospholipid fatty acid (PLFA) profiles. Bacteria are known to alter their membrane fatty acid components in response to environmental stress, thereby generating characteristic PLFA stress signatures. Phenotypic changes in PLFA profiles in response to starvation stress have been observed in a number of bacteria, including *Escherichia coli*, *Vibrio vulnificus* (26), and *Vibrio cholerae* (18), a psychrophilic marine *Vibrio* strain (ANT-300) (29), methylotrophic bacteria (19), and a barophilic marine spirillum (32). Changes that are typically found in PLFA profiles when gram-negative bacteria are starved include an increase in the ratio of saturated to unsaturated fatty acids (18, 26, 32), an increase in the ratio of the *trans*- to *cis*-monoenoic unsaturated fatty acids (18), and increases in the moles percent of cyclopropyl fatty acids (18, 19). In contrast to the results reported for various gram-negative bacteria, Kostiw et al. (24) reported negligible PLFA changes in starved *Arthrobacter crystallopoietes*.

Stress signatures are useful in that they provide in situ profiles of the metabolic status of natural microbial communities (37, 41, 42). The study reported here was undertaken to determine what, if any, phenotypic changes in PLFAs occur in bacteria isolated from deep subsurface environments. The bacteria tested were a *Pseudomonas aureofaciens* strain and an *Arthrobacter protophormiae* strain, each isolated from vadose (unsaturated) zone sediments at the Hanford site in south-central Washington State. The effects of starvation on total numbers of bacteria, culturability, and fatty acid profiles were tested in a porous medium under moist conditions as well as under desiccated conditions. To date, PLFA changes in desiccated cells have been tested only with a cyanobacterium (31). We wanted to test the effects of lowered water availability on

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these organisms, since they were isolated from an unsaturated zone and were likely originally derived from surface soils. We also quantified diglyceride fatty acids (DGFAs). It has been proposed that DGFAs are indicators of nonviable cells (40). Upon cell death (or cell lysis) phospholipases are thought to cleave the polar head groups from membrane PLFAs, thereby forming diglycerides. Thus, the ratio of DGFAs to PLFAs may provide an estimate of the ratio of nonviable to viable cells.

MATERIALS AND METHODS

Bacterial strains and preparation of cells. These two bacterial strains were isolated by T. O. Stevens of Battelle's Pacific Northwest Laboratories from samples collected from the Yakima Barricade borehole at the Hanford site in south-central Washington State. The samples were collected from the vadose zone in the Hanford formation. The geology of this site and the sampling methods have been described previously (21). The growth medium used for isolation contained 1% PTYG agar (4) (0.1 g of glucose, 0.1 g of yeast extract, 0.05 g of peptone, 0.05 g of tryptone [all from Difco], 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.07 g of $CaCl_2 \cdot 2H_2O$, and 15 g of agar [Difco] liter of deionized H_2O^{-1}). The isolates were identified by fatty acid methyl ester analysis using the MIDI system (Microbial ID, Inc., Newark, Del.) by T. O. Stevens. The *P. aureofaciens* strain was isolated from a depth of 99 m and has been designated culture H326H25 in the Subsurface Microbiology Culture Collection (SMCC), maintained by David Balkwill at Florida State University, Tallahassee, Fla. The *A. protophormiae* strain (SMCC culture H212H12) was isolated from a depth of 65 m. For starvation experiments, the isolates were cultured in 1.0-liter batches in 10% PTYG broth, containing 10 times the concentration of organic components used in 1% PTYG agar and without added agar but with the same concentration of inorganic constituents. Cultures were incubated at 22°C with intermittent shaking until late log phase (18 h). The cells were then washed twice, each time by centrifuging (9,400 × *g* for 20 min) and resuspending the pellet in 10 mM Tris buffer, pH 7.0. The concentration of cells was measured by phase-contrast microscopy with a Petroff-Hausser counting chamber. The cell concentration was adjusted to 10^8 cells ml^{-1}. The washed cell suspensions were then mixed into moist quartz sand samples.

Starvation-desiccation treatments. The porous medium used for starvation and desiccation treatments was white quartz sand (−50 to +70 mesh; Sigma, St. Louis, Mo.). The sand in 120-g dry weight portions was placed into two types of glass containers: crystallizing dishes (50 cm high by 10 cm in diameter) and 8-oz. Mason jars. The crystallizing dishes were used for desiccation treatments. The Mason jars were used for treatments that remained moist. Two types of moist treatments were used: one in which washed cells were placed into sand with sterile water as described below and the other in which washed cells were placed into sand with a 1% PTYG broth solution. Sterilization of the sand in the glass containers at 450°C overnight ensured combustion of any residual organic matter as well. The 120-g sand samples were moistened to approximately field capacity with 41 ml of sterile NANOpure water (Barnstead, Dubuque, Iowa), and then 16.5 ml of the cell suspension was mixed into each of the samples in the petri dishes and the Mason jars. The Mason jars were sealed to keep the samples moist. The samples in the petri dishes were exposed daily for 16 days for periods of 30 to 65 min in a flow of sterile HEPA-filtered air to remove moisture at a slow steady rate. The samples were mixed with a spatula immediately after each period of air drying. Thus, there were five different treatments: (i) moist, inoculated; (ii) moist, inoculated, with nutrients; (iii) moist, sterile; (iv) dried, inoculated; and (v) dried, sterile. Triplicate samples were collected from each of the microcosms. All treatment samples were incubated at 22°C.

Water potential and water content. Water contents were measured by weighing the sand samples before and after heating for 24 h at 105°C. Water potentials were measured by thermocouple psychrometry (Decagon Devices, Pullman, Wash.). Water contents and water potentials of dried samples were measured daily immediately after air drying and mixing.

Direct counts and plate counts. Samples were collected for microbial analysis immediately after the sand was inoculated and after 8 and 16 days of incubation. Total numbers of cells in the sand were estimated by a modification of the acridine-orange direct counting method (16, 36) as described by Brockman et al. (10) and Kieft et al. (21). Cells were counted in 20 microscopic fields on each of five microscope slides (previously combusted at 450°C for 24 h), with a Zeiss Axioskop epifluorescence microscope equipped at ×630 total magnification, yielding a minimum detection limit of approximately 3.0×10^4 cells g (wet weight) of $sediment^{-1}$. Sterile controls consisting of porous media combusted at 550°C for 3 h were prepared in parallel with the samples. The analyses were rejected and repeated if control slides from that batch showed any cells.

The culturability of cells was monitored by a plate count procedure. Samples (1.0 g wet weight) were mixed by vortex for 1 min in 10 ml of 0.1 M $Na_4P_2O_7$ (pH 7.0). Subsequent 10-fold dilutions were made in a phosphate-buffered saline solution consisting of 1.18 g of Na_2HPO_4, 0.223 g of $NaH_2PO_4 \cdot H_2O$, and 8.5 g of NaCl liter of H_2O^{-1} (43). The standard plate count medium was 1% PTYG agar. Plates were incubated at 20°C for a minimum of 8 weeks.

Fatty acid analysis. Samples for PLFA analysis were quick-frozen at −80°C. Sand samples (8.5 to 10.3 g wet weight) were extracted in a single-phase organic solvent (8) modified to include a phosphate buffer (40). The sand samples were extracted at room temperature for 3 h in 30.8 ml of extractant, after which 10 ml each of chloroform and NANOpure water (chloroform extracted) were added. After allowing the phases to separate for 8 h, the lipid-containing phase was collected and dried by rotary evaporation. The lipid extract was then fractionated on a silicic acid column (17). The phospholipid-containing polar lipid fraction was subjected to a mild alkaline methanolysis, transesterifying the fatty acids into methyl esters (17).

Fatty acid methyl esters were further separated and quantified by gas chromatography-mass spectrometry. Samples were dissolved in isooctane containing 50 pmol of methylnonadecanoate μl^{-1} as an internal standard. Aliquots of 1 μl were injected into a Hewlett-Packard HP5890 series II gas chromatograph interfaced with a Hewlett-Packard HP971 series mass selective detector. The gas chromatograph was equipped with a 60-m nonpolar capillary column (Rt_x-1, 0.25 mm internal diameter; film thickness, 0.1 μm; Restek, Bellefonte, Pa.). The column was programmed from an initial temperature of 100 to 150°C at 10°C min^{-1}, held at this temperature for 1 min, and then raised at 3°C min^{-1} to a final temperature of 280°C at which it was held for 5 min. The injector and source housing temperatures were maintained at 270 and 290°C, respectively. Mass spectra were collected at an electron energy of 70 eV. A single-ion monitoring program which scanned for the base peaks of saturated, monounsaturated, and polyunsat-

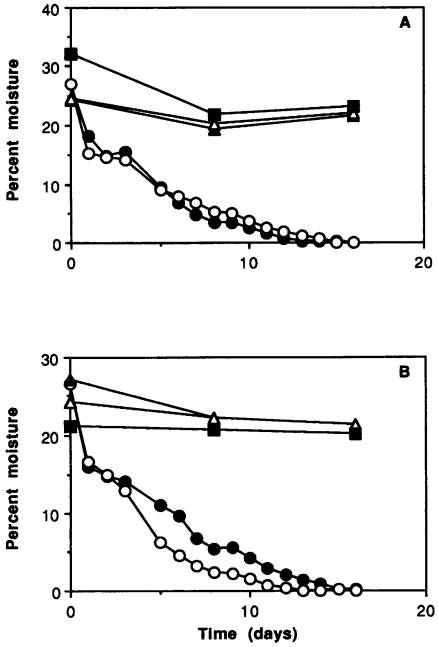

FIG. 1. Changes in percent moisture by weight during incubation in sand porous medium of *P. aureofaciens* (A) and *A. protophormiae* (B). ▲, inoculated, moist treatment; ●, inoculated, desiccated treatment; △, inoculated, moist treatment amended with 1% PTYG broth; ■, sterile, moist control; ○, sterile, dried control.

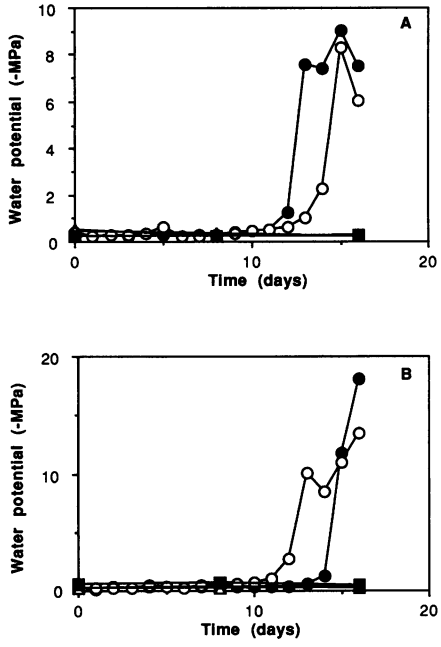

FIG. 2. Changes in water potential during incubation in sand porous medium of *P. aureofaciens* (A) and *A. protophormiae* (B). ▲, inoculated, moist treatment; ●, inoculated, desiccated treatment; △, inoculated, moist treatment amended with 1% PTYG broth; ■, sterile, moist control; ○, sterile, dried control.

urated fatty acid methyl esters throughout the run was used. Fatty acids were identified both by relative retention times compared with authentic standards (Matreya, Inc., Pleasant Gap, Pa.) and by the mass spectra. Fatty acids are designated as described by Ringelberg et al. (33).

DGFAs were recovered after the chloroform fraction from the silicic acid column was spotted on a thin-layer chromatography plate (60 A, 250 μm thick; Whatman, Clifton, N.J.). The plate was developed in hexane-diethyl ether (80:20, vol/vol). The fatty acid methyl esters were formed in the same manner as described for the phospholipids and were identified and quantified by using the same gas chromatography-mass spectrometry program and internal standard as described above.

RESULTS

Water content and water potential. The water contents in the desiccated treatment samples declined to nearly 0% moisture during the 16 days of incubation (Fig. 1). Water potentials in the desiccated treatment samples declined to approximately −7 MPa in the *P. aureofaciens* experiment and approximately −15 MPa in the *A. protophormiae* experiment (Fig. 2), with the most severe desiccation occurring after the day 8 sampling. The sealed samples showed only a slight moisture loss, which probably occurred during the brief sampling intervals. The water potential data demonstrate that although significant amounts of moisture were lost from the air-dried samples during the first week, the water potential did not change measurably during this interval. Water potential is a better measure than water content for estimating the degree of water stress experienced by microorganisms in a porous medium (30).

Direct counts and plate counts. Direct microscopic counts remained relatively constant for all three treatments of both *P. aureofaciens* and *A. protophormiae* (Fig. 3 and 4), dropping approximately 1 order of magnitude during the 16-day experiment. The culturability of both organisms was strongly affected by desiccation and the availability of nutrients. The numbers of culturable *P. aureofaciens* CFU dropped to below the lower limit of detection (approximately 10^2 CFU per g [dry weight] of sand) by day 16 of incubation in the nutrient-deprived treatment samples under both moist and dried conditions; the number of culturable *P. aureofaciens* CFU increased slightly from the initial value in the moist treatment that included nutrients (Fig. 3). The numbers of culturable *A. protophormiae* CFU declined to below the detection limit by day 8 of incubation in the desiccated, nutrient-deprived treatment and by day 16 in the moist, nutrient-deprived treatment; the number of culturable *A. protophormiae* CFU increased slightly in the moist, nutrient-amended treatment (Fig. 4). The numbers of culturable cells in the uninoculated treatment samples remained below the detection limit throughout the experiment.

Total PLFAs. When the total PLFA data were used to estimate total numbers of cells (with a conversion factor of 5.9 × 10^4 cells pmol of $PLFA^{-1}$), the numbers were in approximate agreement with the direct microscopic counts (Fig. 3 and 4). The conversion factor was derived from an average value of 10^4 mol of PLFA g (dry weight) of $cells^{-1}$ (39) and 5.9 × 10^{12} cells g^{-1} (dry weight) (38). The PLFA-calculated numbers of cells remained relatively constant at approximately 10^8 cells g (dry weight) of $sand^{-1}$ throughout the experiment. For both organisms, the nutrient-amended treatment samples had the highest concentration of PLFAs and the desiccated, nutrient-deprived treatment samples had the lowest concentration of PLFAs. PLFAs were detected in very low concentrations (mean = 40.7 pmol g [dry weight] of $sand^{-1}$) in the uninocu-

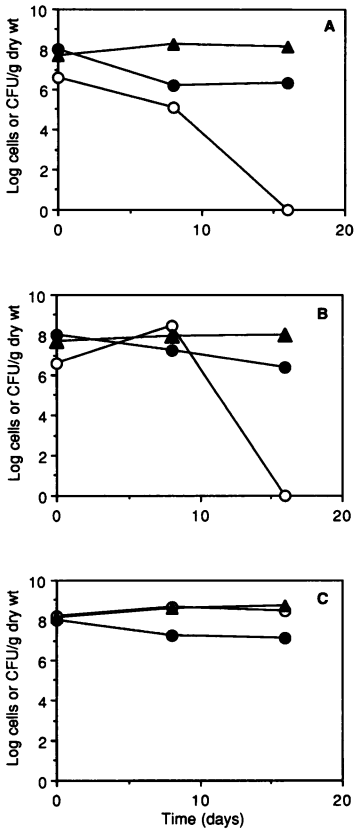

FIG. 3. Direct microscopic counts (●), plate counts (○), and PLFA data converted to cells per gram (dry weight) (▲) for *P. aureofaciens* starved under moist conditions (A), starved and desiccated (B), and incubated under moist conditions and amended with 1% PTYG broth (C).

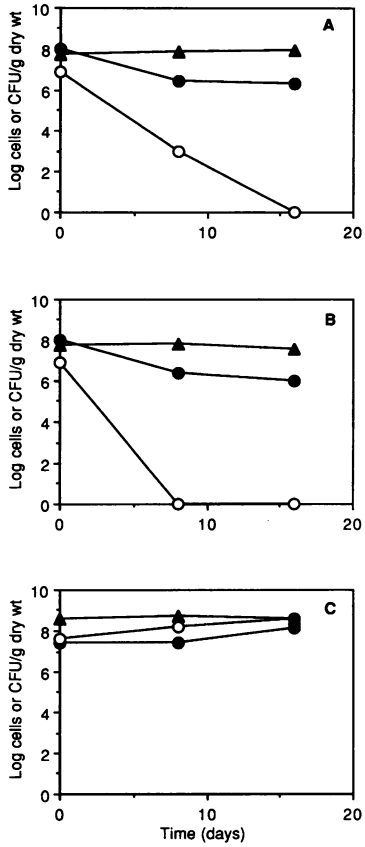

FIG. 4. Direct microscopic counts (●), plate counts (○), and total PLFA data converted to cells per gram (dry weight) (▲) for *A. protophormiae* starved under moist conditions (A), starved and desiccated (B), and incubated under moist conditions and amended with 1% PTYG broth (C).

lated control samples. The predominant fatty acids in the uninoculated controls were palmitic acid (16:0) and stearic acid (18:0), which were probably contaminants in the NANOpure water and/or the solvents used in the extraction process. Concentrations of PLFAs in the blanks averaged less than 1% of the total fatty acids in the inoculated samples and thus had negligible influence on PLFA profiles.

***P. aureofaciens* PLFA profiles.** The PLFA profiles of *P. aureofaciens* were strongly affected by moisture and nutrient availabilities. Desiccation resulted in an increase in the ratio of saturated to unsaturated fatty acids (Fig. 5A), increases in the ratios of *trans*- to *cis*-monoenoic fatty acids (Fig. 5B and C), and increases in the ratios of cyclopropyl saturated fatty acids to their monoenoic precursors (i.e., increases in the cy17:0/16:1ω7c and cy19:0/18:1ω7c ratios) (Fig. 5D and E). The *P. aureofaciens* in the nutrient-amended treatment showed a decreased ratio of saturated to unsaturated fatty acids but an increase in the ratio of cyclopropyl saturated fatty acids to their monoenoic precursors. The moist, nutrient-deprived *P. aureofaciens* showed the least change in PLFA profiles during the 16-day experiment. The sizes of the fatty acid chains, as indicated by such indices as the 16:1ω7c/18:1ω7c ratio, remained relatively unchanged for all treatments throughout the experiment (data not shown).

***A. protophormiae* PLFA profiles.** The *A. protophormiae* showed relatively few changes in PLFA profiles in response to either nutrient deprivation or desiccation. In all treatments and at all sample times, the PLFA profiles of the *A. protophormiae* were dominated by anteisobranched 15:0 saturated fatty acid, with the proportion of this fatty acid ranging from 68 to 82%. Subtle changes did occur in other species of fatty acids, which consisted primarily of i15:0, i16:0, 16:0, a17:0, and 18:0 in this organism. The PLFA changes that occurred during the 16-day experiment were greatest in the moist, nutrient-amended treatment; these included an increase in the a17:0/a15:0 ratio of branched saturated fatty acids (Fig. 6A), an increase in the i15:0/a15:0 ratio of fatty acids (Fig. 6B), and an increase in the i17:0/a17:0 ratio of fatty acids (Fig. 6C).

Diglycerides. The concentration of DGFAs in both the *P. aureofaciens* and the *A. protophormiae* isolates remained relatively constant throughout the experiment (Fig. 7A and 8A). DGFA concentrations were at least 1 order of magnitude lower than the PLFA concentrations for both isolates (Fig. 7B and 8B). The ratio of DGFA to PLFA in the *P. aureofaciens* remained constant at 0.12 or below (Fig. 7C). An increase in the ratio to 0.2 occurred in the *A. protophormiae* isolate after 16 days of desiccation and nutrient deprivation, while the cells in the other two treatments maintained the same low ratios (≤0.10) throughout the experiment (Fig. 8C).

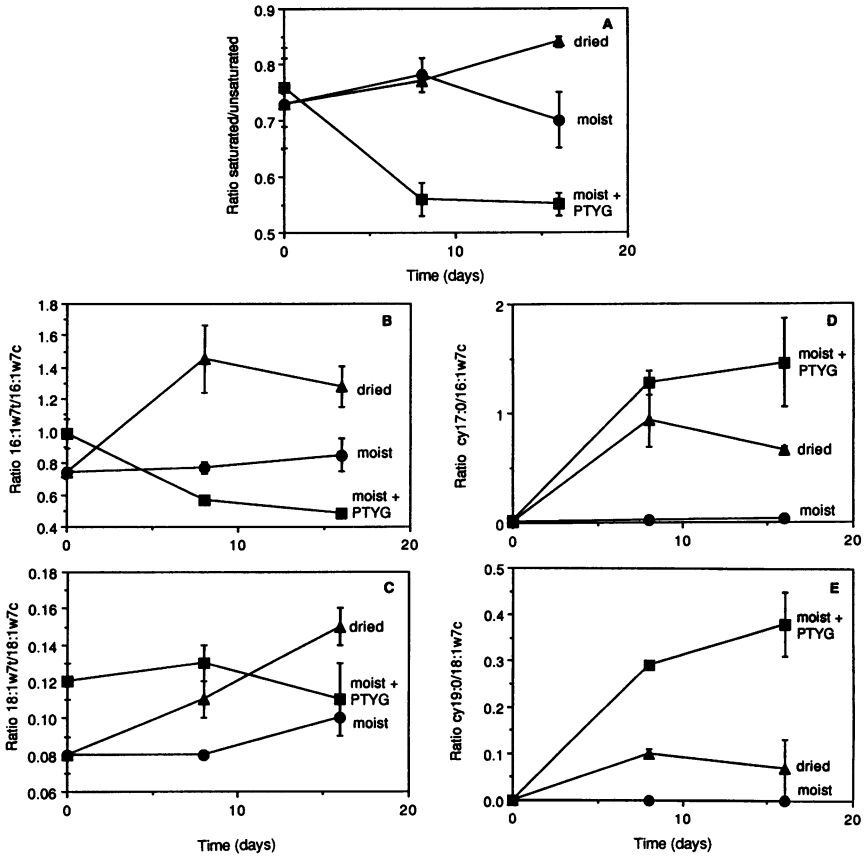

FIG. 5. Changes in PLFA profiles observed for *P. aureofaciens*: the ratio of saturated to unsaturated fatty acids (A), the ratio of 16:1ω7t to 16:1ω7c fatty acids (B), the ratio of 18:1ω7t to 18:1ω7c fatty acids (C), the ratio of cy17:0 to 16:1ω7c (D), and the ratio of cy19:0 to 18:1ω7c (E). Error bars represent 1 standard deviation.

DISCUSSION

The two subsurface isolates used in this study appeared to demonstrate surprisingly poor survival characteristics under the starvation and desiccation conditions of this experiment. Bacteria of the genus *Arthrobacter* are generally very well adapted for starvation survival under both moist and dry conditions (9, 11). Many strains of *Pseudomonas* have also been shown to maintain viability in at least portions of populations subjected to nutrient deprivation (25, 27); some strains of *Pseudomonas* are even able to remain viable during soil desiccation, though they are generally much less drought tolerant than *Arthrobacter* strains (12, 34). Amy et al. (1) have shown that several subsurface isolates exhibit starvation survival patterns similar to those reported for marine heterotrophs (3); i.e., the numbers of viable cells initially rise because of fragmentation (division without growth), subsequently diminish, and finally approach a stable level at which cells appear to remain viable indefinitely. There are several possible explanations for the apparently poor survival observed in this experiment. It is possible that these bacteria are poorly adapted for starvation survival. This is unlikely, because the tracer data from the subsurface sampling operation indicate that these isolates are truly subsurface microorganisms and it is axiomatic that subsurface isolates must be able to survive long-term nutrient deprivation, at least under in situ conditions. Alternative explanations for the apparent lack of survival are more likely. One of these is that the particular conditions of this starvation experiment were significantly different from those encountered in situ by the bacteria: the mineralogy of the porous medium in this experiment was different from the sediments of the Hanford formation, and furthermore, starvation was imposed very suddenly in the experiment by washing the cells in nonnutrient buffer. While this method of imposing starvation on the microbes is similar to methods used by other investigators (1, 3, 28, 29), it may have been too harsh for these subsurface bacteria. A second plausible explanation is that the microbes entered a viable but nonculturable state under starvation conditions. This is a common phenomenon among bacteria in many natural environments (35), and there is evidence that at least a portion of the cells in deep subsurface environments are viable in situ but do not respond to the usual methods of cell cultivation (21). The relatively constant ratio of DGFAs to PLFAs suggests that the majority of cells of both types remained viable (or at least nonlysed) throughout the experiment under all incubation conditions. Only the *A. protophormiae* desiccated for 16 days showed a marked increase in DGFAs; the increase in the DGFA/PLFA ratio to 0.21 coincided with nonculturability and with the most stressful conditions (−15 MPa matric water potential) for this isolate.

The changes in PLFA profiles observed for the *P. aureofaciens* when it was desiccated are consistent with those reported for other gram-negative bacteria under physiologically stressful conditions. In fact, this isolate evinced all of the previously

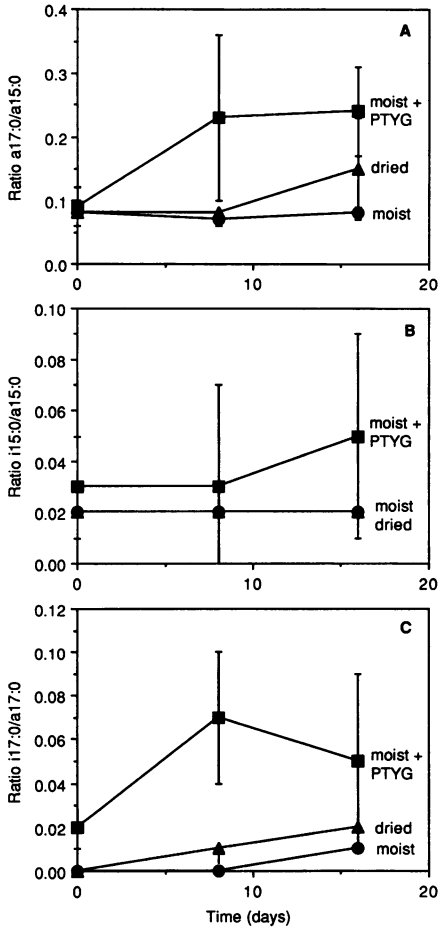

FIG. 6. Changes in PLFA profiles of *A. protophormiae*: the ratio of a17:0 to a15:0 branched saturated fatty acids (A), the ratio of i15:0 to a15:0 fatty acids (B), and the ratio of i17:0 to a17:0 fatty acids (C). Error bars represent 1 standard deviation.

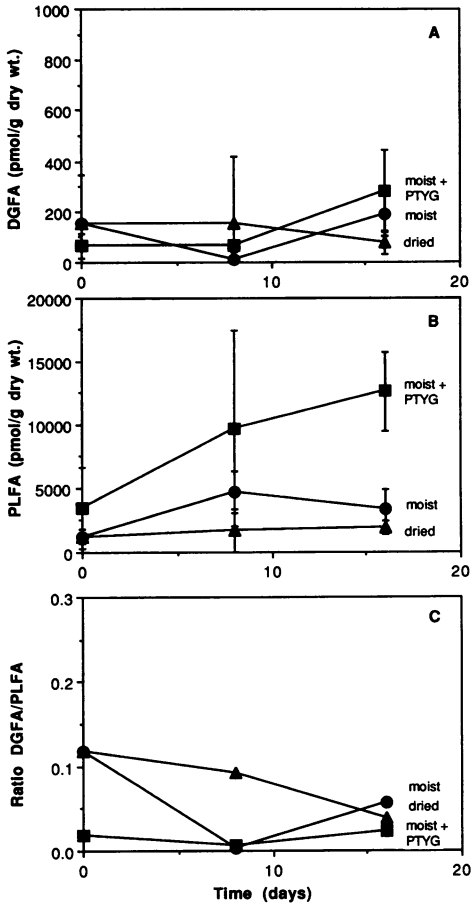

FIG. 7. Changes in DGFAs (A), PLFAs (B), and DGFA/PLFA ratios (C) in *P. aureofaciens*. Error bars represent 1 standard deviation.

reported stress indicators for gram-negative bacteria: an increase in the ratio of saturated to unsaturated fatty acids, an increase in the ratio of *trans*- to *cis*-monoenoic fatty acids, and an increase in the ratio of cyclopropyl fatty acids to their monoenoic precursors. This information is useful because it can be compared with the fatty acid profiles observed for extracts of natural subsurface microbial communities. High values for these same three stress indicators have been observed in natural subsurface microbial communities (42). The observation of these PLFA changes in pure cultures of bacteria under the controlled conditions of laboratory experiments provides support for the interpretation of PLFA profiles in natural microbial communities.

The conversion of monoenoic fatty acids to cyclopropyl fatty acid products was observed under two very different sets of conditions in this experiment: desiccation without added nutrients and moist conditions with nutrient amendment. The moist, nutrient-amended treatment was intended to generate well-nourished, actively dividing cells, at least in the early portion of the experiment. The cell enumeration data indicated that the population grew during the initial period of the experiment. This initial period of population growth could easily have been followed by a period of nutrient deprivation; however, the plate count data indicate that a large proportion of the cells subjected to nutrient-amended treatment remained viable throughout the experiment. Another effect of the nutrient amendment was likely an increase in the biochemical oxygen demand and a concomitant decrease in the oxygen concentration. Oxygen depletion could, in turn, have triggered an increase in cyclopropyl fatty acids in the *P. aureofaciens*. Anaerobic incubation of a procaryotic estuarine sediment community has been shown to increase the proportion of cyclopropyl fatty acids to aerobically incubated sediments (17). Guckert et al. (18) also demonstrated that an increase in cyclopropyl PLFAs occurred in a *V. cholerae* culture starved for 7 days. Thus, the increase in cyclopropyl PLFAs can be associated with cell age, nutrient deprivation, and/or anaerobiosis.

The lack of major changes in fatty acid make-up observed in the *A. protophormiae* in response to nutrient deprivation confirms and expands on results previously observed by Kostiw et al. (24) for *A. crystallopoietes* and also observed by us in another subsurface *Arthrobacter* sp. (SMCC culture ZAL001) (22). *Arthrobacter* spp. are known for their changes in cell morphology in response to nutrient deprivation (11), and we have observed typical conversion from rod shape to coccus shape and reduction in cell size in this particular strain under starvation conditions (data not shown). Evidently, changes in cell shape are not accompanied by changes in membrane PLFAs.

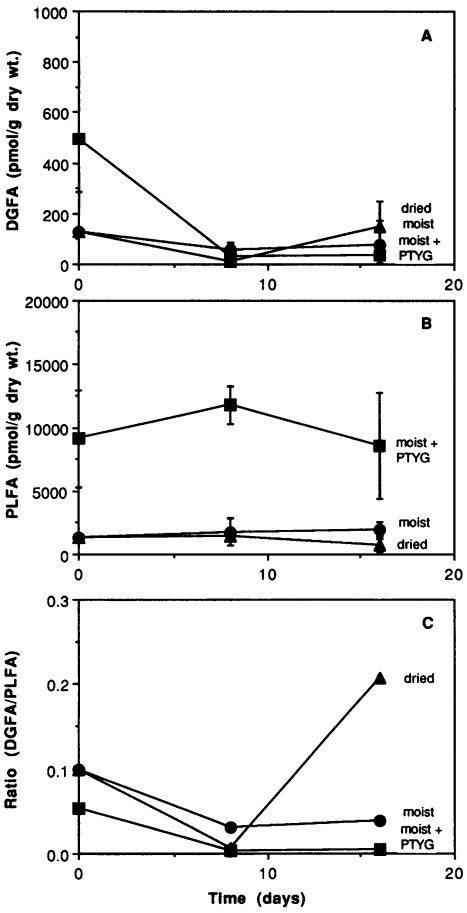

FIG. 8. Changes in DGFAs (A), PLFAs (B), and DGFA/PLFA ratios (C) in *A. protophormiae*. Error bars represent 1 standard deviation.

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