Deep-Sea Bacteria: Growth and Utilization of Hydrocarbons at Ambient and In Situ Pressure

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Microorganisms present in Atlantic Ocean sediment samples collected at a depth of 4,940 m were found to be capable of utilizing hydrocarbons under both ambient and in situ pressures. The rate of utilization under in situ pressure (500 atm) and ambient temperature (20 C) was found to be significantly less compared with hydrocarbon utilization examined under conditions of ambient temperature (20 C) and pressure (1 atm).

Microorganisms that have been reported to occur under pressures and temperatures found in the deep sea, but at relatively low rates. Compounds utilized in studies reported to date have included: acetate (1, 2), agar (2), amino acids (2), carbon dioxide (7), gelatin (2), hydrocarbons (6), nitrate (10, 12), starch (2, 11, 12), sugars (2), and sulfur-containing substances (9). The question remains whether those bacteria found in the deepest parts of the world oceans are slow growing because of limited nutrient supply or high pressure and low temperature effects on metabolic activities.

Currently, there is a rising concern as to the ability of the marine environment to accommodate the ever-increasing amounts of petroleum products entering the oceans. Petroleum may enter the deep ocean in weathered form by adhering to material heavier than water or through natural seepage. This study was undertaken to investigate microbial degradation of hydrocarbons under simulated deep-sea pressures.

MATERIALS AND METHODS

Organisms. The bacterial strains used were from core samples collected aboard the research vessel EASTWARD at a location 150 miles east of Cape Kennedy, Fla. and at 4,940-m depth. One milliliter of the core slurry from the sediment-water interface was aseptically introduced into 24 ml of a salts medium (1.0 g of NH₄NO₃; 0.3 g of KH₂PO₄; 0.7 g of KH₂PO₄; 3.0 g of NaCl; 1.5 g of MgSO₄; 1,000 ml of distilled water) containing 0.37% (vol/vol) n-hexadecane which provided a two-phase system. The sample was incubated at 20 C and growth was monitored by plating 0.1-ml portions onto a seawater-agar medium and enumerating TVC (total viable count) (6).

The n-hexadecane enrichment procedure included three serial transfers, accomplished by aseptic removal of 1 ml of stationary phase culture and transfer of this portion to fresh salts medium containing 0.37% (vol/vol) n-hexadecane. Percent n-hexadecane utilized at stationary phase was determined by GLC (gas-liquid chromatography) for each of the three enrichments.

GLC analyses. Atmospheric and 500-atm samples were removed in duplicate and extracted with one-half volume of n-hexane after the addition of 0.1 ml of dodecanol (internal standard). Ten grams of Na₂SO₄ were added to each flask, after which the n-hexane layer was removed and retained. This step was repeated and the second n-hexane layer thus obtained was added to the first. The extracts were saturated with nitrogen gas, sealed, and allowed to stand overnight. The n-hexane extracts were concentrated at 35 C to 8 ml in a rotary flash evaporator, after which they were again concentrated to 1.5 ml by blowing nitrogen gas over the concentrate. Procedures of GLC analyses have been described in detail elsewhere (6).

Pressure experiments. The mixed culture obtained after the third enrichment was diluted with salts medium, without n-hexadecane, to about 3 × 10⁸ cells per ml. Small glass vessels, capped at each end with rubber serum vial stoppers (4), were filled with 2.15 ml of diluted culture. Eight microliters of n-hexadecane, containing 3 × 10⁶ dpm of [1-¹⁴C]n-hexadecane, were injected aseptically as finely dispersed droplets into the glass vessels. Eight vessels were placed into each of five pressure reactors (High Pressure Equip., Erie, Pa.) and 500-atm hydrostatic pressure was applied. The pressure reactors and atmospheric control samples, each of which was run in duplicate, were incubated at 20 C. Two additional sets of controls, one inoculated but “poisoned” with 100 ppm of HgCl₂ at the initiation of the experiment and which was uninoculated, were run to measure abiotic alteration of the n-hexadecane and loss of n-hexadecane from the medium through evaporation and absorption by the rubber vial stoppers.

Uptake and utilization. At 1-week sampling intervals, pressure reactors containing inoculated culture vessels, poisoned culture vessels, and uninoculated control vessels were taken from the incubator, the
pressure was slowly released, and the contents of the glass vessels were emptied into separate stoppered flasks. Cellular metabolism was blocked instantly by addition of 1 N H₂SO₄ which, in effect, reduced the pH to about 2. Radioactive CO₂ from biological conversion and hexadecane vapors from evaporation were trapped on phenethylamine-saturated fluted filter paper suspended in plastic wells above the liquid. Cells were removed by membrane filtration (Millipore Corp.) and washed with 15 ml of methanol to remove hydrocarbon loosely bound to the cell surface. Material on the phenethylamine-saturated filter paper, cells, and spent medium from inoculated cultures, poisoned cultures, and uninoculated controls were counted in an Intertechnique SL-40 liquid scintillation spectrometer, using channels ratio for the filters and external standard ratio for the spent media for quench correction. Growth was monitored by plating duplicate nonpoisoned pressure and control samples at given time intervals on modified sea water yeast extract agar (3). The radioactivity present in the different fractions of the poisoned cultures was used to determine the extent of abiotic hexadecane alteration; the uninoculated controls were used to quan-
ticate the loss of hexadecane from the medium by evaporation, by adsorption to the glass, and absorption by the rubber stoppers. These values were used to correct the atmospheric pressure and pressure data given in Tables 2 and 3.

RESULTS

Enrichment of mixed cultures on n-hexadecane. Growth of the sediment-water slurry inoculum on n-hexadecane is shown in Table 1. The time required for the mixed culture to reach stationary phase decreased from 7 to 5 days, from the first to the third serial transfer. The lag phase associated with each serial transfer, i.e., enrichment, decreased from 3 days to 1 day for the first and third transfer, respectively. At the same time, the TVC measured at stationary phase for each enrichment increased about fivefold. The increase in TVC was accompanied by an increase in n-hexadecane utilization (38.6 to 99.7%). Additional enrichments on n-hexadecane did not increase either the TVC at stationary phase or the extent of n-hexadecane utilization determined by GLC analysis.

Growth and utilization of n-hexadecane at 1 and 500 atm. Growth characteristics of the mixed culture at 1 and 500 atm are shown in Fig. 1. The curve representing the culture grown under atmospheric pressure exhibited an increase in TVC, reaching a maximum at 14 days. The curve representing growth on n-hexadecane at 500 atm showed an initial decrease in TVC, followed by a sharp increase paralleling results at 1 atm. The TVC reached a maximum at 21 days and decreased thereafter. GLC analyses of n-hexadecane utilization are summarized in Table 2. The culture held at 1 atm showed about 50% degradation of the available n-hexadecane within the first 7 days and about 92% at 14 days. Hydrocarbon utilization at 1 atm paralleled results for growth shown in Fig. 1. GLC analysis of samples of the culture incubated at 500 atm revealed very slight hydrocarbon utilization at 7 days, quickly followed by

| Enrichment on n-hexadecane | Lag phase (days) | Time required to reach stationary phase (days) | Viable count at stationary phase (cells/ml) | n-hexadecane utilized at stationary phase |
|----------------------------|-----------------|-----------------------------------------------|-------------------------------------------|------------------------------------------|
| Original ...              | 3               | 7                                             | 2.06 x 10^6                              | 38.6                                     |
| Second ...                | 2               | 6                                             | 4.22 x 10^6                              | 68.7                                     |
| Third ...                 | 1               | 5                                             | 1.03 x 10^6                              | 99.7                                     |

* The salts medium used contained 0.37% n-hexadecane (vol/vol).

FIG. 1. Growth of a mixed culture of strains of deep-sea sediment bacteria on n-hexadecane at 20 C and 1 and 500 atm, expressed as total viable counts after enumeration on modified sea water yeast extract agar plates.
increased utilization which peaked at 21 days, also paralleling results for growth shown in Fig. 1. The GLC tracings did not indicate any accumulation of smaller chain breakdown products of the n-hexadecane.

Utilization and fate of [1-14C]n-hexadecane at 1 atm and 500 atm. Data are given in Table 3 which show utilization of radioactive n-hexadecane occurring under ambient and elevated pressures. Total recovery of labeled material averaged 93.8%. Although both 1 and 500 atm, day 7 samples yielded approximately the same percent uptake (CO₂ and incorporation), the amounts of n-hexadecane respired as CO₂ differed markedly. From day 14, n-hexadecane was utilized with almost total respiration in the form of CO₂, the values of which corresponded with TVC (Fig. 1) and GLC data (Table 2).

Mixed hydrocarbon substrate degradation. The mixed culture used in this study was also found to degrade a 19 component mixed hydrocarbon substrate previously described by Walker and Colwell (8). All of the components were degraded after incubation for 4 weeks at 20 C. Hydrocarbon substrate remaining after degradation, as determined by GLC analysis and given as percent of the initial concentration, was as follows: cyclohexane, 23.04%; n-decane, 14.26; cumene, 18.71; n-undecane, 12.78; n-dodecane, 12.04; n-tridecane, 13.35; naphthalene, 17.61; n-tetradecane, 25.08; n-pentadecane, 28.48; n-hexadecane, 31.26; pristane, 37.42; n-heptadecane, 33.84; n-octadecane, 34.00; n-nonadecane, 33.69; n-eicosane, 31.71; phenanthrene, 26.43; 1,2-benzanthracene, 25.21; perylene, 24.34; and pyrene, 27.01.

DISCUSSION

The enrichment procedure followed in this study yielded seven isolates at the stationary phase after three serial transfers. These have been presumptively identified as Aeromonas, Pseudomonas, and Vibrio spp. (6). After incubation of the mixed culture comprising these seven strains at 500 atm, only five of the seven strains survived. Interestingly, the five strains could not be individually cultured on n-hexadecane salts medium. Hence, a metabolic synergism appeared to be in effect for these strains. The growth curve for the mixed culture held at 500 atm (Fig. 1) showed a decrease in TVC at 7 days, which may represent a die-off of pressure-sensitive variants. This marked decrease in TVC probably obscures the reproduction rate of other species. However, the drop in TVC was reproducible. The sharp decrease in TVC for cultures held at 500 atm was observed to occur between 21 and 35 days, representing loss of the two strains from the mixed culture. The absence of the two strains was unequivocal at day 28.

GLC analysis of n-hexadecane utilization by cultures held at 1 and 500 atm (Table 2)
revealed that degradation of the hydrocarbon by the mixed cultures paralleled TVC data as shown in Fig. 1. Breakdown products were not evident from the GLC profiles. The conclusion drawn from these results is that the hydrocarbon was assimilated to cell material and converted to CO₂ and/or products not detectable in the GLC system employed. The fate of the n-hexadecane which was utilized was not directly discernible from growth and GLC analyses. Therefore, radioactive hydrocarbon ([¹⁴C]n-hexadecane) was employed in subsequent experiments. From uptake data given in Table 3, i.e., CO₂ production and hydrocarbon incorporation, at day 7, both 1- and 500-atm samples appeared to have removed the same amount of n-hexadecane from the medium (ca. 50%). However, most of the radioactivity was present in the incorporated fraction of 500-atm samples at day 7 and not in the ^1CO₂ respired fraction. In the case of the sample incubated at 1 atm, the major portion of the radioactivity was accounted for in the respired fraction. However, the GLC data for the 500-atm samples did not reveal degradation products, from which is inferred that there was total conversion of the available hydrocarbon to CO₂.

The apparent discrepancy between the GLC data given in Table 2 and results of the radioactive label experiments, shown in Table 3, for day 7 most likely represents buildup of an intracellular pool of radioactive n-hexadecane, which is very slowly metabolized in the initial stages of growth. Such findings have been noted for amino acid incorporation under pressure (5). Data given in Table 3 for analyses performed at indicated times after the seventh day of incubation nicely parallel results shown in Table 2, i.e., rapid utilization of n-hexadecane at 500 atm, between day 7 and day 21, with a plateau in activity at day 21 and thereafter. Essentially all of the available hydrocarbon was converted to CO₂.

It should be emphasized, however, that the amount of n-hexadecane introduced into the medium (0.37% vol/vol) is far in excess (ca. 1,000×) of that which is theoretically capable of being completely oxidized to CO₂ and water, using the oxygen made available to the culture in the system employed in this study. Much of the injected n-hexadecane is inaccessible to the bacteria owing to abiotic alteration or to the affinity of the hydrocarbon for the rubber of the serum vial stoppers. It was necessary, therefore, to introduce an overabundance of n-hexadecane into the system. Subsequently, in determining the extent of hydrocarbon utilization, as given in Tables 2 and 3, the amount originally injected was corrected for the significant losses occurring through absorption by the rubber stoppers or through loss by evaporation after acid was added to collect the CO₂.

Degradation of all components of the mixed hydrocarbon substrate was observed when tested at 20 C. Utilization of individual hydrocarbons and a mixed hydrocarbon substrate, employing in situ conditions of pressure (500 atm) and temperature (4 C), are currently under investigation.

The bacterial isolates obtained in this study and examined both in mixed and pure culture most likely represent those bacteria capable of surviving decompression and rise in temperature associated with sample retrieval, hence may be only a small fraction of the deep-sea sediment population. However, it is clear from results of this study that deep-sea bacteria are capable of utilizing hydrocarbons under conditions of elevated pressure. Future work in our laboratory will include examination of deep-sea sediment samples without decompression or temperature change since work is underway, in collaboration with the National Bureau of Standards, Equation of State Section, on construction of a deep ocean environmental sampler. The compelling questions of deep-sea microbial activities will thus be answerable.

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