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Hydrocarbon Mineralization in Sediments and Plasmid Incidence in Sediment Bacteria from the Campeche Bank

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Rates of degradation of radiolabeled hydrocarbons and incidence of bacterial plasmid DNA were investigated in sediment samples collected from the Campeche Bank, Gulf of Mexico, site of an offshore oil field containing several petroleum platforms. Overall rates of mineralization of [^{14}C]hexadecane and [^{14}C]phenanthrene measured for sediments were negligible; <1% of the substrate was converted to CO_2 in all cases. Low mineralization rates are ascribed to nutrient limitations and to lack of adaptation by microbial communities to hydrocarbon contaminants. Plasmid frequency data for sediment bacteria similarly showed no correlation with proximity to the oil field, but, instead, showed correlation with water column depth at each sampling site. Significant differences between sites were observed for proportion of isolates carrying single or multiple plasmids and mean number of plasmids per isolate, each of which increased as a function of depth.

The Campeche Bank region of the southern Gulf of Mexico is an area of extensive offshore oil drilling and, as such, is subject to continuous low-level contamination with petroleum hydrocarbons (4, 25). Accidental release of large quantities of oil during drilling operations may also occur, as was the case in June 1979, when the IXTOC I well blew out, spilling more than 500,000 tons of oil over a period of 10 months. (1). Chronic oil pollution from petroleum platforms, residual effects of the IXTOC I blowout, and input of hydrocarbons into the environment from other sources, e.g., tanker operations (11, 12), coastal refineries (12), and river-borne terrigenous biogenic hydrocarbons (4), are all factors which would be expected to contribute to hydrocarbon contamination of the region and, consequently, to have a significant impact on autochthonous microbial communities.

Heterotrophic bacteria have been observed to exhibit a higher incidence of plasmid DNA in hydrocarbon-contaminated environments, such as offshore oil fields (15), riverine sediment polluted by coking plant discharges (5, 10), groundwater contaminated by aromatic hydrocarbons (29), and a dystrophic lake containing naturally high concentrations of aromatic humic compounds (33). Additional effects of hydrocarbon contamination that have been reported include increases in the fraction of hydrocarbon-utilizing microorganisms and in the capacity of the microbial community to degrade hydrocarbons (2, 7, 13, 31).

The objective of the present study was to investigate sediments of the Campeche Bank for rates of hydrocarbon degradation as well as frequency and distribution of plasmid DNA in heterotrophic bacteria, both being potential indicators of effects of chronic oil pollution. Sampling sites within an area of several oil-drilling platforms are presumed to be subject to a greater degree of hydrocarbon contamination than sites distant from the platforms. Sediment, rather than the water column, was sampled in this study because sediment, in general, has been shown to contain larger numbers of hydrocarbon-utilizing bacteria (8, 24, 25). In addition, sediments accumulate particulate and insoluble hydrocarbons via deposition (4, 30). Thus, benthic microbial populations may be more sensitive indicators of long-term contamination by petroleum than the pelagic community. In this study, hydrocarbon degradation was measured as mineralization of ^{14}C-labeled hexadecane and phenanthrene, considered to be representative of aliphatic and aromatic fractions, respectively, of crude oil.

MATERIALS AND METHODS

Sampling sites. Isolation of bacteria for plasmid incidence and collection of sediment samples for hydrocarbon degradation studies were carried out aboard the B/O *Justo Sierra* during a cruise conducted, in collaboration with scientists at the Universidad Nacional Autonoma de Mexico, in the Campeche Bank region of the Gulf of Mexico, in March 1986. Analyses were subsequently completed at the University of Maryland, College Park. Dissolved and dispersed hydrocarbons in the water column were determined concurrently by other investigators on the cruise (23). Four of five sampling stations (Fig. 1) were established in approximately a north-northwest transect from the western outlet of the Laguna de Terminos and included the following: one nearshore station, 17, ca. 25 nautical miles distant from the oil platform area; two stations, C and D, within 1 nautical mile of major platforms; and one deeper-water station, 23, 15 nautical miles from the area. The fifth station, 29, was located ca. 40 nautical miles to the southwest.

Sample collection. Procedures used for sample collection and processing were as follows. Water was recovered from the surface with a sampling apparatus constructed of a stainless-steel jar contained within a metal box support. A Smith-MacIntyre grab was used to retrieve sediment. Approximately 1 cm of the top of the sediment sample was removed with a sterile spatula and discarded. Sediment from below the top 1 cm of the sample was then collected with a spatula and weighed for use in the plate count procedure. Samples for ^{14}C-labeled hydrocarbon mineralization experiments, collected as 25-cm^3 cores from grab samples, were extruded into 250-ml Mallinkrodt glass bottles to which 100 ml of filtered (Gelman Metricel GA-8, 0.2 μm) seawater was added. Bottles were loosely capped and stored at 5°C for 6 days, at which time they were transported overnight on ice to the University of Maryland.

Enumeration and isolation of bacteria. Ca. 10 g (wet

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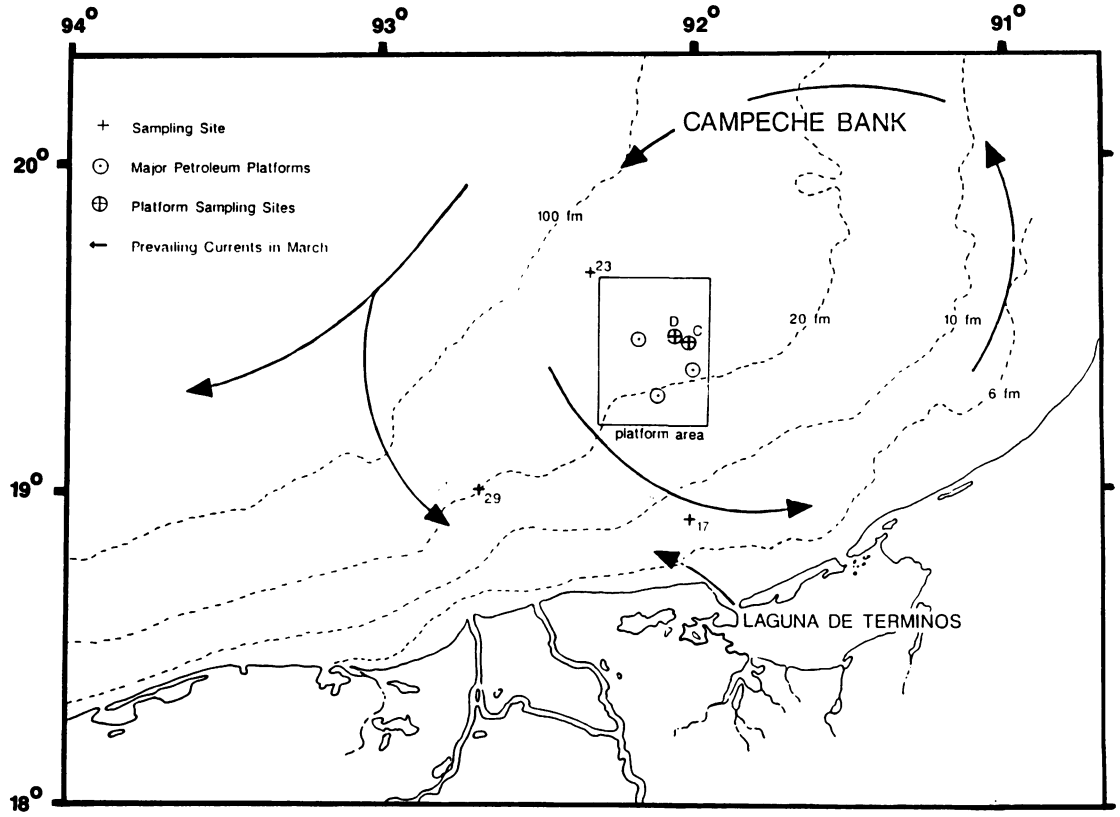

FIG. 1. Map of area of study, including sampling sites and major petroleum platforms.

weight) of sediment was shaken in flasks containing 90 ml of artificial seawater (26). Appropriate sample dilutions were spread onto marine agar 2216 plates (Difco Laboratories). Bacterial colonies were enumerated after incubation for 48 h at 25°C. Plates were stored at 5°C until they could be transported overnight, on ice, to the University of Maryland.

Ca. 30 to 100 colonies were picked from plates with 30 or more colonies. Each was streaked for isolation on a marine agar 2216 plate. Isolates were grown in marine broth 2216 (Difco Laboratories) for 3 days at 25°C, with shaking at 100 rpm, and subsequently preserved under liquid nitrogen in cryovials as 1:1 solutions of culture broth–24% glycerol.

Mineralization of ^{14}C-labeled hydrocarbons in sediment. Mineralization experiments were performed by using an incubation/trap system modified from Somerville et al. (35). Sediment samples were prepared by blending sediment-water mixtures for 1 min in a Waring blender. Mineralization of *n*-[1-^{14}C]hexadecane (Amersham Corp.) or [9-^{14}C]phenanthrene (Pathfinder Laboratories) in 1-ml samples of slurried sediment was determined by liquid scintillation counting (Beckman LS 7500) of $^{14}CO_2$ trapped in 1 N NaOH (1 ml), using aqueous counting scintillant (20 ml; Amersham).

For each of the four stations sampled in this study (stations 17, C, D, and 23), duplicate or triplicate tests were performed. Hexadecane data were not obtained for station C. Incubation times used were 0, 0.25, 0.50, 3.75, and 7.00 days for [^{14}C]hexadecane and 0, 0.50, 1.00, and 7.00 days for [^{14}C]phenanthrene. Concentrations of [^{14}C]hexadecane used were 0.06, 0.11, 0.53, and 1.05 μg/ml of sediment slurry. The [^{14}C]phenanthrene concentrations used were 0.06, 0.10, 0.51, and 1.05 μg/ml.

Plasmid DNA analysis. Isolates to be screened for plasmids were cultured in marine broth 2216. Plasmid DNA was extracted by the boiling lysis procedure of Holmes and Quigley (19), and electrophoresis was performed on a horizontal 0.8% agarose gel. *Escherichia coli* V517 plasmid DNA (27), purified by ultracentrifugation in a cesium chloride gradient and dialysis against TE buffer (pH 8.0) (28), was used as a source of eight molecular size standards for supercoiled DNA (1.4, 1.8, 2.0, 2.6, 3.4, 3.7, 4.8, and 35.8 megadaltons [MDa]). Commercially available *Hin*dIII-digested lambda DNA (Bethesda Research Laboratories) was used as a source of linear DNA standards (1.3, 1.5, 2.8, 4.3, 6.1, and 15.0 MDa). Plasmid DNAs of isolates exhibiting bands of 2 MDa or less were determined to be high-molecular-weight RNA species by treatment with RNase A (Boehringer Mannheim Biochemicals).

Statistical methods. Statistical analysis of plasmid incidence data was performed with the SAS statistical package (32) on the University of Maryland IBM 4381 computer. The chi-square test of association (34) was used to detect significant differences in the proportion of isolates carrying plasmids or multiple plasmids. Specific differences between sites were identified with a Bonferroni multiple-comparison procedure (20). Numbers of plasmids were compared by using the Kruskal-Wallis test (34) and the Dunn multiple-comparison procedure (9).

RESULTS

Figure 1 depicts the location of sites from which sediment samples were collected for analysis of plasmid incidence and hydrocarbon degradation studies. Plate counts for heterotrophic bacteria were relatively uniform for samples collected

TABLE 1. ^{14}C-labeled hydrocarbon mineralized in sediment slurries (incubation for 7 days at 20°C)

Site	% Mineralized							
	Hexadecane (μg/ml)				Phenanthrene (μg/ml)			
	0.06	0.11	0.53	1.05	0.06	0.10	0.51	1.05
17	0.2	0.7	0.8	0.6	0.1	0.3	0.1	0.1
C	ND[a]	ND	ND	ND	0.3	0.4	0.3	0.0
D	0.0	0.1	0.0	0.0	0.1	0.1	0.1	0.0
23	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1

[a] ND, Not done.

at all sites and ranged from 10^4 to 10^5 CFU/g (wet weight) of sediment. Concentrations of dissolved and dispersed hydrocarbons in the water column were low and varied from 1.11 μg/liter at station C to 1.66 μg/liter at station D (23). The absence of extensive oil slick formation in surface waters, taken together with the low observed hydrocarbon concentrations, suggested that no recent large-scale oil spillages had occurred in the sampling areas.

Data for the mineralization of ^{14}C-labeled hexadecane and phenanthrene in slurried sediment samples are presented in Table 1. Observed rates of mineralization were extremely low, with few apparent differences between either sampling sites or hydrocarbons. Given the purity of the radiochemicals in these experiments (98%), mineralization of at least 2 to 4% of the hydrocarbon substrate would be required to be considered significant.

Plasmid incidence was examined for a total of 242 heterotrophic bacterial strains isolated from the sediment samples collected in the study. Data for stations C and D were pooled to increase statistical power. The distribution of plasmid DNA in the bacterial strains isolated, by site and size of plasmids, is shown in Fig. 2. Plasmid data are grouped by $\log_{10}$ molecular weight and normalized by dividing the number of plasmids in each size class by the number of isolates screened for each site, so that direct comparison of frequencies between sites can be made. Plasmids were similarly bimodally distributed for sites C+D and 23, with plasmids of 3.2 to 10.0 MDa most frequently isolated and large plasmids of >31.6 MDa somewhat less so. Station 29 isolates, by contrast, possessed few small plasmids and

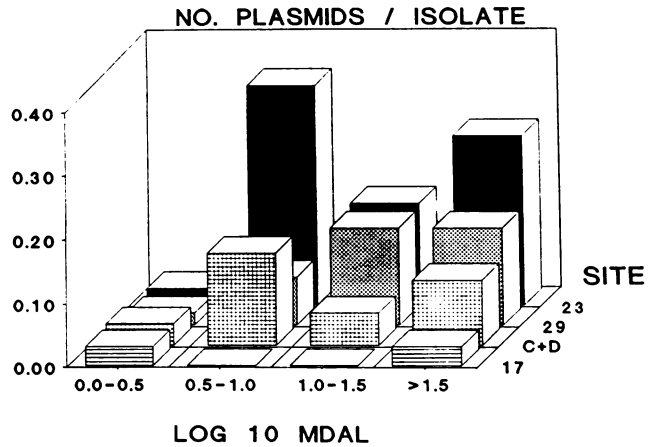

FIG. 2. Frequency of occurrence of plasmids, $\log_{10}$ molecular size, in bacteria isolated from sediment collected at sites in the Campeche Bank.

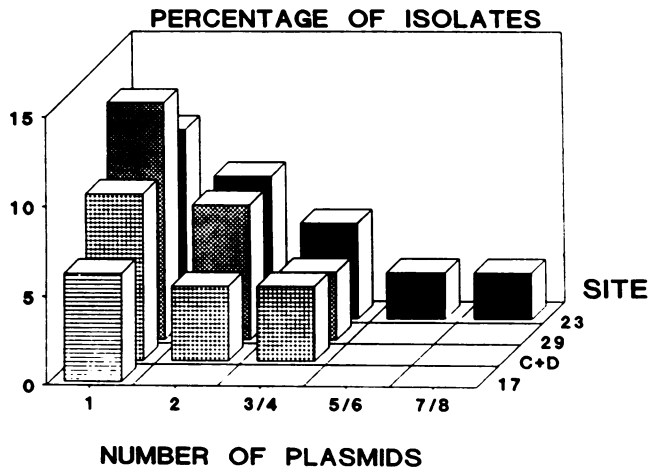

FIG. 3. Frequency of occurrence of number of plasmids in bacteria isolated from sediment collected at sites in the Campeche Bank.

approximately equal frequencies for plasmids 10.0 to 31.6 MDa and those >31.6 MDa. Very small plasmids, i.e., those of <3.2 MDa, were rare in isolates from all sites. The overall frequency of plasmids for station 17 isolates was so low as to preclude comparisons of size distribution with the other sites.

Figure 3 shows incidence of multiple plasmid carriage. Data are grouped by site and number of plasmids carried. Frequencies are expressed as a percentage of isolates from each site, normalizing the data and allowing direct comparison between sites. A clear pattern of plasmid multiplicity is apparent, but is unrelated to the proximity of sampling sites to the oil field. Rather, the trend of increasing multiplicity of plasmids appears to vary as a function of the site depth, which was 18 m at station 17, 46 m (average) at stations C+D, 52 m at station 29, and 117 m at station 23. None of the isolates obtained from station 17, the near-shore shallow-water site, possessed multiple plasmids. Stations C+D and 29 were of similar depth and exhibited similar frequencies of plasmid multiplicity, with generally decreasing numbers of isolates possessing one, two, and three to four plasmids. The same general pattern is evident for station 23, the deep-water station, with the exception that a small number of isolates carried as many as five to eight plasmids.

Plasmid frequency data were compared and found to be significantly different between sites, in terms of proportion of isolates carrying plasmids ($P < 0.05$), proportion of isolates with multiple plasmids ($P < 0.05$), and mean number of plasmids per isolate ($P < 0.10$). Multiple-comparison tests revealed that a significantly smaller proportion of isolates from station 17 carried plasmids ($P < 0.10$) or multiple plasmids ($P < 0.10$) than isolates from all other sites. These data are presented as a function of depth in Fig. 4. The percentage of isolates with plasmids or multiple plasmids, as well as mean number of plasmids per isolate, all clearly increased with increasing depth of the sampling site. The increases were nearly linear when log-transformed data were plotted (Fig. 4).

DISCUSSION

The objective of the present study was to determine whether sediment microbial communities in the Campeche

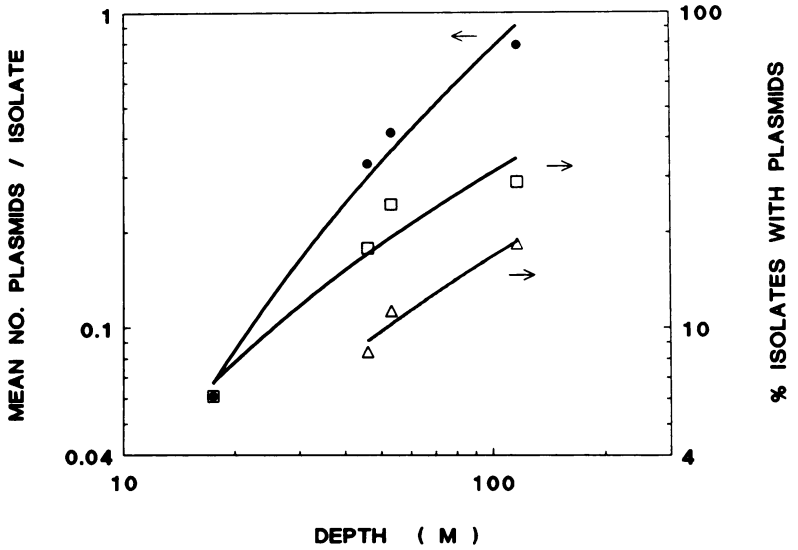

FIG. 4. Relationship of percent isolates carrying plasmids (□) or multiple plasmids (△) and mean number of plasmids per isolate (●) to water column depths of sampling sites.

Bank had adapted to chronic input of petroleum hydrocarbons originating from an active oil field. Hydrocarbon degradation rates in sediment and the incidence of plasmid DNA in bacteria isolated from sediment samples were compared for sites inside and outside the oil platform area. The underlying assumption was that sediments within the area would be more heavily polluted by oil and that the autochthonous microbial flora would exhibit higher degradation rates and a higher incidence of plasmid DNA, as consequences of "adaptation" (3, 36) of the community to elevated levels of hydrocarbons. Bacteria isolated from oil-polluted environments have previously been shown to be more effective in degrading crude oil than bacteria from unpolluted environments (8), and plasmid frequency has been shown to increase in various hydrocarbon-contaminated environments (5, 10, 15, 29, 33).

Negligibly low rates of mineralization of both hydrocarbons were observed for all sites sampled in this study. Only 0.8% of [^{14}C]hexadecane and 0.4% of [^{14}C]phenanthrene were converted to $^{14}CO_2$ in sediment samples exhibiting the highest rates; in most cases, values were 0.1% or less. These results are consistent with those reported by Atlas (1) for mineralization of ^{14}C-labeled hydrocarbons in water samples collected in the Campeche Bank area after the IXTOC I blowout. Biodegradation potentials of 0.5% were recorded for hexadecane but no observable mineralization of 9-methylanthracene, a 3-ring aromatic compound, was observed. In a concurrent investigation, Boehm and Fiest (4) concluded that the biodegradation of IXTOC I oil was occurring, but only very slowly, in sediment. The authors, in each case, concluded that microbial degradation of petroleum hydrocarbons was inhibited by severe limitations of nitrogen and phosphorus. In the present investigation, the failure to observe higher rates of hydrocarbon mineralization in sediments from the platform area compared with other sites, i.e., the apparent lack of measurable adaptation by the microbial community, was likely due to both nutrient limitation and the overall unpolluted state of sites in the Campeche Bank encountered during the study. The concentrations of hydrocarbons in the water column were very low and relatively uniform for all sites, with an average concentration of 1.46 ± 0.44 (standard deviation) μg/liter, the lowest recorded for the Campeche Bank (23). Hydrocarbon levels, therefore, may have been below "threshold" concentrations necessary for adaptation of the community to occur (36).

Plasmid frequency data were in agreement with the results of mineralization experiments in that increases in incidence were not associated with platform area bacteria compared with isolates from other sites. This may also be a consequence of the low observed concentrations of hydrocarbons; other authors have shown that threshold concentrations of chemical compounds are required to effect changes in plasmid incidence (38). Results reported here are in contrast to those obtained by Hada and Sizemore (15) in a study conducted in the northwestern Gulf of Mexico; they found that a higher proportion of *Vibrio* spp. from an oil field carried plasmids than did isolates from control sites. The low degree of hydrocarbon contamination of the Campeche Bank may be a possible explanation for the disparity between the two studies.

Significant differences in plasmid frequency and distribution among bacterial isolates were observed, but were correlated not with proximity of sites to the oil field, but to the depth of sampling sites. Log-transformed plasmid incidence data, expressed as percentage of isolates carrying plasmids or multiple plasmids and mean number of plasmids per isolate, increased as a nearly linear function of the logarithm of site depth. The distribution of multiple plasmids also varied regularly with depth, with isolates from the deep-water site possessing the highest numbers of plasmids while none of the isolates from the near-shore station exhibited more than one plasmid. The size distribution of plasmids, by contrast, was qualitatively similar for all sites and appeared to be generally unrelated to the depth. It should be emphasized that the limitations of the technique used to isolate plasmid DNA may have precluded the detection of very large plasmids, such as the CAM plasmid (150 MDa) (6), resulting in an underestimation of plasmid frequency. Although we were unable to identify the exact upper limit of detection for lack of a suitable standard, several plasmids that were isolated were estimated to be at least twice as large

as the 35.8-MDa plasmid from *E. coil* V517, based on relative mobilities.

The study reported here is the first in which the incidence of plasmid DNA in heterotrophic bacteria of marine sediments has been observed to increase with increasing depth of the water column. Plasmid carriage has been shown previously to increase in a variety of "stressed" environments, such as the marine air-water interface (18), deep terrestrial sediments (14), and the Antarctic (21), and, therefore, may constitute a means by which microbial communities adapt to the suboptimal conditions characteristic of these environments. Bacteria endemic to marine sediments must cope, primarily, with nutrient availability, since both quantity (16) and quality (17) of organic carbon decrease as a function of total water column depth. Plasmid DNA carried by sediment bacteria from deeper waters may encode traits which allow higher growth rates under oligotrophic conditions, as suggested by Kobori et al. (21), or catabolism of the largely refractory substrates which are available (37), or both. Alternatively, environmental conditions may select for the increased mobility of genes, thereby conferring a selective advantage to genes carried on plasmids. Further study is required to both confirm and adequately explain the greater occurrence of plasmid DNA among heterotrophic bacteria of deep-water sediments.

In conclusion, the microbial communities endemic to sediments of the Campeche Bank failed to exhibit adaptation to petroleum hydrocarbon contamination when hydrocarbon mineralization rates and incidence of plasmid DNA in heterotrophic bacteria were used as indicators of adaptation. The lack of adaptation may be a result of both nutrient limitations and the largely unpolluted state of the Campeche Bank at the time of sampling. The frequency and multiplicity of plasmids among bacterial isolates did increase, however, with depth of sampling site, a hitherto unreported phenomenon. The results of this study emphasize the importance of local environmental and geographic conditions as variables when considering environmental adaptation of microbial populations.

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