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Free-Living Amoebae Used to Isolate Consortia Capable of Degrading Trichloroethylene

Scientific Note

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INTRODUCTION

The interaction of protozoa with prokaryotes is well documented (*1–13*). These interactions can be either ecto- or endosymbiotic. An example of photosynthetic symbiosis is the well-defined interaction between paramecium and entrapped Chlorella (*2,7,9*). Paramecium can also form symbiotic relationships with gram-negative heterotrophic bacteria (*8,10*). Jeon (*6*) has described an interaction between amoebae and engulfed bacteria, that eventuated into a dependency of the amoebae on the presence of the engulfed bacterium. Free-living amoebae and tetrahymena can engulf and subsequently provide the necessary niche for the replication of *Legionella* (*1,4,11,13*). *Acanthamoebae* trophozoites and cysts can harbor and support the replication of unidentified gram-negative bacteria (*5,12*). King (*14*) has recently shown that bacteria associated with free-living amoebae are more resistant to toxic environments.

Various methylotrophic and/or heterotrophic bacteria from numerous environmental sources (*15–18*) can degrade trichloroethylene (TCE). Fliermans (*19*) has recently isolated bacterial consortia capable of degrading

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TCE at concentrations as high as 150 ppm. A methanotrophic bacterium capable of degrading TCE was isolated by Little et al. (*16*) from well water at a waste disposal site in the Oak Ridge, Tennessee, area.

Assuming that methylotrophic bacteria *in situ* are a part of a mixed community, and based on our observations that bacteria associated with protozoa may not be easily isolated by standard techniques, we attempted to use protozoa as a tool to isolate TCE-degrading bacteria. Since free-living amoebae constitute a significant fraction, if not the majority of soil and water protozoa, and since there is evidence for the symbiosis between protozoa and methanotrophic bacteria (*20*), we selected for amoebic-bacterial consortia by initially isolating the amoebae and subsequently culturing associated microbes that had been harbored within the amoebae. This was done in an attempt to unveil a different community of TCE-degrading bacteria from a well that had previously been sampled by Little et al. (*16*) for TCE degraders. In so doing, we hoped to find consortia with unique and/or advantageous abilities in terms of survival, stability, or degradation. This report describes the preliminary results on the isolation and characterization of such consortia.

MATERIALS AND METHODS

Water samples were obtained from several wells that were used to monitor a waste disposal site near Oak Ridge, TN. The test site was used previously for the dumping of a variety of organic solvents, including TCE. The number, depth, and approximate concentrations of TCE of the test wells were as follows: well 14, 13 ft., 2,100 ppb; well 27, 30 ft., 13,000 ppb; and well 46, 20 ft., 230 ppb. Water samples were aseptically collected by a nitrogen displacement sampling device (Well Wizard 3013; Q. E. D. Environmental Systems, Inc., Ann Arbor, MI). Samples were collected only after the well lines had been cleared through several cycles of pumping. The water samples were filtered through 1.2 μm cellulose nitrate/acetate filters (Millipore Corp., Bedford, MA) that were then inverted and placed on mineral salts (NATE) agar (*16*). Prior to the addition of the filter pads, the plates were spread with a lawn of live *E. coli*. The test plates were incubated in air at room temperature (23–25°C) for 7–14 d. When amoebic outgrowths had migrated to the edge of the petri dishes, the plates were transferred to desiccator jars flushed with 10% methane in air, as previously described (*16*). The resultant microbial consortia that appeared after 2 wk in the methane atmosphere along the area of amoebic outgrowth were aseptically transferred every 3–4 wk onto NATE agar media plates incubated in a methane in air atmosphere. Heterotrophic bacteria from the consortia were isolated and maintained at room temperature on trypticase soy agar (TSA). Amoebae from the consortia were grown at room temperature by replating consortia on agar plates spread with *E. coli* (NNAE).

Methanotrophs were maintained on NATE plates in a methane in air atmosphere.

For electron microscopic examination, consortium samples were negatively stained on formvar-coated, carbon stabilized grids, with a 0.5% solution of potassium phosphotungstate. Similar samples were fixed for thin-section analysis in a mixture of one part 2.5% glutaraldehyde and one part 5% osmium tetroxide in 0.1 *M* phosphate buffer in an ice bath (*21*). Samples were dehydrated with a graded series of ethanol, followed by propylene oxide, and embedded in Epon 812. Sections were cut on a Porter-Blum MT-1 microtome with a sapphire knife, stained with uranyl acetate and lead citrate, and viewed on a Hitachi H-600 transmission electron microscope.

The ability of the consortia or their amoebic and bacterial components to degrade TCE in a methane in air atmosphere was tested as previously described (*16*). The microorganisms were incubated in 100 mL of liquid NATE medium in 250 mL bottles fitted with teflon septa. The test bottles were injected with 12 mL of filter sterilized methane (0.536 mmol) in 150 mL of headspace. They were supplemented with [1,2-^{14}C] trichloro-ethylene (3.0 mCi/mmol [111 MBq/mmol], 95% pure by GC, Pathfinder Laboratories, St. Louis, MO). The test bottles were inverted and incubated for 12–14 d at 22–24°C on a shaker platform. The contents were analyzed for TCE periodically. Autoclaved cultures were used as negative controls. Other controls included incubating cultures in air without methane and testing of *E. coli* in place of the consortia. After incubation, the content of ^{14}C-TCE in cellular material, CO_2, and culture fluid was determined as described by Little et al. (*16*).

RESULTS

Water from all three test wells yielded free-living amoebae on the NATE plates spread with *E. coli* as a food source. Our supposition that amoebae could harbor methanotrophic bacteria was demonstrated when bacterial growth occurred in the methane atmosphere along the area of amoebic migration. Conversely, control plates without *E. coli*, which allows for amoebic migration, did not support methanotrophic growth away from the filter. Bacterial growth subsequently occurred on transfer of the amoebic populations to fresh NATE plates without added *E. coli* in a methane in air atmosphere. These consortia have been maintained for over four years by subculture on NATE in a methane in air atmosphere. Heterotropic and methanotrophic bacteria and amoebae continually coexist in the consortia. Microscopic examination of the amoebic trophozoites and cysts indicated they were *Hartmannella*. The presence of heterotrophs in these consortia grown in a methane in air atmosphere was evident on transfer of aliquots from NATE to TSA. Microscopic and enzymatic analy-

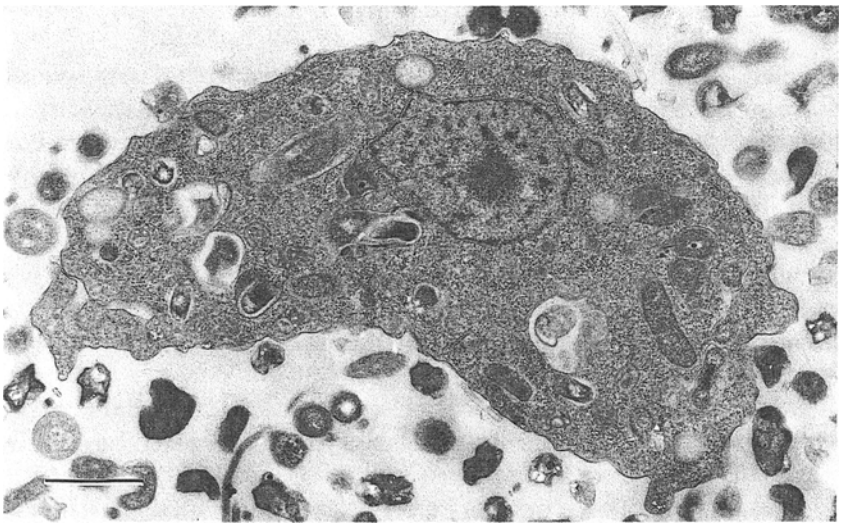

Fig. 1. Free-living amoebae showing "bulls eye" nucleolus and bacteria in the cytoplasm. Bar; 1.0μm.

sis showed the resultant heterotrophic populations were a mixture of genera, including, but not limited to, *Pseudomonas, Alcalignes, Bacillus,* and *Hyphomicrobium.* When suspensions of the consortia were filtered through a 0.8μ filter, and the filtrate subcultured three times on TSA, the resultant heterotrophs could no longer grow when replated on NATE in a methane in air atmosphere. Conversely, when amoebic populations from the NATE plates in a methane atmosphere were subcultured three times in air on NNAE plates, and the newly generated peripheral amoebic populations were replaced on NATE in methane, bacterial growth—presumably methanotrophs—occasionally reoccurred. In addition, when amoebic population grown on NNAE plates were stored for several weeks, such that encystation occurred, viable methanotrophic and heterotrophic bacteria were still present, as evident from bacterial growth when the amoebic cysts were transferred to NATE media in a methane atmosphere.

When consortia, maintained on NATE in methane in air, were filtered through 0.8 μm filters and the filtrate was replated on NATE in methane, methanotrophic and heterotrophic colonies free of detectable amoebae were occasionally obtained.

Thus, we have been able to free heterotrophic bacteria from methanotrophic bacteria and amoebae, and have been able to free the amoebae from methanotrophic and heterotrophic bacteria. We have not as yet, however, been able to disassociate the methanotrophic from the heterotrophic bacteria.

Electron microscopic examination of the consortia and the methanotroph/heterotroph mixture (Figs. 1–4) showed no evidence of a Type I methanotroph previously isolated from this site by Little et al (*16*). Instead, examination of the methanotroph/heterotroph mixture showed some

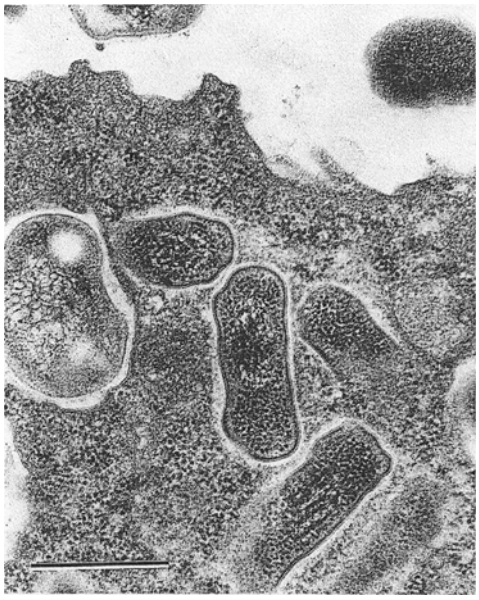

Fig. 2. Bacteria in amoebae trophozoite cytoplasm. Bar; 0.5μm.

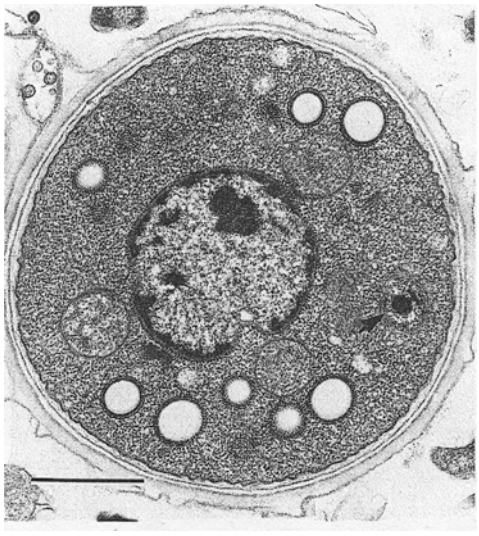

Fig. 3. Cross-section of bacteria in amoebae cyst (arrow). Bar; 1.0 and 0.5μm, respectively.

bacteria with typical gram-negative morphology, and many cells with morphologic characteristics of *Hyphomicrobium* (Fig. 4). Typical free-living amoebae with the expected "bulls-eye" nucleolus were evident (Fig. 1). Bacteria were commonly seen in the amoebic cytoplasm, both in the trophozoite (Fig. 2) and cyst (Fig. 3) stages. In both conditions, the bacteria were in membrane-bound vacuoles.

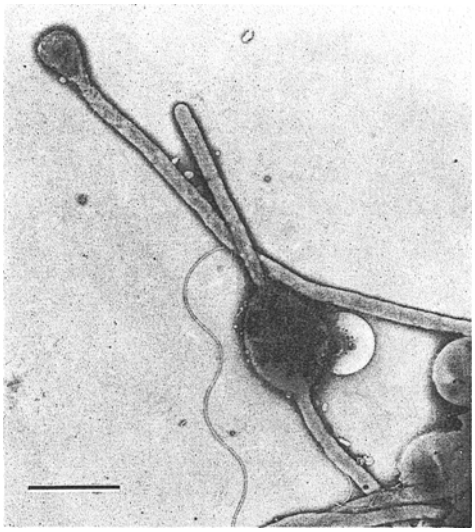

Fig. 4. *Hyphomicrobium* with typical hyphae. Bar; 1.0μm.

Table 1
Degradation of ^{14}C-Trichloroethylene (TCE) by Microbial Consortia

Consortia[a]	No. of Expts.	Average percent ^{14}C per fraction[b]		
		Cell Pellet	CO_2	Soluble
14	2	13(0-25)	87(75-100)	0
27B	3	11(6-16)	49(25-89)	40(0-69)
27P	2	11	73(57-89)	16(0-32)
46N	5	10(0-19)	75(50-100)	15(0-37)

[a]Numerical designation indicates well water of origin.

[b]Determined after subtracting *E. coli* or autoclaved controls which were about 10% of that observed with consortia. Percent of ^{14}C-TCE degraded by the consortia ranged from 30-40% after 12 days incubation at 25°C.

TCE was degraded by the various consortia, as indicated by the fate of ^{14}C-labeled TCE exposed to the consortia in a methane in air atmosphere (Table 1). TCE degradation was also evident on exposure to the mixture of methanotrophic/heterotrophic bacteria. Neither the heterotrophic bacteria nor amoebae populations without associated methylotrophic bacteria degraded TCE (Table 2). TCE was not appreciably degraded by consortia or methanotrophs in air. Neither autoclaved consortia nor *E. coli* degraded appreciable amounts of ^{14}C-TCE.

Table 2
Degradation of ^{14}C-Trichloroethylene (TCE) by Components of the 46N Microbial Consortia

Consortium Component	No. of Expts.	Counts of ^{14}C per fraction[a] Cell Pellet	CO_2	Soluble
Original consortium	2	656	2844	2010
Methanotrophs/ Heterotrophs[b]	2	854	3037	2185
Amoebae[c]	2	142	145	570
Heterotrophs[d]	2	129	105	600
E. coli control	2	129	113	550

[a]Average total counts added to test system was 12,875. Consortia, amoebae, methanotrophs and heterotrophs incubated with TCE in air in the absence of methane showed counts similar to *E. coli* control.

[b]The original consortium was filtered through a 0.8 micron filter and passaged three times on a mineral salts media in a methane in air atmosphere. When the resultant microbial growth was tested for amoebae and heterotrophs no amoebae were detected but heterotrophs were still present.

[c]Amoebae were passaged three times on nonnutrient agar spread with a lawn of *E. coli*. When replanted on mineral salts in a methane atmosphere no bacterial growth was apparent.

[d]Heterotrophs were passaged three times on TSA and were free of amoebae and methanotrophs.

DISCUSSION

That selecting initially for free-living amoebae may result in microbes with degradative capabilities not necessarily found by more traditional culture techniques is seen in a comparison of results from this study and that of Little et al. (*16*). Unlike that study, Type I methanotrophs could not be identified by either electron microscopy or by culture from the amoebae/bacterial consortia described in this study. However, degradation of TCE by the total consortia, but not by heterotrophs or amoebae populations *per se*, suggests that the methanotrophic bacteria are the

TCE-degrading component of the consortia. Unlike the degradation of TCE by the Type I methanotroph isolated by Little (*16*) from the same site, a greater proportion of the ^{14}C was found in CO_2, as opposed to cellular and soluble fractions when TCE was degraded by the consortia in the present study. Also, comparative studies by Palumbo et al. (*22*) showed the amoebae/bacterial consortia described in this study had a greater propensity for mineralization of TCE, compared with the methanotroph isolated by Little et al. (*16*), as well as other TCE degrading bacteria or consortia from other sites.

The nature of these stable amoebae/bacteria associations are, as yet, not completely understood, but they would appear to be in part, symbiotic. This is indicated by the continuing presence of both amoebae and heterotrophs on a minimal salts medium in a methane in air atmosphere. Neither heterotrophs nor amoebae alone should persist under these conditions. Indeed, the heterotrophs and amoebae isolated from the consortia could no longer grow under such conditions. The most likely explanation of the stability of the amoebic–bacterial consortia on NATE in a methane atmosphere is the growth of the methanotrophic bacteria permitting the persistence of the amoebae and heterotrophs.

The electron microscopic examination of the consortia and methanotroph/heterotroph mixtures also helped explain some of the cultural observations and difficulties. In light of the intra-amoebic presence of bacteria, it is not surprising that the initial isolation of amoebic cultures yielded bacterial isolates on subsequent transfer of the amoebae to a methane in air environment. It also explains the difficulties in trying to free the amoebae of the methanotrophs and heterotrophs. Similarly, the difficult, and as yet unrealized, separation of methanotrophs and heterotrophs is explained by the abundance of *Hyphomicrobium* that appears to make up the majority of the bacterial component. This genus of microorganism is noted for its proclivity for associating with other microorganisms, such that their isolation in pure culture has been rarely attained. *Hyphomicrobium* can grow in the presence of single-carbon sources and mineral salts, as used in this study. They can be found in association with methanotrophs, and may degrade methanol produced by methanotrophs, preventing the toxic accumulation of methanol. Whether the *Hyphomicrobium* are degrading TCE in the present study is not presently testable, since we have not as yet separated them from the other bacteria, in spite of trying density gradients and other techniques to do so.

The amoebae in the trophozoite or cyst form may provide a more stable niche for the metabolic activities of the associated methanotrophs and heterotrophs. For instance, the ability of free-living amoebae to protect associated bacteria from the killing effects of chlorine has recently been demonstrated (*14*). Thus, the isolation of the methanotrophs from encysted, and subsequently, excysted amoebae, may be pertinent. Tests are presently underway to evaluate the resistance to various insults of the methanotrophs, in association with, or freed from, their amoebic companions.

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