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Isolation and Characterization of Quinoline-Degrading Bacteria from Subsurface Sediments

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Two gram-negative, motile bacteria isolated from deep subsurface sediments mineralized the nitrogen-containing polyaromatic hydrocarbon quinoline under aerobic conditions and transformed quinoline to soluble intermediates under anaerobic conditions. Many aromatic compounds were also able to serve as the sole source of carbon and energy under aerobic conditions. Rapid aerobic mineralization of quinoline at concentrations as low as 0.002 μg ml^{-1} indicates that these organisms possess a high-affinity uptake and utilization system, which may reflect the oligotrophic nature of deep subsurface environments. Both bacteria harbored four plasmids of identical size, ranging from 50 to 440 kilobases.

Recent microbiological characterization of subsurface sediments from the Department of Energy Savannah River Plant, near Aiken, S.C., indicated an abundant population of aerobic heterotrophs at depths of up to 260 m (10; D. L. Balkwill, Geomicrobiol. J., in press). Microorganisms present in subsurface sediments were able to mineralize a wide range of substrates including quinoline, a heterocyclic nitrogen-containing polyaromatic hydrocarbon (R. J. Hicks and J. K. Fredrickson, Geomicrobiol. J., in press; R. J. Hicks, B. A. Denovan, and J. K. Fredrickson, submitted). Quinoline is a common contaminant in many aquifers affected by fossil fuel processing activities (24) and is also a suspected carcinogen (23). We report here the isolation of bacteria able to degrade quinoline under aerobic and anaerobic conditions, from sediment samples taken from two separate boreholes at depths of 176 and 203 m.

Drilling procedures and sample collection (T. J. Phelps, C. B. Fliermans, S. M. Pfiffner, T. Garland, and D. C. White, J. Microbiol. Methods, in press) and the protocol for biodegradation studies (Hicks et al., submitted) are described in detail elsewhere. Briefly, all down-hole tools were autoclaved or steam-cleaned before use, and drilling fluid did not circulate through the liner holding the sediment core during sampling of sediments for microbiological analysis. Multiple quality assurance methods were incorporated into the sampling of sediments to monitor contamination from drilling fluids, which would contain bacteria from elsewhere. These methods indicated that drilling-mud contamination of the sediments was below a 10-mg kg^{-1} limit of detection.

Modified soil respiration flasks (15) containing 47.5 ml of mineral salts (MS) medium (19) were inoculated with approximately 1 g of sediment. Flasks were amended with 10 μg of carbon ml^{-1} as quinoline by using a mixture of unlabeled quinoline (Sigma Chemical Co., St. Louis, Mo.) and [U-^{14}C]quinoline (Pathfinder Laboratories, St. Louis, Mo.), for a final specific activity of 4.4×10^3 dpm ml^{-1}. For anaerobic degradation, 50 ml of deoxygenated medium was added to serum bottles in an anaerobic chamber (Forma Scientific, Mallinckrodt Inc., Marietta, Ohio), and the bottles were sealed, removed from the chamber, and autoclaved. Bottles were returned to the chamber, amended with quinoline as described above, and inoculated with 0.1 g of sediment. Aerobic and anaerobic degradation were assayed by measuring evolved $^{14}CO_2$ and (after removal of cells and dissolved CO_2) loss of ^{14}C from solution, respectively. Quinoline was degraded under aerobic and anaerobic conditions in two sediment samples from the Middendorf formation, a sand aquifer beneath the Southeast coastal plain, sampled from two separate boreholes (Fig. 1).

To further enrich for quinoline-degrading bacteria, 10 μg of quinoline ml^{-1} was added to the two aerobic samples, which were then incubated for three weeks and plated to MS medium lacking a source of nitrogen with a 2.5-cm-diameter paper disc (no. 541; Whatman, Inc., Clifton, N.J.) containing 10 μl of quinoline placed on the medium as a source of quinoline vapor. A single colony type was evident in enrichments from both sediment samples, and isolates (866A and 957A) utilized quinoline as the sole source of carbon, nitrogen, and energy. These two isolates produced identical physiological characterization profiles when analyzed by the Oxi-Ferm Tube System (Roche Diagnostics, Div. Hoffman-La Roche Inc., Nutley, N.J.). The two isolates were oxidase-positive, gram-negative, motile rods able to use citrate and xylose as the sole carbon and energy sources, hydrolyze urea, and decarboxylate arginine. They did not use dextrose as the sole carbon and energy source either aerobically or fermentatively and did not fix N_2 gas, reduce indole to indigo, or reduce sulfate to hydrogen sulfide. These bacteria could not be classified by using the Oxi-Ferm Identification System but were considered the same strain or very similar strains. Extensive taxonomic identification was not attempted, although the bacteria appeared to belong to the genus *Pseudomonas*.

Alternative substrate stock solutions (0.5 mg ml^{-1}) were made in triple-distilled reverse-osmosis water and assayed at concentrations of 10 and 50 μg ml^{-1} in shake tubes containing 10 ml of modified MS medium (also made with triple-distilled reverse-osmosis water). The modified medium contained $FeCl_3$ and $MgSO_4$ at 1/10 concentration, and medium was diluted 10-fold before use. NH_4Cl and KNO_3 were omitted from the medium for substrates containing a nitrogen atom. The sulfate salts of Mg, Cu, and Zn were substituted for the chloride salts, and $FeCl_3$ was substituted for $FeCl_2$. Cells were grown in 0.3% tryptic soy broth (Difco Laboratories, Detroit, Mich.), washed three times in sterile saline (0.5%), and inoculated at a final density of 10^5 cells ml^{-1}, and the tubes were incubated at 30°C with shaking. Controls consisted of MS plus substrate and MS plus cells.

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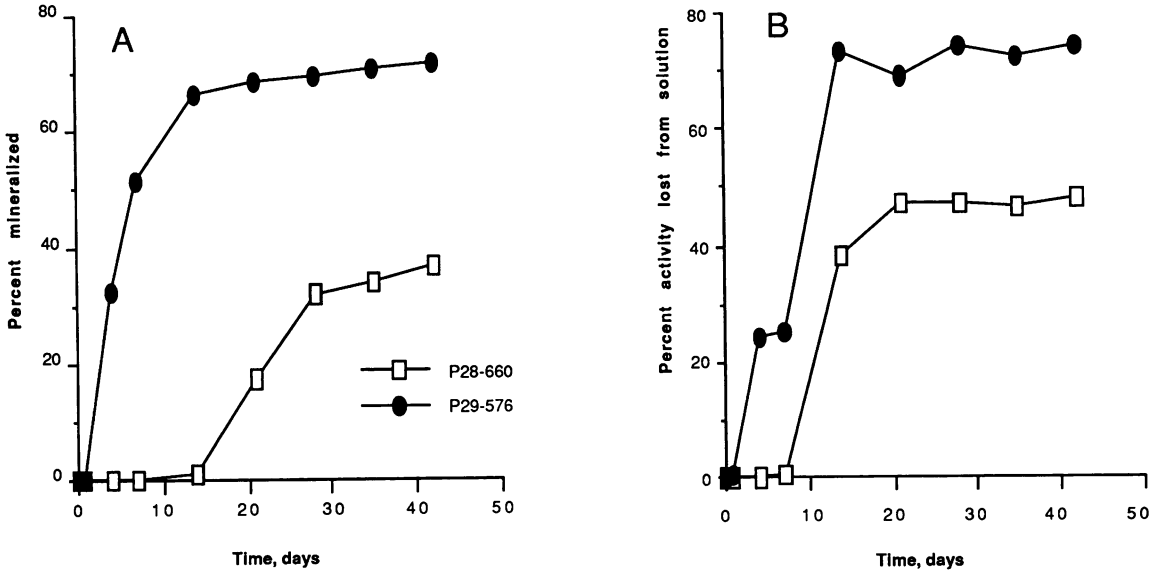

FIG. 1. Biodegradation of quinoline in sediment samples incubated under aerobic (A) and anaerobic (B) conditions. Samples P29-576 and P28-660 were from depths of 176 and 203 m, respectively. Datum points are the average of three replicates minus the value obtained for the $HgCl_2$-poisoned control.

To determine the substrate range of the bacteria, three levels of degradation were defined, as follows: degraded, turbid cultures within 24 h; slowly degraded, slightly turbid cultures and partial or complete disappearance of the UV absorbance spectrum after 1 week relative to the MS-plus-substrate control; and not degraded, UV absorbance spectrum identical to that of the MS-plus-substrate control after 2 weeks. The substrate range of the quinoline-degrading bacteria was as follows: 2-hydroxyquinoline (Aldrich Chemical Co., Milwaukee, Wis.), quinaldic acid, kyrenic acid, benzoic acid, salicylic acid, catechol, parahydroxybenzoic acid, protocatechuic acid, gentisic acid, alpha resorcyclic acid, and gamma resorcyclic acid (all from Sigma) were degraded (protocatechuic acid was degraded via *ortho* cleavage [21]); naphthalene (Sigma), biphenyl (Sigma), and benzene (J. T. Phillips Chemical Co., Phillipsburg, N.J.) were slowly degraded; and 4-hydroxyquinoline (Aldrich), 5-hydroxyquinoline (Aldrich), 8-hydroxyquinoline (Aldrich), 2-methylquinoline (Sigma), isoquinoline (Aldrich), pyridine (Sigma), 2-hydroxypyridine (Aldrich), 4-hydroxypyridine (Aldrich), nicotinic acid (Sigma), nicotinic acid *N*-oxide (Aldrich), 2-hydroxynicotinic acid (Sigma), 6-hydroxynicotinic acid (Sigma), picolinic acid (Sigma), picolinic acid *N*-oxide (Aldrich), 3-hydroxypicolinic acid (Sigma), 2,3-pyridine carboxylic acid (Aldrich), benzo[*f*]quinoline (Phillips), benzo[*h*]quinoline (Aldrich), acridine (Sigma), toluene (Phillips), and *meta*-toluic acid (Aldrich) were not degraded.

The inability to use pyridine and pyridine derivatives was not consistent with the ability of the organism to use quinoline as the sole source of nitrogen for growth, since the sole nitrogen atom is present in the pyridine ring. To test the hypothesis that the initial steps of quinoline utilization induced the ability to cleave the pyridine ring, substrate utilization assays were repeated with quinoline-adapted cells. Quinoline-adapted cultures were grown by shaking the cells in 50 ml of modified MS medium in 250-ml center-well flasks, with the center well containing a strip of Whatman 541 paper containing 100 μl of quinoline to supply the medium with quinoline vapor. Cells were washed and inoculated as described above. Moderately turbid cultures and shifts in the UV absorbance spectrum of the individual compounds were observed at 24 h on nicotinic acid and 6-hydroxynicotinic acid, and slightly turbid cultures and partial disappearance of the UV absorbance spectrum were observed at 1 week on 2-hydroxynicotinic acid, nicotinic acid *N*-oxide, 2,3-pyridine dicarboxylic acid, picolinic acid, 3-hydroxypicolinic acid, and picolinic acid *N*-oxide. Pyridine, 2-hydroxypyridine, and 4-hydroxypyridine were not degraded at 1 week. Thus, there appears to be a requirement for a carboxyl group at one of the ring junction carbons in order for a pyridine derivative to be utilized as substrate by quinoline-adapted cells. The ability of quinoline-adapted cells to degrade pyridine derivatives while tryptic soy broth-grown cells degraded quinoline but not pyridine derivatives suggests an initial cleavage of the benzene ring of quinoline, followed by an induction of pyridine ring cleavage (possibly by quinoline or an intermediate). The identification of intermediates is currently under way.

To assess the efficiency of aerobic quinoline uptake and utilization by these bacteria, the mineralization of quinoline at various concentrations was examined. Quinoline-adapted cells were washed three times and suspended in sterile saline for 2 days at 27°C to metabolize stored substrate. These cells were used to inoculate 50 ml of MS medium in biometric flasks at a final density of 10^7 cells ml^{-1}, quinoline (specific activity of 300 dpm ml^{-1}) was added at concentrations ranging from 0.002 to 1.1 μg ml^{-1}, and the flasks were gently swirled for 30 s and incubated at 27°C without shaking. Controls were amended with $HgCl_2$ to 100 μg ml^{-1} 8 h before the addition of quinoline. Four replicates and one $HgCl_2$-poisoned control were used for each concentration of quinoline. At 0.002 and 0.02 μg of quinoline ml^{-1}, 42 and 11% of the labeled carbon was evolved as $^{14}CO_2$ within the first 6 min. Approximately 60% of the labeled carbon had been mineralized by 8 h at all of the concentrations tested. The results were similar for both isolates. In separate experiments, tryptic soy broth-grown cells transferred to MS amended with quinoline required 20 h for induction of quinoline uptake. Cells transferred to MS amended with

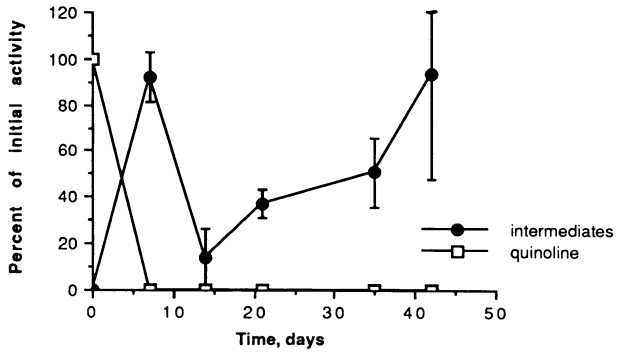

FIG. 2. Anaerobic biodegradation of quinoline by isolate 957A. Datum points are the average of three replicates minus the value obtained for the $HgCl_2$-poisoned control. Error bars represent one standard deviation.

chloramphenicol and quinoline were unable to take up quinoline. The data indicate that these bacteria possess an inducible, high-affinity, rapid-uptake system which probably reflects the low organic carbon content of most deep subsurface environments. In other experiments, the bacteria readily mineralized quinoline at 165 μg ml^{-1}. Higher concentrations were not tested.

Although these bacteria were isolated and purified in aerobic culture, the degradation of quinoline under anaerobic conditions in the same sediment samples from which these organisms were isolated (Fig. 1) suggested that the bacteria might also degrade quinoline anaerobically. Therefore, the ability of these bacteria to degrade quinoline anaerobically was also studied. MS medium amended with 40 μg of NH_4Cl_2 and 50 μg of KNO_3 ml^{-1} was boiled under a stream of pure dinitrogen gas and transferred to an anaerobic chamber (Forma Scientific) where 15-ml volumes were distributed to serum vials. The vials were capped, autoclaved, and immediately returned to the anaerobic chamber. Quinoline-adapted cells of 957A were washed twice, transferred to the anaerobic chamber, suspended in deoxygenated MS medium, amended with quinoline (final activity of 2.2×10^3 dpm ml^{-1}) to obtain 10 μg ml^{-1} upon inoculation into the vials, vortexed, and inoculated into vials at a final density of 10^7 cells ml^{-1}. Poisoned controls were amended with $HgCl_2$ to 100 μg ml^{-1}. Vials were incubated in the anaerobic chamber at 27°C. For each sampling point, three vials and one control vial were sacrificed. Evolution of $^{14}CO_2$ and ^{14}C remaining in solution was measured by the procedure of Little et al. (14). Disappearance of quinoline from solution was determined by reverse-phase high-pressure liquid chromatography by using UV detection (C-18 μBondapak column; Waters Associates, Inc., Milford, Mass.). Activity present as soluble intermediates was calculated by subtracting the activity present as quinoline from the total activity remaining in solution (after filtration of cells and removal of dissolved CO_2). At day 7, quinoline had disappeared and nearly all activity was present as a single unidentified soluble intermediate (Fig. 2). By day 14, a large fraction of the soluble intermediate had been removed from solution. With increasing incubation, soluble intermediates increased and the retention times of the soluble intermediates were different from those of the intermediates present at day 7. Activity present in the cells was not measured; however, the decrease and subsequent increase in soluble intermediates could indicate that cell uptake of a metabolite and the subsequent production of an apparently nonutilizable metabolite had occurred. The mineralization of quinoline was not observed; live-cell suspensions did not evolve significantly more $^{14}CO_2$ than the poisoned controls. These results show that 957A is able to degrade quinoline anaerobically and could have caused the loss of ^{14}C from solution in the original sediment samples (Fig. 1). The possibility exists that sediment samples may have contained other organisms capable of utilizing (and possibly mineralizing) quinoline or the intermediates produced by 957A.

In a separate experiment with tryptic soy broth as the medium, both isolates grew anaerobically, as measured by a large increase in turbidity over a 7-day period, indicating that the bacteria were capable of fermentative growth under anaerobic conditions. To confirm the role of nitrate as the hypothesized electron acceptor in the anaerobic degradation of quinoline in MS medium, experiments were conducted with 957A in the presence and absence of 50 μg of KNO_3 ml^{-1} in MS plus quinoline. Control treatments consisted of the same media lacking inoculum. Reduction to nitrogen gas was blocked by adding acetylene to 5% of the headspace. Disappearance of quinoline in the MS media was shown by high-pressure liquid chromatography; however, the denitrification products NO and N_2O were not detected by gas chromatography (automated headspace sampler [Hewlett-Packard Co., Palo Alto, Calif.] interfaced to a 5880 gas chromatograph with a GSQ column [J and W Inc., Folsom, Calif.]) at 2, 8, 12, or 16 weeks. In addition, ion chromatography (model 10 with AG3-AS3 column; Dionex Corp., Sunnyvale, Calif.) and colorimetric assays (1) at 12 weeks showed that nitrite did not accumulate and nitrate remained at the initial concentration, indicating that the bacterium did not respire nitrate under the conditions tested (data not shown). The terminal electron acceptor (inorganic or metabolite) utilized by this bacterium in the anaerobic degradation of quinoline in MS medium is currently under investigation.

Bacteria able to utilize quinoline aerobically as a sole source of carbon and energy have been isolated from sewage, garden soil, and creosote-contaminated soil (3, 12, 20). Initial attack on the benzene ring and on the pyridine ring of quinoline have both been reported (12, 20). In these studies, the initial product of quinoline degradation is 2-hydroxyquinoline, or its tautomer 2(1H)quinolinone. Pereira et al. (17), using methanogenic consortia, have recently demonstrated aerobic and anaerobic degradation of quinoline to 2(1H) quinolinone, with incorporation of oxygen from water under both conditions. The production of soluble intermediates by 957A under anaerobic conditions may be the result of a similar hydroxylation reaction. Under aerobic conditions, hydroxylation of quinoline on the pyridine ring (2-hydroxyquinoline) sets up the benzene ring of quinoline for the addition of molecular oxygen and ring cleavage (12). In the absence of oxygen, subsequent oxygenated cleavage of the benzene ring of quinoline by 957A would be prevented, resulting in the observed accumulation of a soluble intermediate(s).

The bacteria were examined for the presence of plasmid DNA, because the catabolism of a number of aromatic compounds is known to be encoded on plasmids (2, 5, 7, 13, 16, 22). The Eckhart plasmid visualization method (8) was used, with modifications (9). Both isolates contained four plasmids with estimated sizes of 50, 100, 320, and 440 kilobases (kb), based on plasmid markers of known size (Fig. 3). Previous characterization of the plasmid content of bacteria from the Savannah River Plant showed that 33% of the subsurface isolates contained plasmids, with over half of the plasmids larger than 150 kb (11). Several isolates also

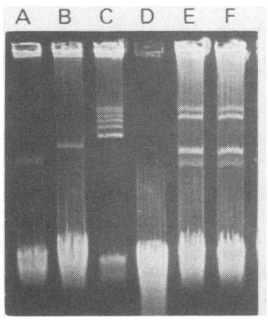

FIG. 3. Visualization of plasmids in a 0.9% agarose gel. Plasmid size markers include pRK2013::Tn*1721*, 62 kb (A. Summers, personal communication (A); TOL plasmid of *Pseudomonas putida* mt-2, 117 kb (6) (B); and plasmids of approximately 155, 200, 255, 300, 440, and 480 kb present in *Rhizobium leguminosarum* T87K3 (4, 18) (C). (D) Strain lacking plasmids. Estimated plasmid sizes for both isolate 866A (E) and isolate 957A (F) are 50, 100, 320, and 440 kb.

harbored plasmids with homology to pWW0, a catabolic plasmid coding for degradation of toluene. The role of plasmids in quinoline catabolism is being evaluated by plasmid curing and transposon mutagenesis studies.

These results demonstrate the presence of bacteria in deep aquifers that can degrade a variety of aromatic compounds. Also, these organisms are apparently adapted to oligotrophic conditions, as demonstrated by their rapid catabolism of quinoline at low concentrations. A greater understanding of the physiology and metabolic potential of deep subsurface microorganisms is needed before attenuation rates of organic contaminants in deep aquifers can be determined or biological in situ remediation can be attempted.

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