Petroleum Pollution Bioremediation Using Water-Insoluble Uric Acid as the Nitrogen Source

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The biodegradation of hydrocarbon pollutants in open systems is limited by the availability of a utilizable nitrogen source. This limitation can be overcome by using uric acid. Enrichment cultures grown on crude oil-uric acid media yielded mixed and pure cultures that degraded petroleum. In a simulated open system, uric acid bound to crude oil and was available for bacterial growth and petroleum biodegradation.

The rate-limiting step in the microbial degradation of petroleum hydrocarbon pollutants in open systems, such as lakes, oceans, and wastelands, is generally a utilizable source of nitrogen (1, 2, 13, 14). Since petroleum contains only traces of nitrogen, the required nitrogen must come from the surrounding environment. In the laboratory, the nitrogen requirement for optimum growth of hydrocarbon oxidizers can be readily satisfied with urea or salts that contain ammonium or nitrate ions. However, these nitrogen sources have a high water solubility, which reduces their effectiveness in open systems because of rapid dilution. Thus, there is at present no practical microbial solution to the reoccurring problem of petroleum pollution in the sea. To overcome the N limitation for petroleum degradation in open systems, Atlas and Bartha (3) studied the effectiveness of several oleophilic nitrogen compounds with low C/N ratios. Subsequently, an oleophilic fertilizer (Inipol EAP 22) was used in the bioremediation of polluted shorelines after the Exxon Valdez spill (1, 11). Initial reports of success (7, 12) have been challenged (5).

Another approach has been the use of a water-insoluble polymer, based on a urea-formaldehyde formulation, which adheres to oil (15, 16).

Uric acid is the major nitrogen waste product of birds, terrestrial reptiles, and many insects. It has a low solubility in water and is the major component of guano fertilizer, suggesting that it might be a useful nitrogen source for the bioremediation of petroleum pollutants in open systems. Many different species of bacteria are known to degrade uric acid (4, 6, 19, 20). We report here the isolation and characterization of a strain of *Acinetobacter* that can grow on crude oil with uric acid used as the sole nitrogen source. Furthermore, we show that uric acid binds to crude oil and is therefore available for bacteria which grow at the hydrocarbon-water interface.

**Isolation and characterization of *Acinetobacter* sp. strain OK1.** We used weathered crude oil as the carbon source, uric acid as the nitrogen source, and pigeon droppings as the inoculum to obtain a mixed culture after several transfers that emulsified and partially degraded the crude oil. Plating on Luria-Bertani agar yielded five colony types: two were unable to grow, two grew poorly, and one grew well on the hydrocarbon-uric acid medium. The strain that grew well, referred to as OK1, was chosen for further study. A comparison of the growth of strain OK1 and the mixed culture on the crude oil-uric acid medium is shown in Fig. 1. Strain OK1 is a gram-negative, strictly aerobic, nonmotile, oxidase-negative short rod. These properties are typical of the genus *Acinetobacter* (8, 10). The Biolog (Biolog Inc., Haywood, Calif.) and API-20 (BioMerieux, Marcy l’Etoile, France) identification kits indicated that strain OK1 was an *Acinetobacter* strain, most closely related to *Acinetobacter baumannii* (identity, 76%). The 16S rRNA gene sequence of strain OK1 (821 bp in length; GenBank accession no. AY260854) had the closest similarity to *A. baumannii* (DSM 3008), with an identity of 813 of 821 nucleotides (99%). In consideration of all the phenotypic and genotypic tests performed, it appears that strain OK1 is a new *Acinetobacter* species which is most closely related to *A. baumannii*.

![FIG. 1. Growth of a mixed enrichment culture (○) and strain OK1 (●) on crude oil and uric acid. Bacteria were inoculated into salts medium containing 5 mg of crude oil per ml and 0.5 mg of uric acid per ml and incubated at 30°C with shaking.](http://aem.asm.org/)
Adhesion of uric acid and strain OK1 to crude oil. Uric acid crystals adhered to droplets of crude oil in the culture medium (Fig. 2). When low ratios of uric acid to crude oil (1/20, wt/wt) were used, the crude oil-uric acid complex had a density less than 1.0 and floated to the surface of the medium, whereas at higher ratios (1/2), the complexes sedimented. These data also demonstrated the interaction between uric acid and crude oil. The BATH test (17) showed that strain OK1 adheres avidly to hexadecane.

Growth of strain OK1 on crude oil and uric acid following removal of water-soluble nutrients. The major assumption in this study is that uric acid binds to crude oil and is not diluted into the surrounding water. To test this assumption, the growth of strain OK1 was measured on crude oil-uric acid media after the media were mixed and after the aqueous phase had been removed and replaced with water three times (a simulated open system). Table 1 demonstrates that high cell yields (2 × 10^8 to 6 × 10^8 cells per ml) and petroleum degradation (48 to 50%) occurred with initial concentrations of 0.25 to 1.0 mg of uric acid per ml. The unwashed control, containing 1 mg of uric acid per ml, yielded 9 × 10^8 cells per ml and 67% petroleum degradation. The use of water-soluble ammonium sulfate and performance of the same washout procedure yielded only 5 × 10^6 cells per ml and 2% petroleum degradation. As is typical of many Acinetobacter strains (18), the growth of strain OK1 on crude oil was accompanied by emulsification of the oil. Preliminary data indicate that the extracellular emulsifier is a glucosamine-containing polysaccharide.

Growth yield as a function of crude oil and uric acid concentrations. To determine growth yields of *Acinetobacter* sp. strain OK1 as a function of petroleum and uric acid concentrations, an overnight culture was inoculated into salts medium containing various concentrations of crude oil and uric acid.

![FIG. 2. Adhesion of uric acid to crude oil. Uric acid crystals are seen adhering on the left and upper sides of the oil droplet by scanning electron microscopy. UA, uric acid.](image1.png)

![FIG. 3. Growth of *A. baumannii* strain OK1 as a function of petroleum concentration and uric acid concentration. (A) *A. baumannii* OK1 was grown in salts medium containing 0.5 mg of uric acid per ml and various concentrations of crude oil. (B) *A. baumannii* OK1 was grown in salts medium containing 5 mg of crude oil per ml and various concentrations of uric acid. Cell concentrations were determined after incubation at 30°C.](image2.png)
After incubation with shaking for 96 h at 30°C, cell yields were determined by spreading appropriate dilutions on Luria-Bertani agar. Growth was proportional to crude oil concentrations ranging from 0 to 5 mg/ml (Fig. 3A) and uric acid concentrations ranging from 0 to 0.5 mg/ml (Fig. 3B), ultimately reaching 9 × 10⁸ cells per ml. The minimum doubling time during the exponential phase on the crude oil-uric acid medium was ca. 50 min.

**Hydrocarbon substrate specificity.** The ability of strain OK1 to utilize various aliphatic and aromatic hydrocarbons as carbon sources was examined in salts medium containing 0.5 mg of uric acid per ml and 2 mg of the test hydrocarbon per ml. The tests revealed that strain OK1 grows on straight and branched-chain (phytane and pristane) aliphatic hydrocarbons containing 12 or more carbons. It failed to grow on shorter alkanes or the 16 aromatic hydrocarbons tested. The Agha Jari crude oil used in the above washout experiment contained only 14% aromatics (9).

The major aim of this study was to test the hypothesis that uric acid can (i) serve as a nitrogen source for hydrocarbon-degrading bacteria and (ii) bind to crude oil, thereby making it a potentially useful nitrogen fertilizer for the bioremediation of petroleum pollution in open systems. The data reported here for the isolated *Acinetobacter* sp. strain OK1 support this hypothesis. Binding of uric acid to crude oil was demonstrated visually and by a simulated open-system growth experiment. Clearly, in a true open system, such as a lake, the complex interactions between marine bacteria and dissolved-phase and beached hydrocarbons would be exposed to a much larger body of water, and the uric acid would have to remain bound to the oil for at least a few days. Although the study reported here dealt exclusively with strain OK1, we have isolated many different bacterial species that can grow on crude oil and uric acid, including some that utilize polycyclic aromatics. Thus, uric acid can serve as a general water-insoluble nitrogen source for hydrocarbon oxidizers. The most important parameter to study in order to convert the in-principle concept developed here to a useful technology is probably the rate of transfer of hydrocarbon-bound uric acid to the aqueous phase. Without these data, attempting to use uric acid for petroleum bioremediation in an open system would be premature.

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**REFERENCES**