Letter

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Growth and Attachment-Facilitated Entry of Bacteria into Sub-Micron Pores Can Enhance Bioremediation and Oil Recovery in Low Permeability and Microporous Media

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Abstract

Bioremediation endpoints for biodegradable hydrophobic compounds in soil aggregates are regulated by bacterial accessibility to different pore sizes. We evaluated the accessibility of the nonmotile hydrocarbon-degrading bacteria, *Dietzia maris* (d=1 μm at stationary phase), to 0.4 μm pores. A significant fraction (22%) of the pore volume of the clayey soil from which the bacteria was isolated, were associated with pores of 0.4-1 μm diameter. Bacterial entry into the pores was observed by electron microscopy and by monitoring mineralization of ^{14}C-hexadecane placed well above membranes with fixed pore sizes (0.4 or 3 μm), in a bioreactor. The membranes were used as a surrogate for soil pores of fixed diameters. When membranes were not wetted, or wetted with non-biodegradable heptamethylnonane, bacteria did not penetrate pores even if they attached to the membrane. However, bacteria penetrated pores when membranes were wetted with hexadecane, as growth on hexadecane yielded a crowd of smaller rod-shaped cells (d_{min}= 0.54±0.14 μm) in biofilms formed on the membrane. A morphological progression with time from smaller, elongated cells at early growth phase to cocci-shaped cells was observed. The results suggest proliferation accompanied with morphological changes as a mechanism of bacterial migration in sub-micron pores and low permeability media.

Introduction

In petroleum-contaminated soils, a significant fraction of hydrocarbons enter pores smaller than 1 μm over time.[1] It has been suggested that limited accessibility of bacteria to hydrocarbons entrapped in pores smaller than, or of similar size to bacteria may control the magnitude of the residual hydrocarbon fraction after extended bioremediation.[2, 3] The lack of bioaccessibility may also influence the efficiency of processes such as microbial enhanced oil recovery and *in-situ* bio-cracking of high molecular weight hydrocarbons in deep oil and gas reservoirs.[4, 5]

Only a few studies have addressed the question of what is the minimum pore diameter bacteria can access.[6, 7] Recently, chemotactic swimming of planktonic, motile cells of *Escherichia coli* (d=0.76 μm) and *Bacillus subtilis* (d=0.86 μm) through channels as small as 1.1 μm was reported by Mannik *et al.* where the chemoattractant (Luria-Bertani media) and bacteria were introduced from opposite ends of the pore.[8] Furthermore, it was observed that if a Gram-negative *E. coli* cell was lodged in the mouth of a pore as small as 0.4 μm, it produced daughter cells in the pore which colonized along the length of the pore. The cells in the pore were deformed and nonmotile, but after emerging from the pore by colonizing along the pore reproduced healthy, motile cells. The authors related such changes in cell physiology to the flexible and thin cell wall of Gram-negative bacteria.[8]

There are no reported studies demonstrating the entry of hydrocarbon degrading bacteria into sub-micron or sterically restrictive pore sizes. In this study, we evaluated the passage of *Dietzia maris*, a Gram-positive, nonmotile, hydrocarbon-degrading bacterial strain[9] through 0.4 μm pores, a size much smaller than the diameter of *D. maris* in stationary phase in growth cultures. Experiments were conducted in specially designed bioreactors with membranes separating the bacterial suspension in the lower chamber from an aqueous phase in the upper chamber

containing a layer of ^{14}C-labelled hexadecane placed well above the membrane. Compounds such as hexadecane (aqueous solubility ~ 4 ppb, melting point =18.2 ºC)[10] and those with higher molecular weights are expected to reside almost indefinitely in soil pores , unless bacteria come in direct contact with the hydrocarbon liquid phase. Hexadecane is easily degradable by *D. maris,* which attach to the hexadecane-water interface for direct uptake and to overcome limited bioavailability of such poorly soluble hydrocarbons.

Different mechanisms for surface translocation of motile bacteria such as swimming, swarming, twitching and sliding have been reported. Sliding is defined as passive transport of motile bacteria on a substratum as a result of natural expansive forces during colony formation and growth in single layers, and changes in cellular microenvironments such as due to surfactant production which can decrease cell-substrate friction forces.[11, 12] However, Mannik *et al.* observed that during passage by proliferation along sub-micron pores, motility did not play a critical role.[8]

We hypothesized that even nonmotile *D. maris* cells could enter submicron pores (≥0.4 μm) in the membrane if they could attach, grow and crowd on an oil-water interface at or near the pore mouth. Forces induced from colony expansion and biosurfactant production by *D. maris* likely also facilitate pore entry. To assess the hypothesis, we conducted parallel experiments with (i) hydrocarbon-free membranes on which there was no growth or attachment, and (ii) membranes wetted with non-biodegradable heptamethylnonane where there was no growth but there was attachment, and (iii) membranes wetted with hexadecane where there was both growth and attachment.

Materials and Methods

Bacterial strain. The bacterial strain used in experiments was isolated from a weathered petroleum-contaminated soil and identified as (*Dietzia maris*; CA160; GQ870425 (99%)). *D. maris* cultured in Bushnell-Hass (BH) mineral media amended with 1.0% (v/v) hexadecane and incubated at 25 ºC for four days, was used as inocula for the bioreactors. *D. maris* cells were nonmotile, and negatively charged in the media (-33.83 mV).[3] Surface tension in *D. maris* cultures measured by a du Nouy ring tensiometer (CSC Scientific) decreased from 69.4±0.1 to 34.6±0.4 dynes/cm in 10 days, indicating biosurfactant production activity by bacteria.

Mineralization experiments. Mineralization experiments and environmental scanning electron microscopy (ESEM) were conducted as described elsewhere.[3] Briefly, the bioreactors (Fig. 1), consisted of a lower and an upper chamber separated by a nuclepore track-etched membrane (GE Healthcare Bio-Sciences) with pore sizes of 0.4, 3 or 5 μm. Bacterial cells were washed two times and inoculated in the lower chamber. Then, 20 μL of ^{14}C-hexadecane was added on top of the aqueous phase in the upper chamber. A side arm containing NaOH solution was attached to the upper chamber to collect $^{14}CO_2$ produced from mineralization of hexadecane. The radioactivity of samples from NaOH trap were periodically measured using a scintillation counter (LS3600, Beckman Coulter).

Membranes were made of polycarbonate, coated with polyvinylpyrrolidone (PVP) and were easily wetted in water. The membrane was slightly negative (-4.82 mV) in the media and the air-water contact angle was 50.1°.[3] The membrane thickness was approximately 9 μm.

For experiments with hexadecane or 2,2,4,4,6,8,8-heptamethylnonane (HMN)-wetted membranes, membranes were first pre-treated by rinsing with methanol to remove the water-wetting agent (PVP), and then 0.1 μL of non-labeled hexadecane or HMN was added to the

center of the membrane surface. As membranes were wetted with non-labeled hexadecane only, mineralization of the non-labeled hexadecane on the membrane generated $^{12}CO_2$ and therefore the measured $^{14}CO_2$ in basic traps were only attributable to ^{14}C-hexadecane on top of the aqueous layer in top chamber. ^{14}C mass balance performed at the end of experiments by accounting for $^{14}CO_2$ generated and residual activity in the non-aqueous phase liquid and aqueous phases, yielded more than 90% recovery in all systems. A control system with hexadecane-wetted membrane but not inoculated with *D. maris* showed no mineralization.

Results and Discussion

Statistically significant ($p<0.05$) and comparable mineralization (12% after 40 days) was observed in the systems with 0.4 μm or 3 μm membranes wetted with non-labeled hexadecane (Fig. 2). The observed mineralization activity indicates that *D. maris* cells were able to migrate through the membranes of either pore size, reach to oil layer on top of the aqueous phase in upper chamber by diffusion and then uptake ^{14}C-hexadecane. It appears that there was no delay in the passage of cells through the smaller, sterically restrictive pore size of 0.4 μm compared to the 3 μm pore. Biofilm formation was observed at the hexadecane-water interface in the upper chamber and on both sides of hexadecane-wetted membrane.

However, biodegradation was not observed in the systems with 0.4 μm HMN-wetted membranes. Microbial Adhesion to Hydrocarbons (MATH) tests[13] indicated comparable extents of cell adhesion on HMN and hexadecane (53±3% and 65±2 %, respectively). This suggests that growth during attachment, rather than attachment on the hexadecane-wetted membranes alone, enabled bacteria to penetrate into and pass through submicron pores and eventually reach the radiolabeled oil-water interface.

Contrary to our results with hexadecane-wetted 0.4 and 3 μm membranes, our previous study showed that systems with non-wetted membranes generated mineralization activity only when pore diameters were 5 μm or larger. We demonstrated that this was due to aggregation of *D. maris* cells under nutritional stress in these bioreactors ($d_{aggregate}$=4.8 ± 1.12).[3] However, the initial hexadecane mineralization rate (rate constant = 0.0034±0.0002 day^{-1} (R^2=0.75) calculated for days 7-41) was similar in all systems where mineralization occurred. A hexadecane mineralization extent of 54±4% over 220 days was obtained in the 5 μm system (Fig. S1). The relatively low mineralization rate could be attributed to limited mixing and oil-water interfacial areas in the bioreactors.

ESEM of hexadecane-free membranes, and hexadecane-wetted membranes removed from the bioreactors are shown in Figure 3A to 3D. At day 1 none of the bacterial cells appeared on the permeate (top) side (note: bacteria were inoculated on feed-side) of hexadecane-wetted membranes (Fig. 3A). However, on day 7, a few *D. maris* cells appeared (Fig. 3B), and by day 10 (Fig. 3C, D) significant numbers of cells were observed on the permeate side, likely due to the growth of *D. maris* cells after entry through the pores. The average of the smaller diameter (d_{min}) of rod-like cells which were observed on the permeate side of the membrane at day 7 were 0.54±0.14 μm (n=20 cells from four images). After 10 days, the bacteria observed on permeate side of membrane were more spheroidal. Consistent with the lack of mineralization activity, no bacteria were observed on permeate side of HMN-wetted (hexadecane-free) membranes over 10 days (Fig. 3E) even though cells were attached on the feed side of the membrane (Fig. 3F).

Bacterial morphology and size in the parent culture maintained in culture flasks containing hexadecane and BH under well-mixed conditions showed a change from rod-shaped cells during the initial exponential growth phase (aspect ratio, d_{min}/d_{max}=0.60±0.14, n=167), to coccoid cells

(d_{min}/d_{max}=0.86±0.10, n=153) in the stationary phase (Fig. 4). Similar observations of morphological changes during growth have been previously reported for *Rhodococcus equi*[14] and *E. coli* [15]. It has been suggested that bacteria may alter its shape in response to changes in nutrient levels in its environment and/or to regulate its attachment on a nutritional surface.[16] The d_{min} of *D. maris* cells was 0.71±0.07 (0. 50-0.93) μm during the early growth phase (day 1) in culture flasks and increased by 40% by the stationary phase.

Generally, bacterial movement and in particular their accessibility to nutrients and substrates in soils is related to their motility and chemotaxis.[17] However, our results show that nonmotile cells may access substrates in submicron pores through proliferation and penetration, accompanied by morphological changes associated with growth to be accommodated in narrow pores. The penetration mechanism in our study involved initial bacterial attachment and growth on hexadecane. Forces imposed by further growth, crowding and colony extension and reductions in surface tension as a result of biosurfactant production during growth likely contributed to bacterial translocation and passage through the pores. The Gram-positive *D. maris* entered 0.4 μm pores during growth, likely because of the cell shape and size during the early growth phase. This is different from the observation by Mannik *et al.* that Gram-negative *E. coli* bacteria migrated through 0.4 μm pores had aberrant cell shapes.[8]

In the context of bioremediation of hydrocarbon-contaminated soils, our results suggest if growth substrates are available at or near the pore mouth, which is more likely in early stages after oil spill or for high saturation of oil in soil aggregates, a wider range of pore sizes will be accessible to hydrocarbon degrading bacteria. For example, considering 0.4 μm as the limit for bacterial accessibility, 72% of the pore volume would be bioaccessible in the hydrocarbon-contaminated sub-Arctic soils from which *D. maris* was isolated, compared to 50% pore volume accessibility

assuming 1 μm as the accessibility cut off. These values were computed from mercury intrusion porosimetry data for aggregates of the field soils (Fig. S2).

In enhanced oil and shale gas recovery, targeted enhancement of growth of bacteria such as *D. maris* could enhance bioaccessibility of residual hydrocarbon compounds entrapped in small pores of oil reservoirs. This could enable biocracking of hydrocarbons and consequently enhance hydrocarbon recovery due to viscosity changes or hydrocarbon gas formation.[4, 5] This may lead to a more sustainable approach for enhanced hydrocarbon recovery than subsurface injection of surfactants to mobilize trapped oils.

Our results could also explain biofilm formation and fouling on the permeate side of membranes in water or wastewater treatment plants.[18] The implications of bacterial entry into sub-micron pores due to alterations of size, shape and growth activity in medicine include bacterial growth and infection in sub-micron gaps between imbuement and dental implants,[19] or bacterial penetration through restrictive blood vessel pores (0.3– 4.7 μm)[20] and deep into tissues to deliver therapeutic cytotoxic proteins to tumors or to directly kill cancer cells.[21, 22]

Supporting Information

Results of long term mineralization in bioreactors, and pore volume distribution in two representative clayey and sandy soils. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interests.

Acknowledgments

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Figure 1.

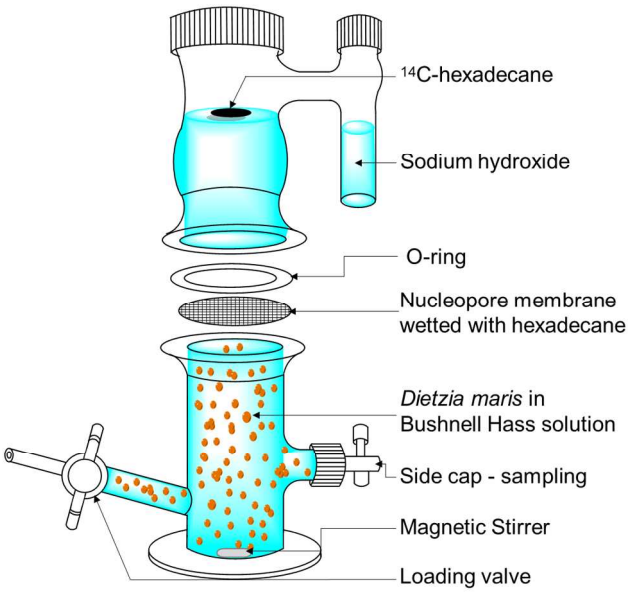

Figure 1. Schematic of the bioreactor used in the experiments.

Figure 2.

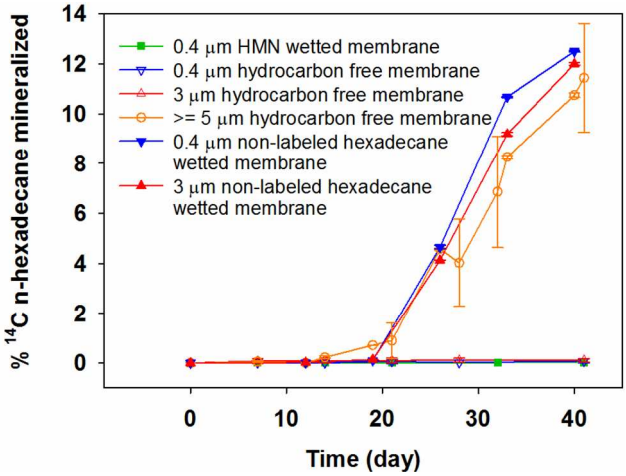

Figure 2. Hexadecane wetting of membranes increased bacterial entry into pores. Cumulative percent of $^{14}CO_2$ generation over time from mineralization of ^{14}C-hexadecane in bioreactors with hydrocarbon-free, and hexadecane- and HMN-wetted membranes with pore sizes of 0.4 and 3 μm. No mineralization was observed in non-inoculated controls. The data are average of at least two samples from at least two bioreactors (n=4). Data from systems with hexadecane and HMN wetted membranes are new results, and data from hydrocarbon-free membranes are represented from our previous study[3] for comparison. Mineralization data from bioreactors without membrane or with hydrocarbon-free membranes with 5, 8 and 12 μm pore diameters are grouped as a single data series as the trends were very similar.

Figure 3.

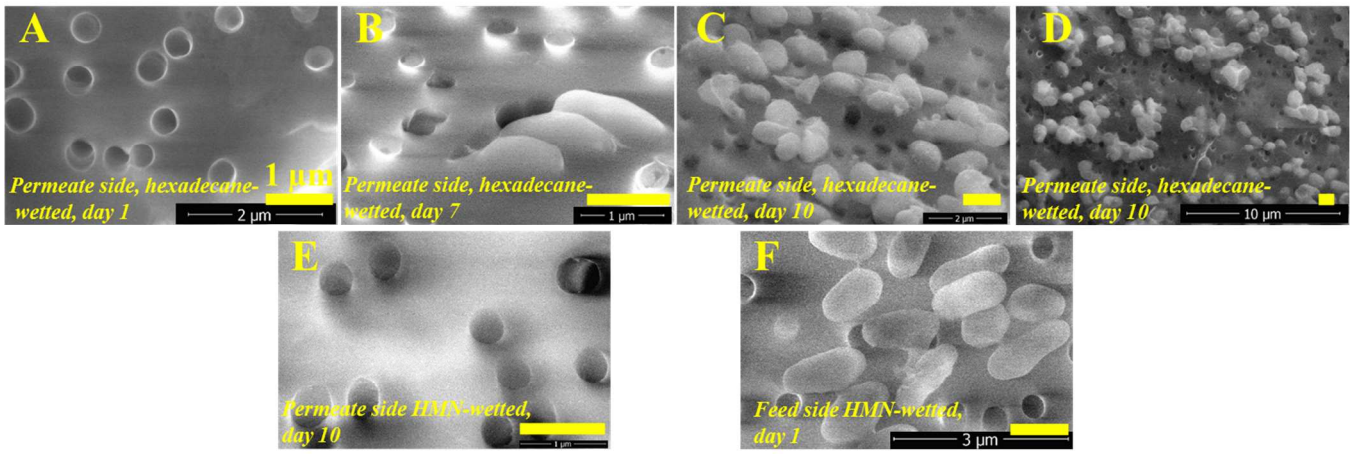

Figure 3. ESEM images of nuclepore track-etched polycarbonate membranes with pore size 0.4 µm reveals bacteria only pass through narrow pores of membranes wetted with hexadecane. Permeate side of hexadecane wetted membranes with 0.4 µm pores at day 1 (A), day 7 (B), day 10 (C, D) and HMN-wetted membrane permeate side at day 10 (E) and feed side at day 1 (F). Yellow scale bars in figures represent 1 µm.

Figure 4.

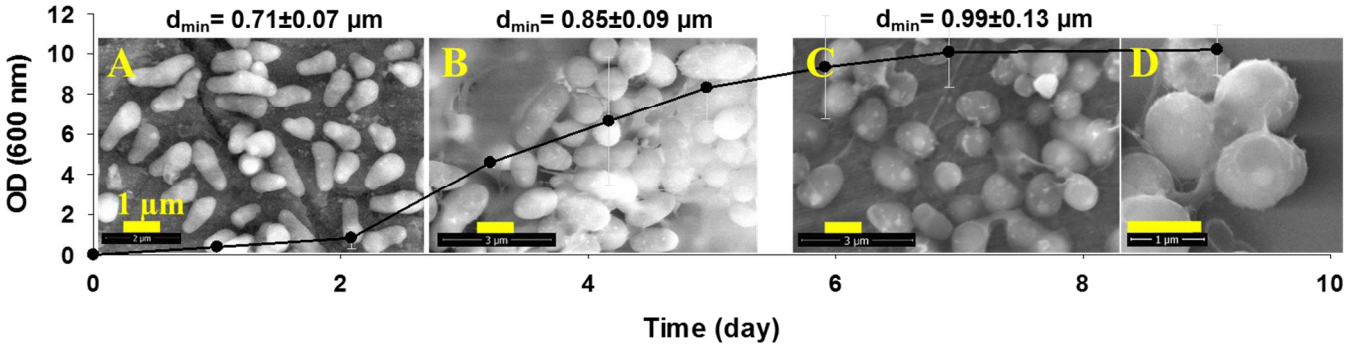

Figure 4. ESEM of *Dietiza maris* cells illustrating bacterial cell size changes at different growth phases in culture media; log phase, day 1 (A), log phase, day 3 (B), and stationary phase, day 10 (C,D). The d_{min} values are average of smaller cell dimensions of 167 cells measured from 11 ESEM images (A), 89 cells from 4 images (B), and 157 cells from 7 images (C). At least two culture flasks samples were used for each sampling day. Yellow scale bars in figures represent 1 µm.

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