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Degradation of Pyrene at Low Defined Oxygen Concentrations by a *Mycobacterium* sp.

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In a fermentor, a *Mycobacterium* sp. was grown on pyrene at defined oxygen concentrations in a range from 11.4 to 227 μM. The maximal growth rate (μ_{max} = 0.057 h^{-1}) and the dissolved oxygen half-saturation constant (K_{DO} = 5.9 μM) were calculated. At 3.4 μM, the growth rate (μ = 0.011 h^{-1}) was only half of what was expected from the kinetic data. Apparently, this was due to limitation of an oxygenase of pyrene degradation.

It is generally accepted that oxygen deficiencies in soils or sediments severely limit the biological degradation of hydrocarbons, including polycyclic aromatic hydrocarbons (PAH) (3, 4, 13). While naphthalene and acenaphthene are slowly degraded under denitrifying conditions (12), bioremediation of PAH with more than two rings appears to depend on aerobic conditions. Therefore, low oxygen concentrations, which often occur in waterlogged soils, might hinder bioremediation of contaminated sites. Low oxygen concentrations might also pose an increased risk of groundwater contamination by partially oxidized, water-soluble metabolites of PAH degradation. To my knowledge, no data to evaluate this risk are available. Molecular oxygen is utilized as an electron acceptor by cytochrome oxidase and as a source of oxygen atoms incorporated into hydrocarbons by mono- or dioxygenases. A limitation of the initial oxygenase would lead to reduced degradation and growth without the accumulation of intermediates. If another oxygenase of PAH degradation (e.g., an aromatic ring cleavage oxygenase) has a low affinity for oxygen, larger amounts of intermediates will be produced at low oxygen concentrations. If an oxidase reaction limits growth at low oxygen concentrations, a decrease in growth will be observed with both PAH and nonaromatic substrates. The aim of the present investigation was to determine the limits of pyrene degradation at low oxygen concentrations and a possible shift in the pattern of metabolites observed in the culture medium.

A PAH-degrading *Mycobacterium* sp. isolate was isolated from soil of a former coal gasification site. It is closely related to *Mycobacterium gilvum* (>99.8% homology of 16S rRNA [1a]). It utilizes phenanthrene, pyrene, and fluoranthene as sole sources of carbon and energy (1).

Investigations of exponential, non-carbon-limited growth are necessary to study the influence of low oxygen concentrations on the degradation of PAH for two reasons. On one hand, adaptation of bacteria to low oxygen concentrations has been observed (8, 16). On the other hand, degradation of these substances is often limited by the rate of mass transfer into the water phase because of the low solubility of PAH (e.g., 0.16 mg $liter^{-1}$ for pyrene [10]). The dissolution of crystalline or adsorbed substrates is governed by physical and chemical parameters rather than by biological factors. Consequently, degradation kinetics are often linear, and the cells are carbon limited (1, 17). Under these conditions, determination of the effect of low oxygen concentrations might be misleading.

We recently described a fermentor system that allows exponential, non-substrate-limited growth on various PAH (1). The system makes use of a high stirring rate and small PAH particles (high surface area) to obtain a high mass transfer rate. Using a quadrupole mass spectrometer as a sensitive measuring device for CO_2 formation, we determined the growth rates of the *Mycobacterium* sp. on various PAH. Growth conditions and analytical procedures of the present investigations were the same as those described previously (1). To maintain various oxygen concentrations, synthetic air was mixed with N_2 in defined proportions with a mass flow controller. Air-saturated medium contained 227 μM oxygen (7.26 mg $liter^{-1}$) under previously described growth conditions (14). Readings of a mass spectrometer data and of a Clark-type oxygen electrode showed good agreement with calculated results from the gas mixture. Growth rates calculated from CO_2 evolution, O_2 uptake, protein concentration, and optical density correlated well. Exponential growth was observed over a 30- to 50-fold increase in CO_2 evolution on pyrene. The maximal growth rate (μ_{max}) and dissolved oxygen half-saturation coefficient (K_{DO}) were calculated with Monod kinetics. Monod kinetics are consistent with the effect of low oxygen concentrations on microbial growth (2, 6, 15) and respiration (9).

During growth of the *Mycobacterium* sp. on pyrene at low oxygen concentrations, no metabolites other than those found at a high oxygen concentration (227 μM) were observed. The metabolites were not identified. Cinnamic acid and phthalate, which are metabolites of pyrene degradation by another *Mycobacterium* sp. (7), were not observed in the supernatants. Several metabolites of pyrene degradation by a *Mycobacterium* sp. and a *Rhodococcus* sp. were identified (7, 18), but the complete degradation sequence is not yet known.

The data from the present study show that Monod kinetics applied to growth of a *Mycobacterium* sp. on acetate in batch cultures. Exponential growth on acetate was found at oxygen concentrations from 3.4 to 227 μM. On acetate as the growth substrate, the K_{DO} was 3.8 μM, and the μ_{max} was 0.114 h^{-1} (r = 0.99) (Fig. 1). On pyrene as the growth substrate, Monod kinetics did not fit the results as well. Calculations from the data in the range of 11.4 to 227 μM oxygen resulted in a K_{DO} of 5.9 μM and a μ_{max} of 0.057 h^{-1} (r = 0.968) (Fig. 1). Despite this result, the measured growth rate (μ) at 3.4 μM was much lower (0.011 h^{-1}) than the one calculated from the coefficients

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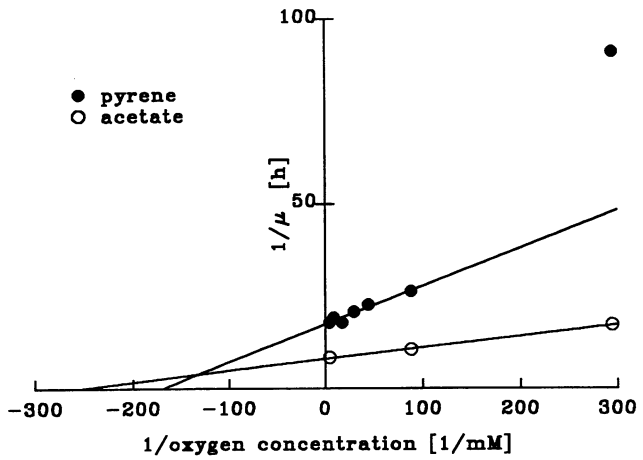

FIG. 1. Reciprocal plot of growth rate of a *Mycobacterium* sp. on pyrene and acetate as a function of dissolved oxygen concentration with linear regression. Growth on pyrene at 3.4 μM oxygen was omitted from the linear regression.

(0.021 h^{-1}). Growth at this oxygen concentration was influenced significantly by small fluctuations (±0.3 μM) in the oxygen concentration. These fluctuations were due to slow fluctuations in the mixture of the gas supply. Therefore, the growth rate was estimated from periods of growth during which the oxygen concentration was precisely 3.4 μM as determined by mass spectrometry. No growth was observed at 2.9 μM O_2 or lower concentrations.

The K_{DO} of whole cells does not necessarily reflect the Michaelis constant of a single limiting enzyme at low oxygen concentrations. Molecular oxygen is used as an electron acceptor and as a source of oxygen atoms incorporated into PAH. The degradation of phenanthrene shows that more oxygen is needed as an electron acceptor for respiration than as a substrate for the dioxygenases involved (5). This should also be true for pyrene degradation. Therefore, the substrate affinity of cytochrome oxidase might govern the growth rate until the degradation ceases because of limitation by an oxygenase. The K_{DO} (5.4 μM) of cells grown on pyrene thus reflects the activity of the cytochrome oxidase. The K_{DO} of cells grown on acetate was similar (3.8 μM). Other K_{DO} values for cells range from 0.4 to 44 μM (summarized in reference 15). The fact that no additional metabolites were observed during growth on 3.4 μM oxygen indicated that the initial oxygenase of pyrene degradation might be the rate-limiting enzyme at low concentrations. From the data of Heitkamp et al. (7), it can be concluded that the first enzyme of pyrene degradation that supports growth in the mycobacteria is a dioxygenase which transforms pyrene to pyrene-*cis*-4,5-dihydrodiol. The K_s of the initial oxygenase of our strain should be higher than 5.4 μM but could not be determined from our data. The Michaelis constants of some other oxygenases not involved in PAH metabolism range from 10 to 70 μM (summarized in reference 15). When a culture growing at 3.4 μM oxygen was switched to a lower concentration of 2.9 μM, an additional metabolite was observed in the supernatant, but CO_2 evolution ceased soon afterward. The metabolite was identified as protocatechuic acid. The occurrence of protocatechuic acid at 2.9 μM oxygen might reflect a limitation of protocatechuic acid dioxygenase while intracellular metabolites were still processed to form this substance.

Exponential growth of the *Mycobacterium* sp. used in this study was observed at oxygen concentrations as low as 3.4 μM. If the bacterium is able to grow exponentially, the demand for the carbon source will exceed the mass transfer of the hydrocarbon at some time and degradation will proceed at a linear rate. At low oxygen concentrations, the time at which linear growth occurs will be delayed because of the lower growth rate. If the initial exponential growth cannot be detected because of a low sensitivity of the measuring device and/or low mass transfer, a longer lag phase will be observed and will be followed by a linear degradation independent of oxygen concentration. The same should hold true for the degradation of pyrene by the *Mycobacterium* sp. in soil. Investigations of hexadecane degradation at low oxygen concentrations show similar results. Above a 1% dissolved oxygen tension (approximately 2.1 μM), the oxygen concentration does not influence hexadecane degradation. At a 0.4% dissolved oxygen tension, a longer lag phase was observed, but the substrate was degraded at a similar rate (11).

The present data show that during degradation of pyrene by a *Mycobacterium* sp. at low oxygen concentrations, no additional metabolites were produced. Thus, limited oxygen should not be an additional risk in terms of evolution of partly oxidized, water-soluble metabolites. To my knowledge, no other data of PAH degradation at low oxygen concentrations which indicate whether this holds true for other bacteria and other PAH as well are available.

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