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Responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene

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Abstract Several recent reports have indicated that some bacteria may have adapted to the low bioavailability of hydrophobic environmental chemicals and that generalizations about the bioavailability of compounds such as polycyclic aromatic hydrocarbons (PAHs) may be inappropriate. Experimental evidence and theoretical considerations show that the utilization of PAHs requires bioavailability-enhancing mechanisms of the bacteria such as: (1) high-affinity uptake systems, (2) adhesion to the solid substrate, and (3) biosurfactant excretion. We examined possible specific physiological responses of anthracene-degrading *Mycobacterium* sp. LB501T to poorly water-soluble anthracene in batch cultures, using solid anthracene as a sole carbon source. *Mycobacterium* sp. LB501T exhibited a high specific affinity for anthracene (a^o_A=32,500 l g^{-1} protein h^{-1}) and grew as a confluent biofilm on solid anthracene present as sole carbon source. No biofilm formation on anthracene was observed when excess glucose was provided as an additional substrate. This difference could be attributed to a modification of the cell surface of the bacterium. Anthracene-grown cells were significantly more hydrophobic and more negatively charged than glucose-grown cells. In adhesion experiments, anthracene-grown cells adhered 1.5- to 8.0-fold better to hydrophobic Teflon and up to 70-fold better to anthracene surfaces than glucose-grown cells. However, no production of biosurfactants was observed. Our results thus indicate that attachment and biofilm formation may be a specific response of *Mycobacterium* sp. LB501T to optimize substrate bioavailability.

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Introduction

The often observed low availability of soil contaminants for biological degradation appears to be primarily a physically controlled phenomenon (Alexander 1999). Attempts to improve bioavailability have therefore made use mostly of physical interventions, such as mixing or slurrying of soil. Recent observations, however, have indicated that specific bacterial adaptations to low contaminant availability may also exist and that generalizations about the availability of hydrophobic organic compounds (HOC) to bacteria may be inappropriate (Guerin and Boyd 1992; Bastiaens et al. 2000; Friedrich et al. 2000; Grosser et al. 2000). Many soil bacteria have been found to degrade and to live on HOC, despite the generally low bioavailability of these substrates. Some bacteria degrade HOC, either sorbed to a solid matrix or dissolved in an organic phase, faster than desorption or partitioning rates in the absence of bacteria would suggest (Harms and Zehnder 1995; Calvillo and Alexander 1996; Lahlou and Ortega-Calvo 1999). Moreover, different isolates degrade sorbed HOC at different rates, indicating organism-specific bioavailability (Guerin and Boyd 1992, 1997; Crocker et al. 1995; Tang et al. 1998; Grosser et al. 2000). Other recent reports indicate that sorption-limited bioavailability of polycyclic aromatic hydrocarbons (PAHs) is an important selective factor for PAH-degrading bacteria in enrichment cultures and that different bacteria inhabiting the same soil may be favored by the different degrees of PAH bioavailability (Tang et al. 1998; Bastiaens et al. 2000; Friedrich et al. 2000). It is in accordance with these findings that the bioavailability of HOC has been recognized to be influenced by physical and biological factors (Bosma et al. 1997). Bioavailability is described as the extent of mass transfer to microbial cells relative to the intrinsic catabolic potential of these organisms. This view accounts for the dynamics of biodegradation and points at the importance of continuous substrate supply for the progress of biodegradation. On the microscopic scale, it is the concurrence of an unequal spatial distribution of microorganisms and HOC and the

physically retarded transfer of HOC, for instance by sorption to organic matter, that is believed to cause low bioavailability and affect bioremediation measures (Luthy et al. 1994; Bosma et al. 1997; Zhang et al. 1998). As pollutant transfer in soil occurs mainly by diffusion, increased mass transfer may basically result from steeper concentration gradients between a substrate reservoir and catabolically active cells (Harms and Bosma 1997). HOC-degrading bacteria thus may improve the substrate transfer either by shortening their distance to a substrate source, for instance by attaching to it, or by developing high-affinity uptake systems that reduce more efficiently the aqueous phase substrate concentration in their surroundings (Wick et al. 2001a). Both mechanisms would lead to increased rates of substrate diffusion, desorption, or dissolution.

The purpose of the present study was to analyze possible specific physiological responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene, with an emphasis on the above-mentioned mechanisms. *Mycobacterium* sp. LB501T had been isolated using a new Teflon membrane-based extraction method that appeared to select strongly hydrophobic bacteria (Bastiaens et al. 2000). Previous experiments had shown that the provision of low amounts of solid anthracene as sole carbon source led to mass-transfer-limited pseudo-linear growth, with growth rates directly depending on the amount of solid substrate added (Wick et al. 2001b). The possibility to provoke limited bioavailability in a controlled way by adding defined, small amounts of solid anthracene made this bacterium a good candidate for this study.

Materials and methods

Bacterium and culture conditions

Mycobacterium sp. LB501T is an aerobic, rod-shaped bacterium capable of degrading and growing on anthracene (Bastiaens et al. 2000). *Mycobacterium* sp. LB501T was grown in a minimal medium (Harms and Zehnder 1994) containing 0.5% of a trace elements solution (Pfennig and Lippert 1966) in the presence of various amounts of solid anthracene or 2 g glucose l^{-1} as sole carbon sources. Anthracene (>98% by HPLC, Fluka, Buchs, Switzerland) was provided as crystals with diameters ranging over 0.2–0.5 mm, obtained by fractionation through a meshed wire sieve (DIN 4188; Retsch, Haan, Germany). Unless stated otherwise, cultures were grown at 25 °C on a gyratory shaker at 130 rpm in 300-ml Erlenmeyer flasks containing 100 ml of medium. Glucose-grown cells were harvested in the late exponential phase and washed three times in cold 10 mM phosphate-buffered saline (PBS) and used within 4 h. Cultures growing linearly on small amounts of solid anthracene (Wick et al. 2001b) were harvested after about 15 days growth. All solutions were prepared with deionized water (Nanopure cartridge system; SKAN, Basel, Switzerland). Water-dissolved anthracene concentrations during growth of *Mycobacterium* sp. LB501T in batch cultures containing 2 g anthracene l^{-1} were measured as follows. At different stages during cultivation, samples were taken with a 1-ml glass syringe (Hamilton Company, Reno, Nev., U.S.A.) and filtered through a 0.2-µm regenerated cellulose filter (RC 58; Schleicher & Schuell, Dassel, Germany) in a stainless-steel filter holder (Millipore, Bedford, Mass., U.S.A.) in order to remove suspended cells and anthracene crystals. Losses of dissolved anthracene due to sorption to the filters were minimal, as was determined in control experiments where solutions of known anthracene concentration were filtered and analyzed. The aqueous solubility at 25 °C of anthracene (C_w^{sat}) is 3.47×10^{-7} mol l^{-1} (Schwarzenbach et al. 1993).

Analytical methods

HPLC (Kontron Instruments, Zürich, Switzerland) analysis was performed on a RP-8 LiChroCART 125-4 column (E. Merck, Darmstadt, Germany), with a mobile phase of MeOH/water (70:30, v/v) and a flow of 1 ml min^{-1}; and anthracene was detected by fluorimetry (λ_{ex}=251 nm, λ_{em}=400 nm). Dissolved organic carbon (DOC) was measured with a TOCOR 2 (Mayhak, Hamburg, Germany) DOC analyzer. Scanning electron microscopy (SEM) was performed on Jeol JSM-6300S (Jeol, Tokyo, Japan) after gold-covering of the sample. Environmental SEM was performed on a Philips XL30 ESEM-FEG (Philips Electron Optics, Eindhoven, The Netherlands) without any sample preparation. Surface tensions of cell-free culture supernatants were measured with a plate device (DCA-322 dynamic contact angle analyzer; CAHN Instruments, Madison, Wis., U.S.A.), according to the Wilhelmy technique (Adamson 1990). Turbidity (optical density, OD) as a measure for the density of cell suspensions was determined with a Hitachi U2100 spectrophotometer (Hitachi Instruments, Tokyo, Japan) at 578 nm crystal, after carefully shaking the samples. Due to their size, anthracene crystals sedimented quickly and did not interfere with the OD measurements. Cell suspensions of OD_{578}>0.5 were diluted before measurement. Protein content of adhered and suspended biomass was quantified by the method of Spector (1978), using bovine serum albumin as protein standard.

Characterization of bacterial cell-surface properties

The electrophoretic mobility of bacterial suspensions in 10 mM PBS at pH 7.2 was determined in a Doppler electrophoretic light-scattering analyzer (Zetamaster; Malvern Instruments, Malvern, UK) at 100 V. The zeta potential (ζ)as an indirect measure of cell-surface charge was calculated from the electrophoretic mobility according to the method of Helmholtz-Smoluchowski (Hiementz 1986). Bacterial lawns needed for contact angle measurements were prepared by collecting cell suspensions in 10 mM PBS on 0.45-µm pore-size Micropore filters (Schleicher & Schuell, Dassel, Germany), mounting the filters on glass slides, and drying them for 2 h at room temperature. Cell-surface hydrophobicities were derived from the contact angles (θ_w) of water drops on the bacterial lawns, using a microscope with a goniometric eye-piece (Krüss, Hamburg, Germany) (van Loosdrecht et al. 1987). Cell radii (R) of acridine orange-stained cells were determined by epifluorescence microscopy (Olympus Optical Co., Tokyo, Japan) as $R=0.5(l\times w^2)^{1/3}$ with l and w being the length and width of the bacterium. The microscope was equipped with a digital camera (SenSys; Photometrics, Tucson, Ariz., U.S.A.) and a computer running the MetaView image-analysis software (Visitron Systems; Universal Imaging Corp., Puchheim, Germany). The number of cells per OD_{280} unit was determined by counting in a Thoma chamber under a light microscope.

Adhesion experiments

Solid surfaces

Coupons of Teflon 350 (a co-polymer of perfluoroalkoxyheptafluoropropylene, polytetrafluoroethylene) with a water contact angle (θ_w) of 105±1 °, glass slides (θ_w=12±2 °), and glass slides covered with thin layers of solid anthracene (θ_w=99±3 °) were used as model surfaces to study bacterial adhesion in batch experiments. In column experiments, Teflon granules with diameters of 382±50 µm (Dolder, Basel, Switzerland) and glass beads with diameters of 225±25 µm (Roth, Reinach, Switzerland) were used. Glass and Teflon were cleaned in chromosulfuric acid for 24 h and

subsequently rinsed, first with 0.5 M KCl and then with deionized water. Anthracene granules with average diameters of 450±50 µm were obtained by fractionation, using a meshed-wire sieve (DIN 4188; Retsch, Haan, Germany).

Batch experiments

Batch adhesion experiments were carried out in duplicate in sealed glass vials, according to a method described by Rijnaarts et al. (1993). Briefly, 15-ml glass vials containing flat pieces of Teflon, glass, or glass slides coated with anthracene were filled to the brim with 0.1 M PBS (glass, Teflon) or anthracene-saturated 0.1 M PBS (anthracene-coated glass slides) and sealed, avoiding any headspace. Various amounts of concentrated cell suspensions were gently injected through the stoppers into the vials in order to obtain four different initial suspended cell concentrations (C_0). The vials were immediately positioned on a slantwise-rotating wheel (8 rpm, amplitude of 0.1 m) and incubated at room temperature. After 2 h of incubation the vials were opened, 1 ml of the suspension was removed to determine its OD_{578}, and the number of attached cells were counted by epifluorescence microscopy after staining with acridine orange.

Column experiments

Column percolation experiments were performed at 25 °C, according to the method of Rijnaarts et al. (1993). Glass columns of 9.2 cm or 2.2 cm length and internal diameters of 1 cm were wet-packed with either glass beads, Teflon beads, or anthracene granules. The columns were connected to a peristaltic pump and suspensions of glucose- or anthracene-grown cells in 0.1 M PBS with $0.6<OD_{280}<0.7$ (1.2×10^8–1.5×10^8 cells ml^{-1}) were pumped through the columns at a constant flow rate in down-flow mode. The use of identical, empty bed flow rates led to different hydraulic flow velocities, depending on the respective porosities. Hydraulic flow rate, porosity, and pore volume were 0.43± 0.02 cm min^{-1}, 0.49±0.04, and 3.61±0.07 ml for glass, 0.43± 0.12 cm min^{-1}, 0.51±0.01, and 3.11±0.07 ml for Teflon, and 0.41± 0.05 cm min^{-1}, 0.72±0.02, and 1.23±0.03 ml for anthracene. Removal of bacterial cells was expressed as normalized steady-state effluent concentration (C/C_0). Experiments were performed in duplicate or triplicate. The omission of nutrients in the suspensions prevented significant bacterial proliferation during the short-term experiments.

Calculations

The adhesion efficiency of bacteria (α_t) is commonly defined as the ratio of the rate of attachment (η_t) to the rate of bacterial transport to the surfaces (η_{trans}; Elimelech and O'Melia 1990):

$$\alpha_t = \eta_t / \eta_{trans} \quad (1)$$

In the absence of repulsive forces, α_t reaches unity, whereas it falls below unity in the presence of repulsive forces. For columns filled with spheres of uniform size, a calculation method for η_{trans} was used that takes into account the contributions of convection, diffusion, sedimentation, and van der Waals attraction of the cells (Rajagopalan and Tien 1976; Martin et al. 1992; Rijnaarts et al. 1993). Experiments with glass and Teflon served to compare bacterial adhesion to a hydrophilic and a hydrophobic surface. For the case of solid anthracene, we were mainly interested in comparing adhesion efficiencies of differently pre-cultivated cells and we used Eq. 1, although our anthracene crystals were not spherical.

Degradation of dissolved anthracene by resting suspended cells

Aqueous anthracene solutions were prepared by adding crystalline anthracene to 0.1 M PBS, pH 7.3, containing 10% or 15% dimethylsulfoxid (DMSO) in screw-cap Erlenmeyer flasks. The DMSO served as a co-solvent. Control experiments showed that DMSO at this concentration had no influence on the activity of *Mycobacterium* sp. LB501T. The flasks were incubated on a gyratory shaker at 180 rpm for at least 24 h, to achieve saturation. Residual crystals were removed by filtration through glass frits. To prevent sorption losses, all solutions containing anthracene were handled with glass pipettes or glass syringes only. Degradation experiments were started by adding 1–2 ml of anthracene-pregrown cell suspension in 0.1 M PBS to the anthracene-saturated PBS/DMSO solutions to give final cell densities of 1.5×10^7–3×10^7 cells ml^{-1}. Samples of 1 ml were taken, analyzed, and used to calculate specific degradation rates as a function of anthracene concentration. As determined by controls that were poisoned with 15 mg $HgCl_2$ l^{-1}, total losses by abiotic degradation, evaporation, or sorption to cells or recipient walls were below 5% h^{-1} and thus negligible.

Quantification of adhered and suspended biomass during growth on solid anthracene

Eighteen batch cultures of *Mycobacterium* sp. LB501T containing 3 g solid anthracene l^{-1} were cultivated under identical growth conditions. At given intervals, duplicate cultures were picked randomly, their OD measured, and they were filtered through a fritted funnel covered with a paper filter (No. 589/1; Schleicher & Schuell, Dassel, Germany), to separate suspended cells from cells attached to solid anthracene. The protein content of the suspended biomass was analyzed. The filtered crystals were removed from the paper filter and resuspended in 5 ml 0.9% NaCl solution in a 10-ml test tube. The test tube was exposed to a Branson 1210 ultrasonic bath (Branson Ultrasonics, Danbury, Conn., U.S.A.) for 1.5 min, to release attached cells from the crystal surfaces; and the resulting suspension was analyzed for its protein content.

^{3}H-Leucine incorporation by attached and suspended bacterial cells

To compare the metabolic activity of suspended cells and biofilm cells associated with solid anthracene, ^{3}H-leucine incorporation tests were performed. Batch cultures at 9 days and 12 days old (OD_{578}=0.24, 0.54, respectively) pregrown on 2 g anthracene l^{-1} were filtered through a fritted funnel covered with a paper filter to separate suspended cells from anthracene-associated cells. Anthracene-associated cells accounted for about 37% of the total biomass in the batch studied. Replicate samples of 5 ml of the suspended biomass and 10–13 mg of overgrown anthracene crystals were incubated separately with 50 nM of ^{3}H-leucine (48 Ci $mmol^{-1}$; Amersham Life Science, Cleveland, Ohio, U.S.A.) for 30 min in 15-ml screw-cap tubes under reduced light conditions. The incubation was stopped by the addition of six drops of a 36% formaldehyde solution (Fluka). The samples were filtered through a 0.22-µm nitrocellulose filter (25 mm, Millipore) and exposed to 10 ml of ice-cold 5% trichloroacetic acid (TCA) for 15 min, filtered again, and rinsed twice with about 3 ml chilled TCA (5%). Then, 10 ml of Filter Count (Packard, Canberra Co.) scintillation cocktail were added, shaken vigorously, and allowed to stand overnight. The radioactivity was measured in a Kontron BETA-matic I liquid scintillation counter (Kontron Instruments, Zürich, Switzerland). Blanks contained about 0.1% formaldehyde. No quenching by the anthracene crystals was observed, as the scintillation cocktail dissolved most of the crystals.

Results

Growth of *Mycobacterium* sp. LB501T on solid anthracene

When *Mycobacterium* sp. LB501T was grown in shaken batch cultures with 3 g anthracene crystals l^{-1} as the sole carbon source, biofilm formation on the crystals con-

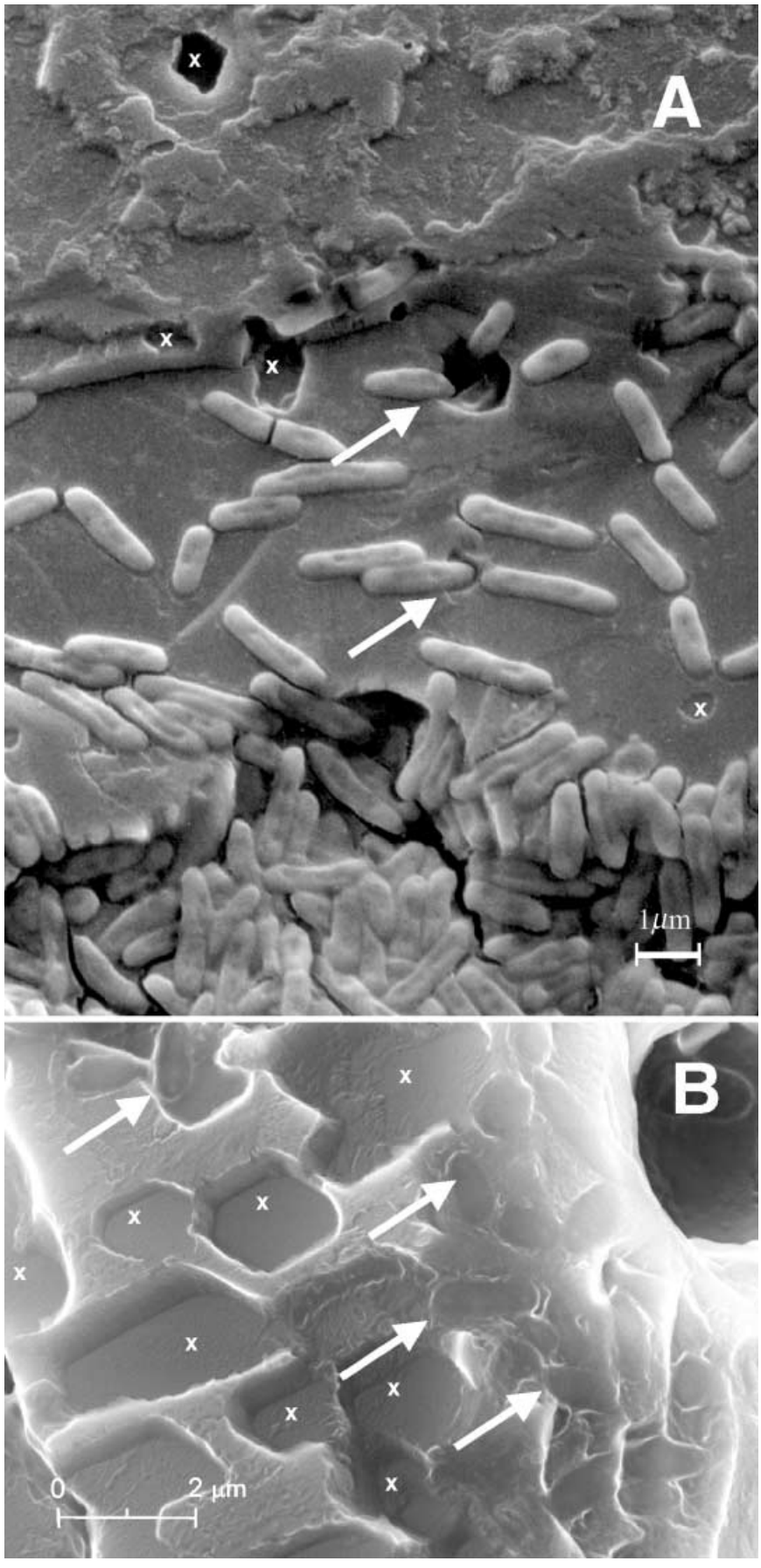

Fig. 1 Scanning electron micrograph (**A**) and environmental scanning electron micrograph (**B**) of *Mycobacterium* sp. LB501T on an anthracene surface, taken during early stages of biofilm formation by *Mycobacterium* sp. LB501T. Craters on the anthracene surface (*x*) were absent from fresh anthracene crystals (not shown) and thus can contribute to anthracene consumption by surface-associated bacterial cells (*arrows*)

Table 1 Optical density at 578 nm (OD_{578}), surface tension and protein content of the adhered and suspended biomass of *Mycobacterium* sp. LB501T growing in shaken batch cultures on 3 g solid anthracene l^{-1}. Values are average ±SD of duplicate measurements. Protein *crys* content of the biomass adhered to 0.3 g of solid anthracene, protein *sus* content of the biomass suspended in 0.1 l of liquid culture, protein *tot* content of adhered and suspended biomass, *%Crystal* fraction of the total biomass adhered to solid anthracene

OD_{578}	Surface tension (μN cm^{-1})	$Protein_{crys}$ (μg)	$Protein_{sus}$ (μg)	$Protein_{tot}$ (μg)	$\%_{Crystal}$
0.007±0	730±10	0	21±0	21±0	0.0±0
0.012±0.005	740±0	51±13	99±66	146±79	35±9
0.042±0.035	730±2	87±20	234±272	321±252	27±6
0.108±0.031	730±18	209±75	405±184	614±259	34±12
0.205±0.015	720±0	248±27	463±311	711±284	35±4
0.551±0.105	670±12	397±207	632±99	1,029±306	39±20
0.696±0.070	665±7	363±276	1,344±230	1,706±46	21±16
1.346±0.053	665±49	1,559±17	2,252±211	3,811±228	41±0.5
1.400±0.035	705±28	975±947	5,011±1603	5,986±656	16±15

tributed up to 40% to the total biomass during all growth phases (Table 1). When grown in unshaken batch cultures, more than 95% of the total biomass was crystal-bound (data not shown). This indicates that growth preferentially took place on the crystal surface and that suspended cells represented biofilm cells that were shed-off. SEM images taken at early stages of biofilm formation showed craters on the anthracene surface, some of which enclosed bacterial cells (Fig. 1). These craters were absent from fresh anthracene crystals (data not shown) and can be attributed to anthracene dissolution, driven by anthracene consumption by surface-associated bacteria. To test whether anthracene was available to suspended cells, we followed the water-dissolved anthracene concentration during growth on 2 g crystalline anthracene l^{-1} (Fig. 2). During the lag phase, dissolved anthracene remained within the range of aqueous solubility but, with commencing growth, it decreased rapidly and dropped below the detection limit of $\leq 10^{-8}$ mol l^{-1} when $OD_{578}>0.1$. Despite the virtual absence of anthracene in the water phase, the suspended cell concentrations increased to $OD_{578}>1.5$ (Wick et al. 2001b). Interestingly, *Mycobacterium* sp. LB501T did not colonize anthracene crystals when excess dissolved glucose as additional carbon source was present or when anthracene was provided in quantities of 25 g l^{-1} or higher.

To compare the metabolic activity of suspended cells and biofilm cells, ^{3}H-leucine incorporation experiments were performed. Surprisingly, suspended cells incorporated more ^{3}H-leucine (7 μg Ci mg^{-1} protein h^{-1}) than biofilm cells (3 μg Ci mg^{-1} protein h^{-1}), indicating that they had a high metabolic potential, even in the absence of significant water-dissolved anthracene. As we did not disperse the biofilm prior to ^{3}H-leucine exposure, we cannot exclude that cells in both states were equally active and that reduced ^{3}H-leucine incorporation by attached cells was due to lower availability of ^{3}H-leucine to biofilm cells.

Mycobacterium sp. LB501T did not excrete surface-active agents, as was seen from the absence of a decrease in surface tension of cultures growing on 2–3 g solid anthracene l^{-1} (Table 1).

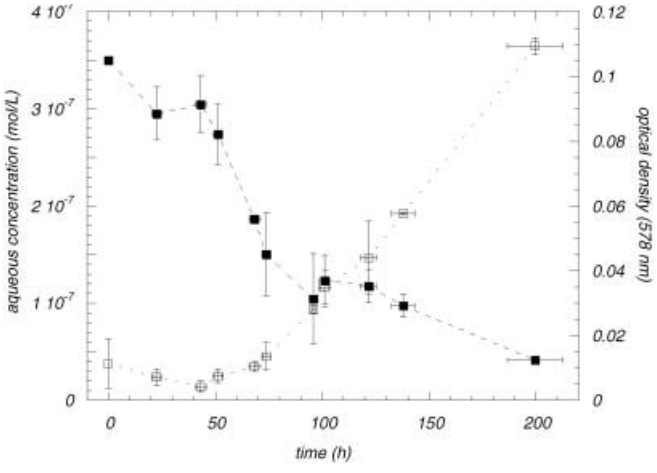

Fig. 2 Time course of the aqueous anthracene concentration (*filled squares*) during growth (measured as the optical density at 578 nm) of *Mycobacterium* sp. LB501T (*open squares*) in a batch culture with 2 g solid anthracene l^{-1} as sole carbon source. Standard deviations (n=3) of anthracene concentrations are indicated by error bars (some hidden by the symbols). *L* Liter

Degradation of dissolved anthracene by *Mycobacterium* sp. LB501T

In degradation experiments where only water-dissolved anthracene was provided, a good agreement between the concentration needed to saturate the anthracene uptake system of *Mycobacterium* sp. LB501T and the aqueous solubility of anthracene (62 μg l^{-1}) was found. Approximately 80% of the maximal rate of substrate uptake (q_{max}) of anthracene degradation was reached with a saturated aqueous anthracene solution. Increasing the solubility of anthracene with the co-solvent DMSO had only little effect on the uptake rate, as q_{max} in the presence of DMSO ($7.1\times10^{-6}\pm6\times10^{-7}$ mol g^{-1} protein h^{-1}) was similar to q_{max} in the absence of DMSO (7.8×10^{-6} mol g^{-1} protein h^{-1}; Wick et al. 2001b). From the activity versus concentration plot, the first order rate constant, also referred to as the specific affinity (a_A^0; l g^{-1} protein h^{-1}), was calculated by dividing q_{max} by the half saturation constant (K_m;

Table 2 Effect of the growth substrate on the size and physicochemical surface properties of *Mycobacterium* sp. LB501T. Values are means ±SD from n=145 (anthracene) and n=90 (glucose). The mean radius was calculated as: $R=0.5(l\times w^2)^{1/3}$, l and w being the length and width of the bacterium. For water contact angle (Θ_w) and zeta potential (ζ), n=4 (anthracene) and n=3 (glucose); and determinations were performed at pH 7.2

Substrate (μm)	Cell width (μm)	Cell length (μm)	Mean radius (degrees)	Θ_w (mV)	ζ
Anthracene	0.71±0.11	1.3±0.08	0.43±0.02	66±2	–53.9±5.3
Glucose	0.79±0.1	1.45±0.06	0.48±0.03	49±5	–31.7±4.7

Table 3 Influence of the growth substrate on attachment of *Mycobacterium* sp. LB501T in saturated columns containing various packing materials. Values are means ±SD (n=4 measurements at various bacterial concentrations)

Substrate	Adhesion efficiency (α_t) on surface		
	Glass	Teflon	Anthracene
Anthracene	0.78±0.19	1.70±0.19	2.77±0.23
Glucose	0.79±0.05	1.1±0.18	1.81±0.10

mol l^{-3}). The a^o_A of *Mycobacterium* sp. LB501T for anthracene was 32,500 l g^{-1} protein h^{-1} (Wick et al. 2001b).

Substrate influence on cell-surface characteristics and bacterial adhesion

The observation that biofilm formation occurred with small amounts of anthracene as the sole substrate source suggests that the substrate regime may affect the surface properties of *Mycobacterium* sp. LB501T. Comparison of the cell-surface hydrophobicity as a measure of surface free energy and ζ as an indirect measure of cell-surface charge gave significant differences between anthracene- and glucose-grown cells (Table 2). Cells grown on glucose were slightly larger, significantly more hydrophilic, and less negatively charged than anthracene-grown cells. The growth substrate also influenced the efficiency of the adhesion of *Mycobacterium* sp. LB501T cells to glass, Teflon, anthracene, and anthracene-covered surfaces. Column percolation experiments (Table 3) and static batch adhesion experiments (Table 4) showed clear differences between anthracene- and glucose-grown cells. Anthracene-grown cells adhered better than glucose-grown cells to the hydrophobic surfaces, Teflon (1.5-fold in columns, 8-fold in batches) and anthracene (1.5-fold in columns, 70-fold in batches). In contrast, glucose-grown cells adhered 5-fold better to glass in the batch system than anthracene-grown cells. In the column system, anthracene- and glucose-grown cells adhered equally well to glass. The column system was generally much less discriminative than the batch system. This difference resulted probably from the dominant transport mechanisms being advective diffusion in the column system and diffusion in the batch system. Anthracene-grown cells preferred hydrophobic Teflon to hydrophilic glass by factors of 2.2 in columns and 38 in batches, whereas glucose-grown cells showed only a slight preference (1.4-fold) for Teflon in columns and none in batches. In the batch system, anthracene-grown cells adhered 245-fold better to anthracene-covered surfaces than to glass, and 7-fold better than to Teflon. In contrast, glucose-grown cells adhered similarly well to all three surfaces. As pointed out before, α_t values obtained with irregular anthracene surfaces in column experiments are not directly comparable with the corresponding α_t obtained with the regularly shaped surfaces of glass and Teflon. It should be noted that α_t values obtained with anthracene-grown cells and Teflon exceeded unity, which is in apparent contradiction of theory. As the calculation of η_{trans} and consequently α_t does not account for the active mobility of bacterial cells, we may have underestimated the transport of cells toward the surfaces and thus somewhat overestimated the adhesion efficiency of *Mycobacterium* sp. LB501T.

Discussion

Colonization of solid anthracene by *Mycobacterium* sp. LB501T

When provided with low amounts of solid anthracene, *Mycobacterium* sp. LB501T attaches to anthracene crystals, where it develops a biofilm. No such biofilm formation is found with low amounts of solid anthracene in the

Table 4 Influence of growth substrate on the attachment of *Mycobacterium* sp. LB501T in batch systems to glass, Teflon, and anthracene-covered surfaces. Values are means ±SD (n=4 measurements at various bacterial concentrations). N_a Number of cells adhering cm^{-2} after 2 h, N_s initial number of cells ml^{-1} in suspension

Substrate	Relative number of adhered bacterial cells (N_a/N_s) on surface		
	Glass	Teflon	Anthracene
Anthracene	$2.0\times10^{-4}\pm9.2\times10^{-5}$	$7.7\times10^{-3}\pm2.4\times10^{-3}$	$4.9\times10^{-2}\pm2.7\times10^{-2}$
Glucose	$1.0\times10^{-3}\pm4.8\times10^{-4}$	$9.4\times10^{-4}\pm6.4\times10^{-5}$	$7.0\times10^{-4}\pm4.3\times10^{-4}$

presence of glucose as a second carbon substrate, or when high amounts of solid anthracene (resulting in high anthracene dissolution fluxes) are provided. Hence, it appears that biofilm formation does not occur under high substrate regimes. This in turn suggests that biofilm formation may be required for growth under conditions of low substrate availability. This view is corroborated by the observed influence of the growth regime on the cell wall characteristics of *Mycobacterium* sp. LB501T. Particularly, the increased hydrophobicity of anthracene-grown cells, as opposed to glucose-grown cells, is in accordance with their tendency to form biofilms on hydrophobic anthracene.

Cryo-SEM images of *Mycobacterium* sp. LB501T biofilms exhibited direct contact of extracellular polymeric substances (EPS) with the anthracene surface (Wick et al. 2001b). The distance of the nearest bacterium from the surface was ≤1 µm, but direct contact of cell bodies with anthracene was not observed. SEM and environmental SEM images nevertheless showed traces of the local utilization of anthracene (Fig. 1), indicating an increased, consumption-driven anthracene dissolution. Theoretical considerations and experimental evidence indeed show that the small distance between a biofilm and a solid substrate favors diffusive mass transfer and, consequently, dissolution, by giving rise to steep aqueous concentration gradients between the surfaces of active cells and the surface of their substrate source (Harms and Zehnder 1995). Moreover, the biofilm cells appeared to consume most of the dissolving solid anthracene, as was seen from very low dissolved anthracene concentrations in the bulk medium (Fig. 2). It seems therefore that growth predominantly took place on the crystals, from which biofilm cells were released into the medium. The permanently low aqueous anthracene concentrations furthermore confirmed that *Mycobacterium* sp. LB501T did not excrete biosurfactants, which would have transferred anthracene into the aqueous phase by solubilization. Surprisingly, suspended cells had a high metabolic potential. We therefore estimated whether the observed dissolved anthracene concentrations, below 10^{-8} mol l^{-1}, would be sufficient to fulfill the maintenance requirements of anthracene-grown cells and thereby keep them active. Solving the extended Monod equation (van Uden 1967) for the aqueous anthracene concentration (C) at maintenance, by using the reported half-saturation constant (K_m=2.4×10^{-4} mol m^{-3}), the maximum specific growth rate (μ_{max}=0.036 h^{-1}), and the maintenance rate coefficient (b=0.00069 h^{-1}) of *Mycobacterium* sp. LB501T (Wick et al. 2001b) resulted in an aqueous maintenance anthracene concentration of 4.7×10^{-9} mol l^{-1}. This concentration may have been available to suspended cells, as it is 50% below our detection limit. Maintenance of suspended cells with dissolved anthracene and possibly even slow growth in suspension can therefore not be excluded.

Substrate affinity of *Mycobacterium* sp. LB501T

Another important characteristic of bacteria subjected to low substrate is their specific affinity (a_A^0; Button 1985), i.e. the efficiency to reduce the substrate concentration at their surfaces, in relation to the bulk aqueous substrate concentration. High specific affinities create steep concentration gradients and fast, diffusive substrate transfer. For instance, the observed value of 32,500 l g^{-1} protein h^{-1} is in the range of the a_A^0 values of the efficient dibenzofuran-degrader *Sphingomonas* sp. HH19k for the HOC, dibenzofuran (62,700 l g^{-1} protein h^{-1}; Harms and Zehnder 1994), but above the a_A^0 values of *Pseudomonas* sp. T2 (1,000 l g^{-1} protein h^{-1}; Button 1985) and *P. putida* G7 (5,000 l g^{-1} protein h^{-1}; Ahn et al. 1998) for the much better and soluble toluene (C_w^{sat}=5.62×10^{-3} mol l^{-1}) and naphthalene (C_w^{sat}=2.45×10^{-4} mol l^{-1}). It is also well above the a_A^0 values (20–140 l g^{-1} protein h^{-1}) of mixed cultures degrading naphthalene, phenanthrene (C_w^{sat}=6.36×10^{-6} mol l^{-1}), and pyrene (C_w^{sat}=6.76×10^{-7} mol l^{-1}; Guha et al. 1999). This indicates that strain *Mycobacterium* sp. LB501T is well adapted to the naturally occurring low range of aqueous anthracene concentrations (C_w^{sat}=3.47×10^{-7} mol l^{-1}).

Implications for anthracene utilization

Living on anthracene is a challenge for a soil bacterium like *Mycobacterium* sp. LB501T. This compound causes mass transfer limitation, even under the most favorable conditions, for instance when it is supplied as accessible crystals in liquid medium. However, it appears that *Mycobacterium* sp. LB501T responds specifically to the fact that anthracene is hydrophobic and/or enriches in hydrophobic phases. *Mycobacterium* sp. LB501T changes its cell-surface properties and adhesion tendency, depending on the growth substrate; and it attaches to HOC crystals only when a low anthracene dissolution flux prevails. This indicates that attachment is an actively regulated strategy to optimize substrate availability and that, by forming biofilms, *Mycobacterium* sp. LB501T optimizes the anthracene dissolution flux within the physically determined limits. Biofilm formation and attachment to pure solid, liquid, non-aqueous-phase liquid-dissolved, and sorbed chemicals have been reported repeatedly (e.g. Goswami and Singh 1990; Bouchez et al. 1995; Tongpim and Pickard 1996; Linos et al. 2000). Mycobacteria and related genera are known for the particular hydrophobicity of their cell surfaces, due to compounds such as mycolic acids in their cell walls. These molecules are believed to stimulate attachment to hydrophobic surfaces (Bendinger et al. 1993). Its preference for hydrophobic surfaces may explain why *Mycobacterium* sp. LB501T was isolated when a Teflon membrane was used to enrich anthracene-degrading bacteria from soil and not when a conventional enrichment protocol was applied to the same soil (Bastiaens et al. 2000). Besides the peculiarities of its cell surface, *Mycobacterium* sp. LB501T appears to have further adaptations for the utilization of substrates of permanently low bioavailability. It exhibits a high specific affinity for anthracene, combined with very low substrate requirements for cell maintenance. However, unlike many bacteria utilizing sparingly soluble compounds (Hommel

1990; Neu 1996), *Mycobacterium* sp. LB501T does not excrete surface-active compounds. It may be speculated as to whether this strategy would be successful in soils, unless they contained a non-aqueous contaminant phase. Given the enormous total interfacial area in a soil and the narrowness of water-filled pores, biosurfactant excretion would be very costly, due to heavy losses of biosurfactant molecules by sorption to interfaces. This view is corroborated by the frequently negative experiences in the application of surfactants in engineered soil remediation.

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