Direct Characterization of the Physicochemical Properties of Fungal Spores Using Functionalized AFM Probes

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ABSTRACT A new method is described for characterizing the physicochemical properties of native microbial cells by using atomic force microscopy (AFM) with chemically functionalized probes. Adhesion forces were measured, under deionized water, between probes and model substrata functionalized with alkanethiol self-assembled monolayers terminated with OH and CH_3 groups. These were found to be 6 ± 2 nN ($n = 1024$), 0.9 ± 0.4 nN, and ~0 nN, for CH_3/CH_3, CH_3/OH, and OH/OH surfaces, respectively, and were not significantly influenced by changes of ionic strength (0.1 M NaCl versus deionized water). This shows that functionalized probes are very sensitive to changes of surface hydrophobicity. Using OH- and CH_3-terminated probes, patterns of rodlets, ~10 nm in diameter, were visualized, under physiological conditions, at the surface of spores of *Phanerochaete chrysosporium*. Multiple (1024) force-distance curves recorded over 500×500-nm areas at the spore surface, either in deionized water or in 0.1 M NaCl solutions, always showed no adhesion for both OH- and CH_3-terminated probes. Control experiments indicated that the lack of adhesion is not due to transfer of cellular material onto the probe, but to the hydrophilic nature of the spore surface.

INTRODUCTION

Microbial cell adhesion and aggregation play a crucial role in natural environments as well as in industrial processes (Savage and Fletcher, 1985; Characklis and Marshall, 1990). Understanding the fundamental mechanisms underlying these processes requires knowledge of the cell physicochemical properties, i.e., surface hydrophobicity and surface electrical properties, as well as of molecular interactions acting at the cell surface, including van der Waals and electrostatic interactions, solvation interactions, and macromolecular interactions. The osmotic stress method (Le Neveu et al., 1976), the surface forces apparatus (Marra and Israelachvili, 1985), and the micropipette technique (Evans, 1980), have provided valuable insight into interaction forces at biological interfaces. However, these techniques are not suited for mapping forces at the surface of single cells due to their poor lateral resolution. Hence, there is a great interest in developing methods capable of mapping molecular interactions at the surface of microbial cells at high spatial resolution.

During the last decade, atomic force microscopy (AFM) has emerged as a valuable tool for visualizing the morphology of biological surfaces (Weisenhorn et al., 1990; Butt et al., 1990; Radmacher et al., 1992; Southam et al., 1993; Schabert et al., 1995; Pum and Sleytr, 1996; Dufrêne et al., 1997, 1999) at high spatial resolution and for probing interaction forces (Ducker et al., 1991; Lee et al., 1994; Butt et al., 1995). Quantitative force measurements with the AFM require probes of well-defined surface chemistry. This can be achieved by functionalizing gold-coated probes with self-assembled monolayers (SAMs) of organic thiols terminated with selected chemical groups. Although such an approach has already been used to characterize a variety of thin organic films (Frisbie et al., 1994; van der Vegte and Hadziioannou, 1997; Vezenov et al., 1997) and polymer surfaces (Sinniah et al., 1996), applications dealing with the surface of native microbial cells have not been reported.

In this paper I present the direct characterization of the surface properties (morphology, molecular interactions) of microbial cells by using AFM with chemically functionalized probes. Probes and model substrata are functionalized with alkanethiol SAMs terminated with OH (hydrophilic) and CH_3 (hydrophobic) groups. The surface chemical composition of substrata is determined using x-ray photoelectron spectroscopy, and force-distance curves are recorded between functionalized probes and substrata in aqueous solutions. Functionalized probes are then used to investigate the surface properties of spores of the fungus *Phanerochaete chrysosporium*. Previous investigations have shown that the spore surface is coated with a thin layer of regularly arranged rodlets that are mainly made of proteins (Gerin et al., 1993, 1994). However, little is known about the spore physical properties, and in particular about their surface hydrophobicity, due to the lack of direct surface measurements.

MATERIALS AND METHODS

Oxide-sharpened microfabricated Si_3N_4 cantilevers (Park Scientific Instruments, Mountain View, CA), with spring constants of 0.03 N/m, and silicon wafers (Wacker Chemitronic, Burghausen, Germany) were coated by electron beam thermal evaporation with a 4-nm-thick titanium layer followed by a 30-nm-thick gold layer. The coated surfaces were immersed for 18 h in 1 mM solutions of $HS(CH_2)_{11}OH$ and $HS(CH_2)_{17}CH_3$ in ethanol and then rinsed with ethanol. Functionalized probes and substrata were always used immediately after they were prepared.

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The surface chemical composition of functionalized substrata was determined by x-ray photoelectron spectroscopy (XPS), using an SSI X-Probe (SSX-100/206) photoelectron spectrometer from Fisons, interfaced with a Hewlett Packard 9000/310 computer allowing instrument control, data accumulation, and data treatment. The pressure during analysis was between 2.5×10^{-6} Pa and 2.5×10^{-7} Pa. The spectrometer used monochromatized aluminum K_α x-ray radiation (1486.6 eV). The irradiated zone was an elliptic spot, with a shorter axis of 1000 μm. The constant pass energy in the hemispherical analyzer was 150 eV. In these conditions, the resolution determined by the full-width at half-maximum of the $Au_{4f7/2}$ peak of a standard gold sample was ~1.5 eV. Binding energies were determined by reference to the C_{1s} component due to carbon bound only to carbon and hydrogen, set at 284.8 eV. The background was subtracted linearly. Data treatment was performed with the ESCA 8.3 D software provided by the spectrometer manufacturer. Atom fractions were calculated using the peak areas normalized on the basis of acquisition parameters and of sensitivity factors proposed by the manufacturer (mean free path varying according to the 0.7th power of the photoelectron kinetic energy; Scofield cross-sections (Scofield, 1976); constant transmission function).

AFM measurements were made at room temperature (20°C), either in deionized water or in 0.1 M NaCl solutions, using an optical lever microscope equipped with a liquid cell (Nanoscope III, Digital Instruments, Santa Barbara, CA). High-resolution images of the spore surface were recorded in deflection mode. The applied force was maintained below 1 nN and the scan rate was ~2 Hz. The sensitivity of the AFM detector was estimated using the slope of the retraction force curves in the region where probe and sample are in contact. Adhesion force histograms were obtained by recording 32×32 force-distance curves on areas of given size and calculating the pull-off force measured for each force curve.

Conidiospores of *P. chrysosporium* INA-12 (Collection Nationale de Culture de Microorganismes I-398, Institut Pasteur, Paris, France) were collected from mycelial mats as described earlier (Gerin et al., 1993). They were immobilized by mechanical trapping in an Isopore polycarbonate membrane (Millipore) with pore size similar to the spore size (Kasas and Ikai, 1995). After filtering a spore suspension (10 ml; 10^6 cells per ml), the filter was carefully cut (1×1 cm) and transferred into the AFM liquid cell.

RESULTS AND DISCUSSION

To validate the surface functionalization procedure, model silicon substrata were treated in the same way as the probes and analyzed by XPS. The surface chemical composition of gold-coated substrata after self-assembly of CH_3- and OH-terminated alkanethiols is shown in Table 1. The apparent carbon concentration is high, and sulfur is detected in significant concentration at the surface. For the OH-functionalized substratum, a significant amount of oxygen is found, consistent with the presence of hydroxyl groups at the surface. Interestingly, the S/C ratios determined by XPS for CH_3- and OH-terminated monolayers (0.024 and 0.05, respectively) are much lower than the S/C ratios deduced from the stoichiometric composition of the molecules (0.06 and 0.09, respectively). This indicates that alkanethiols form oriented monolayers in which the sulfur atoms are in contact with gold, in agreement with the literature data (Bain et al., 1989). The water contact angles measured for CH_3- and OH-terminated monolayers, given in Table 1, are indicative of hydrophobic and hydrophilic surfaces, respectively.

The forces measured between functionalized probes and substrata in deionized water are plotted as a function of surface separation in Fig. 1. Multiple (1024) force curves were recorded on 1×1-μm areas at different locations and the adhesion forces upon retraction were measured for each force curve. The resulting adhesion histograms, Fig. 1, show fairly narrow distributions, indicating reproducibility of the measured forces and their excellent representativity for the three probe/substratum combinations. For the CH_3/CH_3 combination, large adhesion forces, of 6 ± 2 nN ($n = 1024$) magnitude, are observed upon retraction. In contrast, the curves recorded between the CH_3-terminated probe and OH-terminated surface show much lower adhesion forces, of 0.9 ± 0.4 nN magnitude, while forces ~0 nN are recorded between like OH-terminated surfaces. The trend observed when comparing the different probe/substratum combinations are consistent with previous studies (Sinniah et al., 1996; Vezenov et al., 1997).

The role of electrostatic interactions was tested by recording force-distance curves in 0.1 M NaCl. In these conditions, adhesion forces of 7 ± 2 nN ($n = 1024$) magnitude are measured between two CH_3-terminated surfaces, while no adhesion is always observed between OH-terminated surfaces. Hence, the results are not significantly affected by changes of ionic strength, pointing to the fact that electrostatic interactions play a minor role in determining the measured adhesion forces. These observations are consistent with the expectation that both hydroxyl- and methyl-terminated probes should have a net zero surface charge. Accordingly, the above results demonstrate that chemically functionalized AFM probes can differentiate between solids having distinct surface hydrophobicities or surface energies.

Functionalized probes were then used to characterize the surface of spores of *P. chrysosporium* under aqueous conditions. High-resolution images of spores recorded with

TABLE 1 Chemical composition and water contact angle of gold-coated silicon substrata after self-assembly of alkanethiols

	Atom fraction (%)*				
	$Au_{4f7/2}$	C_{1s}	S_{2p}	O_{1s}	θ_w(deg)†
Gold-coated silicon + $HS(CH_2)_{17}CH_3$	22.2 (1.1)	76.0 (1.2)	1.8 (0.05)	<0.2	111 (0.5)
Gold-coated silicon + $HS(CH_2)_{11}OH$	31.5 (0.7)	58.1 (0.8)	2.8 (0.01)	7.8 (0.1)	20 (2)

*Determined by XPS; mean value of two independent sets of measurements with the difference between duplicates in parentheses.

†Water contact angle measured by the sessile drop technique; mean value of two independent sets of measurements with the standard deviation in parentheses.

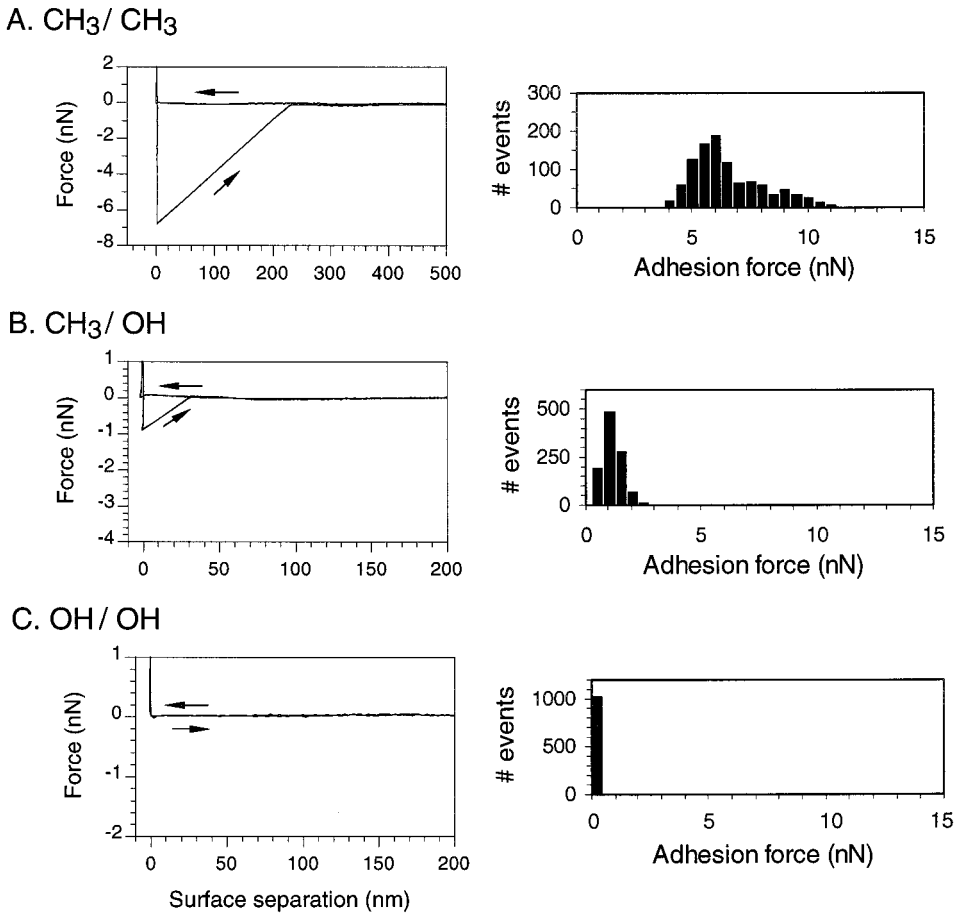

FIGURE 1 AFM force-distance curves (*left*) and adhesion force histograms (*right*) obtained in deionized water for CH_3/CH_3 (*A*), CH_3/OH (*B*), and OH/OH (*C*) functionalized probe/substratum combinations. Adhesion force histograms were generated by recording 1024 force-distance curves over 1 × 1-μm areas. The absolute values of the adhesion forces for the CH_3/CH_3 and CH_3/OH measurements can vary from one experiment to another due to variations in the radius of curvature of the probe and in the cantilever spring constant.

CH_3- and OH-terminated probes in deionized water are shown in Fig. 2, *A* and *B*, respectively. The spore surface is covered with rodlet structures several hundred nanometers in length and ~10 nm in diameter, which agrees well with previous data obtained by transmission electron microscopy combined with freeze-etching (Gerin et al., 1994). It is worth noting that the functionalized probes provide nanoscale lateral resolution (10 nm), which is close to the resolution typically achieved with silicon nitride probes.

Force-distance curves measured between the spore surface and CH_3- and OH-terminated probes in deionized water are plotted in Fig. 2, *C* and *D*, respectively. No significant difference is found between the two types of probes. Strikingly, the lack of curvature in the contact region shows that the sample is not deformed by the AFM probe, which is in contrast with force curves obtained on soft, living mammalian cells (Radmacher et al., 1996). No significant adhesion force is measured upon retraction, which reflects a lack of attractive interactions at the probe/substratum interface. Multiple (1024) force-distance curves recorded over 500 × 500-nm areas always show no adhesion (Fig. 2, *E* and *F*), indicating that the spore surface chemistry is fairly homogeneous. Similar curves were obtained in 0.1 M NaCl (adhesion force ~0 nN over 500 × 500-nm areas), pointing to the fact that the measured forces are not of electrostatic origin.

Comparison of these data with those obtained on functionalized substrata suggests that the spore surface is hydrophilic. However, one may argue that cellular material, or macromolecular substances present in the aqueous phase, may adsorb onto the probe surface; this would change the probe geometry and chemistry, thereby making it difficult to interpret the force measurements. To address this question, a control experiment was performed in which force-distance curves were recorded between probes that were previously used to image the spore surface and substrata functionalized with CH_3- and OH-terminated monolayers. As shown in Fig. 3, the adhesion forces measured for the CH_3/CH_3 (7 ± 1 nN; n = 1024) and OH/OH (~0 nN) combinations are similar to those obtained with "fresh" probes (Fig. 1), i.e., probes that had never been in contact with the spore samples. These data show that the lack of adhesion measured between the spore and the CH_3-terminated probe is not due to the adsorption of hydrophilic material on the probe surface. There are further arguments supporting the notion that the probe is not significantly modified upon contacting the spore surface: 1) nanometer-size rodlets are well-resolved, with dimensions in agree-

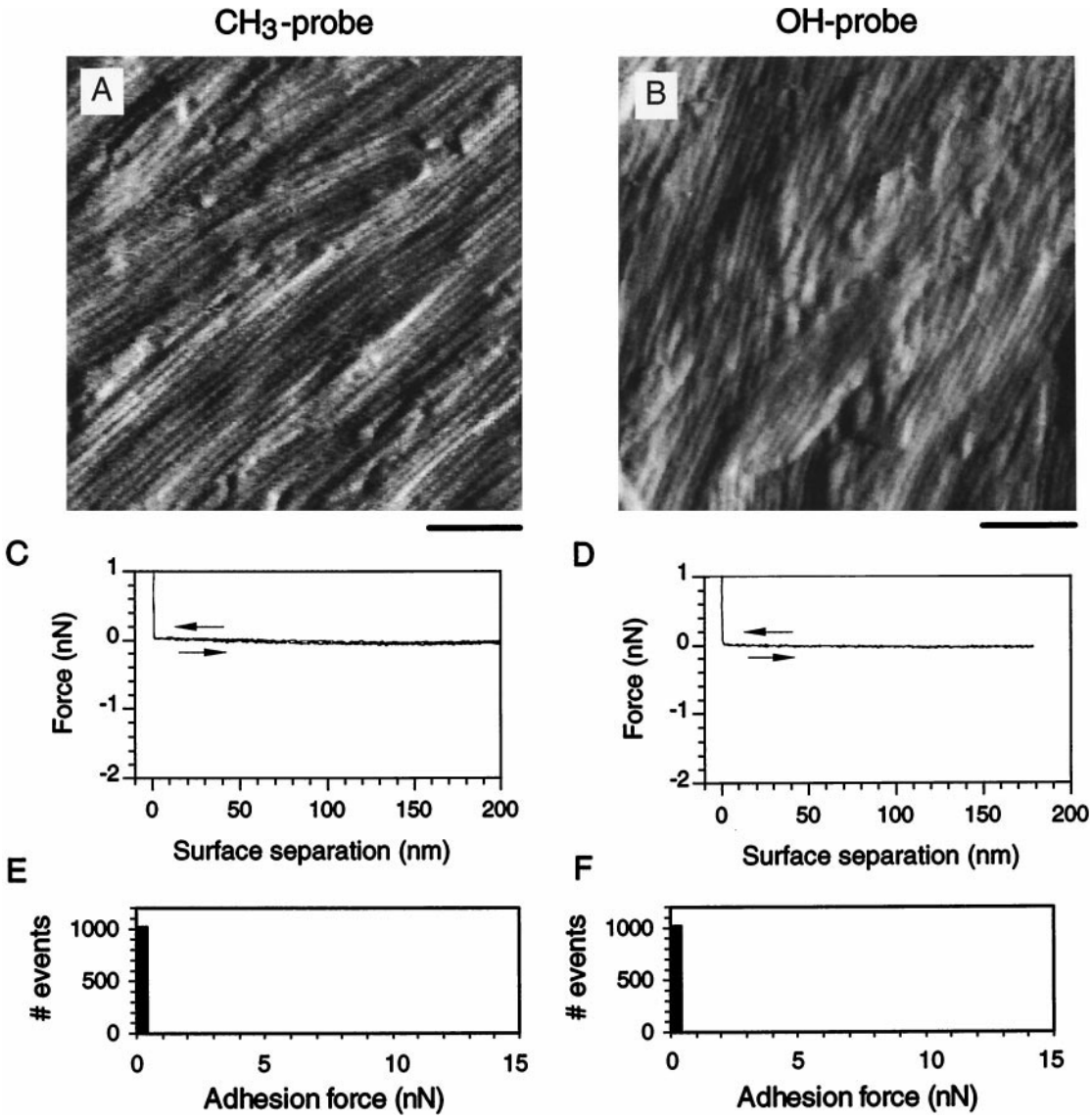

FIGURE 2 AFM deflection images (*A*, *B*; scale bars = 100 nm), force-distance curves (*C*, *D*), and adhesion force histograms (*E*, *F*) obtained, under deionized water, for the surface of *P. chrysosporium* spores with CH_3- (*A*, *C*, *E*) and OH- (*B*, *D*, *F*) terminated probes. Adhesion force histograms were generated by recording 1024 force-distance curves over 500 × 500-nm areas. The data shown are representative of results obtained on >10 spores, using different probes and independent preparations.

ment with electron microscopy data; 2) force-distance curves show that the spores are fairly stiff, contrary to mammalian and bacterial cells.

Taken together, the above observations lead me to conclude that the force measurements presented in this study provide direct evidence for the hydrophilic character of the spore surface. During the past decades, microbial cell surface hydrophobicity has attracted considerable attention in view of its role in microbial adhesion processes (Doyle and Rosenberg, 1990). A variety of experimental methods (van der Mei et al., 1991) have been developed to assess microbial hydrophobicity, including water contact angle measurements on cell lawns, adhesion to hydrocarbons, partitioning in aqueous two-phase systems, and hydrophobic interaction chromatography. However, these assays only provide information on the overall hydrophobic character of a large number of cells; furthermore, some of them do not unambiguously measure surface hydrophobicity, but are also strongly influenced by electrostatic interactions. The novel method presented here circumvents these limitations by enabling nanoscale probing of the surface hydrophobicity of single cells.

Further work is needed to assess to what extent the spore surface properties are affected by changes in the environmental conditions. In particular, one may anticipate that storage under dry conditions will promote the exposure of

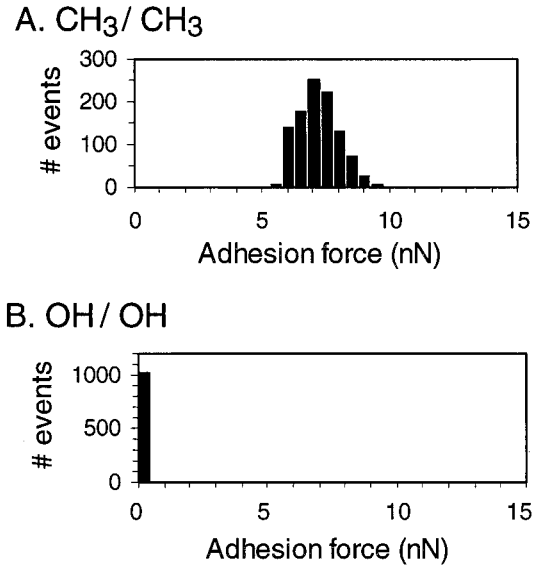

FIGURE 3 Control experiment: adhesion force histograms (1024 events; 1×1-μm areas) obtained, in deionized water, for CH_3/CH_3 (*A*) and OH/OH (*B*) probe/substratum combinations using functionalized probes that were previously used to examine the spore surface.

hydrophobic moieties at the surface, thereby rendering the overall spore surface more hydrophobic.

CONCLUSION

This paper shows that AFM with chemically functionalized probes is a versatile and powerful technique for characterizing molecular interactions and surface physicochemical properties of microbial cells in the native state. Functionalization makes the probe very sensitive to surface hydrophobicity, the magnitude of the adhesion forces measured between functionalized probes and substrata decreasing in the order $CH_3/CH_3 > CH_3/OH >$ OH/OH. Functionalized probes enable direct visualization of the surface ultrastructure (rodlets) of *P. chrysosporium* spores to a lateral resolution of 10 nm. The spore surface is homogeneously hydrophilic, as revealed by the lack of adhesion forces measured between the spore and both CH_3- and OH-terminated probes. This non-adhesive character may play an important role in determining the biological functions of fungal spores, namely protection and dispersion. Potential applications of the methodology presented here include the nanoscale mapping of hydrophobicity, electrical properties, solvated macromolecules, and specific recognition sites at the surface of prokaryotic, animal, and plant cells, using probes functionalized with relevant chemical groups or biomolecules.

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