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Hydrophobic Characteristics of *Bacillus* Spores

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Abstract. Spores from several *Bacillus* species displayed a strong affinity for hexadecane and other hydrophobic solvents. The binding of *Bacillus subtilis* spore suspensions to octyl-Sepharose was enhanced by ammonium sulfate and other salts, but was inhibited by detergents. Treatment of spore suspensions with strong denaturants promoted their adherence to hexadecane, presumably by exposing hydrophobic residues in coat proteins. The hydrophobic characteristics of spores may be important in the ecological adaptation of certain bacteria.

It is now well accepted that many bacterial species possess hydrophobic surface characteristics. The pathogenesis of several different kinds of bacteria may depend on hydrophobic interactions between the bacterium and host components [3, 6, 9, 12, 14, 17]. The binding of bacteria to hydrophobic surfaces has been ascribed to a variety of cellular components. Jonsson and Wadstrom [6] have suggested that protein A contributes to the hydrophobicity of *Staphylococcus aureus*. Ofek et al. [13], Tylewska et al. [17], and Miorner et al. [9] maintain that M protein and lipoteichoic acids can impart hydrophobic characteristics to *Streptococcus pyogenes*. Fimbriae appear to be important hydrophobic structures on certain Gram-negative bacteria [15]. It has been known for years that bacterial spores do not absorb water as well as protoplasts or intact cells, suggesting that the spores are also hydrophobic (see Murrell [11] for review).

In work related to determinative assays for intact and vegetative cells of *Bacillus anthracis*, we noted that spore preparations tended to adhere strongly to plastic microtiter plates [2] (unpublished results). This observation suggested that spores of *Bacillus* species, unlike intact cells [12], could be hydrophobic. We now present results which show that *Bacillus* spores are indeed hydrophobic and that the hydrophobicity can be modified by protein denaturants and by enzymes. This is the first report detailing some of the hydrophobic characteristics of mature spores, although Rosenberg (M. Rosenberg, University of Tel-Aviv, personal communication) has shown that the spores of a *B. cereus* strain can adhere to hexadecane droplets.

Materials and Methods

Chemicals. Hexadecane, octane, and toluene were the highest grade available from Aldrich Chemical Company (Milwaukee, WI) or Eastman Organic Chemicals (Rochester, NY). Octyl-Sepharose, lysozyme, trypsin, subtilisin, and mutanolysin were products of Sigma Chemical Company (St. Louis, MO). Bacteriological media were obtained from Difco Laboratories (Detroit, MI). Amersham Radiochemicals (Arlington Heights, IL) supplied a uniformly labeled [^{14}C] mixture of amino acids (10 mCi/mmol). Salts and other reagents were from Fisher Scientific (Louisville, KY).

Bacteria and growth conditions. Species and strains of *Bacillus* have been described previously [2]. Preparations of vegetative cells were obtained from exponential cultures in brain–heart infusion broth. Sporulation was accomplished by growth at 37°C, with vigorous aeration for four days in a medium consisting of brain–heart infusion, 0.5% (wt/vol) yeast extract, 1.0 mM Ca^{2+}, and 50 μM Mn^{2+} [7]. Pellets obtained upon centrifugation were suspended in distilled water and centrifuged through 55% (wt/vol) sucrose. This process was repeated until a preparation was obtained that was judged to be free of intact cells as determined by the Gram stain. Usually, 4–6 centrifugations in sucrose were necessary. Finally, the spores were washed three times in cold distilled water.

Assay for hydrophobicity. The procedures described by Rosenberg et al. [14] were generally followed. Cell or spore suspensions in aqueous media were mixed with a nonaqueous solvent, and the amount of adherence to the hydrocarbon was determined by turbidity measurements. Usually, a 3.0-ml portion of cells or spores in saline was mixed with a 0.6-ml volume of hexadecane or other hydrocarbon by vortexing vigorously for 30 s. After the phases had separated (approximately 15 min), the contents of the aqueous phases were analyzed for loss of turbidity. The turbidities of starting spore or cell suspensions were usually 0.4–0.5 absorbance units at 450 nm, 1-cm pathlength. Samples were run in duplicate. Replicate assays agreed within ±5%.

Chromatography on octyl-Sepharose. Columns of octyl-Sepha-

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Table 1. Binding of *Bacillus* spores and vegetative cells to hexadecane[a]

Organism	Adherence to hexadecane (%)	
	Spores	Vegetative cells
B. anthracis 14185	36.5	6.0
B. anthracis UMRL	24.0	5.8
B. cereus 9634	56.1	3.7
B. cereus T	63.8	3.8
B. mycoides 6462	37.2	2.3
B. mycoides MWC	21.0	2.0
B. subtilis 168	13.2	1.6
B. thuringiensis 4040	61.4	3.9
B. thuringiensis 4055	38.3	4.9

[a] Spores or vegetative cells were suspended in 3.0 ml saline (O.D. approximately 0.45–0.50) and 600 μl hexadecane added. Following mixing, assays were conducted on the separate aqueous phase for turbidity. The percent loss of turbidity from the aqueous phase was then calculated.

rose were prepared in Pasteur pipettes. Bed volumes were 0.9–1.3 ml. Samples for chromatography were standardized at 1.0 absorbance units (450 nm, 1 cm), and 200 μl were loaded onto the columns. Under these conditions the columns were not saturated with spores. The amount of spore material adhering to a column was monitored by liquid scintillation counting. The extent of adherence was calculated by determining the radioactivity recovered from the column and by a knowledge of the amount of radioactivity added. For *B. subtilis* 168, the spore suspensions contained 27,560 CPM/ml, for *B. anthracis* 14185, the amount of radioactivity in the suspensions was 45,530 CPM/ml.

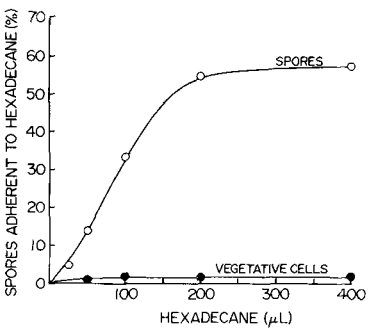

Fig. 1. Adherence of *Bacillus cereus* 9634 spores to hexadecane. Spores or cells in saline (3 ml) were mixed with hexadecane and the amount of adherence assayed by turbidity changes in the aqueous phase.

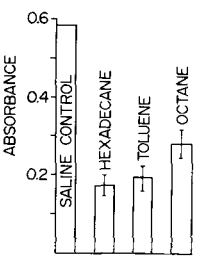

Fig. 2. Partitioning of spores of *Bacillus cereus* T by nonaqueous solvents. Final volumes were 3.0 ml spores in saline and 600 μl organic solvent. Error bars represent the standard deviation for assays conducted in duplicate on three separate days.

Results and Discussion

Spores from several species of *Bacillus* were suspended in saline and assayed for adherence to hexadecane. The results (Table 1) revealed that all spore preparations were partially partitioned into the hydrocarbon phase. The spores exhibited heterogeneity with respect to their hydrophobicities. *Bacillus subtilis* 168 was the least hydrophobic with approximately 13% of the spores adhering to hexadecane, whereas *B. cereus* T, *B. cereus* 9634, and *B. thuringiensis* 4040 were the most hydrophobic. When intact cells of the species and strains were mixed with hexadecane, there was no tendency for the organisms to adhere to the nonaqueous phase. Figure 1 shows that adherence to hexadecane depended on the amount of hydrocarbon used. A ratio of approximately 133 μl hydrocarbon/ml cell suspension was found to be sufficient for maximal adherence. Most assays were run at a ratio of 200 μl organic solvent/ml aqueous cell suspension in order to ensure saturation.

Spores of *B. cereus* T were interacted with several hydrocarbons, including hexadecane, toluene, and octane, and the extent of adherence determined (Fig. 2). The best partitioning solvent appeared to be hexadecane, although toluene was highly effective in partitioning the spores. Results from other laboratories have previously shown that intact bacterial cells may have high affinities for some solvents, such as hexadecane or toluene, but not for other solvents [14]. When nonadherent spores were reextracted with hexadecane, the turbidity of the aqueous suspension decreased (not shown), suggesting that the entire population of spores contained hydrophobic sites.

The foregoing results established that spores, but not their parent intact cells, could bind to hydrophobic solvents. Vegetative cells of *Bacillus* species are not thought to possess significant amounts of surface protein [10]. Spores, on the other hand, contain proteinaceous coats [18] that could be providing the sites for adherence to hydrocarbon. When adsorption of water vapor to spore components is considered, the coat proteins appear to be the most refractory to hydration [11, 19]. It was reasoned that protein structure-disrupting agents, such as guanidine hydrochloride or sodium

Table 2. Effect of chemical and enzymatic treatments on the hydrophobicity of *Bacillus subtilis* 168 and *Bacillus thuringiensis* 4040

Treatment	% Adherence to hexadecane[a]	
	B. subtilis 168	*B. thuringiensis* 4040
None, control spores	14	61
Water, 100°C, 15 min	28	71
Guanidine hydrochloride (6 *M*)[b]	23	81
Sodium dodecyl sulfate (SDS, 10 mg/ml)	40	84
Lysozyme	35	59
Mutanolysin	16	63
Trypsin	22	56

[a] Spore suspensions (3.0 ml in saline) were mixed with 600 µl hexadecane in the standard assay procedure. Spores were 0.45–0.47 absorbance units (500 nm, 1 cm).

[b] Treatments with guanidine hydrochloride, sodium dodecyl sulfate, lysozyme, mutanolysin, and trypsin were for 4 h at 37°C, followed by centrifugation and washings in water.

Table 3. Binding of *Bacillus* spores to octyl-Sepharose

Eluant	% Adherent to column[a]	
	B. subtilis 168	*B. anthracis* 14185
Water	1	99
0.1 *M* ammonium sulfate	45	80
0.5 *M* ammonium sulfate	9.0	85
2.0 *M* ammonium sulfate	0	99
Sodium dodecyl sulfate (10 mg/ml)	0	36
0.15 *M* sodium chloride	87	68
Triton X-100 (1%, vol/vol)	51	95

[a] Assays were conducted by scintillation counting of [^{14}C]-labeled spores. The spores were made radioactive by the addition of 1.0 µCi/ml (final activity) of a uniformly labeled mixture of amino acids. The [^{14}C] amino acids were added at the end of 18 h growth of the cells in sporulation medium.

dodecyl sulfate (SDS), or enzymes, could modify the hydrophobicities of spores. For *B. subtilis* 168, extraction with hot water, 6 *M* guanidine hydrochloride, or SDS resulted in an enhancement of the hydrophobic characteristics of the spores. For example, spores in saline were only 14% adherent to hexadecane, but, after extraction with SDS, 40% of the spores adhered to the hydrocarbon (Table 2). Treatment of spores with lysozyme, but not mutanolysin, also resulted in a significant increase in adherence. In addition, trypsin slightly increased the tendency of *B. subtilis* 168 spores to bind to hexadecane. Similar kinds of results were obtained for *B. thuringiensis* 4040 in that the protein extractants and the hot water increased the tendency of the spores to adhere. Enzymes, however, did not modify the hydrophobicity of *B. thuringiensis* 4040 spores.

For *B. subtilis*, it is assumed that lysozyme removed peptidoglycan-containing components on the spore surface. It may be possible that cell wall turnover products from vegetative cells bind to the surfaces of spores, thereby masking hydrophobic sites. *B. thuringiensis* cells are refractory to the effect of lysozyme but can be slowly lysed by mutanolysin (G. Zipperle, unpublished results).

When *B. subtilis* 168 spores were passed through an octyl-Sepharose column, it was observed that only a small percentage of the spores adhered when water was the solvent (Table 3). Spores suspended in saline or ammonium sulfate solutions were much more adherent to octyl-Sepharose. Both SDS and Triton X-100 greatly reduced, compared with the salts and buffers, the adherence of spores to octyl-Sepharose. These results are in agreement with others who have shown that ions enhance hydrophobic interactions and that detergents inhibit binding of cells to hydrocarbons [4, 8, 16]. *Bacillus anthracis* 14195, in contrast, adhered firmly to octyl-Sepharose in the absence of ions (Table 3). This observation suggests that the hydrophobic species on the surface of spores of *B. anthracis* are different from those on spores of *B. subtilis*. Neither sodium dodecyl sulfate nor Triton X-100 prevented the binding of *B. anthracis* spores to the octyl-agarose column. Although it is generally accepted that salts tend to promote hydrophobic interactions, it is also recognized that some proteins will adhere strongly to hydrophobic ligands in the absence of the salts [4, 16]. In the chromatography experiments (Table 3), control runs using unsubstituted agarose established that hydrophobic groups were necessary for adherence.

The interactions between spores and hydrophobic groups may be important in the ecologies of the bacteria. Spore attachment to hydrophobic side chains on an environmental protein may provide a means for providing nitrogen source during germination and outgrowth. Proteases in spore coats [5] would then have ready access to substrates. Hydrophobicity assays may also be useful in the study of sporulation. It would be interesting to equate the appearance of hydrophobicity with a particular spore component during the vegetative cell-to-spore transition. Finally, the binding of spores to hydrophobic surfaces may offer a means of separat-

ing the spores from vegetative cells. Support for this view comes from the findings of Boyles and Lincoln [1], who found that spores of *B. anthracis* tended to collect in foam (more hydrophobic surface) in sparged cultures.

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