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Surface Hydrophobicity of Spores of *Bacillus* spp.

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The surface hydrophobicity of 12 strains of *Bacillus* spp. was examined in a hexadecane–aqueous partition system. Mature and germinated spores of *Bacillus megaterium* QM B1551 transferred to the hexadecane layer, while vegetative and sporulating cells did not. Wild-type spores were more hydrophobic than spores of an exosporium-deficient mutant of *B. megaterium* QM B1551, although the mutant spores were shown to be hydrophobic to some extent by using increased volumes of hexadecane. This result suggests that the exosporium is more hydrophobic than the spore coat and that the surface hydrophobicity of spores depends mainly on components of the exosporium. The surface hydrophobicity of spores of nine other species of *Bacillus* was also examined, and spores having an exosporium were more hydrophobic than those lacking an exosporium. Thus measurement of the hydrophobicity of spores by the hexadecane partition method may provide a simple and rapid preliminary means of determining the presence or absence of an exosporium.

INTRODUCTION

The surface hydrophobicity of bacterial cells has been determined by several methods, based on the precipitation of cells by salts (Lindahl *et al.*, 1981), hydrophobic interaction chromatography (Smyth *et al.*, 1978; Doyle *et al.*, 1984), and adherence to various liquid hydrocarbons including hexadecane (Rosenberg *et al.*, 1980; Doyle *et al.*, 1984; Craven & Blankenship, 1987; Kutima & Foegeding, 1987). The use of a hexadecane–aqueous partition system is one of the simplest and most rapid methods.

Spores of *Bacillus cereus* (Doyle *et al.*, 1984; Kutima & Foegeding, 1987), *Clostridium perfringens* (Craven & Blankenship, 1987) and *Bacillus subtilis* (Doyle *et al.*, 1984) have hydrophobic characteristics, but their affinities for liquid hydrocarbons are different. Rosenberg *et al.* (1985) reported that gramicidin S was responsible for the hydrophobicity of *B. brevis* spores, but the structural components involved are still unknown. The factors responsible for the hydrophobicity of spores of other species of *Bacillus* have not been reported. In morphological classification, spores may be divided into two groups based on the presence of an exosporium. Since the chemical composition of the exosporium (Matz *et al.*, 1970; Takumi *et al.*, 1979) is different from that of the spore coat (Warth *et al.*, 1963; Aronson & Pandey, 1978), the hydrophobic properties of spores with and without exosporium may be different. If so, it could be possible to determine the presence or absence of an exosporium on the basis of differences in surface hydrophobicity of spores.

In the present study we used a hexadecane–aqueous partition system to examine the hydrophobic characteristics of *Bacillus megaterium* QM B1551 at different stages of its life cycle, and those of spores of nine other species of *Bacillus*. We also examined the link between hydrophobicity and the presence of an exosporium.

Table 1. *Cultivation conditions and concentrations of Urografin used for purification of spores*

Organism*	Medium†	Incubation time (d)	Incubation temp. (°C)	Concn of Urografin (g ml^{-1})
B. brevis IFO 3331	TYEA	7	30	1·263
B. cereus T	TYEA	7	30	1·210
B. circulans IFO 3329	TYEA	7	30	1·210
B. coagulans IFO 3557	BHIA	21	37	1·233
B. licheniformis IFO 12195	TYEA	7	30	1·263
B. macerans IFO 3490	TYEA	7	37	1·210
B. megaterium QM B1551	SNB	3	30	1·210
	TYEA	3	30	1·210
B. megaterium ATCC 33729	SNB	3	30	1·210
	TYEA	3	30	1·210
B. megaterium ATCC 19213	TYEA	3	30	1·210
B. pumilus IFO 3813	TYEA	7	30	1·191
B. subtilis ATCC 6051	TYEA	3	37	1·210
B. thuringiensis IFO 13865	TYEA	7	37	1·263

* IFO, Institute for Fermentation, Osaka; ATCC, American Type Culture Collection.

† SNB, supplemented nutrient broth; TYEA, tryptose yeast extract agar medium; BHIA, brain heart infusion agar medium.

METHODS

Organisms and media. The *Bacillus* strains used in this study, and their cultivation media, are listed in Table 1. Brain heart infusion agar (BHIA) contained (g l^{-1}): brain heart infusion (Difco), 37; yeast extract (Difco), 5; and agar, 15; in salt solution (pH 7·0). Tryptose yeast extract agar (TYEA) contained (g l^{-1}): tryptose (Difco), 10; yeast extract, 2; and agar, 15; in salt solution (pH 7·0). The salt solution consisted of (g l^{-1}): $MgSO_4.7H_2O$, 0·25; $MnCl_2.4H_2O$, 0·025; $CaCl_2.2H_2O$, 0·15; and $FeSO_4.7H_2O$, 0·0003. Supplemented nutrient broth (SNB) was prepared as described by Setlow & Kornberg (1969).

Preparation of spores. The cultivation conditions are given in Table 1. Spores were harvested and collected by centrifugation at 1500 *g* for 15 min. The pellets were suspended in deionized water, layered on top of a solution of Urografin (Schering; the concentrations of Urografin used are indicated in Table 1) and centrifuged at 1500 *g* for 30 min. The pellets were resuspended in deionized water, and the suspension was layered on top of a fresh Urografin solution and centrifuged. This procedure was repeated several times to remove vegetative cells and cell debris. The clean spores thus obtained were washed five times with chilled deionized water by centrifugation at 1500 *g* for 15 min.

Preparation of vegetative cells, sporulating cells and germinated spores of B. megaterium QM B1551. Cells were inoculated into 100 ml supplemented nutrient broth and incubated at 30 °C with aeration by shaking for 7 h (vegetative cells) and for 16 h (sporulating cells). The cells were then harvested and washed several times with saline by centrifugation at 1500 *g* for 15 min. After 16 h cultivation, more than 95% of forespores within the mother cells were refractile when observed by phase-contrast microscopy. The spore suspensions were heat-activated at 60 °C for 30 min. Germinated spores (more than 90% of spores having lost refractility) were prepared by incubating heat-activated spores with 10 mM-KNO_3 at 37 °C for 1 h.

Measurement of the surface hydrophobicity of spores and cells. The surface hydrophobicity of mature spores was measured by the method of Rosenberg *et al.* (1980) with a minor modification. Spores were suspended in deionized water to prevent ionic germination. Various volumes of hexadecane (up to 1·0 ml) were added to 3 ml of spore suspensions (OD_{610} 0·5) in test tubes (18 × 180 mm), and the mixtures were agitated vigorously by Vortex mixer for 1 min at room temperature. After the two layers had separated completely on standing for about 15 min, the OD_{610} of the aqueous phase was measured with a Shimazu UV-240 spectrophotometer. Results are presented as percentage transfer to the hexadecane layer, calculated by the formula $[(D_i - D_f)/D_i] \times 100$, where D_i is the optical density of the initial spore suspension and D_f is that of the aqueous phase after agitation with hexadecane. Hydrophobicity measurements were carried out in duplicate. The mean values from three experiments are reported; the calculated values from three experiments agreed within ±3%.

Hydrophobicities of vegetative cells, sporulating cells and germinated spores were determined using saline instead of deionized water as a suspension medium.

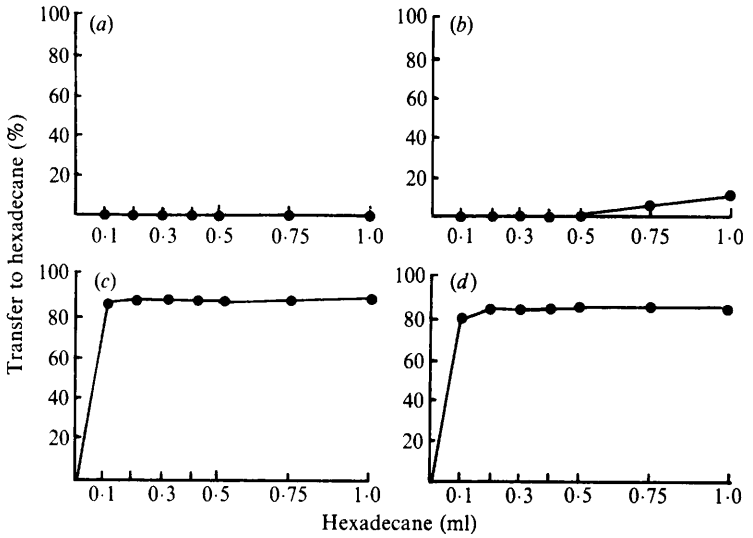

Fig. 1. Surface hydrophobic properties of (*a*) vegetative cells, (*b*) sporulating cells, (*c*) mature spores and (*d*) germinated spores of *B. megaterium* QM B1551. The cell or spore suspension (3 ml), prepared as described in Methods, was mixed with hexadecane (from 0 to 1·0 ml). The percentage transfer to hexadecane was determined by measuring the OD_{610} of the aqueous phase (see Methods). Each point represents the mean of three experiments (the same applies in Figs 2 and 3).

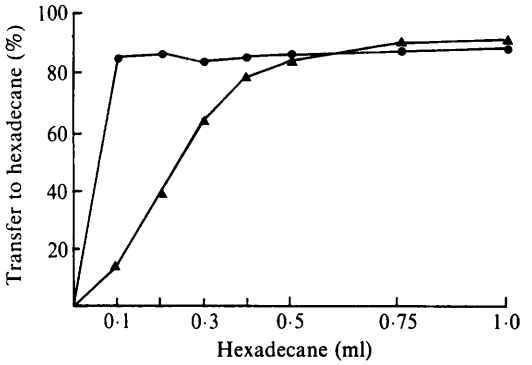

Fig. 2. Comparison of surface hydrophobic properties of wild-type (●) and exosporium-deficient (▲) spores of *B. megaterium* QM B1551. Spores were prepared as described in Methods. Percentage transfer to hexadecane was determined as for Fig. 1.

RESULTS

Surface hydrophobicity of spores and cells of B. megaterium QM B1551

The surface hydrophobic properties of various types of *B. megaterium* QM B1551 cells are shown in Fig. 1. No vegetative cells transferred to the hexadecane layer. Sporulating cells did not transfer to hexadecane when the volume of hexadecane was 0·5 ml or less, but minor transfer (less than 10%) was observed with 0·75 and 1·0 ml hexadecane. In contrast, more than 80% of the mature and germinated spores transferred to 0·1 ml hexadecane in this partition system, which indicates that spores, either mature or germinated, are considerably hydrophobic.

Comparison of the hydrophobicity of wild-type and exosporium-deficient mutant spores of B. megaterium QM B1551

The spore structure of *B. megaterium* QM B1551 consists of the core, inner membrane, cortex,

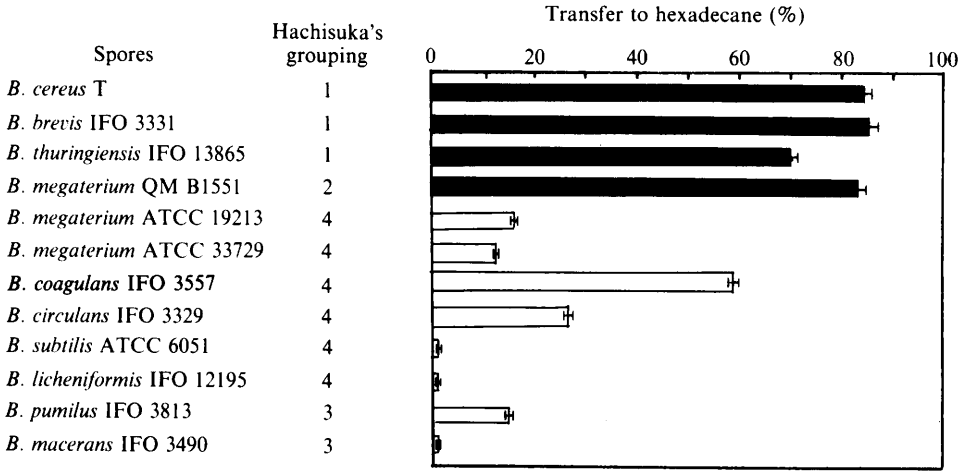

Fig. 3. Relationship between surface hydrophobicity and presence of exosporium. Spores in deionized water (3 ml) were mixed with 0·1 ml hexadecane. Percentage transfer to hexadecane was determined as for Fig. 1. ■, Presence of exosporium based on more than 70% transfer; □, absence of exosporium based on less than 70% transfer; the error bars represent SE. The classification of spores according to Hachisuka *et al.* (1984) is shown. The groups are defined as follows: group 1, exosporium and appendages present; group 2, exosporium only; group 3, appendages only; group 4, neither exosporium nor appendages present.

outer membrane, spore coat and exosporium (Koshikawa *et al.*, 1984). In order to study the role of the exosporium in hydrophobicity, the surface hydrophobicity of exosporium-deficient mutant spores (Koshikawa *et al.*, 1984) of *B. megaterium* QM B1551 was compared with that of wild-type spores (Fig. 2). The wild-type spores transferred easily to the hexadecane layer with only 0·1 ml of hexadecane, while little transfer of the exosporium-deficient spores occurred with this volume of hexadecane. Transfer of the exosporium-deficient spores into hexadecane gradually increased with increasing volumes of hexadecane, but transfer was less than 80% below 0·4 ml hexadecane. Transfer of the exosporium-deficient spores reached the same level as that obtained by the wild-type spores with 0·5 ml hexadecane or more. These results indicate that the hydrophobic property of spores is mainly due to the exosporium, while the spore coat has a (weaker) hydrophobic property.

Comparison of surface hydrophobicity of spores of various Bacillus species

Transfer to hexadecane was determined for spores of three strains of *B. megaterium* and nine other species of *Bacillus*. According to their surface hydrophobic characteristics, these spores were divided into three types. The spores of *B. megaterium* QM B1551, *B. coagulans* IFO 3557, *B. thuringiensis* IFO 13865, *B. cereus* T and *B. brevis* IFO 3331 were the most hydrophobic: more than 60% transfer occurred with 0·1 ml hexadecane. Spores of *B. megaterium* ATCC 33729 and ATCC 19213, *B. pumilus* IFO 3813 and *B. circulans* IFO 3329 had moderate hydrophobicities, requiring 0·3–0·5 ml hexadecane for 60% transfer. The transfer of spores of *B. macerans* IFO 3490, *B. licheniformis* IFO 12195 and *B. subtilis* ATCC 6051 was less than 20% even with 1·0 ml hexadecane, indicating that these bacterial spores were the least hydrophobic.

The composition of medium and the cultivation temperature affect structures and properties of spores (Hitchins *et al.*, 1972; Waites *et al.*, 1979; Bayliss *et al.*, 1981; Foegeding & Fulp, 1988). To examine the effect of culture medium on surface hydrophobicity, *B. megaterium* QM B1551 and ATCC 33729, *B. licheniformis, B. pumilus* and *B. cereus* T were cultivated in supplemented nutrient broth and on tryptose yeast extract agar and the resulting spores were tested for their hydrophobicity. The hydrophobic properties of these bacterial spores were not affected by the cultural media (data not shown).

We also examined the relationship between the presence of an exosporium and the surface

hydrophobicity of the spores (Fig. 3). With spores possessing an exosporium (*B. cereus, B. brevis, B. thuringiensis* and *B. megaterium* QM B1551), transfer was more than 70%, whereas for strains lacking an exosporium (except *B. coagulans*) transfer was less than 30%.

DISCUSSION

We have studied the surface hydrophobic characteristics of spore-forming bacilli and also examined whether the measurement of surface hydrophobicity of spores could be used as indicator of the presence of an exosporium.

Mature and germinated spores of *B. megaterium* QM B1551 were strongly hydrophobic (Fig. 1). Experiments with an exosporium-deficient mutant of *B. megaterium* QM B1551 (Fig. 2) indicated that the hydrophobic property of spores is largely due to the exosporium, but that the spore coat makes a small contribution to hydrophobicity. In the case of the spores of *B. brevis*, gramicidin S was reported to be responsible for the high surface hydrophobicity (Rosenberg *et al.*, 1985). However, the relation of gramicidin S polypeptide to the exosporium has not been established, and it is not known whether such polypeptides are produced by *B. megaterium*.

There is little information on the chemical composition of isolated exosporia. Isolated exosporium fractions from *C. botulinum* type A (Takumi *et al.*, 1979) and *B. cereus* (Matz *et al.*, 1970) were composed mainly of protein and lipid. The fact that, in our experiments, spores possessing an exosporium exhibited strong hydrophobicity may be due to the protein (probably hydrophobic) and the lipid components of the exosporium.

The spore coat consists mainly of hydrophobic proteins (Warth *et al.*, 1963; Aronson & Pandey, 1978). Doyle *et al.* (1984) found that agents which disrupt protein structure modified the hydrophobicity of spores, suggesting that the spore coat also has hydrophobic sites.

Hachisuka *et al.* (1984) divided spores of *Bacillus* species into four groups on the basis of the presence of the exosporium and the appendages (see legend to Fig. 3). In our experiments, spores of *B. brevis, B. cereus* and *B. thuringiensis* (members of Hachisuka's group 1, with both exosporium and appendages) showed the most hydrophobic properties. The strong hydrophobic properties of these spores are believed to derive mainly from the exosporium. The contribution of appendages to their hydrophobicity is not clear at present. The reported chemical properties of the main component of isolated appendages from *B. cereus* IAM 1110 (Kozuka & Tochikubo, 1985) would suggest that appendages may not have strong hydrophobic properties in comparison with the exosporium.

The spores of *B. megaterium* QM B1551 (Hachisuka's group 2, with exosporium only) showed almost the same hydrophobicity as those in group 1, which suggests that the strong hydrophobicity originates from the exosporium. Doyle *et al.* (1984) found that spores of *B. anthracis* (also in group 2) showed strong hydrophobicity in a hydrophobic chromatography experiment, a result which supports our conclusion.

Spores of *B. macerans* and *B. pumilus* (Hachisuka's group 3, with appendages only) had low and moderate surface hydrophobicities, respectively. The differences in surface hydrophobicities of these spores may be due to the amount of appendages or hydrophobic components in the spore coat.

Spores of *B. megaterium* ATCC 33729 and ATCC 19213, *B. circulans, B. licheniformis, B. subtilis* ATCC 6051 and *B. coagulans* belong to Hachisuka's group 4, and lack both exosporia and appendages. Their surface hydrophobicities, except for *B. coagulans*, were moderate or low. Although spores of *B. coagulans* had almost the same hydrophobicity as spores with an exosporium, the exosporium was apparently absent (Ohey & Murrell, 1962). We do not know why *B. coagulans* spores showed strong hydrophobicity. A possible explanation is that the bacterium may have a tightly fitting exosporium-like layer as reported for *B. subtilis* PM9 (Sousa *et al.*, 1976). The variation in hydrophobicity of spores in group 4 may reflect the relative proportion of nonpolar and polar groups on the surface of the spore coat.

Since spores possessing an exosporium showed high affinities for hexadecane, measurement of hydrophobicity with hexadecane seems to be a simple and rapid method for detecting the presence of an exosporium in *Bacillus* species.

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