THE UNIVERSITY OF MANITOBA

The surface properties of *Bacillus* spores and their relationship to germination inhibition by eugenol.

BY

Darryl Melnyk

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

WINNIPEG, MANITOBA

OCTOBER, 1987 ©

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ISBN 0-315-37332-6

THE SURFACE PROPERTIES OF *BACILLUS* SPORES AND THEIR RELATIONSHIP TO GERMINATION INHIBITION BY EUGENOL

BY

DARRYL MELNYK

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge the many people who helped make this thesis a reality. I am indebted to my advisors Dr. G. Blank and Dr. M.A.H. Ismond who initiated this study and provided me with guidance and advice not only during the investigation but also in the preparation of this manuscript. I would also like to thank Dr. Lyric for his suggestions and keen eye for taxonomic aberrations.

In addition, many of the staff and students of the Food Science department contributed either directly or indirectly to the completion of this project. Specifically I would like to thank Dr. Murray for his enthusiasm and inspiration, Margaret "Tables" Anderson for sacrificing many of her evenings and weekends to prepare this manuscript, Jim Rogers for his technical expertise, Paul Stephen for his computer wizardry, Aniko Bernatsky for her fluorescence work, Blaine Snell for his help with the photography and the remainder of the students and staff members for tolerating my bad jokes and poor renditions of "Happy Birthday".

The financial support provided by Natural Sciences and Engineering Research Council and the Food Science department was greatly appreciated.

Finally, I would like to dedicate this thesis to my parents.

ABSTRACT

Melnyk, Darryl. M.Sc. The University of Manitoba. October, 1987. The surface properties of *Bacillus* spores and their relationship to germination inhibition by eugenol. Major Professors: Dr. M.A.H. Ismond and Dr. G. Blank.

The hydrophobic behavior of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans* and *Bacillus stearothermophilus* var *calidolactis* spores was examined using bacterial adherence to hydrocarbons and hydrophobic interaction chromatography. Additional hydrophobicity determinations were performed on *B. subtilis* and *B. licheniformis* spores using the salt aggregation test, adherence to polystyrene, and two phase aqueous polymer methods. Spore hydrophobicity varied among species and also among crops of the same species. Correlation among the hydrophobicity values determined using the different methods was evident in all species except *B. coagulans*. The presence of ammonium sulfate (0.01 M) increased the hydrophobic behavior of the spores. Both *B. subtilis* and *B. licheniformis* spores possessed an equivalent net negative charge as determined by electrostatic interaction chromatography and microelectrophoresis. In the presence of ammonium sulfate, *B. subtilis* spores exhibited high adherence to cation exchange resins. In addition, ammonium sulfate reduced the spore zeta potential as well as the adherence of spores to a weak anion exchange resin. *B. licheniformis* spore germination was inhibited by 0.03% (v/v) eugenol while *B. subtilis* spore germina-

tion was not inhibited by eugenol concentrations up to 0.14% (v/v). Since B. licheniformis spores were determined to be more hydrophobic than B. subtilis spores, the sensitivity of spores to germination inhibition by eugenol may be related to their hydrophobicity.

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INTRODUCTION

Bacterial spores possess a relatively nonpolar or hydrophobic surface (Gaudin et al., 1960; Sacks and Alderton, 1961) which may vary among species (Sacks, 1969) and even strains (Doyle et al., 1984). In addition, the spores may also vary with respect to surface charge density (Douglas, 1955).

The surface properties of spores may influence their interactions with the surrounding physicochemical environment. Yasuda-Yasaki et al. (1978) and Yasuda et al. (1982) have suggested that germination inhibition by long chain alcohols, fatty acids and phenolics may be related to interaction of the inhibitor with hydrophobic or charged sites on the spore surface.

In this investigation the hydrophobicity of several Bacillus spore species was determined using five different assay methods. These methods included: bacterial adherence to hydrocarbons (BATH), hydrophobic interaction chromatography (HIC), salt aggregation test (SAT), adherence to polystyrene and the two phase aqueous polymer system. Since charge may influence hydrophobic interactions, the surface charge of two Bacillus spore species was also determined using electrostatic interaction chromatography (EIC) and microelectrophoresis. Finally, the ability of a phenolic compound, eugenol, to inhibit spore germination of two Bacillus species was evaluated and discussed with reference to the hydrophobic and charge properties of the spores.

LITERATURE REVIEW

(1) Hydrophobicity

As a result of their polar nature, water molecules interact with each other through hydrogen bonding. In order for a substance to become soluble among water molecules it must disrupt some of these hydrogen bonds. If the substance is polar or ionic in nature, it can engage in hydrogen or dipole bonding with the surrounding water molecules and to some extent reestablish the bonding matrix. However, in the case of nonpolar substances such as hydrocarbons, the hydrogen bonding cannot be reestablished between the solute and the surrounding molecules. As a result, hydrogen bonding between water molecules surrounding nonpolar substances becomes more intense and highly ordered (Frank and Evans, 1945). The decrease in entropy renders such a system unstable; nonpolar molecules are forced to leave the aqueous phase in such a manner that their contact with water molecules is minimized. The association of nonpolar molecules is facilitated through van der Waals forces. The tendency of molecules to be removed from water due to their nonpolar nature is described as their hydrophobic character or hydrophobicity.

(a) Bacterial hydrophobicity

The surface layers of bacteria are composed of a complex arrangement of carbohydrates, proteins and lipids (Reaveley and Burge, 1972). Some of these compounds are hydrophobic in nature. For example, many lipids possess long nonpolar hydrocarbon chains

which display varying degrees of hydrophobicity. The proteins may also contain hydrophobic regions due to the presence of amino acids with nonpolar side chains. Cells which contain such hydrophobic areas on their surface often behave in a hydrophobic manner. This was first recognized by Mudd and Mudd (1924a,b) who observed bacteria adhering to oil-water interfaces. Marshall and Cruikshank (1973) suggested that this interfacial adherence was a result of cell surface hydrophobicity.

In the last ten years, extensive hydrophobicity determinations have been carried out on bacterial cells using several methods. The results of these studies indicate that bacteria demonstrate great variation in their surface hydrophobicity even within a given species (Smyth et al., 1978). Often these differences in hydrophobicity can be accounted for by variations in the chemical composition of the the surface layers (Smyth et al., 1978; Miorner et al., 1984).

The outer surface of most bacterial cells is the cell wall. Classically, bacteria have been divided into two groups based on the nature of their cell wall as revealed by the Gram stain. Gram-positive bacteria contain 50 to 80% peptidoglycan in their cell walls. The remaining fraction is composed of compounds such as teichoic and teichuronic acids, polysaccharides, surface proteins plus lipoteichoic acid (Shockman and Barret, 1983). The presence of compounds such as lipoteichoic acid have been demonstrated to be responsible for surface hydrophobicity in streptococci (Miorner et al., 1983). Gram-negative cell walls contain

only 5 to 20% peptidoglycan. In addition, they possess an outer membrane constructed of phospholipids, proteins and lipopolysaccharides (Reaveley and Burge, 1972). The amount of lipopolysaccharide in the cell walls of Salmonella has been related to their surface hydrophobicity (Magnusson and Johansson, 1977).

Some bacteria also possess slime layers, capsules and various appendages on their outer surface. Many of these structures can affect cell hydrophobicity. For example, the presence of fimbriae on Escherichia coli surfaces has been shown to correlate with high surface hydrophobicity (Lindahl et al., 1981).

Cell surface hydrophobicity is regarded as an important factor in bacterial adhesion. For example, recent research has indicated that hydrophobic interactions are involved in the adhesion of oral bacteria to hydroxyapatite surfaces (Nesbitt et al., 1982). The surface properties of bacteria are also of interest in relation to their pathogenicity (Smith, 1977). Staphylococcus aureus strains isolated from infections showed much higher hydrophobicity than strains isolated from healthy carriers (Ljungh et al., 1985). However, the relationship between surface hydrophobicity and adherence is highly dependent on the characteristics of the specific bacterial strains studied and the nature of the surface involved. Criado et al. (1985) concluded that adherence of Neisseria meningitidis to buccal cells was a function of surface charge and not hydrophobicity.

(b) Spore hydrophobicity

The nonpolar nature of the spore surface was first revealed through techniques developed to separate spores from vegetative cells. Gaudin et al. (1960) developed a foam floatation method to separate the spores of Bacillus subtilis var. niger from vegetative cells. They demonstrated that spores had a greater affinity for air-liquid interfaces as compared to their respective cells. Sacks and Alderton (1961) also used differences in surface properties to separate spores from vegetative cells. Using a two phase polymer system composed of polyethylene glycol 4000 and potassium phosphate, they observed that spores of Bacillus megaterium entered the polyethylene glycol phase while cells remained in the phosphate rich phase. Sacks (1969) reported, however, that some other species of Bacillus produced spores which did not enter the polyethylene glycol phase. The surface characteristics of the spores appeared to vary depending on the species and possibly on the strain involved. This observation was confirmed by Doyle et al. (1984) who demonstrated that hydrophobicity of spores varied greatly among species and strains. They also reported that spores were more hydrophobic than their corresponding vegetative cells.

In most species the outer surface of the spore is comprised of coats composed primarily of protein with small amounts of lipid and phosphorus (Strange and Dark, 1955; Salton and Marshall, 1959). While the protein compositition appears relatively similar among species (Murrell, 1969), the morphology of the coats varies widely (Bradely and Franklin, 1958). Warth et al.

(1963) reported that the structure and morphology of the outer coat varied among species while the inner coat structure was relatively constant and appeared laminated in nature. In addition, the phosphorus content of the spore coat increased concomitant with the size of the outer coat. Aronson and Fitz-James (1968) proposed that the protein structure in the outer coats is stabilized through hydrophobic interactions.

Various researchers have demonstrated that the surface hydrophobicity of spores is a function of the spore coat. Coat defective spores are much less hydrophobic than normal spores (Kutima and Foegeding, 1987). Craven et al. (1985) reported that spore coat preparations were much more hydrophobic than spores divested of their coats. In addition, spores retain their hydrophobic properties through the germination and outgrowth process up to the time when the spore coat is shed (Rosenberg et al., 1985). The exact nature of the hydrophobic sites on the spore coat is not known, however, the antibiotic gramicidin S has been demonstrated to contribute to the hydrophobicity of Bacillus brevis spores (Rosenberg et al., 1985).

(c) Factors affecting the hydrophobicity of cells and spores

Growth conditions have been reported to affect the surface hydrophobicity of cells (Knox et al., 1985; Hogt et al., 1983) and spores (Rosenberg et al., 1985). Cell hydrophobicity is also dependent upon the phase of growth (Rosenberg et al., 1980a).

Chemical and physical treatments including antibiotics and

antibacterial agents have been demonstrated to alter the cell hydrophobicity (Ramirez-Rhonda et al., 1986; El-Falaha et al., 1985). Doyle et al. (1984) demonstrated that the hydrophobicity of Bacillus subtilis spores was altered by chemical treatments with compounds such as detergents, lysozyme, trypsin, sodium dodecyl sulfate and guanidine hydrochloride. In addition, the presence of salts in cell or spore suspensions can increase hydrophobic behavior (Smyth et al., 1978; Rosenberg, 1984b; Doyle et al., 1984). This increased hydrophobic behavior may be due to two phenomena. First, by increasing the ionic strength, salts reduce electrostatic charge effects allowing hydrophobicity to play a greater role in surface interactions (Lindahl et al., 1981). Secondly, salts like ammonium sulfate increase the structure or order of water molecules thereby encouraging hydrophobic interactions (Hjerten, 1981). Salt enhancement of surface hydrophobicity has been developed as a method for assessing bacterial hydrophobicity (Lindahl et al., 1981). Lindahl et al. (1981) also demonstrated that a decrease in pH will increase the hydrophobicity of the cell surface. At low pH, surface charged groups become protonated. This reduces electrostatic charge effects allowing hydrophobic interactions to become more predominant.

(2) Hydrophobicity measurement

(a) Hydrocarbons

Tanford (1973) assessed the hydrophobicity of hydrocarbon molecules by comparing their free energy of transfer from water

to a hydrocarbon solvent. Based on the extensive solubility measurements of liquid hydrocarbons in water reported by McAuliffe (1966), Tanford reported that the negative free energy of transfer increased in proportion to the length of straight alkane chains. As the length of the hydrocarbon chain was increased, the transfer of the test compound from water to hydrocarbon was more favorable in terms of free energy. This increase in negative free energy of transfer revealed that hydrocarbons were more hydrophobic as their chain length increased.

(b) Proteins

Hydrophobicity measurements of complex compounds such as proteins and lipids are more difficult than hydrocarbons since the former usually contain both hydrophobic and hydrophilic areas. For example, protein molecules are composed of a series of amino acids linked via peptide bonds. Some of the amino acids have hydrophobic side chains while others have hydrophilic side chains. This results in a molecule with a series of hydrophobic and hydrophilic sites. In aqueous solutions, many protein molecules will fold so as to bury the hydrophobic regions within the interior of the molecule, thereby, removing these regions from the water phase.

Several methods of assessing protein hydrophobicity have been developed based on the proportion of hydrophobic and hydrophilic side chains as determined by the amino acid composition. One such approach involves assigning hydrophobic amino acids a

value based on the free energy of transfer of their side chain from water to a nonpolar solvent (Tanford, 1962). The number of residues for each hydrophobic amino acid is multiplied by its hydrophobicity value. The results are totalled to arrive at a total hydrophobicity for the molecule which is divided by the number of residues in the protein to produce an average hydrophobicity value.

Two similar methods involve an assessment of the polarity value as developed by Fisher (1964) and the nonpolar side chain frequency value (NPS) as developed by Waugh (1954). The polarity value is the ratio of the volume of polar amino acids in the molecule to the volume of nonpolar amino acids. Nonpolar side chain frequency is expressed as the number of nonpolar amino acid residues divided by the total number of amino acid residues. These methods which are based on amino acid composition are often termed "total" hydrophobicity methods.

Another approach to determining protein hydrophobicity involves measuring the affinity of the protein molecule for hydrophobic groups or solvents i.e. the tendency of the molecule to engage in hydrophobic interactions. This measurement of protein hydrophobicity is termed "effective" or "surface" hydrophobicity. The total hydrophobicity value and the effective hydrophobicity value for a given protein usually show limited correlation because of protein folding (Keshavarz and Nakai, 1979). In order to achieve a stable low energy state, protein molecules will fold to remove hydrophobic groups from the aqueous phase. As a result, these hydrophobic groups are buried within the molecule where

they interact among themselves to stabilize the folded state. These hydrophobic groups located within the molecule do not affect surface hydrophobicity. Since it is the surface hydrophobicity which determines the hydrophobic behavior of the molecules towards their environment, the effective or surface hydrophobicity assessments are much more valuable in predicting the hydrophobic behavior of protein molecules as compared to the total hydrophobicity values.

Numerous techniques have been developed to assess the surface hydrophobicity of proteins and other biological macromolecules. One of these techniques is known as hydrophobic interaction chromatography. This technique involves the passage of protein molecules through an agarose gel column containing hydrophobic ligands (Er-El et al., 1972). The extent to which proteins interact with the ligands on the column is dependent upon their surface hydrophobicity. Elution of proteins is carried out by lowering the ionic strength of the eluting buffer. In general, proteins which elute initially are hydrophilic or weakly hydrophobic in nature while proteins which bind strongly to the column are eluted last and are hydrophobic in nature.

Another method used to determine surface hydrophobicity of proteins is the two phase aqueous polymer system used by Shanbhag and Axelsson (1975). This system is composed of two polymer phases: a polyethylene glycol phase and a dextran phase. The polyethylene glycol phase contains a small percentage of a hydrophobic polyethylene glycol derivative such as polyethylene glycol

palmitate. A sample protein is introduced into the system, followed by vigorous mixing. The degree to which a protein partitions itself into the polyethylene glycol phase containing the hydrophobic groups is compared to its partitioning into polyethylene glycol phase without hydrophobic derivatives. The difference in partitioning due to the hydrophobic groups is used as an indicator of protein surface hydrophobicity. Other methods which have been developed to assess protein surface hydrophobicity involve various fluorescent probes (Sklar et al., 1977) and fluorescence quenching procedures (Eftink et al., 1977).

(c) Bacteria

Before discussing the methodology of cell hydrophobicity determinations some preliminary comments on bacterial hydrophobicity and its measurement are appropriate. It is somewhat misleading to describe a bacterial cell as being hydrophobic or hydrophilic in nature. The outer surface of most bacterial cells contains both hydrophobic and hydrophilic regions. For example, Marshall and Cruikshank (1973) observed some bacteria adhered to oil-water interfaces only by the ends of the cell. While determining the number and size of such hydrophobic regions may enable one to calculate the proportion of the cell surface area which is hydrophobic in nature, the intensity of hydrophobicity in each region would also have to be considered. Some areas may be strongly hydrophobic whereas others may be only moderately hydrophobic.

No method has yet been developed which allows the size,

number and intensity of the hydrophobic regions on an individual cell surface to be determined. Most current methods are based on measuring the "hydrophobic behavior" of a population of cells and using this behavior as an indicator of the cell surface hydrophobicity. The methods are based on the following premise: if a cell possesses hydrophobic regions on its surface, it will interact with other hydrophobic compounds or surfaces through hydrophobic interactions.

The majority of cell hydrophobicity determination methods measure the proportion of cells in a population which adhere to a specific hydrophobic surface such as a liquid hydrocarbon, a hydrophobic Sepharose derivative, a hydrophobic polymer surface or hydrophobic regions on neighbouring cells. The final result is usually expressed as the percentage of cells in the population which adhere to the hydrophobic surface. If a large proportion of the cells adhere to the surface, the population is considered to be hydrophobic. While the test results do indicate the proportion of cells which possess the degree of hydrophobicity necessary to adhere to the surface, they do not indicate the area of the cell surface which is hydrophobic or the intensity of the hydrophobicity in the hydrophobic regions.

Furthermore, it is very unlikely that hydrophobic interactions are the only factor involved in adherence. Long range forces such as electrostatic interactions are also involved. As a result, using adherence as a measure of hydrophobicity can be misleading since cells differ in other surface characteristics

such as surface charge plus the presence of appendages and various morphological features which can influence adherence. The size, shape and density of the cells may also affect their adherence to hydrophobic surfaces.

(i) Hydrophobic interaction chromatography

This method originally developed for use with proteins has also been applied to bacterial cells. Hydrophobic interaction chromatography (HIC) was used to determine the hydrophobicity of E. coli strains by Smyth et al. (1978). Using hydrophobic Sepharose derivatives packed in Pasteur pipettes with bed volumes of approximately 0.6 mL, the adherence of cells suspended in 4 M sodium chloride or 1 M ammonium sulfate (pH 6.8) was monitored by decreases in absorbance and viable counts. Adherence can also be monitored by radioactively labelling the cells (Dahlback et al., 1981).

E. coli cells possessing the K88 antigen were found to adsorb to the hydrophobic gel while cells without the antigen showed no adsorption. The adsorbed cells were not readily desorbed by decreasing the ionic strength. The cells did not adsorb to Sepharose without attached hydrophobic ligands. When sodium chloride was increased from 0 to 2 M, cells containing the antigen showed increased adsorption while cells without the antigen were not affected. Cells with or without the antigen demonstrated increased adsorption in 1 M ammonium sulfate. The effect of the hydrophobicity of the ligand on cell adsorption was unclear as

cells with the antigen adsorbed highly to phenyl, octyl, naphthoyl and palmitoyl Sepharose derivatives but had much lower levels of adherence to naphthyl and dodecyl derivatives.

Tylewska et al. (1979) reported adsorption of cells on HIC columns decreased in the presence of detergents such as Triton X-100 and Tween 80. Adsorption was also affected by pH and ionic strength. In addition, Tylewska et al. (1979) reported that some cells had high levels of nonspecific adsorption to Sepharose without hydrophobic ligands.

Spore hydrophobicity was assessed using HIC by Doyle et al. (1984). They compared the spore hydrophobicity of B. subtilis 168 with Bacillus anthracis 145 using octyl-Sepharose. B. subtilis spores suspended in water possessed an adsorption level of only 1%. When suspended in 0.1 M ammonium sulfate their adsorption increased to 45%. Approximately 9% adsorption was achieved when the spores were suspended in 0.05 M ammonium sulfate while spores in 2 M ammonium sulfate showed no adsorption. Suspending B. subtilis spores in 0.15 M sodium chloride increased their adsorption to 87%. The use of detergents also increased the adsorption of B. subtilis spores. In contrast to B. subtilis, B. anthracis 145 spores, suspended in water, possessed a 90% adsorption rate. The addition of ammonium sulfate, sodium chloride and detergents decreased the adsorption of B. anthracis spores; treatment of spores with sodium dodecyl sulfate decreased adsorption for both spores.

(ii) Two phase aqueous polymer systems

Another protein hydrophobicity method applied to bacterial cells is the two phase aqueous polymer system. Magnusson and Johansson (1977) used a polyethylene glycol-dextran two phase system with hydrophobic polymers to assess bacterial hydrophobicity. They used polyethylene glycol (PEG) esterified with monocarboxylic acids varying in length from 2 to 28 carbon atoms. In addition, palmitic acid was coupled to the dextran polymer. Radioactively labelled bacterial suspensions were added to equal amounts of the two phase systems - the PEG rich phase and the dextran rich phase. After mixing and separation both phases were analysed for the presence of bacteria. The ability of the bacteria to partition into the PEG phase with and without hydrophobic ligands was used as a measure of their hydrophobicity.

In the PEG-dextran system without hydrophobic ligands, the majority of rough strains of Salmonella were found in the dextran rich phase. The presence of hydrophobic groups with chain lengths of 12 to 16 carbon atoms in the PEG rich phase increased the number of rough cells entering this phase. Smooth Salmonella strains were primarily found in the PEG phase in the absence of hydrophobic ligands. The addition of hydrophobic polymers to the dextran rich phase did not alter the partition of the smooth cells; however, hydrophobic groups added to the PEG rich phase reduced the number of smooth cells entering the PEG phase. The results indicated that smooth strains had lower levels of surface hydrophobicity as compared to the rough strains. In addition, rough strains required different concentrations of hydrophobic

polymers to alter their partitioning which indicated that the levels of hydrophobicity in the rough strains were variable depending on the strain. Partitioning of the cells into phases containing hydrophobic ligands was found to be dependent on several factors including carbon chain length of the hydrophobic group, surface charge of the cell and the concentration of the hydrophobic ligand.

Other factors have also been reported to affect hydrophobicity determinations using the PEG-dextran two phase system. Miorner et al. (1982) reported that heat inactivation reduced the hydrophobicity of streptococci as determined using an aqueous polymer two phase system. The concentration and type of salts present in the two phase systems greatly altered the cell distribution patterns (Walter and Johansson, 1986). Hofsten (1966) reported the partitioning of E. coli in two phase systems was affected by the growth medium but unaffected by heat, detergent or formaldehyde treatment.

Sacks and Alderton (1961) used a two phase aqueous polymer system containing PEG and potassium phosphate to separate Bacillus spores from vegetative cells. They reported that the vegetative cells were partitioned into the phosphate-rich phase while the spores were concentrated at the interface and in the PEG-rich phase. Albertsson (1958) demonstrated that polystyrene, a hydrophobic polymer, also entered the PEG phase. Based on this observation Sacks and Alderton (1961) concluded that spores possess a relatively nonpolar periphery. However, the PEG-phosphate system

has been reported to be a charge sensitive phase system (Walter and Johansson, 1986). The phosphate creates a positively charged PEG phase which attracts negatively charged particles. Sacks and Alderton (1961) reported that spores at the interface had a much higher electrophoretic mobility than spores in the PEG phase. Consequently, it is unclear whether the spores were separated from the cells on the basis of charge or hydrophobicity. The authors also observed germinated spores remained in the PEG phase while spores in the outgrowth phase, which had shed their coats, were no longer found in the PEG phase. In a later study, Sacks (1969) reported spores of some Bacillus species did not partition into the PEG phase unless the molecular weight of the PEG polymers was reduced. This further confirmed the heterogeneity of spore surfaces.

(iii) Bacterial adherence to hydrocarbons

Bacterial adherence to hydrocarbons as a means of assessing hydrophobicity was developed by Rosenberg et al. (1980a). The method involved the addition of a liquid hydrocarbon to a bacterial suspension of known absorbance. The two phases were vortex mixed and after phase separation the absorbance of the suspension was remeasured by removing an aliquot with a Pasteur pipet. The percentage decrease in the absorbance of the suspension was used to calculate the percentage of cells which adhered to the hydrocarbon. The adherence of cells to the hydrocarbon was assumed to be a measurement of their hydrophobic properties.

Rosenberg et al. (1980a) reported that the optimum volume of

hydrocarbon to be added ranged from approximately 10 to 30% of the cell suspension volume. The type of hydrocarbon used did not affect the extent of cell adherence. Using nine species of bacteria they reported that adherence to hydrocarbons varied widely among species. The age of the cells and the composition of the growth medium were found to affect adherence (Rosenberg et al., 1980b).

Recent studies using the BATH assay were reviewed by Rosenberg (1984a). In numerous investigations, many parameters of the assay have been found to influence the extent of adherence, in particular the duration of mixing and the concentration of the bacterial suspension. The suspending medium has also been shown to affect adherence (Richards and Russell, 1986). In addition, the presence of proteins or surfactants has been reported to inhibit adherence (Ofek et al., 1983; Rosenberg et al., 1980a). Lysis of cells of some species has also been reported when using the BATH assay (Lachica and Zink, 1984; Kefford and Marshall, 1986). Another problem encountered with the BATH assay is its inability to assess low levels of cell hydrophobicity. Rosenberg (1984b) proposed the use of ammonium sulfate to enhance the adherence of weakly hydrophobic cells. He reported that ammonium sulfate dramatically increased adherence of cells which previously showed no adherence in standard buffers.

The BATH method has also been employed to measure the hydrophobicity of bacterial spores. Rosenberg (1982), as cited by Rosenberg (1984a), reported that spores of Bacillus cereus read-

ily adhered to hexadecane while vegetative cells showed little adherence. Doyle et al. (1984) reported spore adherence to hydrocarbons varied among species and even among strains of the same species. The spores showed greater adherence than the vegetative cells for all species in the study. Treatment of spores with heat, detergents, lysozyme, trypsin and guanidine hydrochloride were shown to affect their adherence to hydrocarbons. Treatment with sodium dodecyl sulfate was also reported to increase spore adherence. All spore species showed greater adherence to hexadecane as compared to toluene or octane.

Rosenberg et al. (1985) reported that the adherence of B. brevis to hydrocarbons was affected by the composition of the sporulation medium and demonstrated that the adherence of spores was not affected by their germination. However, once outgrowth began, adherence declined rapidly. Adherence appeared to be related to the synthesis of the antibiotic gramicidin S. Spore coats of Clostridium perfringens were reported to be much more adherent to toluene than spores without coats (Craven et al., 1985). Decreasing the pH or increasing the ionic strength has been reported to increase spore adherence to hydrocarbons (Craven and Blankenship, 1986).

(iv) Salt aggregation test

The use of the salt aggregation test (SAT) to measure surface hydrophobicity of bacteria was first proposed by Lindahl et al. (1981). "Salting out", a common method used in protein fractionation, involves addition of salts such as ammonium sulfate to

a protein solution. As the concentration of the salt increases, proteins become less soluble and finally precipitate. Correlation exists between the level of salt required to precipitate a protein and its hydrophobicity as determined by HIC (Hjerten, 1981). The proteins which precipitate at the lowest salt concentrations are the most hydrophobic. As the ionic strength of the solution increases, electrostatic interactions between proteins are repressed and hydrophobic interactions between proteins are favoured (Lindahl et al., 1981). Proteins containing a high number of surface charged groups and/or small numbers of surface hydrophobic groups require greater amounts of salt for precipitation as compared to proteins with lower numbers of surface charged groups and higher numbers of hydrophobic groups on their surface.

Lindahl et al. (1981) applied the salting out method using ammonium sulfate to determine the surface hydrophobicity of enterotoxic E. coli strains and compared the results with those obtained using HIC. The E. coli strains which aggregated at low salt concentrations (< 2.0 M) adsorbed to octyl-Sepharose columns while strains requiring salt concentrations greater than 2 M for aggregation did not adsorb onto the column. In addition those strains possessing surface fimbriae were shown to be very hydrophobic as compared to nonfimbriated strains.

Ljund et al. (1985) used the SAT method to study the surface hydrophobicity of Staphylococcus aureus strains isolated from human infections. They reported that 86 to 91% of the strains isolated from infections were very hydrophobic, aggregating in

phosphate buffer or physiological saline. In contast, only 39% of strains isolated from healthy carriers were very hydrophobic. The composition of the growth medium significantly altered the hydrophobicity of the cells.

Rozgnyi et al. (1985b) reported an improved SAT method which involved the use of hydrophobic paper cards and methylene blue stain. The hydrophobic paper cards provided a convenient disposable support material for the SAT while the methylene blue aided in identifying aggregation. Five different patterns of aggregation were observed during the study.

The effects of several SAT variables on hydrophobicity determinations of Staphylococcus were reported by Rozgnyi et al. (1985a). The density of the cell suspension was of particular importance in order to detect aggregation. Optimum results were obtained with a cell suspension of A_{540} = 20. Both the time required for complete aggregation and the storage conditions of cells influenced their hydrophobicity.

(v) Adherence to polystyrene

Factors involved in the attachment of bacteria to polystyrene were studied by Fletcher (1976, 1977). However, it was Rosenberg (1981) who proposed bacterial adherence to polystyrene as a method for determining bacterial hydrophobicity. In this method, a polystyrene disk was placed onto the surface of an agar plate containing surface colonies. After a specific time period, the plate was removed and rinsed for 2 minutes with tap water to

remove nonadhering cells. Cells remaining on the disk were fixed and stained in order to identify hydrophobic colonies. Rosenberg (1981) reported the results obtained using this method correlated with hydrophobicity values as determined by BATH.

As with other hydrophobicity determination methods, adherence can be affected by several variables. Time allotted for attachment, age of the culture and temperature have been reported to affect the adherence of cells to polystyrene (Fletcher, 1977). In addition, the presence of proteins and surfactants can impair adherence (Fletcher, 1976; Humphries et al., 1986). As in other methods, ammonium sulfate promoted adherence (Rosenberg, 1984b).

(vi) Other hydrophobicity determination methods

Fletcher and Marshall (1982) assessed the hydrophobicity of various inanimate surfaces using the bubble contact angle method which measured surface free energy. An air bubble was injected into a watertight chamber containing the test surface on the top of the chamber. The bubble floated up through the water until it made contact with the test surface. The angle of contact between the air bubble and the test surface was an indication of the difference between the surface tension of the water and the free energy of the surface. An angle of 0^{o} indicated no difference. Angles less than 15^{o} indicated a hydrophilic surface. As the hydrophobicity of the surface increased the contact angle became greater.

Fattom and Shilo (1984) applied this method to measure the

hydrophobicity of cyanobacteria species adsorbed on Millipore filter pads. The results they obtained correlated with determinations made by BATH and HIC.

The adherence of various bacterial species to films of free fatty acids was studied by Kjelleberg and Stenstron (1980). They reported that adherence was a function of the composition of the lipid film, the film and bacterial surface charges and the hydrophobicities of the film and the cells. As a result, the adherence findings could be used as indirect measurement of cell hydrophobicity.

Determination of hydrophobicity of Yersinia enterocolitica by a latex particle agglutination (LPA) test was described by Lachica and Zink (1984). Cell suspensions were mixed with equal volumes of latex particles (5.70 um diameter). A positive test was indicated by a rapid agglutination reaction. This investigation proposed that hydrophobic forces were the main factors involved in the agglutination; however, electrostatic interactions could also be involved. A correlation between plasmid-associated cell surface properties and the latex particle agglutination test was observed. Growth conditions were reported to alter the hydrophobicity of the cells.

In addition to previous methods which largely measure hydrophobicity via cell adherence to hydrophobic surfaces, methods have been developed which assess surface hydrophobicity by measuring the ability of hydrophobic molecules to bind to the cell surface. Unlike previous methods, the binding of these small

hydrophobic molecules to the cell surface provides a quantitative measure of cell surface hydrophobicity. The number of hydrophobic molecules which bind to the cell population is related to the area of the hydrophobic regions on the cell surfaces. These methods also have the advantage of reduced interference from surface charge and other surface characteristics because the molecules are small enough to interact independently with hydrophobic regions on the cell surface. The major disadvantage of these methods is that they do not provide an estimate of the heterogeneity in hydrophobicity among the population.

Kjelleberg et al. (1980) developed a quantitative method for assessing bacterial hydrophobicity by examining the affinity of dodecanoic acid for the cell surface. The dodecanoic acid was radioactively labelled in order to detect its presence on the cells. They reported that Serratia marcescens was strongly hydrophobic. The hydrophobicity varied depending on the phase of growth; late log phase cells were the most hydrophobic. Salmonella typhimurium MRID which has been identified as being hydrophobic by other methods (Magnusson and Johansson, 1977) was reported to be less hydrophobic than Serratia.

Similarly, Noda and Kanemasa (1986) determined hydrophobicity of bacterial surfaces using nonionic surfactants. In this method, surfactants were added to a suspension of bacterial cells. The amount of surfactant adhering to the bacterial surfaces was determined by reacting excess surfactant in the supernatant with potassium ions to form a cationic complex. The complex was extracted into dichlorobenzene using a tetrabromophenol-

phtalein ethylester potassium salt. The tetrabromophenolphtalein ethylester ion was measured by absorbance at 620 nm. The amount of surfactant bound to the bacterial surface was assumed to be directly proportional to its hydrophobicity. Since the surfactant is nonionic, adherence was not influenced by electrostatic interactions.

(3) Surface charges of bacterial cells

As previously discussed, the adherence of bacterial cells to hydrophobic surfaces may be influenced by surface characteristics other than hydrophobicity. In aqueous solutions most surfaces acquire a charge due to the ionization of surface groups and/or adsorption of ions (Richmond and Fisher, 1973). The nature and density of the charge is dependent upon the composition of the aqueous solution including its pH and ionic strength (Douglas, 1959). Most bacteria possess a negatively charged surface at neutral pH (Harden and Harris, 1953). Hexadecane/saline interfaces and polystyrene surfaces are also negatively charged (Todd and Gingell, 1980; Gingell and Vince, 1982). As a result, an electrostatically based repulsive force exists between cells and these hydrophobic surfaces. In order for cells to adhere to these surfaces, an attractive force must exist between the cells and the surface which can overcome the electrostatic repulsive force. Since the charge intensity on bacterial surfaces varies among species and strains (Pedersen, 1981), the magnitude of the repulsive force and the corresponding attractive force required for adherence will be dependent on the species. Increasing the ionic

strength of the suspending medium reduces the long range repulsive forces between two negatively charged surfaces by compressing their double ion layers (Marshall, 1976). As a consequence, less attractive force is required to facilitate adherence.

The effect of pH on the charge of bacteria indicates that the ionizable groups on the cell surface are responsible for the charge profile (Marshall, 1976). These groups may be charged or neutral depending on the pH. Consequently, as the pH changes the charge of the cell will also change. At a neutral pH, most cells possess more negatively charged groups than positively charged groups resulting in a net negative charge. Common ionizable groups which can result in negative charges include carboxyls, hydroxyls, phosphatadyls and sulfhydryls. Groups which may assume positive charges include aminos, guanidyls and imidazoles. A review by Wood (1980) indicated that modification of these ionizable groups resulted in changes in the surface charge of the cell as determined by ion exchange chromatography.

(a) Measuring cell surface charge

Cell surface charge is usually determined by microelectrophoresis (Richmond and Fisher, 1973) - a technique which involves placing the cells in an electric field and monitoring the direction and speed of their migration under a microscope. The direction of the migration reveals the net charge of the cell while the speed of migration serves as an estimate of the cell's charge density. The charge potential at the plane of shear between the

cell and the medium is referred to as the zeta potential.

The relative surface charge of bacterial cells can also be measured by using ion-exchange chromatography. Daniels (1972) described bacteria as macroscopic zwitterions which adsorb to anion and cation exchange resins at pH values above and below their isoelectric points, respectively. The use of ion exchange resins to determine bacterial surface charge was termed "electostatic interaction chromatography" by Pederson (1981). Since most bacteria have isoelectric points below 7, they are negatively charged at neutral pH and adsorb to strongly basic anion exchange resins containing ammonium groups. The proportion of cells in a population which bind to the anion exchange resin is used as a measure of its relative surface charge. Higher adsorption values are assumed to indicate greater surface charge. However, variable adsorption values may only indicate the degree of heterogeneity in surface charge among the population.

(b) Spore surface charge

Surface charges of bacterial spores were first reported by Douglas (1955) using microelectrophoresis. The electrophoretic mobility patterns of B. megaterium and B. subtilis spores resembled the behavior of electrokinetically inert materials which acquire a net negative charge through the adsorption of ions. The electrophoretic mobility patterns also indicated that the two species were significantly different in chemical and/or physical structure.

Douglas and Parker (1958a) reported that the electrophoretic mobility of B. subtilis spores varied with age. The authors also observed that spore charge was homogeneous within populations. Douglas and Parker (1958b) studied the electrophoretic mobility of B. megaterium spores and cells throughout the germination and outgrowth process. They reported a slight decrease in mobility during germination followed by large decrease upon emergence of the vegetative cell. The presence of the carboxyl group, as the principal surface charge group on both spores and cells, was also confirmed by these investigators. This observation repudiated Douglas's (1955) earlier suggestions of an electrokinetically inert spore surface.

Douglas (1959) also confirmed the presence of amino groups on the spore surface. He concluded that the net negative charge of the spore was due to the adsorption of anions by the amino groups. However, Alderton and Snell (1963) proposed that B. megaterium spores could act as weakly acidic cation exchangers, rather than adsorbing anions.

Tochikuba et al. (1975) conclusively demonstrated that the charge of B. subtilis spores was due to carboxyl groups. By blocking amino groups with 2,4,6 trinitrobenzene sulfonic acid or 4-chloro-3,5 dinitrobenzoic acid, they observed little change in electrophoretic mobility indicating the absence of amino groups on the spore surface. However, when carboxyl groups were blocked using diazomethane, the spores exhibited no mobility. These studies indicated that carboxyl groups were responsible for the negative charge of spores.

Tochikubo et al. (1975) also investigated the electrophoretic mobility of germinated spores and reported that they exhibited a lower mobility than refractile spores. When amino groups were blocked, spore mobility increased; blocking the carboxyl groups resulted in spores with a slight positive charge. These results suggested that positively charged amino groups emerge during germination. The authors cited previous work by Kondo and Foster (1967) which reported that the outer layers of B. megaterium spore coats contained high levels of lysine and moderately high levels of aspartic and glutamic acid. Tochikubo et al. (1975) proposed that the charge of the resting spores was due to the carboxyl groups of aspartic and glutamic acid while the appearance of positively charged groups during germination was due to the exposure of lysine residues.

Cheung and Brown (1986) reported that the surface charge of Bacillus stearothermophilus spores was affected by nutrient depletion during sporulation.

(4) Inhibition of spore germination

(a) Germination

Germination is a well defined stage in the development cycle of sporeforming bacteria. The germination process is characterized by rapid (10 minute) irreversible changes in the chemical composition and structure of the spore. Levinson and Hyatt (1966) reported the sequence of germination events as being: loss of

resistance to heat and chemicals, loss of dipicolonic acid, increase in stainability and phase darkening. In addition, protein degradation (Setlow, 1975a,b), peptidoglycan degradation (Dring and Gould, 1971) and excretion of cations (Powell and Strange, 1953; Swerdlow et al., 1981) have been observed to occur during germination.

Initiation of anabolic activity also occurs during spore germination. The spore begins to consume oxygen (Levinson and Hyatt, 1956), accumulate high energy compounds such as ATP, NADH and NADPH (Setlow and Kornberg, 1970a; Setlow and Setlow, 1977) and synthesize macromolecules including RNA and protein (Setlow and Kornberg, 1970b; Setlow, 1975a). Twenty to thirty minutes after the initiation of germination the spore undergoes a swelling process which results in a 20% increase in cell pack volume (Hitchins et al., 1963). If conditions are favourable, the spore will begin the outgrowth process which consists of preemergence swelling (up to 100% increase in cell pack volume), rupture of the spore coat, emergence of the vegetative cell and elongation of the vegetative cell (Hitchins et al., 1963).

Germination can be initiated by either physiological, chemical or mechanical factors (Gould, 1969). Certain metabolizable compounds can physiologically trigger germination. The first evidence of these metabolic initiators was discovered by Hill (1949a,b). He found amino acids such as L-alanine and ribosides such as inosine initiated germination of spores of some Bacillus species. The initiation was very specific for the L form of

alanine. D-alanine would not initiate germination. Other metabolizable compounds such as glucose also initiate germination (Wolf and Thorley, 1957).

Nonmetabolizable chemicals like ions, surfactants, and chelates initiate chemical based germination (Foerster and Foster, 1966; Rode and Foster, 1960a; Riemann and Ordal, 1961). In addition, lysozyme, subtilisin and other enzymes may cause germination-like changes in spores of some Bacillus species (Gould and Hitchins, 1963; Sierra, 1964). Mechanical factors which can initiate germination include abrasion, deformation and hydrostatic pressure (Rode and Foster, 1960b; Clouston and Wills, 1969).

The exact nature of the physiological mechanism which triggers the germination process is not fully understood. Some researchers believe the mechanism is metabolic in nature because germination can be prevented using metabolic inhibitors (Dring and Gould, 1975). Others have suggested that the mechanism is allosteric in nature since nonmetabolizable analogues of nutrient germinants also initiate germination (Vary, 1978).

Regardless of the mechanism involved, compounds which initiate germination must come in contact with the outer surface of the spore. Removal of portions of the outer surface of the spore results in altered germination properties (Waites et al., 1972; Vary, 1973; Stelma et al., 1978; Nakatani et al., 1985; Kutima and Foegeding, 1987). Similarly, changes in the morphology and composition of spore coats within a given species also affects germination properties (Aronson and Fitz-James, 1975; Cheung and

Brown, 1986). These findings indicate that the spore coat plays a role in the germination process.

(b) Inhibition of germination

Inhibition of spore germination can result from either inappropriate environmental conditions or specific inhibitory compounds. Environmental conditions such as pH, temperature, water activity and ionic strength can inhibit spore germination. The range of environmental conditions under which spores can germinate varies greatly depending on the spore species and the nature of the germination medium (Gould, 1969).

In addition to environmental conditions, specific chemicals can inhibit germination. Early germination studies using amino acids indicated that the D-form of the amino acid could inhibit germination initiated by the corresponding L-form amino acid (Woese et al., 1958). The D-form of the amino acid was thought to inhibit the metabolic process initiated by the corresponding L-form. Other metabolic inhibitors were studied. The germination of B. cereus T spores was not affected by agents such as arsenate, arsenite, borate cyanide and monoiodoacetate (Murty and Halvorson, 1957). Hyatt and Levinson (1962) demonstrated the inhibition of germination by these agents was to some extent dependent on the germination medium. In a survey of 14 metabolic inhibitors, Curran and Knaysi (1961) reported only ethanol (10%) was effective in inhibiting spore germination of B. subtilis.

Gould (1964) studied the effects of various food preserva-

tives on the growth of bacteria from spores. Most preservatives did not prevent germination but did inhibit outgrowth. Only nitrite at concentrations of 0.075 to 0.250% (w/v) inhibited germination. Sofos et al. (1979) reported nitrite did not inhibit germination of either B. cereus T or Clostridium botulinum spores in chicken frankfurter emulsions; however, germination was inhibited by potassium sorbate. Other preservatives such as EDTA and methyl or propyl esters of p-hydroxybenzoic acids have been demonstrated to be effective germination inhibitors (Winaro et al., 1971; Parker, 1969). Al-Khayat et al. (1987) reported that phenolic antioxidants such as BHA, BHT and TBHQ were effective inhibitors of B. subtilis spore germination. Sanitizing agents such as chlorine can also prevent germination if used at sufficiently high levels (Wyatt and Waites, 1975).

Halmann and Keynan (1962) reported that octyl alcohol was very effective in inhibiting germination of Bacillus licheniformis spores. Many other alcohols and related compounds have inhibitory activity against spore germination; these include chlorocresol (Parker and Bradely, 1968; Parker 1969a,b), phenylethyl alcohol (Slepecky, 1963), long chain alcohols (Truijillo and Laible, 1970), cinnamyl phenols and related compounds (Lewis and Jurd, 1972) and eugenol (Al-Khayat and Blank, 1985). The inhibitory effects of most of these compounds were reversible by washing the spores with water or surfactants. Parker (1969a,b) believed this reversibility was evidence that the alcohols interfered with an enzyme needed for germination. He was able to demonstrate that chlorocresol inhibited spore germination by

subtilopeptidase-A, a spore lytic enzyme.

Alternatively, Sierra (1970) suggested that chlorocresol interfered with the use of amino acids such as L-alanine. A similar inhibitory mechanism was proposed for germination inhibition by Veronal, sodium 5,5 diethyl barbituate (Sierra and Bowman, 1969). Trujillo and Laible (1970) demonstrated the inhibitory activity of aliphatic alcohols increased with chain length. Yasuda-Yasaki et al. (1978) studied the inhibition of B. subtilis spore germination by various hydrophobic compounds including alcohols; they reported that the inhibitory activity increased as the hydrophobicity of the compounds increased.

In contrast to their inhibitory affects, some alcohols have been reported to increase the rate and extent of spore germination through a process known as activation (Hyatt and Levinson, 1968). Activation is a reversible conditioning process which encourages spore germination. The activation process is usually considered more essential in the germination of young spores than in older spores (Keynan and Evenchik, 1969). Craven and Blankenship (1985) suggested a hydrophobic site is involved in spore activation by alcohols.

Other hydrophobic compounds such as fatty acids have inhibitory activity against spore germination (Foster and Wynne, 1947; Tonge, 1964). Tonge (1964) proposed that fatty acids may inhibit spore germination by coating the spore surface with one or two monolayers. Yasuda et al. (1982) studied the relationship between germination inhibition by fatty acids and phenolic compounds and

their hydrophobic/ionic character. The inhibitory effect of fatty acids correlated directly with their hydrophobic character. The inhibitory effects of phenolic compounds were more complex and thought to be dependent on both hydrophobic and electostatic effects. Since these compounds inhibit L-alanine initiated germination, Yasuda-Yasaki et al. (1978) suggested that the inhibition mechanism may involve the interaction of the inhibitor with a hydrophobic site on the spore coat near the L-alanine receptor site.

MATERIALS AND METHODS

(1) Source of microorganisms and maintenance

Bacillus coagulans (ATCC 7050) and *B. licheniformis* (ATCC 14580) were obtained from the American Type Culture Collection (Rockville, Maryland). *B. subtilis* (ATCC 6063) was obtained as a standardized spore suspension (Difco Laboratories, Detroit, Michigan). *B. stearothermophilus* var *calidolactis* was obtained from spore suspensions used in the Delvotest P ampoules (Gist-Brocades nv, Holland).

All cultures were maintained on nutrient agar slants at 5°C.

(2) Spore preparation

(a) Vegetative cell inocula

B. stearothermophilus was grown in tryptone yeast extract glucose medium (Kim and Naylor, 1966) at 55°C. *B. licheniformis* was grown in nutrient broth (BBL) or tryptic soy broth (TSB) (Gibco) at 32°C. *B. subtilis* and *B. coagulans* were grown in nutrient broth or tryptic soy broth at 37°C. Cell inocula were incubated for 18 to 24 h using a gyratory shaker (150 rpm). Portions (10 mL) from each inoculum were recultured in their respective media for 18 to 24 h and harvested at 4000 g for 30 min at 4°C. The pellets were suspended in 20 mL peptone (Difco, 1%).

(b) Spore crops

Portions (2 to 5 mL) of each cell pellet suspension were evenly distributed over the surface of the sporulation media (200 mL) in 1 L Roux bottles.

Sporulation medium A, used for B. stearothermophilus, consisted of (g/L): nutrient broth, 8.0; yeast extract, 4.0; $MnCl_2 \cdot 4H_2O$, 0.01; agar, 20.0 (Kim and Naylor, 1966). Sporulation medium B, used for the remaining organisms except B. subtilis crop 1, consisted of (g/L): nutrient broth 8.0; yeast extract, 5.0; $CaCl_2 \cdot 2H_2O$, 0.1; $MgCl_2 \cdot 2H_2O$, 0.2; $MnCl_2 \cdot 4H_2O$; 0.01; agar, 20.0 (Krieg, 1981). B. subtilis crop 1 was produced on nutrient agar (Difco). Temperatures of incubation were identical to those used for vegetative cell growth.

The extent of sporulation was monitored using phase optics. Spore crops were harvested after 7 to 18 days of incubation using three 10 mL washes of cold, sterile distilled water. The pooled spores were centrifuged (12 to 16,000 g; 30 min at 4°C). This procedure was repeated two times. Final spore crops were stored in water at 5°C until used.

(c) Spore cleaning

Each of the harvested spore crops contained vegetative cells and cellular debris. This contaminating material was removed by a series of aqueous washing and centrifugation steps. Due to the heterogeneity of the contaminating material, the centrifugation

speed and duration were varied to ensure complete cleaning.

In most cases, spore crops were centrifuged first at 500 to 600 g for one to three hours at 4°C. The surface layers of the pellet which contained a large proportion of cells and debris were removed by gentle washing. The remaining pellet was resuspended and examined microscopically. The centrifugation-washing procedure was repeated five to ten times.

Centrifugation at 4,000 g or higher was sometimes required in order to achieve better cleaning. Usually, two or more distinct layers were formed in the pellet - a dark top layer consisting mostly of vegetative cells plus a light coloured bottom layer consisting primarily of spores. The top layer was removed by gentle shaking or mixing.

(d) Production of initial and subsequent spore crops

In order to assess the hydrophobic consistency of the harvested spores, additional crops of B. subtilis and B. licheniformis were produced. Spores from the original crop (crop 1) were aseptically streaked onto nutrient agar. After incubation at 37°C for 24 h, a colony was inoculated into TSB (37°C for 24 h). The resultant vegetative growth was used as an inoculum for spore crop 2. Alternatively, a spore portion (0.01 mL) from crop 1 was used as the initial inoculum for vegetative growth.

Six spore crops of B. subtilis were produced which included:

1 - original
2 - from crop 1 by single colony inoculum
3 - from crop 2 spore portion
4 - from crop 1 spore portion
5 - from crop 1 spore portion
6 - from crop 1 spore portion.

Three spore crops of B. licheniformis were produced which included:

1 - original
2 - from crop 1 by single colony inoculum
3 - from crop 2 spore portion.

All spore crops were harvested and cleaned as previously outlined.

(3) Hydrophobicity determination methods

(a) Bacterial adherence to hydrocarbons (BATH)

All spore crops were initially standardized to A_{600} = 0.15 to 0.30 (LKB Ultrospec II; 1 cm path length) using either distilled water (pH 7.2 to 7.6), ammonium sulfate (0.01 M, pH 6.0) or physiological saline (pH 5.3). Portions (7 mL) of each spore crop suspension were combined with hexadecane (0.25 mL, analytical grade) in borosilicate glass test tubes (18 mm outer diameter). The spore-hexadecane suspension was vigorously mixed (60 sec) using a vortex mixer. After phase separation (30 min), a portion (3 mL) from the aqueous phase was removed using a Pasteur pipet and the decrease in absorbance determined. In most assays, portions (7 mL) of each spore suspension were also mixed without hexadecane and any decrease in absorbance was recorded. The

decrease in absorbance values was used as a measure of spore adherence. All glassware was cleaned with chromic acid (Chromerge^{R}, Fisher) prior to use.

Using B. subtilis (spore crops 1 and 4) the effects of the following variables on spore adherence were investigated: mixing time (30, 60 and 90 sec), concentration of hexadecane (36, 71 and 143 uL/mL of spore suspension), area of the hexadecane-water interface (test tube sizes: 16, 18 and 20 mm, outer diameter), pH of suspending citrate-phosphate buffer (0.1 M; pH 3.0, 5.0 and 7.0) and type of hydrocarbon (toluene and hexadecane).

In addition, the hexadecane adherence of vegetative cells (B. subtilis cell crop 1; B. licheniformis cell crop 1) and their respective spore crops were compared. Both spores and cells were suspended in physiological saline.

(b) Hydrophobic interaction chromatography

Chromatography columns packed with Sepharose 4B, Sepharose CL-4B, octyl-Sepharose CL-4B or phenyl-Sepharose CL-4B (Pharmacia, Upsala, Sweden) were prepared in Pasteur pipets (5 3/4", Fisher) plugged with glass wool, and in disposable 12 mL plastic columns (Biorad Inc.). Bed volumes used in both types of columns were 1.0 to 1.5 mL.

Spore suspensions, prepared in distilled water or ammonium sulfate (0.01, 0.1 , 0.2 M), were loaded onto the columns using either 100 uL or 5 mL portions having an $A_{600} > 1.0$ or 0.1 to

0.3, respectively. Columns loaded with spore samples were eluted using suspending solutions (5 mL). The A_{600} of the eluate was compared to the control which consisted of an equivalent portion of the spore suspension loaded onto the column plus 5 mL of the suspending buffer. The percentage decrease in absorbance between the eluate and the control was used as measurement of spore adherence. Spore adherence was also assessed in the presence of the following detergents at a 1% concentration: Tween 80; Triton X-100 and hexadecyl trimethyl ammonium bromide.

(c) Salt aggregation test (SAT)

Spore suspensions in distilled water (A_{600} = 2.5 to 3.0) of B. subtilis (crop 4) and B. licheniformis (crop 3) were placed in a series of deep well glass slides with equivalent volumes of ammonium sulfate solutions (0.125, 0.250, 0.50, 1.0, 2.0 and 4.0 M). The slides were rocked gently to allow mixing and then viewed under 10 X magnification using a stereomicroscope. The lowest concentration of ammonium sulfate which caused aggregation was recorded.

(d) Adherence to polystyrene

Suspensions of B. subtilis spores (crop 1) and B. licheniformis spores (crop 3) were prepared in distilled water and ammonium sulfate (0.01 M). Portions (3 mL; A_{600} = 0.1 to 0.2) of each suspension were dispensed into the following containers: test tube (control; 18 mm outer diameter; borosilicate glass), beaker (47 mm outer diameter; borosilicate glass) and culture

dish (polystyrene, 53 mm outer diameter, Falcon). After incubation (60 min, 20°C), the absorbance (600 nm) of the decanted suspensions was measured and compared with that of the control. The glass beaker was used for adherence comparison with the polystyrene dish since both had a similar surface area. The adherence was also measured in the presence of the following detergents at a concentration of 1%: Tween 80, Triton X-100 and hexadecyltrimethyl ammonium bromide.

(e) Polyethylene glycol-phosphate two phase system

A two phase system consisting of polyethylene glycol 4000 (Serva) and phosphate buffer (3 M) was prepared according to system "Y" of Sacks and Alderton (1961). The volume of the upper phase was ca. 65% of the total volume. Additionally, a system was prepared in which 10% (by weight) of the polyethylene glycol 4000 was replaced by a hydrophobic derivative, polyoxyethylene stearate (Serva). A portion (50 to 75 uL) of B. subtilis (crop 1) or B. licheniformis (crop 3) was added to a test tube containing 8 mL of one of the two phase systems. After inverting the tube 40 times, the contents were left to equilibrate for 30 minutes. An aliquot (1 mL) was withdrawn from each phase and added to distilled water (9 mL). The absorbance at 600 nm was recorded and compared to control solutions which contained 100% of the spores in either the top or bottom phase. This enabled the distribution of the spores between the two phases to be calculated.

(4) Surface charge determination methods

(a) Electrostatic interaction chromatography (EIC)

Suspensions (A_{600} = 0.3) of B. subtilis (crop 1) and B. licheniformis (crop 3) were prepared using both distilled water and ammonium sulfate (0.01 M). Ion exchange columns were prepared in plastic columns (12 mL, Biorad, Inc.). Bed volumes were approximately 1.5 mL. The anion exchange resins used were Dowex 1-X8, 100 - 200 mesh (Biorad, Inc.) and Amberlite resin IR-45 (OH) (BDH Chemicals Ltd.). The cation exchange resin used was Dowex 50W-X8, 100 - 200 mesh (Biorad, Inc.). Columns were loaded with 5 mL of the spore suspension followed by a 5 mL wash with the suspending medium. The eluate (10 mL) was collected; the absorbance at 600 nm was measured and compared to a control. The control consisted of equal volumes (5 mL) of the spore suspension and suspending medium. Citrate-phosphate buffer (0.1 M, pH 3) and NaCl (2 M) were used to remove adsorbed spores. In order to obtain consistent results, new cation exchange resin (Dowex 50) was used for each run.

(b) Microelectrophoresis

Spore charge was also assessed by determining zeta potentials using the Lazer Zee Meter (Pen-Kem Inc., Croton N.Y.). Spore suspensions of B. subtilis (crop 4) and B. licheniformis (crop 3) were prepared in water and ammonium sulfate (0.01 M) (A_{600} = 0.15 to 0.30). Zeta potentials were determined at 150 volts. Portions (25 mL) of the spore suspensions were loaded into

the cell and spore electrophoretic mobility was observed under a magnification of 270 X.

(5) Germination and outgrowth inhibition by eugenol

Spore suspensions (A_{600} = 0.15 to 0.30) of B. subtilis and B. licheniformis (crop 1) were germinated at 37°C in sterile tryptic soy broth (Gibco) containing 0.01 to 0.14% (v/v) eugenol (Eastman Kodak) in increments of 0.005%. Unless otherwise stated eugenol was not heat sterilized. At 15 to 30 min intervals, portions were removed and A_{600} recorded. The suspensions were incubated long enough for germination and outgrowth to occur in a control suspension with no eugenol (ca. 2 to 3 h). B. subtilis but not B. licheniformis spores were initially heat activated (10 min, 80°C) in distilled water. The latter organism was not activated since preliminary studies indicated an inhibitory effect.

RESULTS AND DISCUSSION

(1) Hydrophobicity determinations

(a) Bacterial adherence to hydrocarbons

The hexadecane adherence levels for *Bacillus* spore crops suspended in water are given in Table 1. Three mean values are provided for each spore crop - the total adherence observed upon mixing with hexadecane, the adherence observed upon mixing without hexadecane (control) and an adjusted adherence value calculated by subtracting the control from the total. *B. licheniformis* (crops 2 and 3) exhibited high values (*ca.* 50% or greater) of total adherence. *B. licheniformis* (crop 1) and *B. subtilis* (crops 4, 5 and 6) showed moderate values (25-50%) of total adherence. The remainder of the crops demonstrated low total adherence values (<25%). Most control values were similar except for *B. stearothermophilus* and *B. subtilis* (crops 2 and 3) which were significantly lower than the corresponding values for *B. licheniformis* (crops 1 and 3) and *B. subtilis* (crop 6). When total adherence values were adjusted using the control values, *B. licheniformis* (crops 2 and 3) still exhibited high adherence values (*ca.* 50% or greater), however, all the remaining crops had low values (<25%) which were not significantly different ($P \leq 0.05$) except for *B. subtilis* crops 5 and 6 which were significantly higher than crop 3. No turbidity or emulsification was observed in the hexadecane layer following phase separation. Phase microscopy revealed that the spores appeared to adhere to

Table 1. BATH ASSAY: Adherence of *Bacillus* spores to hexadecane. Spores suspended in water.

Species	Spore crop	Adherence (Decrease in absorbance, %) Total[1]	Control[2]	Adjusted[1]
B. subtilis	1	23 ± 3^{ab}	8 ± 4^{abc}	15 ± 6^{ab}
	2	7 ± 6^{cd}	3 ± 3^{bc}	4 ± 4^{ab}
	3	5 ± 2^{d}	2 ± 3^{bc}	3 ± 1^{b}
	4	34 ± 7^{ae}	15 ± 4^{abc}	19 ± 7^{ab}
	5	27 ± 8^{ab}	11 ± 3^{abc}	16 ± 7^{a}
	6	46 ± 13^{e}	22 ± 13^{a}	25 ± 7^{a}
B. licheniformis	1	37 ± 11^{ae}	21 ± 6^{a}	17 ± 12^{ab}
	2	75 ± 6^{f}	19 ± 6^{ab}	57 ± 9^{c}
	3	74 ± 4^{f}	24 ± 7^{a}	49 ± 8^{c}
B. stearothermophilus	1	12 ± 6^{bc}	0 ± 0^{c}	12 ± 6^{ab}
B. coagulans	1	24 ± 26^{ab}	8 ± 9^{abc}	17 ± 33^{ab}

[1] Mean values based on a minimum of three trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply only within columns.

[2] Mean values based on a minimum of two trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

the hexadecane-water interface (Figure 1).

The hexadecane adherence levels (total, control and adjusted) for Bacillus spore crops suspended in ammonium sulfate (0.01 M) are presented in Table 2. Only B. subtilis (crop 2) and B. stearothermophilus exhibited total adherence levels less than 50%. The control values in ammonium sulfate were similar to those found with water suspensions except for those of B. subtilis (crops 4, 5 and 6) which showed large increases in adherence to glass. When the total adherence values were adjusted using the controls, B. subtilis (crops 1, 3 and 5), B. licheniformis (crops 1, 2 and 3) plus B. coagulans still possessed high (>50%) adherence values. B. subtilis (crops 4 and 6) had adjusted adherence values less than 50% due to the high value of their controls (>50%).

The hexadecane adherence levels of B. subtilis and B. licheniformis vegetative cells, and their respective spores are compared in Table 3. Both spores and cells were suspended in physiological saline. The vegetative cells of both species exhibited low total and control adherence values (<10%) however, their respective spore crops showed high (>50%) total and control values. Since the control values for the vegetative cells were higher than their respective total values, adjusted values could not be calculated. No significant differences existed among the adjusted adherence values for the two spore species.

As shown in Table 4, increasing the volume of hexadecane from 36 to 143 uL/mL spore suspension had no significant effect

Figure 1. Adherence of B. licheniformis spores to the surface of hexadecane droplets. Spores suspended in $(NH_4)_2SO_4$ (0.01 M). Bar represents approximately 4 um.

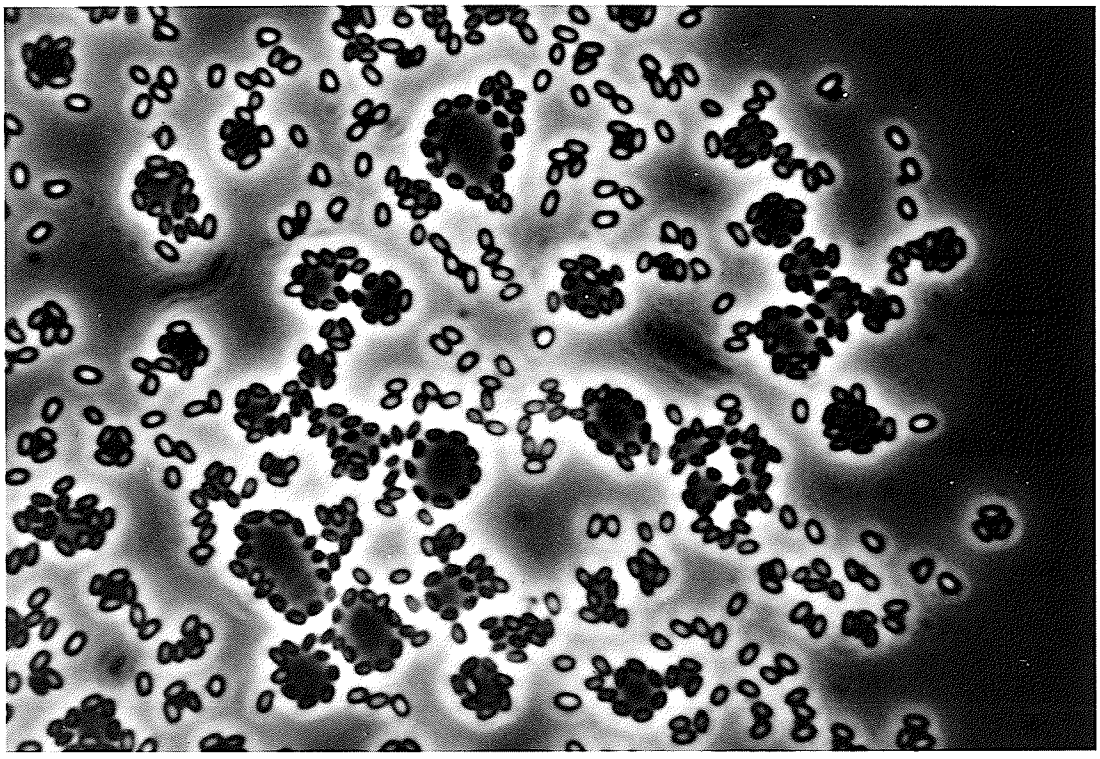


Table 2. BATH ASSAY: Adherence of *Bacillus* spores to hexadecane. Spores suspended in $(NH_4)_2SO_4$ (0.01M).

Species	Spore crop	Adherence (Decrease in absorbance, %) Total[1]	Control[2]	Adjusted[1]
B. subtilis	1	84 ± 2^{a}	17 ± 2^{ab}	67 ± 3^{ab}
	2	23 ± 6^{a}	3 ± 4^{b}	21 ± 8^{c}
	3	59 ± 9	5 ± 3^{b}	54 ± 10^{bd}
	4	96 ± 3^{a}	60 ± 8^{c}	36 ± 6^{e}
	5	93 ± 1^{a}	43 ± 13^{cd}	50 ± 11^{bde}
	6	93 ± 2^{a}	51 ± 0^{de}	42 ± 3^{de}
B. licheniformis	1	87 ± 3^{a}	16 ± 2^{ab}	71 ± 5^{a}
	2	84 ± 14^{a}	11 ± 6^{b}	72 ± 18^{a}
	3	94 ± 1^{a}	31 ± 4^{ad}	64 ± 4^{ab}
B. stearothermophilus	1	35 ± 4	2 ± 1^{b}	34 ± 5^{ce}
B. coagulans	1	91 ± 3^{a}	15 ± 10^{ab}	76 ± 9^{a}

[1] Mean values based on a minimum of four trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply only within columns.

[2] Mean values based on a minimum of two trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

Table 3. BATH ASSAY: Adherence of vegetative cells and their respective spores to hexadecane. Spores suspended in physiological saline.

Species	State	Adherence (Decrease in absorbance, %)		
		Total[1]	Control[1]	Adjusted[1]
B. subtilis (crop 1)	cells	3 ± 5^{a}	16 ± 3^{a}	---
	spores	91 ± 4^{b}	52 ± 11^{c}	39 ± 10^{c}
B. licheniformis (crop 3)	cells	2 ± 2^{a}	6 ± 2^{a}	---
	spores	94 ± 4^{b}	52 ± 19^{c}	42 ± 16^{c}

[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

Table 4. BATH ASSAY: Adherence of B. subtilis spores (crop 1) to varying volumes of hexadecane. Spores suspended in water.

Hexadecane (uL/mL spore suspension)	Adherence (Decrease in absorbance, %)[1]
36	14 ± 3^a
71	17 ± 4^a
143	22 ± 9^a

[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

on adherence of B. subtilis spores. In contrast, adherence of B. subtilis spores increased by more than 100% when hexadecane was replaced by toluene as the hydrocarbon phase (Table 5).

The effect of varying the mixing time from 30 to 90 seconds on B. subtilis spore adherence (total, control and adjusted) is given in Table 6. The total adherence increased significantly when the mixing time was increased from 30 to 60 seconds; however, no significant change in adherence occurred when the mixing time was increased from 60 to 90 seconds. In addition, when the total adherence values were adjusted using the control values, no significant adherence differences were observed among the three time periods.

The effects of cross sectional area of the hexadecane-water interface (controlled by varying the diameter of the mixing tube) on B. subtilis spore adherence are presented in Table 7. The 20 mm tube exhibited higher total and adjusted adherence levels when compared to either the 16 or 18 mm tubes. No differences were observed among the control values.

Adjusting the pH of the spore suspension from 7 to 3 with citrate-phosphate buffer (0.1 M) had no effect on the total, control or adjusted adherence levels B. subtilis spores (Table 8). However, the adherence levels were much higher in the buffer as compared to water.

Previous investigations which employed the BATH method to measure cell hydrophobicity assumed that the decrease in absorbance observed upon mixing cell suspensions with hexadecane was

Table 5. BATH ASSAY: Adherence of B. subtilis spores (crop 1) to hexadecane and toluene. Spores suspended in water.

Hydrocarbon	Adherence (Decrease in absorbance, %)[1]
Hexadecane	14±2
Toluene	35±8

[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the t test ($P \leq 0.05$).

Table 6. BATH ASSAY: Effect of mixing time on adherence of *B. subtilis* spores (crop 1) to hexadecane. Spores suspended in water.

Duration of mixing (sec)	Adherence (Decrease in absorbance, %)		
	Total[1]	Control[1]	Adjusted[1]
30	11 ± 2^{ab}	8 ± 1^{b}	3 ± 2^{d}
60	14 ± 2^{c}	11 ± 1^{ab}	4 ± 1^{d}
90	15 ± 1^{c}	12 ± 2^{ac}	3 ± 2^{d}

[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

Table 7. BATH ASSAY: Effect of cross sectional area of the hexadecane-water interface on the adherence of *B. subtilis* spores (crop 4). Spores suspended in water.

Test tube size (outer diameter, mm)	Adherence (Decrease in Absorbance, %)		
	Total[1]	Control[1]	Adjusted[1]
16	61 ± 7^{a}	21 ± 6^{b}	40 ± 4^{c}
18	59 ± 9^{a}	20 ± 5^{b}	42 ± 4^{c}
20	74 ± 7	18 ± 5^{b}	56 ± 9^{a}

[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

Table 8. BATH ASSAY: Adherence of B. subtilis spores (crop 4) to hexadecane. Spores suspended in citrate-phosphate buffer (0.1 M, pH 3 to 7).

pH	Adherence (Decrease in absorbance, %)		
	Total[1]	Control[1]	Adjusted[1]
7	90±0[a]	40±7[b]	50±7[b]
5	92±1[a]	41±3[b]	51±3[b]
3	92±2[a]	45±10[b]	43±10[b]

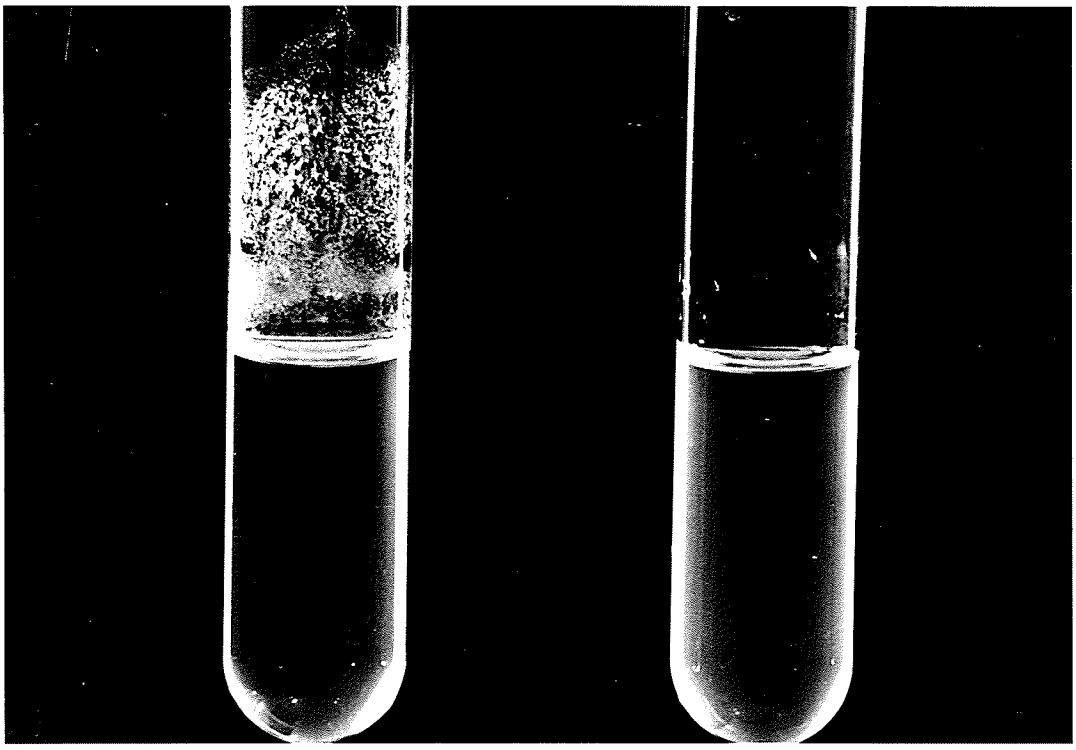
[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

completely due to cell adherence to the hydrocarbon and consequently reported the results as percentage adherence (Rosenberg et al., 1980a,b). However, the control values in Table 1 indicate that the decrease in absorbance may not be due entirely to spore adherence to the hydrocarbon. After the spore suspensions were mixed without hexadecane, a visible film was apparent on the walls of the test tubes (Figures 2, 3). Even though the tubes were gently shaken to remove the film, some spores remained adsorbed to the glass. A slight film was also observed in the tubes mixed with the hexadecane. Consequently, the total adherence value may be a result of spore adherence to both the hydrocarbon and the glass. In order to obtain a more accurate estimate of spore adherence to the hydrocarbon, the total value was adjusted by subtracting the decrease in absorbance found in the control. This adjusted value is based on the assumption that spore adherence to glass is not affected by the presence of hexadecane.

A relationship between spore adherence to glass and to hexadecane appears to exist for most spore crops. Crops with the highest adherence to hexadecane also possessed the highest adherence to glass. This may indicate that hydrophobicity is involved in the adherence of spores to glass, especially since the presence of detergents (Tween 80 1%, v/v) reduced their adherence (Figure 4).

The adjusted adherence values (Table 1) are an indication that variations in adherence exist among spores of different

Figure 2. Adherence of B. licheniformis spores to test tube walls in control tube before (A) and after mixing (B). Spores suspended in $(NH_4)_2SO_4$ (0.01 M).



B A

Figure 3. Film formation on control tube. B. licheniformis spores suspended in $(NH_4)_2SO_4$ (0.01 M).

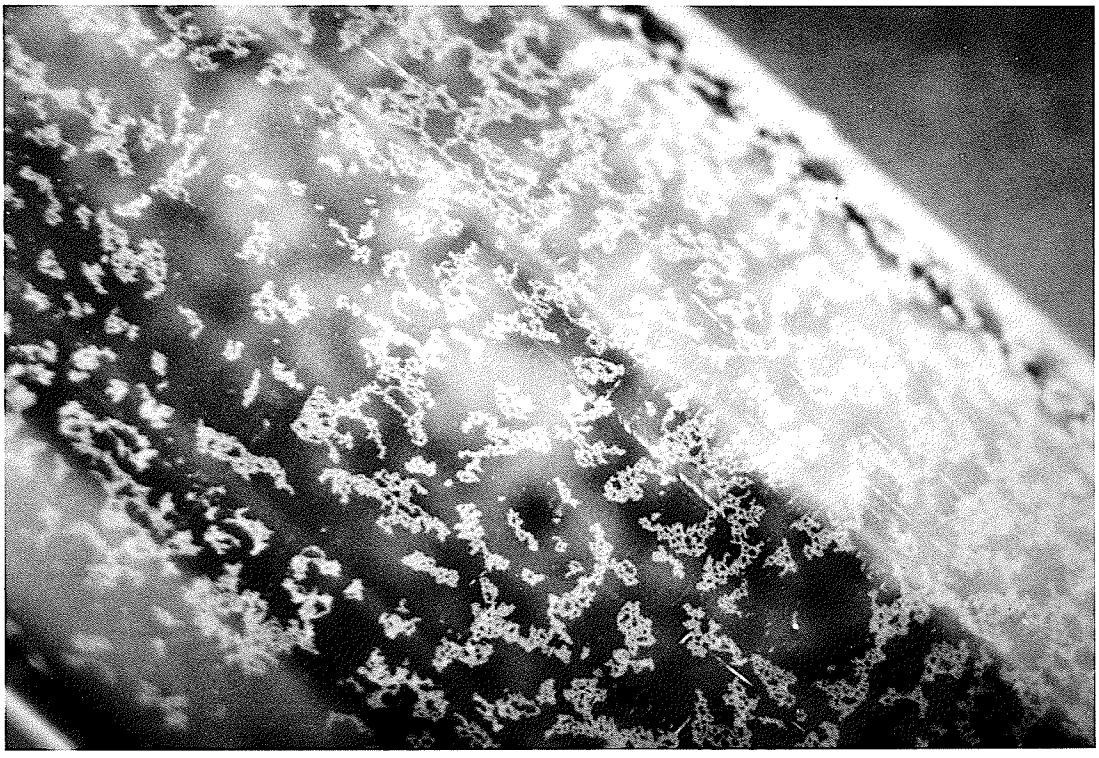
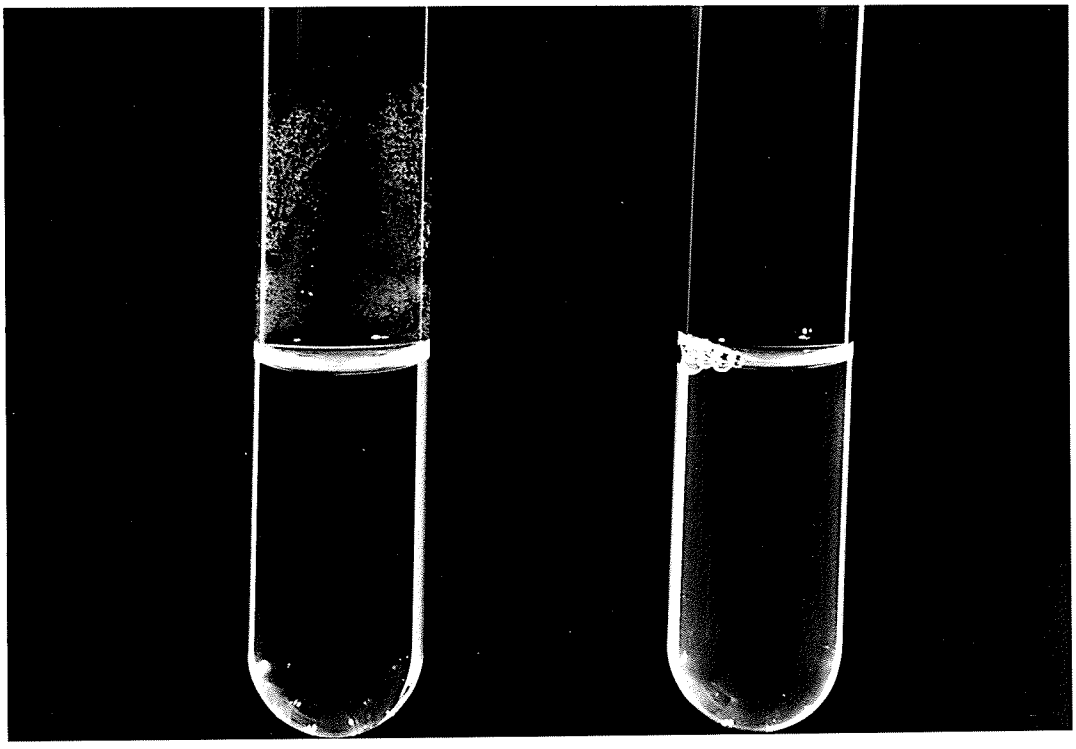


Figure 4. Adherence of B. licheniformis spores to test tube walls in control tube with (A) and without (B) Tween 80 1% (v/v). Spores suspended in $(NH_4)_2SO_4$ (0.01 M).



B A

Bacillus species. B. licheniformis spores exhibited the highest levels of adherence followed by B. subtilis and B. coagulans. B. stearothermophilus spores demonstrated the lowest levels of adherence.

Other investigators have also reported that spore adherence to hydrocarbons varies depending upon the species and strain. Doyle et al. (1984) showed that the hexadecane adherence levels of Bacillus spores suspended in saline ranged from 13 to 64% depending upon the species and strain. Rosenberg et al. (1986) reported that B. sphaericus and B. thuringensis spores suspended in phosphate buffer (0.1 M, pH 7.1) exhibited hexadecane adherence levels of 14 and 40% respectively. The results from these reports cannot however be directly compared with the present results because different spore suspending media were used in each case.

In addition to variation in adherence levels among different species, variation also existed among different crops of the same species. For the six crops of B. subtilis examined, the adherence of crop 3 was significantly lower than crop 6. For the three crops of B. licheniformis evaluated, the adherence of crop 1 was significantly lower than crops 2 and 3. The adherence of spores to hydrocarbons may be influenced by a variety of factors including the environmental conditions employed during sporulation (Rosenberg et al., 1986). In addition it is suspected that the inoculation method used for spore growth is important. For example, all three crops of B. licheniformis were sporulated on identical media using similar environmental conditions. Spore

crop 2, however, was prepared from crop 1 by colony subculture. Crop 3 was subsequently prepared from crop 2. It is possible, therefore, that the first crop contained spores which were heterogeneous with respect to their adherence properties and that the colony isolate used as the inoculum for crop 2 exhibited higher adherence by chance. Selection and propagation of this isolate could explain the increase in adherence of spore crop 2 and its similarity with crop 3. Rundegen and Olsson (1987) also reported that subcultured variant strains of Streptococcus mutans serotype c exhibited altered surface hydrophobicity.

Suspending the spores in ammonium sulfate (0.01 M) increased adherence levels. The extent of the increase however was dependent on the species and the crop. For example B. subtilis (crop 2) and B. stearothermophilus showed only small increases in adherence while B. subtilis (crop 3) and B. coagulans exhibited large increases in adherence. In addition, the presence of ammonium sulfate accentuated differences in adherence among the six crops of B. subtilis. The increased adherence of Bacillus spores to hexadecane in the presence of ammonium sulfate was also reported by Rosenberg (1986), although the concentration of ammonium sulfate used was much higher (2 M).

Spore adherence to hexadecane and to glass also increased in the presence of sodium chloride and citrate-phosphate buffer. This phenomenon of increasing adherence in the presence of specific ions may be due to two factors. First, ammonium sulfate and sodium chloride are nonchaotropic salts which increase the order

of water molecules thereby encouraging the formation of hydrophobic interactions and the exposure of hydrophobic groups on the spore surface. Secondly, salts may also increase adherence by altering electrostatic interactions.

Both spores and hexadecane-saline interfaces are negatively charged (Douglas, 1955; Todd and Gingell, 1980) which results in the creation of a repulsive force. In order for the spores to adhere to the interface, the hydrophobic attractive forces must be stronger than those caused by electrostatic repulsion. Adherence therefore increases in the presence of salts due to a change in the electrostatic repulsive forces, which allows spores with lower hydrophobicity to adhere to the interface.

In the presence of ions, charged surfaces attract oppositely charged ions resulting in the formation of a double layer of ions. As the ionic strength increases the double layer becomes compressed, and the repulsive force between two surfaces with like charges changes. A net repulsive force still exists at small interparticle distances but an attractive force called the secondary minimum is created at wider interparticle distances (Marshall, 1976). Consequently, less attractive force is required for adherence. This may explain why the adherence of negatively charged spores to a negatively charged interface increases in the presence of ions.

The results in Tables 1 and 2 are also an indication that any given population contains both adherent and nonadherent spores. This heterogeneity in spore adherence may be due to

variation in spore hydrophobicity or other spore characteristics which affect adherence.

For example, spore density can vary within a population (Tamir and Gilvarg, 1965) and may be responsible for the heterogeneity in adherence levels. Since the hydrocarbon layer after phase separation is located on the surface of the spore suspension, the density of the spore suspension could affect adherence. For spores with similar surface properties, lighter spores would be expected to adhere to the hydrocarbon layer more easily than heavier spores.

The charge on the bacterial cell has also been reported to influence its adherence to hydrocarbons. For example, Criado et al. (1985) reported that neutralization of the negative charge of Neisseria meningitidis resulted in large increases in adherence. This indicated that the negative charge reduced their ability to interact with hydrocarbons. Since some spore populations are heterogeneous with respect to charge (Sacks and Alderton, 1961), this may be another factor contributing to the heterogeneity of adherence.

An alternate explanation proposed by Doyle et al. (1984) is that the heterogeneity in adherence levels may be caused by limitations in the adherence assay itself. The authors observed that when previously nonadherent spores in a population were reextracted with hexadecane some of them adhered to the hydrocarbon. They suggested this was an indication that the entire population of spores contained hydrophobic sites but access to

the hydrocarbon was limited so only a proportion actually adhered. The phenomenon of adherence of previously nonadherent spores upon reextraction was also observed in this investigation (G. Blank, personal communication).

In order to determine whether assay parameters were limiting adherence, the effects of several factors were investigated. For example, the amount of hydrocarbon used in the BATH assay has been shown to influence adherence. Doyle et al. (1984) reported that the adherence of B. cereus spores to hexadecane was increased by increasing the hexadecane concentration up to 0.133 uL/mL spore suspension. Rosenberg et al. (1985) also reported that adherence of B. brevis spores to hexadecane increased up to 200 uL/mL of spore suspension. In this investigation no significant increase in adherence was observed by increasing the hexadecane concentration up to 143 uL/mL. The lack of agreement with previously published results may be due to the use of spores which possessed lower adherence levels as compared to other investigations. In addition, different spore suspending media were used in previous studies.

The type of hydrocarbon used in the assay did influence adherence levels; B. subtilis spores possessed higher adherence levels to toluene as compared to hexadecane. In contrast, Doyle et al. (1984) reported similar levels of adherence for Bacillus spores mixed with either hexadecane or toluene. Rosenberg (1986) reported that larvicidal spores adhered to corn oil equally well or better than to hexadecane. A recent review of the BATH assay suggested that the reason for this variation in adherence levels

to different hydrocarbons may be due to the viscosity of the hydrocarbon plus the resulting size of drops formed with mixing (Rosenberg, 1984a). Another possibility is that the charge density of hydrocabon/water interfaces may change depending on the hydrocarbon; this could result in variation of the electrostatic repulsion forces between the spore and the interface.

Rosenberg (1984) reported that the mixing time affected the adherence of Acinetobacter calcoaceticus cells. Most of the adherence occurred within the first minute. In this current study, the mixing time did not affect the adjusted adherence levels within the range of 30-90 seconds. Previous spore adherence studies have all used a mixing time greater than 60 seconds with the exception of Doyle et al. (1984) who mixed suspensions for only 30 seconds.

Another parameter examined during this study was the cross sectional area of the hexadecane-water interface. Since spores adhere to the hydrocarbon/water interface, the interfacial area available for adherence may affect adherence values. The interfacial area is ultimately dependent on the diameter of the tube. Since increasing the diameter of the tube resulted in increased adherence levels, interfacial area must have limited adherence in the small diameter tubes. Consequently, test tube diameter is an important parameter when comparing BATH results.

An additional factor which may influence adherence levels is the pH of the suspending medium. The pH would not be expected to affect hydrophobicity directly unless it resulted in the exposure

or withdrawal of hydrophobic groups on the spore surface. However, the pH may be expected to affect the charge of the spore. As pH decreases, acidic groups on the spore surface become protonated and the negative charge of the spore decreases. This was observed by Douglas (1959) who reported that the electrophoretic mobility of various Bacillus spores decreased with decreasing pH. Since the negative charge inhibits cell adherence (Criado et al., 1985), decreases in pH should result in increased adherence. This pH effect has been confirmed for both cells and spores (Nesbitt et al., 1982; Craven and Blankenship, 1986). However, in this study, pH did not affect spore adherence. Due to the ionic strength of the buffer, spores exhibited almost complete adherence at pH 7.0. As a result, detection of increasing adherence at lower pH values was not possible.

In addition to parameters in the BATH assay, adherence may be affected by aging of the spore crops. Keyan and Evenchik (1969) reported that spore populations undergo marked changes with storage such as decreased viability and altered germination patterns. Changes in adherence levels with storage are evident in the two B. subtilis spore crops which were tested extensively. Crop 1 had an adjusted adherence value of 15.3% nine months after harvest (Table 1) but after 23 months its adjusted value had decreased to 3.7% (Table 6). Crop 4 had an adjusted value of 18.7% two weeks after harvesting (Table 1). After six months, its adjusted adherence value was approximately 42.3% (Table 7). It therefore appears that aging may alter spore adherence.

As previously reported (Doyle et al., 1984; Rosenberg et al., 1985), the vegetative cells of B. subtilis and B. licheniformis exhibited little or no adherence to hexadecane even when suspended in physiological saline.

(b) Hydrophobic interaction chromatography

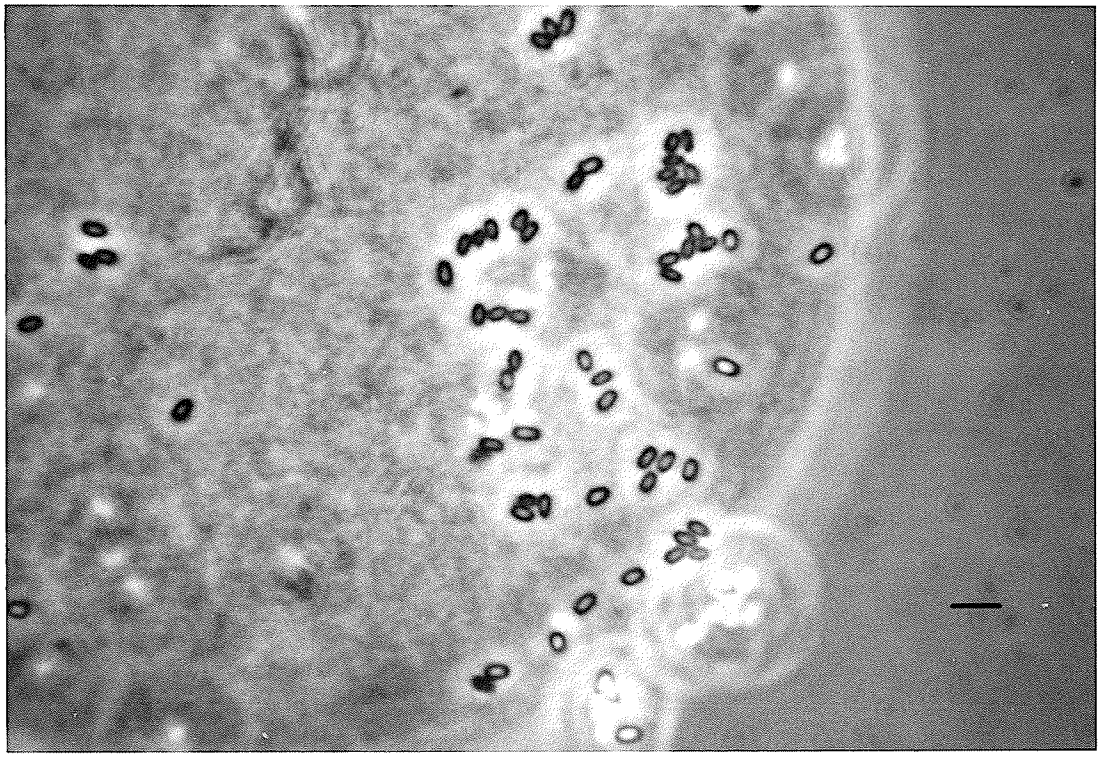
The adherence of B. subtilis spores (crop 1, 100 uL load) to various chromatographic materials equilibrated with water or ammonium sulfate solutions is given in Table 9. Adherence of the spores to a column containing no chromatographic material (control) was increased when water was replaced by ammonium sulfate (0.1 M) as the equilibrating medium. Further increases in spore adherence occurred when the concentration of ammonium sulfate was increased to 0.2 M. The addition of chromatographic material to the column resulted in increased spore adherence. Adherence to Sepharose 4B was not affected by changing the equilibrating medium, while adherence to octyl- and phenyl-Sepharose CL-4B was increased by replacing water with ammonium sulfate. However, increasing the ammonium sulfate from 0.1 M to 0.2 M did not result in increased adherence. In ammonium sulfate (0.1 M), the spores exhibited greater adherence to octyl- and phenyl-Sepharose CL-4B as compared to Sepharose without hydrophobic groups; however, no difference was detected in adherence to octyl- versus phenyl-Sepharose. Microscopic examination revealed spore adherence to the phenyl- and octyl-Sepharose beads (Figure 5); no adherence to Sepharose beads without hydrophobic groups was observed. In the plain Sepharose columns, spore clumps appeared to

Table 9. HIC ASSAY: Adherence of concentrated suspensions of B. subtilis spores (crop 1; 100 uL) to plastic columns containing various chromatographic materials equilibrated with water or $(NH_4)_2SO_4$ solutions.

Equilibrating medium	Adherence (%)[1]			
	Column only	Sepharose 4B	Octyl-Sepharose CL-4B	Phenyl-Sepharose CL-4B
Water	8±3	43±2^c	25±11^a	43±2^c
$(NH_4)_2SO_4$ (0.1 M)	20±5^a	40±5^c	80±1^b	72±3^b
$(NH_4)_2SO_4$ (0.2 M)	24±1^b	46±1^c	84±1^b	84±1^b

[1] Mean values based on a minimum of two trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

Figure 5. Adherence of B. licheniformis spores to the surface of an octyl-Sepharose bead. Spores suspended in $(NH_4)_2SO_4$ (0.1 M). Bar represents approximately 4 um.



be trapped between the beads.

In order to reduce spore clumping and/or physical entrapment by the columns, spore suspensions of B. subtilis (crop 4) and B. licheniformis (crop 3) were initially diluted in water or ammonium sulfate (0.01 M), mixed vigorously and applied to columns of octyl- and phenyl-Sepharose CL-4B in 5 mL aliquots. This procedure reduced nonspecific adherence to the control and Sepharose columns to 10% or less (data not presented). Adherence of spores suspended in water to octyl- and phenyl-Sepharose CL-4B ranged between 8 and 10% (Table 10). No significant differences in adherence were observed between the two species or between the two Sepharose derivatives. Suspending the spores in ammonium sulfate increased adherence to greater than 75% (Table 10). B. licheniformis spores demonstrated significantly greater adherence to phenyl-Sepharose CL-4B than B. subtilis spores.

The adherence levels of various Bacillus species (dilute spore suspensions, 5 mL load) to octyl-Sepharose CL-4B equilibrated in water or ammonium sulfate (0.01 M) are presented in Table 11. All crops exhibited low adherence when suspended in water; no significant differences existed between different crops or species. Ammonium sulfate (0.01 M) increased spore adherence in comparison to water. All three B. licheniformis crops possessed high levels of adherence; no significant differences were detected among crops. In contrast, B. subtilis crops exhibited a wide range of adherence; crop 1 had high adherence, crop 2 had low adherence and crop 3 had moderate adherence. Both B. stearothermophilus and B. coagulans spores demonstrated low levels of

Table 10. HIC ASSAY: Adherence of B.subtilis (crop 4) and B. licheniformis spores (crop 3) (0.01 M) to octyl- and phenyl-Sepharose CL-4B. Spores suspended in water or $(NH_4)_2SO_4$ (0.01 M).

Suspending medium	Species	Adherence (%)[1] Octyl-Sepharose CL-4B	Adherence (%)[1] Phenyl-Sepharose CL-4B
Water	B. subtilis	11±5[a]	10±8[a]
	B. licheniformis	8±3[a]	10±1[a]
$(NH_4)_2SO_4$	B. subtilis	78±1[b]	78±4[b]
	B. licheniformis	87±2[bc]	94±1[c]

[1] Mean values of two trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

Table 11. HIC ASSAY: Adherence of Bacillus spore populations to octyl-Sepharose CL-4B. Spores suspended in water or $(NH_4)_2SO_4$ (0.01 M).

Species	Spore crop	Adherence (%)[1]	
		Water	$(NH_4)_2SO_4$
B. subtilis	1	2 ± 1^a	60 ± 9^c
	2	3 ± 1^a	10 ± 1^a
	3	2 ± 0^a	35 ± 6
B. licheniformis	1	2 ± 3^a	79 ± 3^d
	2	4 ± 3^a	77 ± 1^d
	3	3 ± 2^a	86 ± 0^d
B. stearothermophilus	1	13 ± 3^{ab}	23 ± 1^b
B. coagulans	1	2 ± 1^a	22 ± 7^b

[1] Mean values based on a minimum of two trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

adherence.

The addition of both nonionic and ionic detergents (1%) to B. licheniformis spores suspended in ammonium sulfate (0.01 M) resulted in a large decrease in adherence (Table 12). The type of detergent used did not appear to affect the amount of adherence reduction.

Pasteur pipets plugged with glass wool are the most common columns used in HIC. However, in this study, preliminary investigations indicated that a large number of spores were retained in the empty column. Microscopic examination of the glass wool plug revealed the presence of many spores. The spores appeared physically trapped within the fiber network. Despite the common use of glass wool plugs, no previous reports of cell entrapment within the plug have been found. Since previous discussions of HIC methodology in the literature did not indicate the compactness or the size of the glass wool plug used, it is possible these factors could influence the retention of the cells within the plug. In addition, the concentrated spore suspensions used in the initial investigations may have contained spore clumps which would be more readily trapped within the plug. To minimize physical entrapment, dilute spore suspensions and disposable BioRad plastic columns without glass wool plugs were used in subsequent trials.

In general, the adherence results obtained by HIC using ammonium sulfate correlated well with those results obtained by BATH, with the exception of B. coagulans. This organism exhibited

Table 12. HIC ASSAY: Effect of detergents on adherence of *B. licheniformis* spores (crop 3) to octyl-Sepharose CL-4B. Spores suspended in $(NH_4)_2SO_4$ (0.01 M).

Detergent	Adherence (%)[1]
Control	74±6
Triton X-100[2]	15±7[a]
Tween 80[2]	7±3[a]
Hexadecyl trimethyl[3] ammonium bromide	5±1[a]

[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

[2] 1%, v/v.

[3] 1%, w/v.

high adherence to hexadecane when suspended in ammonium sulfate but low levels of adherence to octyl-Sepharose. Similarly, when spores were suspended in water, adherence levels to octyl-Sepharose were low for all crops; however, hexadecane adherence varied depending on the crop and the species.

Doyle et al. (1984) also reported low levels of adherence for B. subtilis 168 spores to octyl-Sepharose when the spores were suspended in water. While adherence increased in the presence of 0.1 M ammonium sulfate, it was not affected by higher ammonium sulfate concentrations (2.0 M). In contrast, the authors reported that B. anthracis 14185 spores almost completely adhered to the octyl-Sepharose when suspended in water; the addition of ammonium sulfate resulted in a slight decrease in adherence levels. The detergent, sodium dodecyl sulfate, resulted in decreased adherence for both species; Triton X-100 increased adherence for B. subtilis but had little effect on B. anthracis adherence. Since the adherence of spores to octyl-Sepharose is assumed to be based on hydrophobic interactions, the presence of detergents would be expected to reduce adherence as results in this investigation have shown. However, detergents which bind to a hydrophobic cell may have the opposite effect. If the polar end of the detergent binds to the spore, the nonpolar tail will be exposed to the surface thereby increasing the hydrophobicity of the spore. This may explain the increased adherence of B. subtilis in the presence of Triton X-100 observed by Doyle et al. (1984).

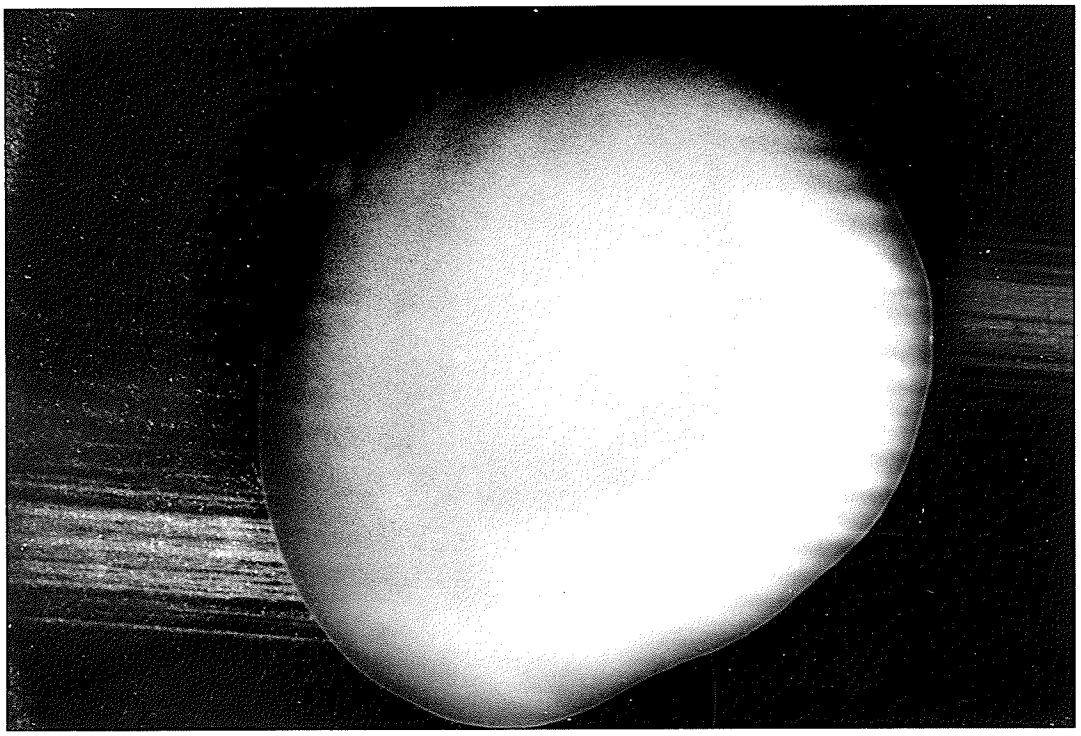
In this investigation, the elution of adherent spores from the octyl-Sepharose columns was not entirely successful. Common elution methods for hydrophobic colums, such as lowering the ionic strength or adding polarity reducing agents, resulted in elution of approximately 50% of the adherent spores. Tylewska et al. (1979) also observed that Streptococcus cells adhering to octyl-Sepharose columns could not be removed with detergents. Similarly, Smyth et al. (1978) reported that E. coli could not be eluted from hydrophobic columns by decreasing the ionic strength. Small et al. (1986) used a variety of agents in an attempt to elute the Trichoplusia virus from octyl- and phenyl-Sepharose columns; only 17% of the adsorbed virus could be eluted. It is possible that the cells or viruses are adsorbed to the column by forces other than hydrophobic interactions . This would explain the inconsistent effects of ammonium sulfate on spore adherence observed by Doyle et al. (1984).

(c) Salt aggregation test

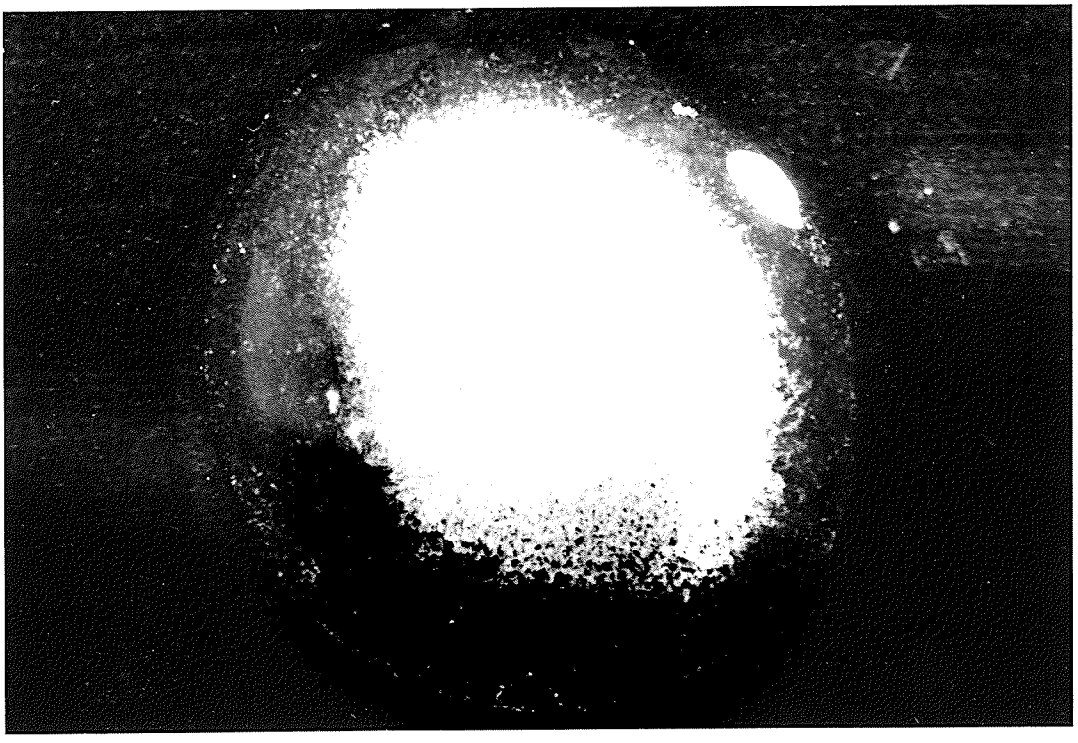
The lowest concentration of ammonium sulfate resulting in aggregation of B. licheniformis spores (crop 3) was 0.625 M; whereas a concentration equal to or greater than 1 M ammonium sulfate was required to aggregate B. subtilis spores (crop 4) (Figure 6).

These results are in agreement with the hydrophobicities determined using the BATH and HIC methods. All methods indicated that B. licheniformis spores were more hydrophobic than those of B. subtilis. However, Doyle et al. (1984) reported that the

Figure 6. Aggregation of B. licheniformis spores in water (A) and $(NH_4)_2SO_4$ (1.0 M) (B).



A



B

effect of ammonium sulfate on spore hydrophobicity (HIC) is not always consistent. Ammonium sulfate may increase or decrease adherence depending on the species and on the concentration of ammonium sulfate used. In addition, nonhydrophobic interactions may be involved in cell aggregation of some species. Jonsson and Wadstrom (1984) reported that detergents and chaotropic salts which reduce hydrophobic interactions did not affect the aggregation of several autoaggregating strains of Staphylococcus aureus. Mozes and Rouxhet (1987) suggested that cell aggregation in ammonium sulfate is more related to surface charge than to hydrophobicity.

(d) Adherence to polystyrene and glass

The adherence levels of B. subtilis (crop 1) and B. licheniformis (crop 3) spores to polystyrene tissue culture dishes and glass beakers is presented in Table 13. Since the surfaces were not rinsed after adherence determinations, the adherence values do not differentiate between loosely and firmly attached spores. B. licheniformis spores demonstrated significantly higher adhesion to polystyrene than those of B. subtilis, both in water and in ammonium sulfate (0.01 M). Ammonium sulfate also increased the adherence of both species. Adherence to glass was less than adherence to polystyrene. When the two species were suspended in water, there were no significant differences in adherence levels to glass. Ammonium sulfate increased the adherence levels of the two species to glass to different extents; B. licheniformis possessed a significantly higher adherence level than B. sub-

Table 13. Adherence of B. subtilis spores (crop 1) and B. licheniformis spores (crop 3) to polystyrene and glass. Spores suspended in water or $(NH_4)_2SO_4$ (0.01 M).

Suspending medium	Species	Adherence (%)	
		Polystyrene[1]	Glass[2]
Water	B. subtilis	21±4[a]	14±11[a]
	B. licheniformis	33±5[b]	20±5[a]
$(NH_4)_2SO_4$	B. subtilis	33±4[b]	22±5[a]
	B. licheniformis	55±7	38±3[b]

[1] Mean values of ten trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

[2] Mean values of six trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

tilis.

The effects of detergents on the adherence levels of B. licheniformis spores (crop 3) suspended in ammonium sulfate (0.01 M) to polystyrene and glass are given in Table 14. None of the detergents significantly affected adherence to polystyrene, but Tween-80 did significantly reduce adherence to glass. In addition, adherence levels to glass and polystyrene were not significantly different in the presence of Triton-X 100 and hexadecyl trimethyl ammonium bromide. The control adherence levels in Table 14 are lower than the corresponding values in Table 13. This may be due to changes in adherence upon storage.

The results of these adherence studies show some correlation with previous methods. B. licheniformis spores were shown by BATH, HIC and SAT to be more hydrophobic than B. subtilis spores; these spores also had greater adherence levels to polystyrene, a hydrophobic surface, than B. subtilis spores. As in previous methods, ammonium sulfate increased adherence. However, detergents which inhibited adherence in HIC did not inhibit adherence to polysytrene indicating that nonhydrophobic interactions may be involved.

In addition the spores also adhered to glass, a hydrophilic surface, as was observed in the BATH studies. In both cases, adherence was reduced in the presence of the detergent Tween 80; as a result, hydrophobicity may play a role in the adherence to glass. For example, Marshall and Cruickshank (1973) reported that two bacterial species which adhered to oil-water interfaces

Table 14. Effect of detergents on the adherence of *B. licheniformis* spores (crop 3) to polystyrene and glass. Spores suspended in $(NH_4)_2SO_4$ (0.01 M).

Detergent	Adherence (%)	
	Polystyrene[1]	Glass[2]
Control	43 ± 1^{a}	30 ± 1^{b}
Triton-X[3]	35 ± 6^{ab}	32 ± 4^{b}
Tween-80[3]	36 ± 3^{ab}	20 ± 1
Hexadecyl trimethyl[4] ammonium bromide	34 ± 3^{ab}	30 ± 1^{b}

[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

[2] Mean values of two trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

[3] 1%, v/v.

[4] 1%, w/v.

also adhered to glass surfaces. The authors suggested that the hydrophobic nature of the cells caused their rejection from the aqueous phase into the glass-water interface. Hydrophobic cells would be transferred from the ultimate hydrophilic surface - water - to a less hydrophilic surface - glass. Marshall and Cruickshank (1973) also reported that Tween 80 reduced cell adherence to glass and to the oil-water interface. Tween 80 would be expected to coat the hydrophobic surfaces of the cell and render them hydrophilic. Hydrophilic surfaces would be less likely to adhere to glass since a short range repulsive force would occur between the two hydrophilic surfaces due to the net increase in the free energy of displaced water (Rutter and Vincent, 1980). Kjelleberg (1984) also suggested that hydrophobic bacteria demonstrate more adherence to solid surfaces than hydrophilic bacteria.

Dewhurst et al. (1986) studied the removal of B. subtilis spores from glass and polystyrene. Using ultrasound, they were able to remove 95.8% of the spores adhering to the glass but only 23.7% of the spores adhering to polystyrene. This suggested a different mechanism of adhesion for the two surfaces. When Tween 80 was added, the percentage of spores which could be removed from the polystyrene surface increased to 42.4% indicating that hydrophobic interactions were likely involved. Paul and Jeffery (1985) also concluded that separate adhesion mechanisms may exist for hydrophilic and hydrophobic surfaces in Vibrio proteolytica.

Other researchers believe no clear correlation exists be-

tween hydrophobicity and cell adherence to surfaces. McEldowney and Fletcher (1986) reported no correlation between bacterial adherence to hydrophobic or hydrophilic surfaces and bacterial hydrophobicity. They suggested that both hydrophobic and electrostatic interactions were probably involved in adhesion to polystyrene. Hogt et al. (1983) reported that adherence to hydrophobic surfaces could not be correlated with either the hydrophobic or electrostatic properties of the cell.

The adherence of B. licheniformis spores to glass increased in the presence of ammonium sulfate. BATH results also indicated increased adherence to glass in the presence of saline or citrate-phosphate buffer. Marshall et al. (1971) noted similar effects of ionic strength on the adherence of Pseudomonas to glass. They attributed the increased adherence to the compression of the double ion layer at higher ionic strengths resulting in a decrease in repulsive forces and the creation of an attractive secondary minimum force. However, this view was not supported by McEldowney and Fletcher (1986) who reported no correlation between the adherence of four bacterial species to hydrophilic or hydrophobic surfaces and the ionic strength of the suspending medium.

(e) Two phase aqueous polymer system

Studies with polyethylene glycol (PEG)-phosphate systems indicated that 92% of B. licheniformis (crop 3) spores and 55% of B. subtilis (crop 1) spores were partitioned into the PEG phase (Table 15). No spores were detected in the phosphate phase; this

Table 15. Distribution of B. subtilis spores (crop 1) and B. licheniformis spores (crop 3) in polyethylene glycol-phosphate two phase systems.

a) polyethylene glycol without hydrophobic groups:

Species	Spores in phosphate phase (%)[1]	Spores in polyethylene glycol phase (%)[1]
B. subtilis	0[2]	54±2[a]
B. licheniformis	0[2]	92±9[b]

b) polyethylene glycol with stearate groups:

Species	Spores in phosphate phase (%)[1]	Spores in polyethylene glycol phase (%)[1]
B. subtilis	0[2]	65±2[a]
B. licheniformis	0[2]	83±7[b]

[1] Mean values of three trials. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

[2] Remaining spores were assumed to be situated at the interface of the two phases.

indicated that the remaining spores were situated at the interface. Introduction of a hydrophobic stearate derivative into the PEG phase did not significantly change the spore distribution.

As previously discussed, either hydrophobic and/or electrostatic forces may be responsible for the distribution of spores in PEG-phosphate systems. Although hydrophobic polymers partition into the PEG phase (Albertson, 1958), Sacks and Alderton (1961) reported spore distribution appeared to be dependent on electrophoretic mobility of the spores. Consequently, the results of this investigation indicate that B. licheniformis spores possessed either a lower electrophoretic mobility and/or a greater hydrophobicity than B. subtilis spores. All previous hydrophobicity methods have shown the latter to be the case. However, the introduction of hydrophobic groups into the PEG phase did not increase the distribution of spores into the PEG phase.

(f) Spore hydrophobicity

Correlation among the different hydrophobicity methods appears to be dependent on the species examined. Each method demonstrated that B. licheniformis spores exhibited greater hydrophobic behavior than B. subtilis spores. However, B. coagulans spores suspended in ammonium sulfate exhibited strong adherence to hexadecane but little adherence to octyl-Sepharose. Other comparisons of bacterial hydrophobicity methods have also reported that the degree of correlation between methods was dependent upon the species investigated (Dillon et al., 1986; Mozes and

Rouxhet, 1987). Mozes and Rouxhet (1987) suggested that the bubble contact angle, HIC and adherence to polystyrene methods were the most suitable hydrophobicity assays while SAT and BATH were described as being the least suitable.

Jonsson and Wadstrom (1984) proposed that hydrophobicity methods may sense hydrophobicity in different ways. In addition, methods may vary in their sensitivity to interference from other surface properties such as charge, adhesive polymers and variations in morphology. For example, Criado et al. (1985) demonstrated that the negative charge of bacterial cells may inhibit their adherence to hydrocarbon-water interfaces. Pederson (1981) reported cells with the same hydrophobicity may vary in charge. As a result, cells of the same hydrophobicity which vary in charge may be incorrectly diagnosed as possessing different hydrophobicities. Consequently, it is important that the results of hydrophobicity determinations be considered in relation to the surface charge of the cell.

(2) Surface charge determinations

The adherence levels of B. subtilis (crop 1) and B. licheniformis (crop 3) spores to Dowex 1, an anion exchange column, are given in Table 16. Both species, regardless of the suspending medium, exhibited nearly total adherence. In contrast, both species exhibited low adherence to Dowex 50, a cation exchange column, when suspended in water. Ammonium sulfate increased adherence to the cation exchange column for both species but adherence levels for B. subtilis were approximately double those

Table 16. EIC ASSAY: Adherence of B. subtilis spores (crop 1) and B. licheniformis spores (crop 3) to Dowex ion exchange resins. Spores suspended in water or $(NH_4)_2SO_4$ (0.01 M).

Suspending medium	Species	Adherence (%)	
		Dowex 1-X8[1]	Dowex 50W-X8[1]
Water	B. subtilis	98±0[a]	9±5[b]
	B. licheniformis	96±1[a]	2±1[b]
$(NH_4)_2SO_4$	B. subtilis	98±1[a]	76±1[a]
	B. licheniformis	98±1[a]	39±6

[1] Mean values of two trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$)

obtained for B. licheniformis. Removal of the adsorbed spores by increasing ionic strength (sodium chloride, 2.0 M) or by decreasing pH (citrate-phosphate buffer, 0.1 M, pH 3) was unsuccessful. Continuous runs (up to 8 trials) did not appear to alter spore adherence to the anion exchange resin; however, continuous runs in the 0.01 M ammonium sulfate did reduce spore adherence to the cation exchange resin. Fresh cation exchange material was therefore used for each trial. Adherence levels of both species to a weakly acidic anion exchange resin, Amberlite IR-45 (OH), are presented in Table 17. When suspended in water, both species demonstrated adherence levels of from 30 to 40%. Ammonium sulfate significantly decreased adherence for each species.

The zeta potentials for B. subtilis (crop 4) and B. licheniformis (crop 3) were -48.9±7.7 and -51.5±10.2 mV respectively when the spores were suspended in water. In ammonium sulfate (0.01 M) the zeta potentials were reduced to -23.4±0.7 mV for B. subtilis and -23.7±4.0 for B. licheniformis.

The complete adherence of spores suspended in water to Dowex 1 and the zeta potential determinations indicated that the spores possessed a net negative charge. The results are in agreement with earlier spore charge studies using microelectrophoresis (Douglas, 1955; 1958a, b). In this investigation, increasing ionic strength did not affect adherence to Dowex but did reduce the zeta potential. Douglas (1959) reported that the electrophoretic mobility of Bacillus spores increased with increasing ionic strength; however, Lindahl et al. (1981) has suggested electro-

Table 17. EIC ASSAY: Adherence of B. subtilis spores (crop 1) and B. licheniformis spores (crop 3) to a weakly basic anion exchange resin [Amberlite IR-45 (OH)]. Spores suspended in water and $(NH_4)_2SO_4$ (0.01 M).

Suspending medium	Species	Adherence (%)[1]
Water	B. subtilis	41 ± 9^{a}
	B. licheniformis	35 ± 2^{ab}
$(NH_4)_2SO_4$	B. subtilis	24 ± 5^{bc}
	B. licheniformis	19 ± 2^{c}

[1] Mean values of three trials each done in duplicate. Mean values with the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

static interactions decreased in the presence of salts. Woods (1980) reported that the effect of salts on bacterial adherence to anion exchange columns was dependent on the bacterial species. Salts inhibited the adherence of Staphylococcus aureus but did not affect the adherence of E. coli. In this investigation the adherence of Bacillus spores to strongly basic anion exchange resins does not appear to be affected by salt concentrations of 0.01 M. As expected, the two species exhibited less adherence to the weakly basic anion exchange column, Amberlite IR-45 (OH); however, no difference in adherence was detected between the species. Ammonium sulfate (0.01 M) significantly decreased adherence indicating that salts may only reduce weak electrostatic interactions.

The low adherence of spores when suspended in water to the cation exchange column, Dowex 50, further confirmed their net negative charge. However, the addition of ammonium sulfate increased adherence especially in the case of B. subtilis. Adsorption of negatively charged cells to cation exchange resins in the presence of salts, has also been observed with other bacteria (Woods, 1980). The adsorption of negatively charged spores to the negatively charged resin in the presence of salts may be explained by a compressed double ion layer formation which creates an attractive surface. Another explanation proposed by Woods (1980) involves the presence of underlying positively charged amino groups which become exposed in the presence of salts. This may be especially true for spores which contain high amounts of lysine in their proteinaceous coats (Kondo and Foster, 1967). The

coats may undergo conformational changes in the presence of salts exposing lysine residues. Alternatively, the rapid saturation of cation exchange resins after only a few runs may indicate that spores are adhering at sites other than at charged groups. In ammonium sulfate, hydrophobic interactions increase and since the polystyrene resin is hydrophobic in nature, these interactions may play a role in spore adherence. However, if this were the case, one would expect B. licheniformis to possess greater adherence to the resin than B. subtilis.

The EIC and microelectrophoresis studies indicate that no charge difference exists between the two species when the spores are suspended in water. In ammonium sulfate (0.01 M), the charge of the spores was decreased as determined by microelectrophoresis. Even though ammonium sulfate did not affect the adherence of spores to the Dowex anion exchange resin, the decreased adherence to the Amberlite anion exchange resin in ammonium sulfate indicated a decrease in the negative charge of the spores. Consequently, the increased adherence of spores in ammonium sulfate to hydrophobic surfaces may be due to a decrease in overall charge.

(3) Inhibition of spore germination and outgrowth by eugenol

Germination and outgrowth of B. licheniformis (crop 1) spores were inhibited by 0.03% eugenol (Table 18). Although outgrowth of B. subtilis (crop 1) spores was inhibited by 0.04% (v/v) eugenol, concentrations up to 0.14% (v/v) did not inhibit germination. At concentrations greater than 0.14%, absorbance measurements became limited due to interference by suspended

Table 18. Concentration of eugenol required to inhibit the germination and outgrowth of B. subtilis spores (crop 1) and B. licheniformis spores (crop 1).

Species	Eugenol concentration (%) required to inhibit	
	Germination	Outgrowth
B. subtilis	>0.14	0.04
B. licheniformis	0.03	0.03

eugenol droplets. The absorbance change over time for each species in tryptic soy broth (Gibco) containing eugenol are illustrated in Figures 7 and 8. The results of this investigation indicate that germination of the hydrophobic species are inhibited by lower concentrations of eugenol as compared to less hydrophobic species.

Similar findings have been reported for other hydrophobic germination inhibitors. Halmann and Keynan (1962) reported that B. licheniformis germination in 7 mM L-alanine could be inhibited by 0.01 M octyl alcohol. However, other investigators reported that 0.1 M octyl alcohol only inhibited approximately 30% of B. subtilis spore germination initiated by phosphate and glucose (Curran and Knaysi, 1961). Foster and Wynne (1947) observed that oleate (100 ug/mL) inhibited the germination of Clostridium botulinum spores in brain heart infusion broth but the same concentration had no effect on the germination of spores of four Bacillus species.

Hydrophobic compounds such as eugenol likely bind to the spore via hydrophobic interactions since the effectiveness of these compounds in disrupting germination has been related to their hydrophobicity. For both alcohols and fatty acids, the more hydrophobic the compound the greater its effectiveness in inhibiting germination (Trujillo and Laible, 1970; Yasuda et al., 1982). The effectiveness of phenolic compounds in disrupting germination appears to be related to both their hydrophobicity and charge (Yasuda et al., 1982). Hydrophobic compounds are

Figure 7. Effect of eugenol on germination and outgrowth of *B. subtilis* spores.

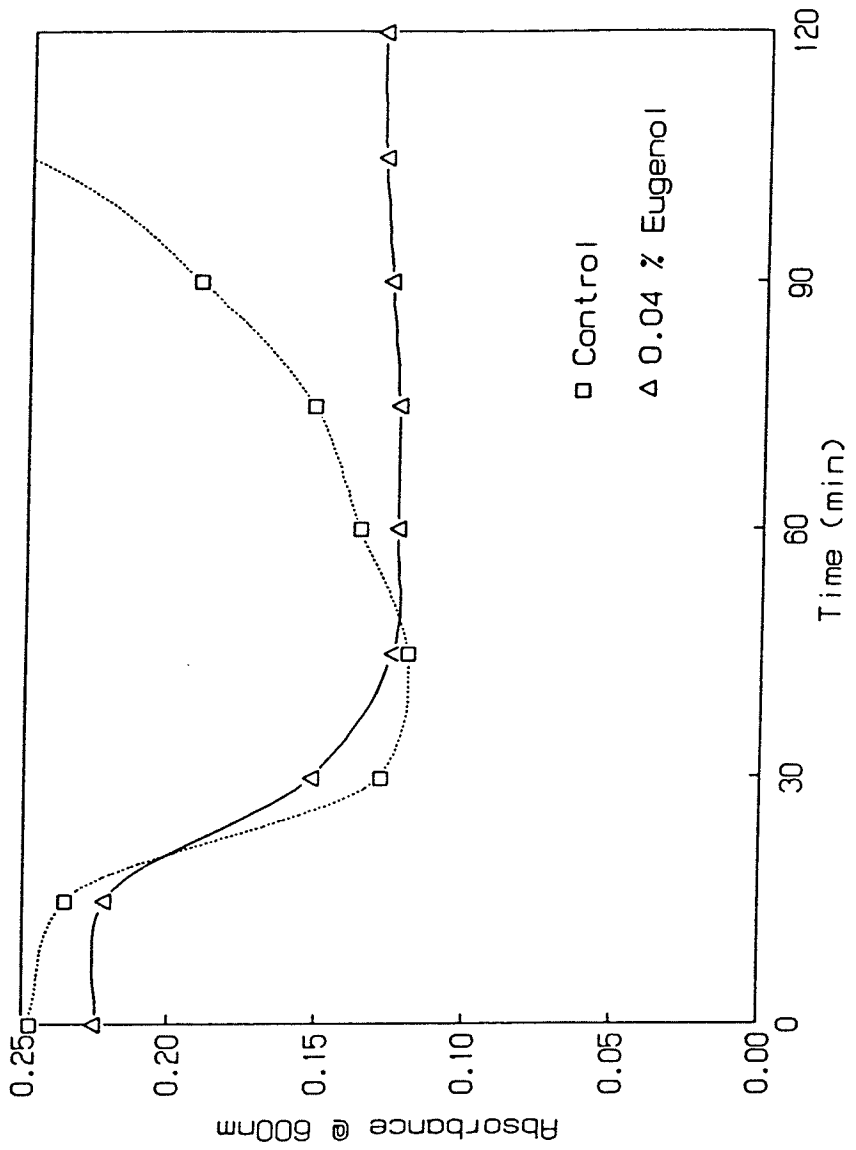
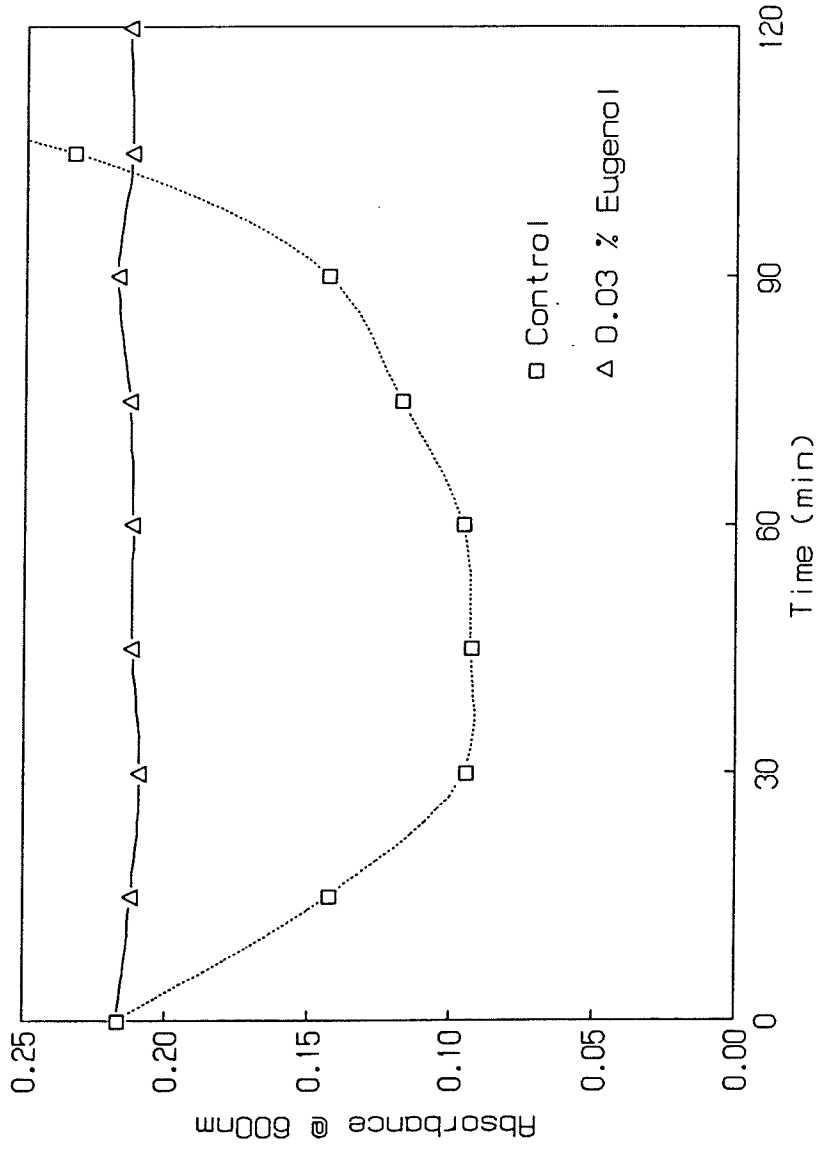

Absorbance @ 600nm
0.25
0.20
0.15
0.10
0.05
0.00
0
30
60
90
120
Time (min)
□ Control
△ 0.04 % Eugenol

Figure 8. Effect of eugenol on germination and outgrowth of B. licheniformis spores.


Absorbance @ 600nm
0.25
0.20
0.15
0.10
0.05
0.00
0
30
60
90
120
Time (min)
□ Control
△ 0.03 % Eugenol

believed to bind to the spore sites responsible for germination initiation (Yasuda-Yasaki et al., 1978). As a result, spores with hydrophobic germination sites may be more sensitive to inhibition by these compounds.

In addition, the inhibition of a germination mechanism by these compounds will be dependent on the ability of the bound compound to disrupt the germination process. Several mechanisms have been suggested to explain how these compounds disrupt germination. Parker (1969) demonstrated that chlorocresol inhibited germination of B. subtilis spores by interfering with the lytic enzyme, subtilopeptidase. Lytic enzymes of different species may vary in their sensitivity to these hydrophobic compounds. Alternatively, Sierra (1970) proposed that chlorocresol inhibited germination by interfering with the germinant L-alanine. The difficulty with this proposal is its failure to explain why species vary in their sensitivity to the hydrophobic agents. Trujillo and Laible (1970) suggested that hydrophobic compounds alter the spore coat since changes in the spore coat have been demonstrated to affect the rate and extent of germination (Kutima and Foegeding, 1987). Since spore coat structure varies among species (Murrell, 1969), hydrophobic compounds could affect various species to different degrees.

Even though a correlation was observed between spore hydrophobicity and sensitivity to germination inhibition by eugenol, no evidence exists that spores with greater hydrophobicity have germination mechanisms which are more hydrophobic or have more affinity for hydrophobic compounds as compared to less hydropho-

bic spores. Even if the germinating mechanism of hydrophobic spores had greater affinity for hydrophobic compounds, the inhibition of germination is not only dependent upon the compound binding to the germinating site but also upon the ability of the compound to disrupt the germinating mechanism. This reasoning is supported by preliminary inhibition studies with B. licheniformis spores (crop 2). If germination inhibition were related to spore hydrophobicity, one would expect that the spores of crop 2, which are considerably more hydrophobic than crop 1 (as determined by BATH), would be inhibited from germinating by lower levels of eugenol than crop 1. Preliminary studies indicated no difference in the levels of eugenol required to inhibit germination in the two crops.

Another difficulty in establishing a relationship between spore hydrophobicity and germination inhibition by eugenol is that the hydrophobic behavior of the spores in tryptic soy broth may not be equivalent to the hydrophobicity determined in water or ammonium sulfate. This investigation has demonstrated that the presence of salts can affect hydrophobic behavior.

The outgrowth of both species was inhibited by similar levels of eugenol indicating that the vegetative cells of both species were equally sensitive. The cells were inhibited by eugenol levels equal to or less than those levels required for germination inhibition. Since the vegetative cells demonstrated little hydrophobicity, cell hydrophobicity cannot be the primary reason for inhibition of vegetative growth by eugenol. However,

it is possible that the target site for outgrpwth of the vegetative cells may be more sensitive compared to the target site for germination of the spores.

SUMMARY AND CONCLUSIONS

In this investigation, the hydrophobic behavior of B. subtilis, B. licheniformis, B. coagulans and B. stearothermophilus spores was examined using both bacterial adherence to hydrocarbons and hydrophobic interaction chromatography. In water, all species with the exception of B. licheniformis (crops 2 and 3), exhibited low adherence to hexadecane. In addition, no populations exhibited substantial adherence to hydrophobic Sepharose derivatives when suspended in water. In the presence of ammonium sulfate or sodium chloride, adherence to hydrocarbons and to hydrophobic Sepharose derivatives increased. With the exception of B. coagulans, the adherence levels determined using the two methods exhibited good correlation for spores suspended in ammonium sulfate. Multiple spore crops of B. subtilis and B. licheniformis demonstrated considerable variation in hydrophobic behavior. Detergents inhibited the adherence of B. licheniformis spores to hydrophobic Sepharose derivatives.

Several factors were determined to increase spore adherence to hydrocarbons including the type of hydrocarbon (toluene adherence greater than hexadecane adherence), the presence of salts (sodium chloride, citrate-phosphate buffer) and an increase in the surface area of the hydrocarbon-water interface. The concentration of hydrocarbon, mixing time or pH did not affect adherence. In addition to hydrocarbon adherence, spores also adhered to the glass sides of the test tube in the absence of hydrocarbon. In contrast to the hydrophobic behavior of some spore popu-

lations, the vegetative cells of B. subtilis and B. licheniformis exhibited little adherence to hydrocarbons.

The results of the hydrophobicity determinations for B. subtilis and B. licheniformis spores using the salt aggregation test, adherence to polystyrene and two phase aqueous polymer systems correlated with previous determinations using bacterial adherence to hydrocarbons and hydrophobic interaction chromatography. A greater proportion of B. licheniformis spores exhibited hydrophobic behavior as compared to B. subtilis spores. Detergents did not inhibit the adherence of spores to polysytrene.

A comparison among the five hydrophobicity determination methods indicated that in most species the methods are assessing the same spore surface properties. However, the results for B. coagulans illustrate correlation is dependent on the species evaluated. Poor correlation may be due to interference of other surface properties such as charge or morphology. In addition, it is questionable whether some methods such as two phase aqueous polymer systems actually measure hydrophobicity or charge. The failure of detergents to inhibit adherence to polystyrene indicates that the value of this assay as a hydrophobicity determination method may be suspect. Furthermore, spores also demonstrated adherence to glass, a hydrophilic surface. The variation in hydrophobicity among subsequent spore crops of the same species indicated that spore hydrophobicity is a variable phenomenon even when spores are produced under the same environmental conditions.

Charge studies with electrostatic interaction chromatography

and microelectrophoresis revealed that both B. subtilis and B. licheniformis spores possessed an equivalent net negative charge in water. Consequently, it is doubtful that the variation in hydrophobic behavior in water is caused by charge. However, the charge was reduced when the spores were suspended in ammonium sulfate. As a result, the increased hydrophobic behavior of spores in ammonium sulfate may be due to reduced electrostatic repulsion.

B. licheniformis spore germination was inhibited by 0.03% eugenol as compared to B. subtilis germination which could not be inhibited by eugenol levels up to 0.14%. This difference in eugenol sensitivity may be related to the hydrophobicity of the two populations since B. licheniformis spores are more hydrophobic than B. subtilis spores. Alternatively, the variation in sensitivity for the two species may be due to differences in the sensitivity of germinating mechanisms to inhibition by eugenol.

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LIST OF ABBREVIATIONS

A	- absorbance
ATCC	- American Type Culture Collection
ATP	- adenosine triphosphate
BATH	- bacterial adherence to hydrocarbons
BBL	- Baltimore Biological Laboratories
BHA	- butylated hydroxyanisole
BHT	- butylated hydroxytoluene
ca.	- approximately
EDTA	- ethylene diamine tetraacetate
EIC	- electrostatic interaction chromatography
HIC	- hydrophobic interaction chromatography
i. e.	- that is
LPA	- latex particle agglutination
M	- molar
NADH	- nicotinamide adenine dinucleotide (reduced form)
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced form)
NPS	- nonpolar side chain frequency
PEG	- polyethylene glycol
RNA	- ribonucleic acid
SAT	- salt aggregation test
TBHQ	- monotertiary butylhydroquinone
TSB	- tryptic soy broth
var	- variety