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Heat Resistance of *Bacillus* Spores When Adhered to Stainless Steel and Its Relationship to Spore Hydrophobicity

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ABSTRACT

Twenty-one strains of *Bacillus* (10 *B. stearothermophilus*, 3 *B. cereus*, and 8 *B. licheniformis* strains) were assayed for spore surface hydrophobicity on the basis of three measures: contact angle measurement (CAM), microbial adhesion to hydrocarbons (MATH), and hydrophobic interaction chromatography (HIC). On the basis of the spore surface characteristics obtained from these assays, along with data on the heat resistance of these spores in water, eight strains of *Bacillus* (three *B. stearothermophilus*, three *B. cereus*, and two *B. licheniformis* strains) either suspended in water or adhering to stainless steel were exposed to sublethal heat treatments at 90 to 110°C to determine heat resistance (*D*-value). Significant increases in heat resistance (ranging from 3 to 400%) were observed for the eight strains adhering to stainless steel. No significant correlation was found between these heat resistance increases and spore surface characteristics as determined by the three hydrophobicity assays. There was a significant positive correlation between the hydrophobicity data obtained by the MATH assay and those obtained by the HIC assay, but these data did not correlate with those obtained by the CAM assay.

Bacillus species are common soil bacteria that are often present in milk. The resistance of their spores to wet heat is a major reason for the development of sterilization techniques such as ultrahigh-temperature (UHT) processing of milk and milk products. Under certain circumstances, exceptionally heat resistant endospores survive UHT processing. Furthermore, the spores of some *Bacillus* species are highly hydrophobic *(8, 12)* and can therefore adhere firmly to food-processing surfaces such as stainless steel *(10, 23)*. Following adhesion to stainless steel, attached spores may germinate, and the resulting vegetative cells may multiply and produce extracellular polysaccharides. Colonies of these organisms continue to grow and progressively cover the surface, forming a biofilm *(5)*. Biofilms are of concern to the food industry because of their high resistance to cleaning procedures *(19, 36)*, which allows bacteria within the biofilm to detach and to cross-contaminate products during processing.

Some strains of *Bacillus* species (*B. subtilis, B. licheniformis*, and *B. pumilus*) survive UHT treatments at 135°C for 10 s *(20)*, and others have been isolated from spoiled UHT-treated milk *(34)*. *B. insolitus, B. cereus/thuringiensis, B. coagulans*, and *B. licheniformis* have been isolated from milk processed at 120 to 132°C *(2)*. More recently, highly heat-resistant spores of *B. sporothermodurans* have been detected in UHT-treated milk from Europe *(25)* and from some non-European dairies *(11)*. Therefore, the attachment of *Bacillus* spores to processing equipment may present a major problem to the dairy industry.

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Bacterial surface hydrophobicity is the key factor in bacterial adhesion to a solid surface *(33)*. Other factors, such as the surface properties of the substrate and the suspending medium, may also play a role in attachment. However, the predominant force involved in the adhesion of *Bacillus* spores may depend on the environmental conditions. For example, with an increasing ethanol concentration, the hydrophobicity of *Bacillus* spores is the major factor governing adhesion to a solid surface *(14)*.

Given the increased resistance of *Bacillus* colonies to detergents when these colonies adhere to surfaces *(31)*, it was thought that a relationship might also exist between the attachment of spores and increases in wet-heat resistance. Since hydrophobicity affects bacterial attachment to substrates, methods for evaluating this surface characteristic were investigated, and the correlation between this surface characteristic and heat resistance was determined. While previous studies have directly correlated cell surface charge and hydrophobicity, no previous studies have correlated spore surface hydrophobicity and resistance to wet heat.

Three cell surface hydrophobicity measures (contact angle measurement [CAM], hydrophobic interaction chromatography [HIC], and microbial adhesion to hydrocarbons [MATH]) were chosen on the basis of the recommendation of Mozes and Rouxhet *(21)*, who suggested the use of multiple measures for evaluating cell surface hydrophobicity. Ahimou et al. *(1)* suggested that the optimal technique for the determination of *B. subtilis* spore hydrophobicity is CAM, since MATH and HIC are influenced by electrostatic interactions. Since other *Bacillus* species were to be investigated, three methods were used in order to establish a suitable test for *Bacillus* spores.

In a French study, the adhesion of *Bacillus* spores (iso-

TABLE 1. *Strains and sources of* Bacillus *species used in this study*

Organism	Strain	Source
Bacillus licheniformis	225	Water
B. cereus	276	Water
B. licheniformis	277	Water
B. cereus	280	Water
B. licheniformis	519	Raw milk
B. cereus	520	Raw milk
B. licheniformis	619	Soil
B. stearothermophilus	14-145	Water supply
B. stearothermophilus	14-158	Cold water
B. stearothermophilus	14-160	Drop hose
B. licheniformis	16-112	Raw milk
B. stearothermophilus	17-92	Feces
B. stearothermophilus	17-97	Dust
B. licheniformis	17-106	Soil
B. licheniformis	17-109	Feed
B. stearothermophilus	BS1	UHT milk
B. stearothermophilus	BS2	UHT milk
B. stearothermophilus	BS3	UHT milk
B. stearothermophilus	BS4	UHT milk
B. stearothermophilus	BS5	UHT milk
B. licheniformis	BL	UHT milk

lated from dairy processing lines) to stainless steel was found to reduce the susceptibility of these spores to thermal destruction. However, the difference between the heat resistance levels of planktonic and adhered spores was not found to be statistically significant *(9)*.

The aims of this study were to correlate heat resistance with spore hydrophobicity and to compare the heat resistance levels of spores of mesophilic and thermophilic strains of *Bacillus* species (isolated from raw milk, dairy farm environments, and UHT milk) adhering to stainless steel with those of spores not adhering to stainless steel. A further aim of this study was to evaluate three surface hydrophobicity measures and their suitability for the evaluation of *Bacillus* spores.

MATERIALS AND METHODS

Test cultures. Twenty-one strains of *Bacillus* (10 *B. stearothermophilus*, 3 *B. cereus*, and 8 *B. licheniformis* strains) previously isolated from Australian raw milk, dairy farm environments, and UHT milk (Table 1) *(7, 15)* were assayed for spore surface hydrophobicity by the three techniques described below. Of these 21 strains, 8 (3 *B. cereus*, 2 *B. licheniformis*, and 3 *B. stearothermophilus* strains) were selected for the adhesion experiments. These strains were selected because they represented a range of levels of spore surface hydrophobicity and heat resistance *(15)*.

Spore suspension preparation. Each strain was inoculated into nutrient broth and incubated for 24 h at 37°C (for *B. cereus* and *B. licheniformis*) or at 55°C (for *B. stearothermophilus*). One milliliter of each overnight culture was inoculated onto nutrient agar supplemented with 3 mg of manganese sulfate per liter and incubated as described above until 80 to 90% sporulation was achieved. Sporulation was monitored by phase-contrast microscopy. Spores were harvested by flooding the agar plate surface with sterile distilled water and scraping with a sterile glass spreader until an opaque suspension was achieved. The spore suspension was then transferred with sterile droppers into sterile McCartney bottles and washed by centrifugation (three times) at 2,500 × *g* for 15 min. Stock suspensions were stored in distilled water at 4°C for further use.

Determination of surface characteristics of spores: CAM. Spore surface hydrophobicity was estimated by the measurement of water contact angle (η_w) on a spore lawn by the sessile drop method *(21)*. The spore lawns were prepared on nitrocellulose-containing filters with 0.22-μm pore diameters (GSWP filter, Millipore Co., Bedford, Mass.) by negative pressure filtration of 3 ml of a ~10^8-spore suspension. Filters with continuous bacterial layers were mounted on glass slides with double-sided adhesive tape and dried in a desiccator containing potassium chloride for 3 h to obtain plateau contact angles. The contact angle of water (a 3-μl drop of Millipore-Q filtered water), delivered by a 50-μl HPLC syringe (Alltech) fitted with a modified flat-tip 24-gauge luer lock syringe tip, to the bacterial surface was measured with a microscope fitted with a digital camera (DC-290, Kodak). Two droplets of water were used for each filter, and the images were acquired within 5 s of the deposition of the drop. The images were analyzed with Adobe Photoshop (Adobe Systems Inc., San Jose, Calif.).

Determination of surface characteristics of spores: HIC. The HIC method with phenyl Sepharose was described by Ismaeel et al. *(16)* and Wiencek et al. *(35)*. Hydrophobic interactions were promoted with the use of spore suspensions in 4 M sodium chloride buffered to pH 6.75 with 0.01 M phosphate buffer to reduce the potential for spore aggregation. Duplicate columns consisted of glass Pasteur pipettes plugged with glass wool and packed to a height of 30 mm with phenyl Sepharose. Columns were washed extensively with 4 M sodium chloride to remove fine particles. Five milliliters of spore suspension was applied to the top of the column and allowed to drain into the gel beds. The absorbance of the eluent was measured at 440 nm and compared with that of the applied spore suspension. The formula $[(D_i - D_f)/D_i] \times 100$, where D_i and D_f are the optical densities of the initial spore suspension and the eluent, respectively, was used to calculate the relative hydrophobicity of the spores.

Determination of surface characteristics of spores: MATH. For MATH, the method of Rosenberg and Doyle *(28)* was used with a minor modification. Hexadecane (1 ml) was added to 3-ml spore suspensions in saline (0.85%) solutions in test tubes (18 by 180 mm). The mixtures were agitated vigorously with a vortex mixer for 1 min. After the two layers had separated completely upon standing for 15 min, the A_{440} of the aqueous phase was measured with a spectrophotometer and compared with the A_{440} of the initial spore suspension. Results were calculated by the formula $[(D_i - D_f)/D_i] \times 100$, where D_i and D_f are, respectively, the optical densities of the initial spore suspension and the aqueous phase after agitation with hexadecane.

Preparation of stainless steel chips. Stainless steel chips were prepared by the method of Intaraphan *(15)*. Stainless steel chips (type 304 L, ca. 1 by 1.5 cm) were submerged in a 5% Decon 90 solution for 24 h to remove grease. The chips were then rinsed with distilled water three times to remove Decon 90 residue. Chips were autoclaved in glass petri dishes at 121°C for 15 min and dried.

Adhesion of spores to stainless steel. For the adhesion study, *Bacillus* spore suspensions (1 ml of a known concentration, ca. 10^7 CFU/ml) were added to sterile screw-cap test tubes (13 by 100 mm, 8 ml; Kimax), each containing a stainless steel chip

of known dimensions. Spores were left to adhere at room temperature (20°C) for 1 h *(9)*. The chips were then removed with sterile forceps and rinsed twice in sterile distilled water (10 ml) to remove weakly adherent spores. After washing, each chip was added to a clean sterile screw-cap test tube containing sterile distilled water (1 ml). Attached spores were removed with a sterile cotton swab and transferred to a test tube containing sterile distilled water (5 ml). The test tube, along with the cut swab tip and stainless steel chip, was vortexed for 2 min to release the spores. Samples were serially diluted with distilled water and enumerated on nutrient agar by the spread plate technique. The rate of adhesion is presented as an adhesion index calculated by the formula $(C_a/C_i) \times 100$, where C_a is the concentration of spores adhering to the stainless steel chip (CFU/cm^2) and C_i is the initial concentration of suspended spores (CFU/ml).

Calculation of decimal reduction times for spores adhering and not adhering to stainless steel. Ten screw-cap test tubes, five prepared as described above and five containing a planktonic spore suspension (1 ml, ca. 10^4 CFU/ml), were heated in an oil bath at specific temperatures (90, 95, 100, and 110°C) to determine heat resistance *(17)*. Two test tubes (one containing a stainless steel chip and the other containing the planktonic spore suspension) were removed every minute for 5 min and cooled in an ice bath for 2 min. Come-up time and cool-down time were monitored with a thermocouple, and in all cases these times were <2 min. Attached spores were enumerated as in the adhesion experiment above. The heat resistance level for each organism was determined in duplicate.

The log value of the number of survivors was plotted against heating time at each temperature to obtain survivor curves. The time required at a given temperature to reduce the microbial population by 90% (*D*-value) was obtained from the reciprocal of the slope of the regression line through the linear section of the curve.

Validation of the method. A coefficient of variance trial was conducted for the aforementioned spore adhesion method with the use of 10 replicates of *B. stearothermophilus* 97 (a highly hydrophobic test strain). Come-up time and cool-down time were repeatedly assayed (10 replicates) to ascertain whether those obtained with the stainless steel chip significantly differed from those obtained without the stainless steel chip. In addition, 20 locations in the oil bath were tested to determine whether a significant temperature differential existed.

Statistical analysis. Analysis of variance was used to determine whether differences between come-up and cool-down times obtained with stainless steel and those obtained without stainless steel were significant. Analysis of variance was also used to determine whether a significant temperature differential existed between the 20 oil bath locations. Student's *t* test was used to determine whether differences between *D*-values obtained with stainless steel and those obtained without stainless steel were significant. All statistical analysis was performed with Minitab release 1.1 for Windows (Minitab Inc., Minneapolis, Minn.).

RESULTS AND DISCUSSION

Validation of the method. The repeatability of the spore adhesion method was confirmed with the coefficient of variance trial. The coefficient of variance was <5%. Differences between come-up and cool-down times obtained with stainless steel and those obtained without stainless steel were not significant ($P > 0.05$), and no significant ($P > 0.05$) temperature differential existed for the 20 oil bath locations.

Spore surface hydrophobicity. The *Bacillus* strains investigated showed variation in spore surface hydrophobicity within species and between methods (Fig. 1). According to the CAM assay, three *B. licheniformis* strains were arbitrarily categorized as very hydrophobic (80 to 100°), three were categorized as moderately hydrophobic (40 to 60°), and the remaining two were categorized as hydrophilic (<40°). One of the *B. cereus* strains was moderately hydrophobic, and the remaining two were hydrophilic. Four *B. stearothermophilus* strains were highly hydrophobic, five were moderately hydrophobic, and two were slightly hydrophilic.

With the use of Pearson's correlation coefficient, significant positive correlations between the MATH and HIC assays at the 95% confidence level were established, indicating substantial agreement between the methods in terms of the relative hydrophobicities of the spores. The CAM method correlated with neither of the other assays according to Pearson's correlation coefficient. This finding suggests that the contact angle measured is affected not only by hydrophobicity but also by other surface characteristics. For example, Mozes and Rouxhet *(21)* reported that surface alteration arising from water removal around cells during equilibration can affect CAM results.

The CAM assay for determining cell surface hydrophobicity has been used widely *(4, 21, 22, 30, 33)*, and variations on this method have involved filter pore sizes, methods for attachment to substrates, methods for equilibrating moisture content, and methods for obtaining angles. Another variation involves the nature of desiccation, which may alter the surface characteristics of cells *(13)*, particularly fimbriae, which are thought to extend from the cell surface into the bulk aqueous phase and may be mediators of bacterial hydrophobicity *(28)*. Mozes and Rouxhet *(21)* suggested that for the detection of hydrophilic organisms the best method is CAM, followed by HIC.

van der Mei et al. *(32)* carried out an extensive assessment of the contact angles and free surface energies of 142 isolates from a range of species and concluded that only CAM (as compared with the MATH) can provide a real estimate of cell surface hydrophobicity. However, no *Bacillus* cells or spores were tested.

MATH has been used by Craven and Blankenship *(6)* for the assessment of *Clostridium* spp. spore coat hydrophobicity. These investigators found that heat treatments (75°C for 20 min) slightly increased the hydrophobicity of intact spores but greatly reduced the isolated spore coat transfer to toluene. These changes were reversible by washing in water. *Escherichia coli* cell hydrophobicity was assessed by MATH but was not found to be significantly correlated with the ability of the organism to adhere to beef muscle. Koshikawa et al. *(18)* investigated eight strains of vegetative, mature, sporulating, and germinated *Bacillus* spp. spores and found *B. licheniformis* to be extremely hydrophilic, while *B. cereus* was highly hydrophobic. These investigators attributed this difference to a higher level of

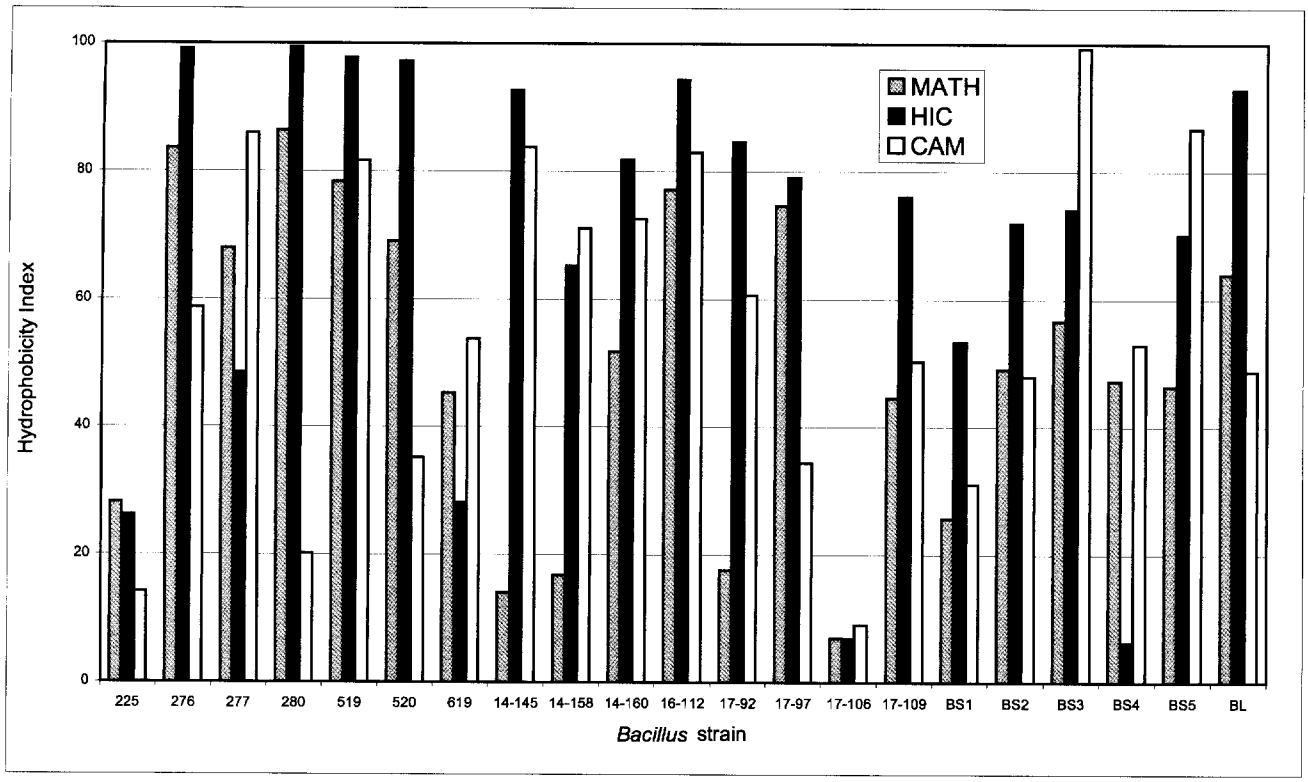

FIGURE 1. *Comparison of hydrophobicity measures for 21 strains of* Bacillus. *Values are averages of duplicate measurements.*

transfer to hexadecane when the organisms possessed exosporia. Spores with hydrophobicity readings of >70% were considered to possess exosporia, whereas for most strains lacking an exosporium, the transfer rate was <30%. On this basis, some of the spores of *B. stearothermophilus* strains used in the present study may lack exosporia.

HIC has been used by a number of researchers for spore research *(24, 27)*. Peng et al. *(24)* established a correlation between the degree of adhesion of *B. cereus* to surfaces and HIC readings, while Ronner et al. *(27)* observed only a trend for five species. Blank et al. *(3)* reported difficulties in assessing *Bacillus* spore hydrophobicity by HIC and MATH owing to the formation of stable films on the walls of test tubes both in the presence and in the absence of test hydrocarbons, but this result was not observed in the present work.

When nine *B. subtilis* strains were assessed in different physiological states by the HIC, MATH, and CAM methods, the HIC and MATH data were found to be influenced by electrostatic interactions *(1)*. This finding supported the findings of van der Mei et al. *(32)* that water CAMs provide a better estimate of cell surface hydrophobicity than the other methods do.

Relationship between rates of adhesion to stainless steel and hydrophobicity for *Bacillus* spores. The spores of all eight *Bacillus* strains adhered strongly to stainless steel, with counts of up to 10^6 CFU/cm^2 being obtained. The adhesion indices ranged from 0.4 to 14.3 (Table 2). When these adhesion indices were correlated with the three measures of hydrophobicity for the eight strains, no significant correlations ($P > 0.05$) were found. This result suggests that hydrophobicity was not the major determinant of the propensity of the spores to adhere to stainless steel. Other factors, such as the presence of appendages on some strains, may have distorted the effect of the hydrophobicity

TABLE 2. *Hydrophobicity levels of* Bacillus *spp. as determined by three methods, along with the average heat resistance increases for organisms adhering to stainless steel*

Organism	MATH %	HIC %	Contact angle θ	Adhesion index	Avg *D*-value increase (%)
B. cereus 276	83.6	99.2	58.6	2.4	214
B. cereus 280	86.5	99.5	20.1	1.1	127
B. cereus 520	69.0	97.2	35.1	1.8	261
B. licheniformis 225	28.2	26.2	14.2	14.3	220
B. licheniformis 619	45.3	28.1	53.8	6.8	125
B. stearothermophilus 14-145	14.1	92.7	83.8	6.3	222
B. stearothermophilus 17-97	74.6	79.0	34.3	1.9	322
B. stearothermophilus BS3	56.6	74.0	99.3	0.4	89

TABLE 3. *Decimal reduction times (D-values) at 90 and 95°C for* B. cereus *and* B. licheniformis *and at 100 and 110°C for* B. stearothermophilus *spores adhering to stainless steel and not adhering to stainless steel (planktonic)*[a]

Organism	D_{90} (min)		D_{95} (min)		D_{100} (min)		D_{110} (min)	
	Planktonic spores	Attached spores	Planktonic spores	Attached spores	Planktonic spores	Attached spores	Planktonic spores	Attached spores
B. cereus 520	1.59	3.87	0.79	2.20				
B. licheniformis 225	1.83	3.93	1.14	2.56				
B. licheniformis 619	4.55	4.69	2.02	2.97				
B. cereus 276	2.13	5.82	1.75	2.71				
B. cereus 280	2.99	3.91	1.49	1.83				
B. stearothermophilus 14-145					4.38	7.95	1.95	5.12
B. stearothermophilus 17-97					0.88	3.53	1.15	2.79
B. stearothermophilus BS3					4.80	5.74	2.91	3.08

[a] In all cases, the mean *D*-values for spores attached to stainless steel were significantly greater than those for spores not attached to stainless steel.

(12). Furthermore, the relative hydrophobicities of the different *Bacillus* spores and the stainless steel surface could have affected spore adhesion differently, since it has been reported that *B. cereus* shows a linear increase in adhesion with increased surface hydrophobicity while the adhesion level of *B. licheniformis* increases from low to medium hydrophobicity but then decreases at high hydrophobicity *(12).*

D-values for spores adhering and not adhering to stainless steel. The *Bacillus* spores assayed in this study have previously been shown to have stronger heat resistance in water than in milk *(15).* Thus, in the present study the heat resistance levels of attached and planktonic spores was measured with the use of distilled water as the suspension medium. For the spores of each of the eight *Bacillus* strains assayed, an increase in heat resistance (*D*-value) was observed when the spores were attached to stainless steel (Table 3).

Increases in heat resistance levels with adherence to stainless steel varied among *Bacillus* strains and ranged from 3% for the D_{90}-value for *B. licheniformis* 619 to 400% for the D_{100}-value for *B. stearothermophilus* 97 (Table 3). For all strains at all assayed temperatures, the heat resistance levels (*D*-values) of the spores attached to stainless steel were significantly higher ($P < 0.05$) than those of the spores not attached to stainless steel.

A similar significant increase (of 200 to 400%) in the heat resistance of *B. stearothermophilus* ATCC 12980 when attached to rubber stoppers has previously been reported *(29).* Faille et al. *(9)* reported that *B. cereus* and *B. licheniformis* strains isolated from French dairy processing lines tended to show increased heat resistance when attached to stainless steel; however, the difference between the heat resistance levels of suspended and adhering spores was not statistically significant. Other investigators found no increase in the heat resistance of *B. cereus* spores at up to 112°C when these spores were attached to stainless steel or rubber surfaces, but large increases in the resistance levels of spores enclosed between these two surfaces were observed *(26).*

Relationship between adhesion rates of *Bacillus* spores and increased heat resistance of these spores when attached to stainless steel. The adhesion rates for spores of the eight *Bacillus* strains investigated were correlated with the increases in *D*-values when the spores were attached to stainless steel. No significant correlation ($P > 0.05$) between adhesion rates and increases in heat resistance was found for the eight strains. This result indicates that the propensity of spores to adhere to stainless steel surfaces cannot account for or predict the increase in the heat resistance of adherent spores.

CONCLUSIONS

The hydrophobicity levels of *Bacillus* spores as measured by the three methods (HIC, MATH, and CAM) varied. While results obtained with HIC and MATH showed some correlation, neither of these measures correlated with the CAM values. The spores adhered firmly to food-grade stainless steel, but their propensity to adhere appeared to be unrelated to their hydrophobicity levels.

When spores were attached to stainless steel, their heat resistance levels were increased significantly, but the number of spores adhering under a given set of conditions was not significantly correlated with the increase in the heat resistance of the spores. Furthermore, no significant correlation between the increases in heat resistance and the relative hydrophobicity levels of the spores could be established regardless of the method used to assess the hydrophobicity.

Bacterial attachment to stainless steel plays an important role in fouling and biofilm formation in the dairy industry. These films harbor and protect bacteria from cleaning and sanitizing processes. The attachment process studied in this project is the precursor to biofilm formation. The extensive changes in the heat tolerance levels of some *Bacillus* spores when attached to stainless steel indicate that such spores could be found within biofilms at certain stages of the UHT process and could act as a reservoir for the contamination of the final product. Such information highlights the need for the effective cleaning of stainless steel surfaces in dairy processing.

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