Biofilm Formation and Sporulation by *Bacillus cereus* on a Stainless Steel Surface and Subsequent Resistance of Vegetative Cells and Spores to Chlorine, Chlorine Dioxide, and a Peroxyacetic Acid–Based Sanitizer

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ABSTRACT

Biofilm formation by *Bacillus cereus* 038-2 on stainless steel coupons, sporulation in the biofilm as affected by nutrient availability, temperature, and relative humidity, and the resistance of vegetative cells and spores in biofilm to sanitizers were investigated. Total counts in biofilm formed on coupons immersed in tryptic soy broth (TSB) at 12 and 22°C consisted of 99.94% of vegetative cells and 0.06% of spores. Coupons on which biofilm had formed were immersed in TSB or exposed to air with 100, 97, 93, or 85% relative humidity. Biofilm on coupons immersed in TSB at 12°C for an additional 6 days or 22°C for an additional 4 days contained 0.30 and 0.02% of spores, respectively, whereas biofilm exposed to air with 100 or 97% relative humidity at 22°C for 4 days contained 10 and 2.5% of spores, respectively. Sporulation did not occur in biofilm exposed to 93 or 85% relative humidity at 22°C. Treatment of biofilm on coupons that had been immersed in TSB at 22°C with chlorine (50 µg/ml), chlorine dioxide (50 µg/ml), and a peroxyacetic acid–based sanitizer (Tsunami 200, 40 µg/ml) for 5 min reduced total cell counts (vegetative cells plus spores) by 4.7, 3.0, and 3.8 log CFU per coupon, respectively; total cell counts in biofilm exposed to air with 100% relative humidity were reduced by 1.5, 2.4, and 1.1 log CFU per coupon, respectively, reflecting the presence of lower numbers of vegetative cells. Spores that survived treatment with chlorine dioxide had reduced resistance to heat. It is concluded that exposure of biofilm formed by *B. cereus* exposed to air at high relative humidity (≥97%) promotes the production of spores. Spores and, to a lesser extent, vegetative cells embedded in biofilm are protected against inactivation by sanitizers. Results provide new insights to developing strategies to achieve more effective sanitation programs to minimize risks associated with *B. cereus* in biofilm formed on food contact surfaces and on foods.

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*Bacillus cereus* can cause food spoilage and foodborne illness by producing emetic and diarrheal toxins (12). The bacterium can be isolated from many soils, dust, and surface water and is present in some types of unprocessed and pasteurized foods (2, 14). *B. cereus* has also been isolated from heat-processed or cooked foods such as baking chocolate (29), baked bread (5), cooked rice, pasta, meats, and dairy products (14). Its presence in raw vegetables (3, 13, 19), salad dressing (18), and seafood (1) has been reported.

One of the most distinct features of *B. cereus* is its ability to produce heat-resistant spores. As a result of sporulation, resistance to wet heat, dry heat, radiation, desiccation, extreme pH, chemicals, enzymes, and high pressure is greatly enhanced (4, 12, 26). This resistance enables the bacterium to survive commercial food pasteurization and cooking at ambient pressure. Sublethal heat treatment of foods containing *B. cereus* spores can select for the pathogen among other microorganisms that might be present.

Biofilm can be defined as an accumulation of microbial cells that have attached to and grown on abiotic or biological surfaces. These cells are frequently embedded in exopolymeric substances. Biofilm formation by non–spore-forming foodborne pathogens and spoilage microorganisms on food contact surfaces is a concern in the food processing industry and has been extensively investigated. The resistance of vegetative bacterial cells in biofilm to environmental stresses such as sanitizers routinely used in the food industry can be profoundly increased (9, 10, 24, 28). It is known that *B. cereus* can form biofilm on food contact surfaces (15, 20, 23). These biofilms can originate from vegetative cells or from spores that become attached to surfaces. It has been reported that spores of *Bacillus* spp. can attach more readily than vegetative cells on stainless steel surfaces because of their hydrophobic properties (8, 22).

The majority of cells in *B. cereus* biofilm are in a vegetative form. However, during the course of biofilm formation and aging, sporulation can occur. The rate and extent of spore production by *B. cereus* in biofilm would be anticipated to be affected by environmental conditions. If *B. cereus* produces spores with a biofilm matrix on food contact surfaces, those spores could have greater resistance to environmental stresses, including sanitizers. Spore formation by *B. cereus* in broth suspensions and on the surfaces of agar media has been studied (25). Spore formation...
by *Bacillus subtilis* in biofilms on glass has been described (16). Formation of spores by *B. cereus* in biofilm on food contact surfaces during exposure to environmental stresses and their subsequent resistance to sanitizers commonly used in the food industry have not been described. It is important to define environmental conditions that promote sporulation of vegetative cells in biofilm and to evaluate the resistance of vegetative cells and spores in these biofilms to environmental stresses.

The objectives of this study were to determine the effects of temperature on biofilm formation by *B. cereus* on stainless steel coupons, to investigate the influence of relative humidity on sporulation of *B. cereus* in the biofilm, and to evaluate the resistance of vegetative cells and spores in biofilm to chlorine, chloramine, peracetic acid, and a peroxyacetic acid–based sanitizer.

**MATERIALS AND METHODS**

Bacterial strain. *B. cereus* strain 038-2, isolated from infant formula, was used. This strain, obtained from Dr. Stephanie Doores (Pennsylvania State University, University Park, Pa.), produces diarrheal and emetic toxins. Cryopreserved spores stored in 15% (wt/vol) glycerol at −25°C were thawed, inoculated (100 μl into 10 ml of tryptic soy broth [TSB], pH 7.0; BBL/Difco, Becton Dickinson, Sparks, Md.), and incubated at 30°C for 24 h. Three consecutive 24-h loop (~10 μl) transfers of TSB cultures incubated at 30°C were made immediately before experiments were conducted.

**Media used.** TSB was used to culture *B. cereus* for use in biofilm studies. Phosphate-buffered saline (PBS, pH 7.4), which contains NaCl (8 g), KCl (0.2 g), Na₂HPO₄ (1.44 g), and KH₂PO₄ (0.24 g) per liter of sterile distilled water was used to prepare suspensions for attachment of cells to the surface of stainless steel coupons. Chlorine solutions were made with potassium phosphate buffer (0.05 M, pH 6.8). Dey-Engley (DE) neutralizing broth (DE broth, pH 7.4; BBL/Difco, Becton Dickinson) was used to neutralize sanitizers after treating planktonic suspensions and biofilms. Vegetative cells and spores were enumerated on brain heart infusion agar (BHIA; BBL/Difco, Becton Dickinson). The concentration of Tsunami 200 was measured with a peroxycacid test kit (Ecolab). Sanitizers (30 ml) were deposited in sterile test tubes (25 by 150 mm) immediately before treatments.

**Preparation of sanitizers.** Chlorinated water (50 and 200 μg/ml, pH 6.8), chloramine solutions (50 and 200 μg/ml, pH 11.0), and a peroxyacetic acid–based sanitizer (40 and 80 μg/ml, pH 3.6; Tsunami 200, Ecolab, St. Paul, Minn.) were prepared. To prepare chlorinated water, NaOCl (Sigma-Aldrich) was combined with potassium phosphate buffer. Aqueous solutions of chloramine dioxide (50 and 200 μg/ml) were prepared from an acidified sodium chloride solution (80%, Sigma-Aldrich). Chlorine solutions were made with potassium phosphate buffer (0.05 M, pH 6.8). Weymouth (WY) neutralizing broth (WY broth, pH 7.4; BBL/Difco, Becton Dickinson) was used to neutralize sanitizers after treating planktonic suspensions and biofilms. Vegetative cells and spores were enumerated on brain heart infusion agar (BHIA; BBL/Difco, Becton Dickinson).

**Preparation of stainless steel coupons.** Stainless steel coupons (type 304 with #4 finish, 2 by 5 cm) were sonicated in 15% phosphoric acid solution at 80°C for 20 min in an ultrasonic water bath (model 250D, VWR, West Chester, Pa.), rinsed in distilled water, sonicated in a hot (80°C) alkali detergent solution (FS ProChlor, Zep, Atlanta, Ga.) for 20 min, and rinsed in boiling distilled water for 10 min. Cleaned coupons were transferred to a beaker and dry sterilized in an autoclave before use.

**Development of biofilm by *B. cereus* on the surface of stainless steel.** After three consecutive 24-h loop (~10 μl) transfers of *B. cereus* grown in 10 ml of TSB at 30°C, cell suspensions (100 μl) were inoculated into TSB (250 ml), incubated at 22°C for 48 h, and centrifuged at 2,300 × g for 10 min. The supernatant was decanted, and cells were resuspended in PBS (2 liters) to give a population of ca. 6.5 log CFU/ml. To attach cells on coupons, 30 ml of cell suspension was deposited in a 50-ml centrifuge tube containing a sterile coupon and incubated at 22°C for 4 h. After incubation, each coupon was rinsed in 400 ml of sterile distilled water by gently moving in a circular motion for 5 s, transferred to a 50-ml centrifuge tube containing 30 ml of TSB, and incubated at 12°C for 6 days or 22°C for 2 days to facilitate the formation of biofilm.

Sporulation of *B. cereus* in biofilm as affected by exposure to air and different relative humidity. Relative humidities of 100, 97, 93, and 85% in 50-ml centrifuge tubes were created by depositing 0.8 ml of sterile deionized water, saturated K₂SO₄, KNO₃, or KCl solutions, respectively, into centrifuge tubes (50 ml), sealing the tubes, and incubating at 12 or 22°C for at least 3 weeks. After biofilms were formed as described above, coupons were kept in the TSB in which they had been incubated or transferred to 50-ml centrifuge tubes, positioned above the saturated salt solutions, and incubated for up to 6 days at 12°C or 4 days at 22°C. At each sampling time, coupons were transferred to centrifuge tubes containing 30 ml of sterile PBS and 3 g of glass beads (425 to 600 μm, Sigma-Aldrich, St. Louis, Mo.), followed by vortexing at maximum speed (G-560, Scientific Industries, Inc., Bohemia, N.Y.) for 1 min. Suspensions were serially diluted in 0.1% peptone water, surface plated (0.1 ml in duplicate) on BHIA, and incubated at 30°C for 24 h to determine total cell counts (CFU per coupon). Total cell counts represent the number of vegetative cells plus the number of spores presented in biofilm because heat treatment of *B. cereus* strain 038-2 spores is not necessary to induce germination and colony formation (25). To determine the number of spores in biofilm, suspensions (4 ml) were heated at 80°C for 10 min, serially diluted in sterile peptone solutions, surface plated (0.1 ml in duplicate) on BHIA, and incubated at 30°C for 24 h. Five replicate experiments were performed, and two coupons were analyzed in each replicate.

**Resistance of vegetative cells and spores in biofilm to sanitizers.** Biofilms of *B. cereus* were developed on coupons immersed in TSB at 22°C for 2 days. The coupons were then retained in TSB or exposed to the air with 100% relative humidity at 22°C for 3 days. Each coupon with biofilm containing various numbers of vegetative cells and spores was immersed in 30 ml of chlorinated water (0, 50, and 200 μg/ml), aqueous chlorine dioxide solution (0, 50, and 200 μg/ml), or Tsunami 200 solution (0, 40, and 80 μg/ml) for 1, 3, and 5 min. After treatment, each coupon was transferred to a 50-ml centrifuge tube containing 30 ml of DE neutralizing broth and 3 g of sterile glass beads and vortexed at maximum speed for 1 min to detach vegetative cells and spores. Suspensions were serially diluted in 0.1% peptone water, surface plated on BHIA, and incubated at 30°C for 24 h to determine total cell counts (vegetative cells plus spores). To determine the number of spores surviving treatment, DE broth suspensions (4 ml) were heated at 80°C for 10 min, serially diluted in 0.1% peptone solution, surface plated on BHIA, and incubated at 30°C for 24 h.
counts (vegetative cells plus spores) and the number of spores produced in the biofilm. The vast majority (99.94%) of CFU initially in PBS suspensions (30 ml, ~6.5 log CFU/ml) in which coupons were immersed to achieve attachment of cells before immersing in TSB were vegetative cells; only about 0.06% of CFU (~3.3 log CFU/ml) were spores. The total cell count on coupons at the end of the 4-h attachment period was ca. 5.8 log CFU per coupon, of which 1.9 to 2.5 log CFU per coupon were spores. When coupons containing attached cells were immersed in TSB and incubated at 12°C, the total cell count did not change significantly (P ≤ 0.05) for 2 days (6.2 log CFU per coupon) but decreased significantly (P ≤ 0.05) to 4.5 log CFU per coupon at 4 days and remained constant for an additional 8 days. The number of spores on coupons incubated at 12°C was in the range of 1.9 to 2.9 log CFU per coupon. When coupons containing attached cells of B. cereus were immersed in TSB and incubated at 22°C, total cell counts in biofilm increased significantly during the first 2 days and remained constant for an additional 4 days. The number of spores in biofilm on coupons incubated at 22°C did not change significantly throughout 6 days of incubation.

Sporulation of B. cereus in biofilm as affected by exposure to air at various relative humidities. The influence of air at 100, 97, 93, and 85% relative humidity on sporulation of B. cereus in biofilm on stainless steel coupons incubated at 12 and 22°C was determined. Biofilms were formed on coupons immersed in TSB at 12°C for 6 days or 22°C for 2 days then exposed to air at various relative humidities at 12°C for 6 days or 22°C for 4 days. Figure 2 shows total cell counts and number of spores in biofilm formed at 12°C on stainless steel coupons exposed to air at various relative humidities. Total cell counts and the number of spores produced in the biofilm decreased significantly (1.2 to 1.5 log CFU per coupon and 1.1 log CFU per coupon, respectively) on exposure to air, regardless of the relative humidity. After exposure to various relative humidities at 12°C for 6 days, total cell counts in biofilm were 2.8 to 3.1 log CFU per coupon and the number of spores was 1.8 log CFU per coupon, regardless of relative humidity.

Figure 3 shows total cell counts and the number of spores produced at 22°C by B. cereus in biofilm on stainless steel coupons exposed to air with various relative humidities for up to 4 days. Compared with biofilm exposed to air at 12°C, several distinct differences in behavior were observed. When biofilm was exposed to air with 100% relative humidity, total cell counts did not change significantly for 4 days, but the number of spores produced in the biofilm dramatically increased from 2.7 (0.02% of total cells) to 5.5 log CFU per coupon (10% of total cells). A similar behavior was observed in biofilm exposed to the air with 97% relative humidity. Total cell counts remained constant for 4 days, but the number of spores increased significantly from 2.7 (0.02% of total cells) to 4.8 log CFU per coupon (2.5% of total cells). When biofilm was exposed to air with 93 and 85% relative humidity for 4 days, the initial total cell count of 6.5 log CFU per coupon decreased significantly to 3.4 and 3.8 log CFU per coupon, respectively, but

**RESULTS**

Development of biofilm by B. cereus on the surface of stainless steel coupons immersed in TSB. Biofilms of B. cereus strain 038-2 were developed on stainless steel coupons immersed in TSB at 12°C for up to 12 days and at 22°C for up to 6 days. Figure 1 shows the total cell counts (vegetative cells plus spores) and the number of spores produced in the biofilm. The vast majority (99.94%) of CFU initially in PBS suspensions (30 ml, ~6.5 log CFU/ml) in which coupons were immersed to achieve attachment of cells before immersing in TSB were vegetative cells; only about 0.06% of CFU (~3.3 log CFU/ml) were spores. The total cell count on coupons at the end of the 4-h attachment period was ca. 5.8 log CFU per coupon, of which 1.9 to 2.5 log CFU per coupon were spores. When coupons containing attached cells were immersed in TSB and incubated at 12°C, the total cell count did not change significantly (P ≤ 0.05) for 2 days (6.2 log CFU per coupon) but decreased significantly (P ≤ 0.05) to 4.5 log CFU per coupon at 4 days and remained constant for an additional 8 days. The number of spores on coupons incubated at 12°C was in the range of 1.9 to 2.9 log CFU per coupon. When coupons containing attached cells of B. cereus were immersed in TSB and incubated at 22°C, total cell counts in biofilm increased significantly during the first 2 days and remained constant for an additional 4 days. The number of spores in biofilm on coupons incubated at 22°C did not change significantly throughout 6 days of incubation.

Sporulation of B. cereus in biofilm as affected by exposure to air at various relative humidities. The influence of air at 100, 97, 93, and 85% relative humidity on sporulation of B. cereus in biofilm on stainless steel coupons incubated at 12 and 22°C was determined. Biofilms were formed on coupons immersed in TSB at 12°C for 6 days or 22°C for 2 days then exposed to air at various relative humidities at 12°C for 6 days or 22°C for 4 days. Figure 2 shows total cell counts and number of spores in biofilm formed at 12°C on stainless steel coupons exposed to air at various relative humidities. Total cell counts and the number of spores produced in the biofilm decreased significantly (1.2 to 1.5 log CFU per coupon and 1.1 log CFU per coupon, respectively) on exposure to air, regardless of the relative humidity. After exposure to various relative humidities at 12°C for 6 days, total cell counts in biofilm were 2.8 to 3.1 log CFU per coupon and the number of spores was 1.8 log CFU per coupon, regardless of relative humidity.

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the number of spores present in the biofilm did not change significantly.

Resistance of vegetative cells and spores in biofilm to sanitizers. Biofilms of \textit{B. cereus} on stainless steel coupons immersed in TSB or exposed to air with 100% relative humidity for 3 days at 22°C were treated with chlorine (0, 50, and 200 mg/ml), chlorine dioxide (0, 50, and 200 μg/ml), and Tsunami 200 (0, 40, and 80 μg/ml). Figure 4 shows the total cell counts (vegetative cells plus spores) recovered from biofilm on coupons treated with chlorine for 1, 3, and 5 min. The numbers of spores initially present in biofilm on coupons that had been immersed in TSB or exposed to 100% relative humidity were 1.7 ± 0.4 and 6.3 ± 0.5 log CFU per coupon, respectively. Total cell counts in biofilm did not change significantly on coupons held in water for 5 min, regardless of previous immersion in TSB or exposure to 100% relative humidity. When biofilm was treated with chlorine at 50 μg/ml, a significant reduction (\( P \leq 0.05 \)) in total cell counts occurred between successive treatment times. Total cell counts in biofilm formed on coupons immersed in TSB decreased significantly from 6.3 initially to 3.8 log CFU per coupon within 1 min, then subsequently to 1.6 log CFU per coupon within 5 min. Total cell counts in biofilm exposed to air with 100% relative humidity did not decrease significantly during the first minute of treatment with 50 μg/ml chlorine, reflecting the presence of higher numbers of spores that, compared with vegetative cells, would exhibit higher resistance to the sanitizer. Within 3 min, however, counts decreased significantly to 5.6 log CFU per coupon. There was not a significant difference in total cell counts detected on coupons treated for 3 or 5 min. When treated with chlorine at 200 μg/ml, total cell counts in biofilm that had been immersed in TSB or exposed to air with 100% relative humidity decreased.
rapidly, indicating lethality to spores as well as vegetative cells. Treatment for 5 min caused decreases in total cell counts to 1.6 to 1.9 log CFU per coupon. In a previous study (7), we observed that treatment of an aqueous suspension of a five-strain mixture of vegetative cells of *B. cereus* with chlorine or chlorine dioxide, both at 200 µg/ml for 5 min, caused reductions of 4.5 and >5.4 log CFU/ml, respectively. Treatment of a five-strain mixture of spores resulted in reduction of 1.8 and >6.4 log CFU/ml, respectively. The addition of organic material to the treatment mixture containing spores reduced the lethality of 200 µg of chlorine dioxide per ml to 2.1 log CFU/ml. Differences in the effectiveness of chlorine and chlorine dioxide in the two studies are attributed in part to different methods used to culture *B. cereus* and differences in test strains. Both studies confirm that the presence of organic material in the milieu surrounding *B. cereus* lessens the lethality of chlorine and chlorine dioxide.

Figure 5 shows total cell counts in biofilm on stainless
steel coupons immersed in TSB or exposed to air with 100% relative humidity at 22°C and treated with 0, 50, and 200 μg of chlorine dioxide per ml. Numbers of spores originally present in biofilm immersed in TSB or exposed to air with 100% relative humidity were 3.1 ± 0.1 and 5.9 ± 0.4 log CFU per coupon, respectively. When biofilm was treated with water for up to 5 min, total cell counts did not change significantly. When treated with 50 μg of chlorine dioxide per ml for 1 min, the total cell count (5.6 log CFU per coupon) in biofilm exposed to air at 100% relative humidity was significantly more than that (4.2 log CFU per coupon) in biofilm on coupons immersed in TSB. When treated with 200 μg of chlorine dioxide per ml, total cell counts of biofilm on coupons immersed in TSB or exposed to 100% relative humidity decreased to 2.4 log CFU per coupon and 3.2 log CFU per coupon, respectively, within 5 min.

Figure 6 shows total cell counts in biofilm on stainless steel coupons immersed in TSB or exposed to air under 100% relative humidity and treated with Tsunami 200 (0, 40, and 80 μg/ml). Numbers of spores in biofilm on coupons immersed in TSB or exposed to air with 100% relative humidity were 2.1 ± 0.1 and 5.4 ± 0.4 log CFU per coupon, respectively. When treated with water for up to 5 min, total cell counts did not change significantly. When treated with 40 or 80 μg of Tsunami 200 per ml, total cell counts in biofilm on coupons immersed in TSB decreased significantly from 6.1 log CFU per coupon initially to 3.8 and 3.6 log CFU per coupon, respectively, within 1 min and to 2.3 and 1.7 log CFU per coupon, respectively, within 5 min. Total cell counts in biofilm exposed to air with 100% relative humidity did not decrease significantly for 3 min, regardless of concentration of Tsunami 200, but eventually decreased significantly after treatment for 5 min.

Spores formed in biofilm exposed to air with 100% relative humidity that subsequently survived treatment with sanitizers were tested for sensitivity to heat. It was assumed that CFU consisted largely of spores (i.e., total cell counts after treatment reflect the number of surviving spores in the biofilm). Table 1 shows the influence of sanitizer treatment on heat resistance of spores. After treatment with chlorine dioxide at 50 or 200 μg/ml, the number of CFU recovered from neutralized, unheated suspensions was significantly higher than the number of CFU recovered from heated (80°C for 10 min) suspensions. Treatment with chlorine or Tsunami, on the other hand, did not significantly affect the heat resistance of spores, which might indicate that a larger percentage of surviving spores was injured by chlorine dioxide, thus decreasing resistance to heat.

**DISCUSSION**

The impetus for this study (i.e., to characterize sporulation of *B. cereus* after biofilm formation as affected by nutrient availability, temperature, and relative humidity and to determine subsequent resistance of vegetative cells and spores to sanitizers) was based on recognizing that vegetative cell growth and the production of biofilm precedes sporulation, resulting in spores enmeshed in exopolymeric substances, thereby potentially being inaccessible to the lethal forms of sanitizers used in the food industry. *B. cereus* spores can attach to inert surfaces, but germination must precede growth and biofilm formation. Studies on biofilm formation and maturation by *Bacillus* spp. have not focused on sporulation or the behavioral characteristics of spores within the biofilm matrix. Vegetative cells can be exposed to various environmental conditions during or after biofilm formation that could stimulate sporulation of *B. cereus*. Our principal hypothesis was that *B. cereus* can gain protection...
TABLE 1. Influence of sanitizer treatment on heat resistance of spores of B. cereus in biofilms after treatment with sanitizers

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<th>Sanitizer</th>
<th>Conc (µg/ml)</th>
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<th>With heat treatment</th>
<th>$R^b$</th>
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<td>0.2</td>
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$^a$ Biofilms were formed on the surface of stainless steel coupons in TSB for 2 days then exposed to air with 100% relative humidity for 4 days at 22°C.

$^b$ Number of spores recovered on BHIA after treatment of biofilm on coupons with water or sanitizers for 5 min. The number of spores recovered after treatment of coupons with water is the number initially detected in the biofilm. Within a given sanitizer, mean values in the same column that are not preceded by the same capital letter are significantly different ($P \leq 0.05$). Within same row, mean values that are not followed by the same lowercase letter are significantly different ($P \leq 0.05$).

$^c$ Treated, neutralized suspensions were not heated before plating.

$^d$ Within sanitizer, reduction compared with the number of spores initially recovered from biofilm on coupons treated with water.

$^e$ Number of spores recovered on BHIA after treatment of biofilm on coupons with sanitizer for 5 min, followed by heating of neutralized suspension at 80°C for 10 min.

$^f$ Within sanitizer, reduction compared with the number of spores initially recovered from biofilm on coupons treated with water and heated at 80°C for 10 min.

did not decrease for 24 days after reaching stationary phase within 4 to 6 days (25). Decreased populations of B. cereus on coupons observed in the study reported here were probably not a result of cell death, but rather cells that became detached and were in the surrounding medium. When coupons with attached cells were immersed in TSB at 22°C for 6 days, visible slime was evident, suggesting that B. cereus produced large amounts of exopolymeric substances as it formed biofilm. Vegetative cells in biofilm that formed in a rich medium (TSB) at 12 or 22°C did not actively produce spores, even with extended incubation. It is concluded from the study on biofilm maturation that 22°C is more favorable than 12°C for biofilm formation by B. cereus on the surface of stainless steel, and the majority of cells in the biofilm were vegetative cells.

The observation that vegetative cells in biofilm formed on coupons immersed in TSB do not produce large numbers of spores led us to examine the effects of other environmental factors on sporulation. We have observed that, although B. cereus produces only a limited numbers of spores in TSB, more than 99% of cells sporulate when cultured on tryptic soy agar (TSA) for 3 days at 22°C (25). Major differences in environmental conditions provided in TSB and on the surface of TSA are oxygen availability and relative humidity. It was hypothesized that available oxygen, relative humidity, or both would influence sporulation of B. cereus in biofilm. To test this hypothesis, biofilms were formed on the surface of stainless steel coupons immersed in TSB at 12 and 22°C for 6 and 2 days, respectively, then exposed to air with various relative humidity at 12 and 22°C for 6 and 4 days, respectively. On exposure of cells to air at high relative humidity, the number of spores...
formed in biofilm at 22°C increased significantly. When biofilm was exposed to low relative humidity or 12°C, cells did not sporulate but, instead, died. This observation suggests that oxygen availability is important for sporulation of *B. cereus* in biofilm but high relative humidity at a temperature higher than 12°C is also necessary. Even though exposing biofilm to air with high relative humidity at 22°C increased spore production by as much as 1,000-fold compared with sporulation in biofilm on coupons immersed in TSB, the percentage of spores in the total cell population in biofilm was only about 10%. Compared with cells grown on TSA, of which 99% sporulated at 22°C (25), the percentage of cells that sporulated in biofilm was low, suggesting that other factors affect sporulation of *B. cereus* in biofilm. If these factors include the availability of nutrients, surfaces of foods would more closely imitate the surface of TSA than the surface of stainless steel in that an essentially unlimited supply of nutrients would be accessible to cells. Given sufficient time at an appropriate temperature, biofilm formation and a substantial level of sporulation of *B. cereus* on foods and food contact surface can occur.

We determined the resistance of vegetative cells and spores of *B. cereus* on treatment with chlorine, chlorine dioxide, and Tsunami 200, a peroxyacetic acid–based sanitizer. The number (CFU) recovered from biofilm exposed to air with 100% relative humidity and treated with chlorine at 50 μg/ml was significantly higher than the number recovered from biofilm on coupons held in TSB and receiving the same treatment, reflecting the presence of a higher number of spores in biofilm exposed to air. Because biofilm that formed on coupons immersed in TSB did not contain more than 1.7 log spores per coupon, total cell counts were decreased to near the detection limit (1.5 log CFU/ml) within a 3-min treatment. Biofilm exposed to air contained higher numbers of spores (6.3 log spores per coupon). Total cell counts in these biofilms after treatment with 50 μg of chlorine per ml for 3 and 5 min decreased to 5.6 and 5.2 log CFU/ml, respectively. Although cells in biofilm were rapidly inactivated on treatment with 200 μg/ml of chlorine, regardless of the number of spores in the biofilm, some cells survived after treatment for 5 min.

Cells (vegetative cells plus spores) in biofilm on coupons exposed to air were more resistant to chlorine dioxide (50 μg/ml) than were cells in biofilm on coupons immersed in TSB. Biofilm that formed on coupons immersed in TSB initially contained 3.1 log spores per coupon. Total cell counts were decreased from 6.1 to 3.3 log CFU per coupon when biofilm was treated for 5 min with chlorine dioxide (50 μg/ml). Biofilm exposed to air with 100% relative humidity initially contained 5.9 log spores per coupon. Total cell counts were reduced from 6.2 to 5.1 log CFU per coupon when biofilm was treated with chlorine dioxide (50 μg/ml). These observations suggest that after treatment with chlorine dioxide for 5 min, the number of survivors in biofilm exposed to air was approximately 1,000 times greater than that in biofilm that had been immersed in TSB. This indicates that the peroxyacetic acid–based sanitizer was less effective than chlorine or chlorine dioxide in killing *B. cereus* spores in biofilm.

Reports on the treatment of biofilm formed by *B. cereus* on stainless steel surfaces with sanitizers are meager. Peng et al. (23) compared with the tolerance of planktonic cells of *B. cereus*, attached cells, and cells in biofilm to treatment with sodium hypochlorite (chlorine) and a quaternary ammonium compound. Cells in a biofilm were most resistant to treatment with chlorine (50 μg/ml), followed by attached single cells and cells in a planktonic state. This supports the hypothesis that *B. cereus* has enhanced resistance to chlorine if attached to a surface or in a biofilm formed on a surface. Although the percentages of spores in planktonic cells, attached cells, and cells in biofilm were not reported, the majority of these cells would likely be vegetative because they were produced in broth culture.

We observed that treatment of *B. cereus* spores with chlorine dioxide reduced their resistance to heat. Young and Setlow (31) demonstrated that chlorine or chlorine dioxide inactivate spores of *B. subtilis* by damaging the inner membrane, resulting in inhibition of germination, outgrowth, or both. Even though the specific mechanism responsible for killing *Bacillus* spp., by treatment with wet heat has not been fully elucidated (26, 27), it is known that one of the factors contributing to resistance of spores to wet heat is the presence of dipicolinic acid in the spore core. The absence of dipicolinic acid in spores increases the sensitivity of spores to heat (6, 21, 30). Damage caused by treatment of oxidizers such as chlorine and chlorine dioxide on the inner membrane of *B. cereus* spores might reduce their resistance to wet heat. Spores killed by other oxidizing agents (e.g., Sterilox and peroxyacetic acid) more readily release dipicolinic acid on subsequent incubation at otherwise sublethal temperatures (11, 17). If this behavior holds true for *B. cereus* spores enmeshed in biofilm on food contact surfaces or foods, the synergistic effect between oxidizing sanitizers and wet heat might contribute to a higher level of lethality.

Further studies will be required to more fully characterize factors affecting attachment of *B. cereus* spores to inert surfaces and possible preferential attachment to inert surfaces exposed to mixtures of vegetative cells and spores. Biofilm formation and subsequent sporulation by *B. cereus* on the surface of foods and the synergistic effect between oxidizing sanitizers and wet heat in killing *B. cereus* in these foods should be investigated.

REFERENCES

