Preconditioning with Cations Increases the Attachment of *Anoxybacillus flavithermus* and *Geobacillus* Species to Stainless Steel

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Preconditioning of *Anoxybacillus flavithermus* E16 and *Geobacillus* sp. strain F75 with cations prior to attachment often significantly increased ($P \leq 0.05$) the number of viable cells that attached to stainless steel (by up to 1.5 log CFU/cm²) compared with unconditioned bacteria. It is proposed that the transition of *A. flavithermus* and *Geobacillus* spp. from milk formulations to stainless steel product contact surfaces in milk powder manufacturing plants is mediated predominantly by bacterial physiological factors (e.g., surface-exposed adhesins) rather than the concentrations of cations in milk formulations surrounding bacteria.

A biofilm is described as microorganisms attached to a surface; they are often embedded in a matrix of polymers and other molecules that either originate from microorganisms in the biofilm or are absorbed from the surrounding environment (1).

Cations have two main effects on the structural integrity and proliferation of a bacterial biofilm. The first is a direct effect, such that cations interact electrostatically with surface-exposed and cell wall-embedded polymers and the surfaces to which they attach (2, 3). The outer surfaces of bacteria generally have an overall negative charge because bacterial cell wall and extracellular matrix polymers have an abundance of negatively charged functional groups (1, 4). Stainless steel also has a negative surface charge (5). Thus, there is an extent of electrostatic repulsion between bacteria and the stainless steel surface to which they attach (3). Factors such as the ionic strength (6), the ratio of the concentrations of monovalent to divalent cations in solution (7), and the proportion of divalent cation bridges in a biofilm matrix (2) have the potential to alter the extent of electrostatic repulsion in a biofilm.

The second effect that cations have on biofilm formation is an indirect effect, such that bacteria may respond to changes in concentrations of cations in their surroundings and adjust their metabolism and physiology (8–11). These bacterial responses may indirectly influence their ability to transition from a planktonic form to an irreversibly attached form and prosper as a biofilm (8–10). Ca²⁺ and Mg²⁺ have been shown to stimulate exopolysaccharide (8, 12, 13) and extracellular protein (9, 14, 15) production by bacteria and often have physiological roles in assisting the initial reversible association of bacteria with a surface or enhancing the cohesion of a biofilm (9, 11, 16). Na⁺ has been shown to stimulate bacteria to increase the proportion of negatively charged, hydrophilic polymers in a wastewater sludge and, therefore, have a detrimental effect on its cohesion (10).

Geobacillus and *A. flavithermus* attach to and form biofilms on stainless steel product contact surfaces in milk powder manufacturing plants and are the predominant bacteria that contaminate milk powder (17, 18). Unprocessed milk typically has total (sum of bound and free) Na⁺, K⁺, Ca²⁺, and Mg²⁺ concentrations of 22, 37, 30, and 5 mM, respectively (19); however, these concentrations can be manipulated during processing. Some milk formulations have total Na⁺ concentrations as high as 100 mM and total Ca²⁺ and Mg²⁺ concentrations as low as 7 and 1 mM, respectively. To gain insight into the extent of the influence that different cation

concentrations and ratios have on biofilm formation by *Geobacillus* and *A. flavithermus*, we investigated the effect of Na⁺, K⁺, Ca²⁺, and Mg²⁺ on attachment to stainless steel and biofilm formation by *A. flavithermus* E16 and *Geobacillus* sp. strain F75 in both a casein digest medium and milk formulations.

Evaluation of the effect of Na⁺, K⁺, Ca²⁺, and Mg²⁺ on attachment and biofilm formation of *A. flavithermus* and *Geobacillus*. *A. flavithermus* E16 and *Geobacillus* sp. F75 were isolated from product contact surfaces at a milk powder manufacturing plant. Prior to attachment and biofilm formation, each bacterial isolate was planktonically preconditioned (to mid-stationary phase after 9 h at 55°C) in medium with one of the following compositions: casein digest medium (1 g/liter) (Difco, BD Biosciences) unsupplemented with cations, referred to as “unconditioned”; casein digest medium supplemented with cations alone (see Table S1 in the supplemental material), referred to as “preconditioned with cations”; casein digest and lactose monohydrate (1 g/liter) (Merck, Darmstadt, Germany) medium supplemented with cations, referred to as “preconditioned with cations and lactose”; milk formulations 1 to 4 (10 g milk powder rehydrated with 90 ml deionized, sterile water) (Fonterra, New Zealand) (see Tables S2 and S3 in the supplemental material), referred to as “preconditioned with milk formulation.”

To remove medium used to planktonically grow and condition the bacteria, cultures, except those preconditioned with milk formulation (1 to 4), were centrifuged at 10,000 × g and the bacterial pellet was resuspended in fresh medium to be used during the attachment and biofilm formation assay, which consisted of casein digest medium (1 g/liter) supplemented with a range of cation concentrations and ratios (see Table S1 in the supplemental material) or milk formulations 1 to 4 (see Tables S2 and S3 in the supplemental material). The resuspended cultures and cultures

Received 10 February 2013 Accepted 25 April 2013

Published ahead of print 3 May 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00462-13>.

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doi:10.1128/AEM.00462-13

preconditioned with milk formulation (1 to 4) were diluted in medium to be used during the attachment and biofilm formation assays to achieve an inoculum of approximately 4.5 log CFU/ml; 1.5 ml of this inoculum was added per well of a 24-well culture plate (BD, Franklin Lakes, NJ). Stainless steel coupons (part number RD128-316; Biosurface Technologies Corporation, Bozeman, MT), which had a surface area of approximately 4 cm², were cleaned and passivated prior to use in the biofilm formation assay, as previously described by Flint et al. (20). One coupon was placed into each inoculum using sterile forceps so that it was completely submerged and horizontal. After the culture plate had been wrapped in a plastic bag to prevent the evaporation of water from the cultures, it was incubated at 55°C for either 30 min or 6 h.

Casein digest medium (1 g/liter) (Difco, BD Biosciences, Sparks, MD) was used because it had low background Na⁺, K⁺, Ca²⁺, and Mg²⁺ concentrations of approximately 1.0, 0.03, 0.004, and 0.002 mM (21), respectively; therefore, the effect of the supplementation of a range of cation concentrations and ratios on attachment and biofilm formation was able to be studied. Casein digest medium was supplemented with analytical grade NaCl, KCl, CaCl₂·2H₂O, or MgCl₂·6H₂O powder (Merck, Darmstadt, Germany). Milk formulations 1 to 4 (Fonterra, New Zealand) had similar fat, protein, and lactose concentrations but different total (sum of bound and free) Na⁺, K⁺, Ca²⁺, and Mg²⁺ concentrations (see Tables S2 and S3 in the supplemental material). The higher the designated milk formulation number (1 to 4), the higher the monovalent-to-divalent cation ratio of the milk formulation (see Table S3 in the supplemental material). Milk formulations 1 to 4 were used to investigate the effect of the monovalent-to-divalent cation ratio on attachment and biofilm formation in milk formulations. Milk formulations 1 to 4 were derived from the same respective batches throughout experimentation. Prior to reconstitution, the milk powders were gamma irradiated (25,000 Gy) to inactivate any contaminating microorganisms so that growth and analysis of the inoculated bacteria of interest were unimpeded.

The bacteria were planktonically preconditioned with a range of cation concentrations and ratios (both in casein digest medium and milk formulations) to allow the bacteria to physiologically adapt to the cationic conditions so that the effect of cation preconditioning on subsequent attachment and biofilm formation could be investigated. This was done to investigate if a physiological response of the bacteria to particular cation concentrations and ratios may abate their ability to attach to and form biofilms in milk powder manufacturing lines. Bacteria preconditioned with cations or milk formulations, except bacteria preconditioned with cations and lactose, for which lactose was omitted from medium in the attachment and biofilm formation assay, were subjected to medium in the attachment and biofilm formation assays that was of the same composition as that used during preconditioning.

To enumerate the attached viable cells per square centimeter on the coupons, the coupons were removed from the cultures using sterile forceps, dipped and rinsed three times in approximately 50 ml of deionized water to remove any loosely attached cells, and placed into a 35-ml plastic container (item code LBS3722W; Thermo Fisher Scientific, New Zealand) with 5 ml of fresh casein digest medium (1 g/liter) and 12 g of glass beads that had a diameter of 6.35 mm (catalogue number 11079635; Biospec Products, Inc., Bartlesville, OK). The plastic containers were vortex mixed vigorously for 2 min to dislodge the attached cells into

the surrounding medium. Standard microbiological plate-counting techniques were used to enumerate the viable CFU/ml in the cell suspension using casein digest medium (1 g/liter) as the diluent and milk plate count agar (MPCA; Oxoid, Basingstoke, United Kingdom). The experiments were carried out on three separate occasions, and mean attached viable cell numbers (CFU/cm²) and 1 standard deviation (σ_{n-1}) are reported. Minitab software was used to calculate population standard errors and 95% confidence intervals ($P \leq 0.05$) to determine significant differences among the mean values.

Effect of ionic strength. Altering the ionic strength between 2 and 125 mM did not significantly influence ($P \leq 0.05$) attachment after 30 min or biofilm formation after 6 h by *A. flavithermus* E16 or *Geobacillus* sp. F75 (Fig. 1 and 2). This was apparent for unconditioned bacteria, bacteria preconditioned with cations, and bacteria preconditioned with cations and lactose (Fig. 1 and 2). Our results contradict those of other studies, which showed that bacterial attachment and biofilm cohesion increase as the ionic strength of the surrounding solution increases (5, 6). Perhaps the addition of cations at concentrations of 2 mM saturated the surfaces of the bacteria and the stainless steel coupons such that further increases in ionic strength may have had no further enhancing effect on biofilm formation. As the ionic strength of milk formulations and most bacterial habitats is greater than 2 mM, our results indicate that the ionic strength of solution does not influence attachment and biofilm formation of *A. flavithermus* and *Geobacillus*.

Effect of the monovalent-to-divalent cation ratio. When comparing the monovalent-to-divalent cation ratios of 2:1 and 10:1 at an ionic strength of either 31 or 125 mM in casein digest medium, there was no significant difference ($P \leq 0.05$) in attachment or biofilm formation by *A. flavithermus* E16 or *Geobacillus* sp. F75 (Fig. 1 and 2, iv to vii). This was apparent when bacteria were unconditioned, preconditioned with cations, and preconditioned with cations and lactose (Fig. 1 and 2, iv to vii). Our findings contrast observations made with wastewater sludge biofilms, in which it has been shown that sludge cohesion decreases as the monovalent-to-divalent cation ratio increases, such that a monovalent-to-divalent cation ratio of 2:1 promotes the greatest extent of cohesion, and at a monovalent-to-divalent cation ratio of 10:1, the cohesion of a sludge is greatly compromised (7). Generally, attachment after 30 min and biofilm formation after 6 h by *A. flavithermus* E16 and *Geobacillus* sp. F75 were similar when comparing milk formulations 1 to 4 (see Fig. S1 and S2 in the supplemental material). However, in agreement with wastewater sludge research, biofilm formation after 6 h by *Geobacillus* sp. F75 tended to decrease as the monovalent-to-divalent cation ratio of the milk formulations increased, particularly by the unconditioned bacteria (see Fig. S2B in the supplemental material). The number of unconditioned, attached viable *Geobacillus* sp. F75 cells after 6 h was approximately 2 log CFU/cm² lower in milk formulation 4 than in milk formulation 2, and the two values were significantly different ($P \leq 0.05$) (see Fig. S2B in the supplemental material). The monovalent-to-divalent cation ratio of milk formulation 4 is likely to be greater than those that exist in any of the cation concentrations and ratios investigated in the casein digest medium, which may explain the apparent inhibitory influence of the high monovalent-to-divalent cation ratio of milk formulation 4 on *Geobacillus* sp. F75 biofilm formation. Milk formulations have higher concentrations of solutes, such as casein and anions, than

FIG 1 Attachment, after 30 min of incubation at 55°C, by viable *A. flavithermus* E16 (A, C, and E) and *Geobacillus* sp. F75 (B, D, and F) cells (log CFU/cm²) on stainless steel coupons completely submerged in casein digest medium (1 g/liter) supplemented with cation compositions of 0 mM (i), 2 mM Ca²⁺ (ii), 2 mM Mg²⁺ (iii), 31 mM 2:1 (iv), 31 mM 10:1 (v), 125 mM 2:1 (vi), and 125 mM 10:1 (vii). Total supplemented cation concentrations of 31 mM (iv and v) and 125 mM (vi and vii) had monovalent-to-divalent cation ratios of 2:1 (iv and vi) and 10:1 (v and vii). Each monovalent-to-divalent cation ratio comprised equal Na⁺ and K⁺ concentrations and equal Ca²⁺ and Mg²⁺ concentrations. Prior to the attachment assay, the bacteria were grown planktonically for 9 h at 55°C in three different media: casein digest medium (1 g/liter) (unconditioned) (A and B), casein digest medium (1 g/liter) supplemented with various cation compositions (preconditioned with cations) (ii to vii) (C and D), and casein digest medium (1 g/liter) supplemented with lactose (1 g/liter) and various cation compositions (preconditioned with cations and lactose) (ii to vii) (E and F). Experiments were repeated as triplicates, and error bars represent one standard deviation (σ_{n-1}). The letters (a to e) represent significantly greater ($P \leq 0.05$) attachment by cation-preconditioned cells (C, D, E, and F) than by unconditioned cells (A and B) for each respective bacterial isolate and each respective cation composition. “a” represents 2 mM Ca (ii), “b” represents 31 mM 2:1 (iv), “c” represents 31 mM 10:1 (v), “d” represents 125 mM 2:1 (vi), and “e” represents 125 mM 10:1 (vii).

casein digest medium. Thus, in milk formulations, a large proportion of Ca²⁺ and Mg²⁺ is chelated, maintaining low free Ca²⁺ and Mg²⁺ concentrations (19, 22). Furthermore, the Na⁺ concentration of milk formulation 4 was greater than the Na⁺ concentration in any of the cation concentrations and ratios used to supplement the casein digest medium; the Na⁺ concentration in milk formulation 4 was 101 mM (see Table S3 in the supplemental material), whereas the highest Na⁺ concentration in the casein digest medium was 56.8 mM (see Table S1 in the supplemental material). Thus, it may have been high Na⁺, low Ca²⁺, low Mg²⁺, or a combination of these factors that inhibited *Geobacillus* sp. F75 biofilm formation in milk formulation 4.

Our results indicate that the monovalent-to-divalent cation ratio generally does not influence attachment and biofilm formation of *A. flavithermus* and *Geobacillus* spp. in processed milk formulations during milk powder manufacture.

Effect of preconditioning with cations. Preconditioning of *A. flavithermus* E16 or *Geobacillus* sp. F75 with cations often significantly increased ($P \leq 0.05$) attachment (by up to 1 log CFU/cm²) after 30 min relative to unconditioned bacteria when the same cation concentrations during attachment were compared (Fig. 1A to D). For example, when *A. flavithermus* E16 attached after 30 min in the presence of 2 mM Ca²⁺, attachment of bacteria preconditioned in 2 mM Ca²⁺ (Fig. 1C, ii) was significantly greater ($P \leq 0.05$) than attachment of unconditioned bacteria (Fig. 1A, ii), with the numbers of attached viable cells at 3.3 and 2.2 log CFU/cm², respectively.

In contrast, preconditioning *A. flavithermus* E16 with cations

did not significantly increase ($P \leq 0.05$) biofilm formation after 6 h relative to that of unconditioned bacteria when the same cation concentrations during biofilm formation were compared (Fig. 2A and C). However, preconditioning had a lasting effect on *Geobacillus* sp. F75 biofilm formation after 6 h; for many cation concentrations and ratios, there was a significant increase ($P \leq 0.05$) (by up to 1 log CFU/cm²) in biofilm formation when bacteria were preconditioned with cations relative to unconditioned bacteria when the same cation concentrations during biofilm formation were compared (Fig. 2B and D).

During preconditioning, the metabolism and physiology of *A. flavithermus* E16 and *Geobacillus* sp. F75 may have been influenced by cations in such a way that subsequent attachment and biofilm formation by the bacteria was enhanced. For example, Ca²⁺ or Mg²⁺ may have stimulated the bacteria to increase their expression of surface-exposed proteins and polysaccharides, which promoted attachment and biofilm formation (9, 12–16). Surface-exposed proteins, such as pili and even flagella, have been implicated in assisting the initial reversible association of bacteria with a surface, and surface-exposed polysaccharides have been implicated in promoting the irreversible attachment of bacteria to a surface and enhancing cohesion among bacteria within a mature biofilm by acting as a cohesive component of the matrix (1).

Since the electrostatic effects of ionic strength and the monovalent-to-divalent cation ratio generally did not influence attachment and biofilm formation of *A. flavithermus* E16 and *Geobacillus* sp. F75 and preconditioning the bacteria with cations often increased attachment, it is proposed that the transition of *A. fla*-

FIG 2 Biofilm formation, after 6 h of incubation at 55°C, by viable *A. flavithermus* E16 (A, C, and E) and *Geobacillus* sp. F75 (B, D, and F) cells (log CFU/cm²) on stainless steel coupons completely submerged in casein digest medium (1 g/liter) supplemented with cation compositions of 0 mM (i), 2 mM Ca²⁺ (ii), 2 mM Mg²⁺ (iii), 31 mM 2:1 (iv), 31 mM 10:1 (v), 125 mM 2:1 (vi), and 125 mM 10:1 (vii). Total supplemented cation concentrations of 31 mM (iv and v) and 125 mM (vi and vii) had monovalent-to-divalent cation ratios of 2:1 (iv and vi) and 10:1 (v and vii). Each monovalent-to-divalent cation ratio comprised equal Na⁺ and K⁺ concentrations and equal Ca²⁺ and Mg²⁺ concentrations. Prior to the biofilm formation assay, the bacteria were grown planktonically for 9 h at 55°C in three different media: casein digest medium (1 g/liter) (unconditioned) (A and B), casein digest medium (1 g/liter) supplemented with various cation compositions (preconditioned with cations) (ii to vii) (C and D), and casein digest medium (1 g/liter) supplemented with lactose (1 g/liter) and various cation compositions (preconditioned with cations and lactose) (ii to vii) (E and F). Experiments were repeated as triplicates, and error bars represent one standard deviation (σ_{n-1}). The letters (a to d) represent significantly greater ($P \leq 0.05$) biofilm formation by cation-preconditioned cells (D and F) than by unconditioned cells (B) by *Geobacillus* sp. F75 for each respective cation composition. “a” represents 2 mM Mg (iii), “b” represents 31 mM 2:1 (iv), “c” represents 31 mM 10:1 (v), and “d” represents 125 mM 2:1 (vi).

vithermus and *Geobacillus* spp. from milk formulations to stainless steel product contact surfaces in milk powder manufacturing plants is mediated predominantly by bacterial physiological factors, e.g., the expression of surface-exposed adhesins, rather than the concentrations of cations in milk formulations.

Preconditioning with cations and lactose decreased attachment by *A. flavithermus* E16. Protein, such as casein, and carbohydrate, such as lactose, are available to bacteria as nutrient sources for biofilm formation during milk powder manufacture (19). To simulate biofilm formation by *A. flavithermus* and *Geobacillus* spp. in milk powder manufacturing lines and to provide bacteria with an adequate carbohydrate source to synthesize surface-exposed polysaccharides (23), lactose was added to cultures during preconditioning. Thus, when bacteria were preconditioned with cations and lactose, the effect of preconditioning bacteria with ranging cation concentrations on their subsequent attachment and biofilm formation was investigated in the presence of both a protein source and a carbohydrate source. The extent of attachment after 30 min by *A. flavithermus* E16 preconditioned with cations and lactose tended to be less than that observed when the bacteria were preconditioned with cations alone (Fig. 1C and E). During preconditioning, *A. flavithermus* E16 may have utilized the lactose in the medium to increase production of surface-exposed polysaccharides, which subsequently decreased attachment. Many studies have shown that surface-exposed polysaccharides can inhibit bacterial attachment (24, 25).

The present study indicated that cation concentrations and ratios in milk formulations generally would not influence attach-

ment and biofilm formation by *A. flavithermus* and *Geobacillus* during the processing of milk formulations in milk powder manufacturing plants. However, as the monovalent-to-divalent cation ratio of milk formulations increased, biofilm formation after 6 h by *Geobacillus* sp. F75 was increasingly inhibited. This implied that either high Na⁺ concentrations or low Ca²⁺ or Mg²⁺ concentrations, or a combination of these factors, have the potential to inhibit biofilm formation by some *A. flavithermus* and *Geobacillus* spp. strains during the processing of milk formulations. Interestingly, preconditioning of planktonic *A. flavithermus* E16 and *Geobacillus* sp. F75 with cations often enhanced attachment and was more influential than the electrostatic effect of cations on the attachment process. It is proposed that cations, such as Ca²⁺ and Mg²⁺, influenced the physiology or metabolism of planktonic *A. flavithermus* and *Geobacillus* spp. such that subsequent attachment was enhanced. It is also proposed that attachment by *A. flavithermus* and *Geobacillus* spp. in milk powder manufacturing lines is mediated predominantly by bacterial physiological factors, e.g., the expression of surface-exposed adhesins, rather than the concentrations of cations in milk formulations.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Business, Innovation, & Employment and Fonterra, New Zealand.

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