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THE ROLE CRANBERRY PROANTHOCYANIDINS PLAY IN THE PRIMARY
ATTACHMENT OF BACTERIA TO SURFACES: *BACILLUS CEREUS* MODEL

by

ANTHONY ROBERT JONES

Under the Direction of Dr. Sidney A. Crow, Jr.

ABSTRACT

The development of a proanthocyanidin (PAC) treatment, along with the understanding of its mechanism of action, would provide an alternative method of preventing attachment to and colonization of surfaces by microorganisms, as well as potentially disrupting preexisting biofilms. The purpose of this research is to examine the role a cranberry proanthocyanidin plays in the primary attachment of *Bacillus cereus* to an abiotic surface. This technology could be employed in food processing plants where a premium is placed on maintaining a sanitized work environment to prevent product contamination. A biofilm assay showed that a surface treated with proanthocyanidins actually promoted rather than prevented the attachment of *Bacillus cereus*. This was further made evident by the fact that the surface hydrophobicities of *B. cereus* cells grown in media supplemented with proanthocyanidins were greater than those grown in its absence. In addition, light microscopy analysis showed a greater degree of sporulation of *B. cereus* cells when grown on TSA plates supplemented with PACs. These results suggest that

proanthocyanidins may be inducing endospore formation in *Bacillus cereus* leading to increased attachment and surface hydrophobicity values.

INDEX WORDS: *Bacillus cereus*, Cranberry proanthocyanidins, Attachment, Biofilm, Hydrophobicity, Motility, Mutagenesis, Proteomics

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ANTHONY ROBERT JONES

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2007

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Anthony Robert Jones
2007

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ATTACHMENT OF BACTERIA TO SURFACES: *BACILLUS CEREUS* MODEL

by

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College of Arts and Sciences
Georgia State University
December 2007

DEDICATION

I dedicate this dissertation to my family especially.....

my Columbus family: Mom, Dad, and Nic

my Atlanta family: Drs. Sandra and Samuel Demons, Sermetria, and Tina

my German family: Opa, Omi, Robert, Harald

Without you all, I would not be where I am now. Please accept this recognition as a token of my endless gratitude.

ACKNOWLEDGEMENTS

I would never have been able to finish my dissertation without the guidance of my committee members. I would like to express my deepest gratitude to my advisor, Dr. Sidney Crow, not only for his excellent guidance, but also for providing me with an excellent atmosphere for doing research. I would like to thank Dr. Pierce, who not only served as a vital member of my committee, but also graciously provided his time and knowledge which has aided me in reaching my research goal. I would also like to thank Dr. Kuk-Jeong Chen for not only providing her lab space and equipment so that I may carry out the molecular portion of my research, but more importantly for her support, encouragement, and willingness to guide me to the final academic/professional destination. Special thanks goes to Dr. Robert Simmons, who was willing to participate in my final defense committee at the last moment. I would like to thank Trudy Tucker, who through shared experiences made the arduous process of finishing a dissertation a much more pleasant/enjoyable undertaking. It would have been a lonely lab without her. I would like to thank Brook Danboise, who as a good friend, was always willing to help and give her best suggestions, and whom without my work would not be complete. Many thanks to Ginger Moulder, Jennifer Hooker, Dr. Poole, Peggy Ogden, my supplemental instruction friends, and laboratory students. Thank you all.

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I. INTRODUCTION

Phytochemical rich cranberry juice has been a staple in the war against in vivo microbial attachment for many years now. Whether preventing the attachment of *Escherichia coli* to uroepithelium, *Helicobacter pylori* to the stomach mucosa, or *Streptococcus mutans* to structures within the oral cavity, it is evident that cranberries are quite effective in a wide range of environments. To further explore their capabilities, cranberry phytochemicals, specifically proanthocyanidins, will be used to determine the ability to reduce primary attachment of *Bacillus cereus* to stainless steel. The mechanism of action will be clarified by observing activity on both the proteomic and genetic levels.

Chemical composition of cranberry proanthocyanidins

To begin, it is important to know the chemical composition of cranberry and how they are synthesized. Cranberry juice is a rich source of polyphenols which have been shown to be biologically active, and effective against attachment of various microorganisms. Although the crude extract of cranberry fruit can prevent attachment, the fraction of the juice which is active is that of proanthocyanidins (Howell *et al.*, 1998). Proanthocyanidins, or condensed tannins, are high molecular weight polymers of flavan-3-ol monomers whose structure consists of three phenyl rings each with various hydroxyl substituents (Johnson-White *et al.*, 2006). Proanthocyanidins are present in the fruits, bark, leaves and seeds of many plants, with their major function being protection against microbial pathogens, insect pests and larger herbivores (Dixon *et al.*, 2004). The deposition of proanthocyanidins in the endothelial layer of the seed coat in many species would appear to be a classic example of a protective barrier, which

functions by binding metals through complexation involving their o-diphenol groups. For example, iron depletion causes severe limitation to bacterial growth, and it has been suggested that the ability to bind iron represents one mechanism for the antibacterial activity of proanthocyanidins (Scalbert, 1991).

The proanthocyanidin fraction consists of molecules with both A and B type linkages. The A-type proanthocyanidins are characterized by linkage between C2 of the upper unit and the oxygen at C7 of the starter unit, in addition to linkage between C4 of the upper unit and positions 6 or 8 of the lower unit (Fig. 1). Apparently, linkage to C4 of the upper unit proceeds first as in the biosynthesis of B-type proanthocyanidins, and A-type proanthocyanidins can be formed from the B-type compounds in vitro (e.g. treatment of proanthocyanidins B1 or B2 (Fig. 1) with an oxidizing agent resulted in formation of proanthocyanidins A1 or A2 (Kondo *et al.*, 2000). On the basis of experiments on the effects of incubation of epicatechin with banana extracts, it has been proposed that the A-type linkages result from polyphenol oxidase (PPO) mediated oxidation of the B-ring to a quinoidal structure, with subsequent addition of the C7 hydroxyl to C2 (Tanaka *et al.*, 2000). In the case of the larger oligomeric A-type proanthocyanidins, it is not known whether an A-linked dimer is formed first, with subsequent addition of further B-linked extension units, or whether the additional C2–C7 bond is formed at a later stage. Ultimately, the A-type linkage is the structure responsible for the antibacterial adhesion properties conferred on the host upon cranberry consumption.

B-type proanthocyanidins are found in apples, grapes, green tea, and dark chocolate, and consists of flavan-3-ol units linked mainly through the C4→C8 bond linkages.

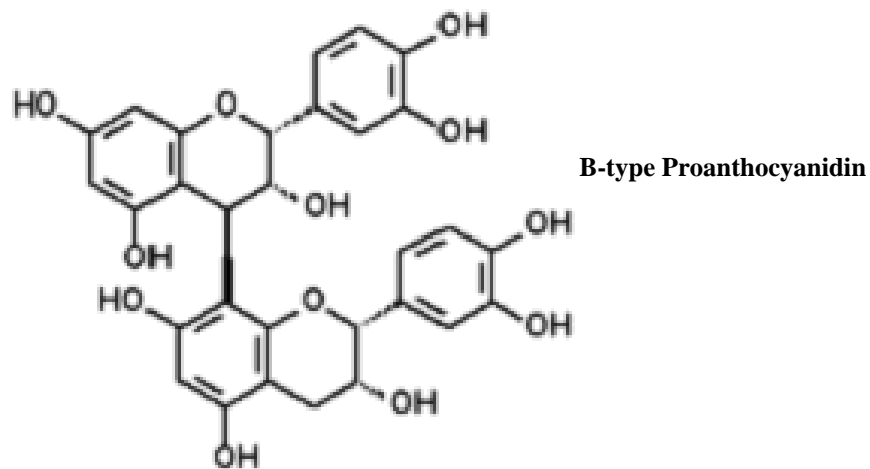
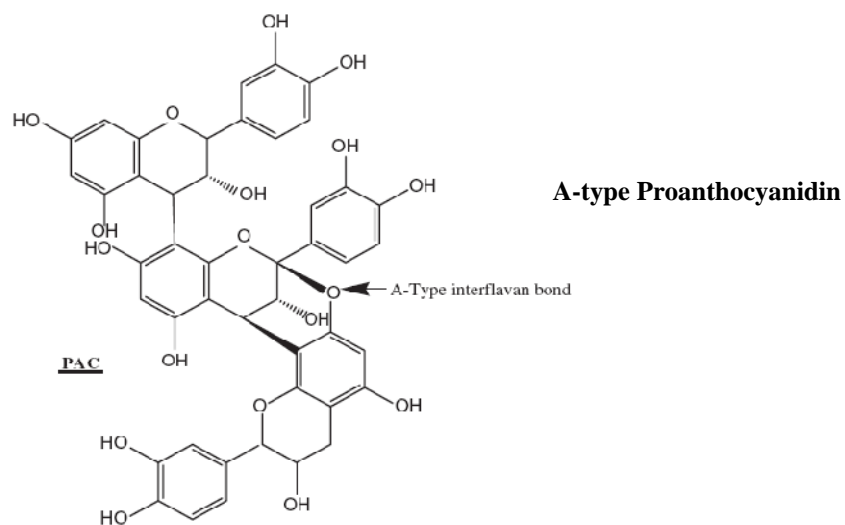


Figure 1. Chemical structure of the cranberry polyphenols of A and B type proanthocyanidins.

How cranberry proanthocyanidins alter the attachment of microorganisms in vivo

Now that the origins of the phytochemicals have been explored, the next logical step would be to examine how cranberry juice, more specifically A-type proanthocyanidins, alters the attachment of microorganisms in the body environment. The majority of research has focused upon cranberry's interaction with *E. coli*. Cranberry juice therapy has been a common method used in the remediation/prevention of urinary tract infections, due to the belief that the high acidity of the juice was the causative agent in this phytotherapy. However, Sobota (1984) showed the urine of mice and humans, after the consumption of cranberry juice, contained the materials that after neutralization of the acidity of the cranberry could still reduce *E. coli* adhesion to epithelial cells. This outcome was further supported by Liu *et al.* (2005) who proposed several mechanisms to explain the interactions between cranberry juice and the surface of *E. coli*: (i) cranberry juice altering the conformation of the P-fimbriae; (ii) cranberry juice blocking the adhesive action of P-fimbriae; (iii) cranberry juice removing P-fimbriae from the cells, and (iv) cranberry juice causing genetic or phenotype-level changes in *E. coli* with P-fimbriae. Ultimately this group discovered that cranberry juice appears to affect *E. coli* strain HB101pDC1 P-fimbriae equilibrium lengths, density, and adhesion forces with a model surface. Of particular interest to this research is that this decrease in the adhesion force could be explained by the fact that the hydrophilic moiety of the proanthocyanidins binds the hydrophobic proteins involved in *E. coli* attachment. Through the use of atomic force microscopy (AFM), Lui *et al.* (2005) speculated that after attachment, the adhesion force between the P-fimbriae of *E. coli* and the AFM silicon nitride tip changed from the interaction between non-polar materials to that between the non-polar tip and the polar, proanthocyanidin modified P-fimbriae. This results in a decrease in the adhesion forces between the model surface and the bacterium. With regards

to B-type proanthocyanidins, Howell *et al.* (2005) discovered that B-type linkages did not protect against bacterial attachment in uroepithelium suggesting that they are not capable of preventing attachment or were not bioavailable. They found that anti-adhesion activity against *E. coli* was detectable only in the urine of those volunteers that consumed a single serving (240 ml) of the 27% cranberry juice cocktail and not those who consumed rich B-linked proanthocyanidin foods. In addition to uroepithelial health, cranberry juice can be employed in maintaining periodontal health through the alteration of attachment properties of oral bacteria.

The proanthocyanidin constituent of the juice exhibits anti-coaggregation activity against a variety of oral bacteria (Weiss *et al.*, 1998). Steinberg *et al.* (2004) have shown that proanthocyanidins strongly affect biofilm formation via a sucrose-dependent mechanism. The effect is mediated by inhibiting the synthesis of the polysaccharides glucan and fructan by immobilized and soluble glucosyltransferases (GTF) and fructosyltransferase (FTF). The inhibitory effect of the proanthocyanidins on the soluble forms of GTF and FTF was more pronounced than on the immobilized forms. By inhibiting GTF and FTF activity, proanthocyanidins reduce the concentration of polysaccharides that mediate the adhesion of *Streptococcus sobrinus* to biofilm. However, they also noted that proanthocyanidins exhibit an anti-adhesion effect also in a sucrose-free environment, but this was less effective than the sucrose-dependent adhesion, suggesting that it affects more than one mechanism of bacterial adhesion. Finally, proanthocyanidins also have exhibited the ability to inhibit the attachment of *Helicobacter pylori* to the mucus constituents in the human stomach which is critical in the prevention of gastric ulcers.

By utilizing urease activity of bacteria bound to immobilized mucus to measure bacterial adhesion, Burger *et al.* (2002) showed that the sialic acid-specific adhesion to human gastric

mucus and to erythrocytes is inhibited by phytochemical constituents derived from cranberry. They found that the 50% inhibitory concentration of proanthocyanidins was 37, 125 and 305 $\mu\text{g ml}^{-1}$ of proanthocyanidins for *H. pylori* strains BZMC-25, EHL-65 and 17874, respectively, that preincubation of proanthocyanidins with mucus had no effect on adhesion of these organisms, and finally that proanthocyanidins at 100 $\mu\text{g ml}^{-1}$ did not cause detachment of the bacteria from mucus. It has been argued that the most common habitat of *H. pylori* is gastric mucus and to cause a disease, generations of *H. pylori* must detach from mucus and reach the underlying epithelium. Burger *et al.* (2002) believe that cranberry juice may affect such de novo adhesion by inhibiting the sialic acid-specific adhesion of *H. pylori* to new sites either in the mucus or on the epithelium.

With the aforementioned examples demonstrating how effective cranberry juice can be in preventing the primary attachment of microorganisms *in vivo*, cranberry proanthocyanidins may be a logical alternative to harsh chemicals used in preventing primary attachment on an abiotic surface such as stainless steel.

Bacillus cereus physiology and attachment properties

The primary test organism in this research is *Bacillus cereus*. *Bacillus cereus* is a Gram-positive, endospore forming bacteria with a distinct ability to adhere to and form biofilms on stainless steel. In addition to being a major culprit in the dairy processing industry, this organism is also found in rice, spaghetti, spices, and vegetables (Klavenes *et al.*, 2002), and can cause food poisoning in the form of a diarrheal type (enterotoxin) and/or a vomiting type (emetic toxin). Burger (19) indicated that great differences exist between the spore surface properties of *B. cereus* strains, mainly in the appendages, size of the exosporium and the composition of

adsorbed proteins, entailing significant differences in their ability to adhere and to resist the clean in place procedure. Consequently, some strains (with a small exosporium and long appendages) may represent a major concern for food processing industries, especially if the future processing practices encountered are more favorable to their survival. To this point, *B. cereus* strain ATCC 14579 was chosen because it is resistant to pasteurization, capable of growth in cold, refrigeration environments (thermoduric psychrotrophy), and can survive alkaline wash solutions employed during CIP cycles. Lindsay *et al.* (2002) found that even upon exposure to an alkaline solution of up to pH 12, the internal cell pH for *B. cereus* remained near neutral. Since it is far less pathogenic, *B. cereus* selection is commonly utilized in place of *B. anthracis*.

Although several studies have been carried out to classify attachment, not much is known about the attachment mechanism of *B. cereus*. Husmark and Ronner (1992) discovered that the strong adhesion of *B. cereus* spores is mainly due to three characteristics: the relatively high hydrophobicity, the low spore surface charge and the spore morphology. Stalheim and Granum (2001) later found that spore appendages of *B. cereus* are complex proteinaceous structures that differ among strains, and that degradation of the *B. cereus* endospore appendages with proteolytic enzymes (found in commercial detergents) appeared to be insufficient for removal of all types of *B. cereus* although it may reduce the problem, especially in flowing systems. Klavenes *et al.* (2002) went on to support this observation by experimentally removing *B. cereus* spore appendages via sonication. They found that under static conditions unaltered spores adhered slightly, but significantly better than spores without appendages. However, when a controlled hydrodynamic shear was introduced, via a radial flow chamber, some strains of *B. cereus* spores actually adhered better whereas others adhered very poorly leading them to conclude that adhesion phenomena vary from strain to strain.

The ability of *B. cereus* to withstand conventional cleaning methods is compounded by the fact that the spores will clump upon exposure to heat treatments. Furukawa *et al.* (2005) found that after 20 minutes of heat treatment at 85°C, the ratio of spore clumps for *B. cereus* increased nearly 3-fold, which allowed greater numbers of microorganism to survive. This result was attributed to an increase in surface hydrophobicity of the spores as demonstrated by a bacterial adherence to hexadecane (BATH) assay. They later verified this supposition.

Oosthuizen *et al.* (2002) took a proteomic approach to reveal differences in protein expression of *B. cereus*. Using glass wool as an attachment surface, this group of researchers identified 15 unique proteins that are expressed in biofilm cells as compared to planktonic cells.

Common chemical sanitizers and their mechanisms of action

Quality defects in refrigerated products and ultimately spoilage are most often the result of microbial contamination, which can be introduced during pre- and post-pasteurization processes growth (Meer *et al.*, 1991). Common sources of microbial contamination may include rinsing with untreated water, improper cleaning, failing to remove the build up of microorganisms on rubber parts, separators, and pipes, or negligent handling of the product (Murphy, 2005). These situations ultimately lead to the introduction of unwanted microorganisms into products, severely limiting their shelf life.

Chemical sanitizers and/or cleaners are often employed to reduce the bioburden, or load of microbial contaminants. These common sanitizers are illustrated in Table 1 (Parkar *et al.*, 2003). These sanitizers must be completely removed from the parts being cleaned or they may end up in the final product (Murphy, 2005), unless post clean-up is executed.

Table 1. List of commercially used chemical cleaners, an example of each, and a description of their mechanism of action.

Class of Agent	Example(s)	Mode of Action
Alkaline cleaning agents	standard caustic soda	Cause the dissolution of cell material
enzyme based cleaning agents	protease and polysaccharidase	Proteolytic activity and a surfactant action
oxidizing agents	as sodium hypochlorite, chloramine, chlorine dioxide, and hydrogen peroxide	Initiate a cascade of oxidative attack on bacterial enzymes, and peroxidative attack on membrane unsaturated fatty acids
quaternary ammonium chloride agents	Bactosolve®	Cause breakdown of cell membranes followed by intracellular potassium efflux and cellular protein/nucleic acid damage
Detergents	Tween-80 and dobanic acid	Surfactant effects enhancing access of other cleaners to the biofilms by increasing their wettability

From a medical standpoint, as more and more microorganisms become resistant to antibiotic therapies, hospitals will witness a dramatic increase in nosocomial infections resulting in severe disease or death. Adhesion is a necessary first step in microbial colonization and most pathogenesis, providing a good theoretical target for new therapies (Bavington and Page, 2005).

The aim of this study was to examine the efficiency of an aerosol composed of cranberry proanthocyanidins in the prevention of attachment of microorganisms to, and/or the disruption of established biofilms on abiotic surfaces such as stainless steel. This technology could be employed in food processing plants where a premium is placed on maintaining a sanitized work environment to prevent product contamination. A cranberry aerosol could also be employed on metal equipment and pipes to reduce attachment and biofilm growth. Investigations at both the physiological and molecular levels of proanthocyanidins effects on attachment by *B. cereus* were examined. Knock-out mutants from wild-type organisms were generated, using a Stratagene QuikChange® Mutagenesis Kit, to determine if cranberry exposure can be mimicked.

II. MATERIALS AND METHODS

Proanthocyanidin extraction

The proanthocyanidin fraction was isolated from blended cranberry fruit using a column chromatography method outlined by Neto *et al* (19). Whole cranberry fruit was macerated in a blender with 500 mL of a 40:40:19:1 (v/v/v/v) mixture of acetone/methanol/water/formic acid. After standing for 1 h, the mixture was filtered and the solids reextracted twice with 250 mL of solvent mixture. The solution was concentrated by rotary evaporation and then loaded on a

Diaion HP-20 column (Supelco) to remove sugars. The column was washed with 300 ml distilled water to remove sugars and the phenolics were then eluted with methanol.

The solution was loaded onto a C18 column to separate small phenolics from flavonoids. The column was washed with 100 mL water, then 15:85 (v/v) methanol/water to elute the small phenolic acids. The flavonoids were eluted with methanol containing 5 mg mL⁻¹ formic acid until no further reddish color appeared on the column. The flavonoid eluate was concentrated via rotary evaporation and applied to a Sephadex LH-20 column (Amersham Biosciences, Uppsala, Sweden) (25 × 290 mm) to separate the proanthocyanidins from flavonols and anthocyanins. Flavonols and anthocyanins were eluted with a 70:25:5 (v/v/v) mixture of methanol/water/formic acid until the eluate was colorless. The proanthocyanidin fraction (PAC) was eluted with 200 mL of 70:30 acetone/water and dried in a SpeedVac. The presence of proanthocyanidins and the absence of other flavonoids were verified by HPLC.

Analysis of proanthocyanidin fraction (PAC)

The presence of proanthocyanidins and absence of other flavonoids such as flavonols and anthocyanins or small phenolic acids in this fraction was verified by using HPLC (PerkinElmer Series 200 HPLC equipped with an autoinjector, HPLC pump, and a UV/VIS Detector set at an excitation wavelength of 280 nm). The sample was analyzed using an EMD Lichrospher® Si 100 column (4.0 x 250 mm, 5 µm particle size) with an injection volume of 10 µL. The ternary mobile phase consisted of (A) dichloromethane, (B) methanol and (C) acetic acid and water (1:1 v/v). Separations were effected by a series of linear gradients of B into A with a constant 4% C at a flow rate of 1 mL/min as follows: elution starting with 14% B in A; 14-28.4% B in A, 0-30 min; 28.4-39.2% B in A, 30-45 min; 39.2-86% B in A, 45-50 min. (Adamson *et al.*, 1999).

Bacillus cereus culture conditions

B. cereus ATCC 14579 was used throughout the study. *B. cereus* cells were routinely grown in tryptic soy broth (TSB) alone or supplemented with cranberry proanthocyanidins (200 µg/ml) and incubated for 12-18 h at 30°C.

Biofilm assay

A biofilm assay was performed using a costar® 24 well culture plate with each plate consisting of a different condition. A chromatography sprayer was used to mist the control plate wells with 95% ethanol, whereas the experimental plate was misted with 95% ethanol containing 5, 10, 20, 40, 80, 160, and 200 µg ml⁻¹ of cranberry proanthocyanidins. Both sets of plates were allowed to dry at room temperature for 30 min. After drying, 2 ml of TSB was added to each well, followed by inoculation with 100 µl of a 10⁸ ml⁻¹ suspension of *B. cereus*. The plates were then placed at 30°C on a shaker set at 190 rpm for 3 and 18 hrs. Upon draining and washing each well 3 times with PBS, crystal violet was added and allowed to stain for approximately 30 minutes. After staining, the crystal violet was removed and each well was rinsed 3 times with DI water. After rinsing, 2 ml of 95% ethanol was added to each well to de-stain. The wells were analyzed with the use of a PerkinElmer VICTOR² set at a 600 nm wavelength.

Bacterial Adherence to Hexadecane (BATH) assay

The relative hydrophobicities of *B. cereus* endospores, vegetative cells, and cells grown in TSB supplemented with proanthocyanidins (5, 10, 20, 40, 80, 160, and 200 µg ml⁻¹) were measured by using the BATH assay of Rosenberg (19). To start, 3 ml of a spore or cell

suspension in PBS at an A_{440} of 0.8 to 1.0, was added to varying amounts of hexadecane (0.1, 0.2, 0.6, and 1.0 ml). The two phases were vortexed for 120 s, and after separation for 15 min, the aqueous phase was carefully removed. The absorbance of the aqueous phase was measured at 440 nm by using a Turner ® SP-830 spectrophotometer. The percent decrease in absorbance, or index of hydrophobicity, was calculated as follows: $100(A_0 - A_f)/A_0$ where A_0 and A_f were the initial absorbance and final absorbance, respectively.

Motility assay

Media for the swarming assay was TSA 0.5% (w/v) agar or TSA 0.5% (w/v) agar supplemented with 5, 10, 20, 40, 80, 160, and 200 $\mu\text{g ml}^{-1}$ proanthocyanidins. Swimming motility media was composed of TSA 0.25% (w/v) agar with or without the cranberry proanthocyanidin concentration mentioned before. The center of the plates were inoculated with an overnight TSB culture of *B. cereus* and incubated at 30°C for 12–16 h. After incubation, the diameter of the migrating colonies was measured and recorded.

Sporulation Assay

B. cereus 14579 spores were prepared by inoculating nutrient agar plates supplemented with MnSO_4 (40 mg liter⁻¹) and CaCl_2 (100 mg liter⁻¹). Tryptic soy agar (TSA) plates and TSA plates supplemented with PACs (100 $\mu\text{g ml}^{-1}$) were also inoculated. All plates were incubated at 30°C for 24 to 48 hours. Sporangia were stained with malachite green, and counterstained with safranin.

SDS-PAGE analysis

The sodium dodecyl sulfate polyacrylamide gel electrophoresis technique was utilized to differentiate protein expression of *B. cereus* cells grown in the presence and absence of proanthocyanidins. After the harvested cells were sonicated in lysis buffer, concentrated (2D quantification Kit), and quantified, the samples were boiled for 90s in a 2X sample treatment buffer (50 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β -mercaptoethanol; and 0.1% bromophenol blue) and the proteins were separated by SDS-PAGE on 12% gels. The gels were stained with silver stain, or Coomassie blue, as appropriate. Amersham protein molecular weight standards were used in both cases. The silver staining was done according to the modified Blum protocol as illustrated on Table 2 (19). The Coomassie blue stain was carried out via the quick hot staining method. A solution was prepared of 0.025% Coomassie R-250 by dissolving 1 PhastGel Blue tablet in 1L of 10% (v/v) acetic acid. This solution was then heated to 90°C and poured over the gel, which was in a stainless steel tray. The tray was then covered with a lid and placed on a laboratory shaker for 10 min. The gels were destained in 10% (v,v) acetic acid in a tray on a shaker for at least 2 hours at room temperature. After staining, the bands of interest were excised, extracted, and digested. The samples were then concentrated with a ZipTip® before spotting onto a MALDI target plate. MALDI-TOF-TOF was carried out at the Microchemical Core Facility, Emory University for identification of the purified proteins. The amino acid sequences of the proteins were determined by mass spectrometry and analyzed by the Mascot™ software at the aforementioned facility.

Table 2. Modified Blum silver staining protocol utilized to stain gels that will undergo MALDI-TOF/MS analysis.

Step	Procedure	Comments
1	Fix gel in 40% EtOH, 10% HAc for 1 h	
2	Wash gel in 30% EtOH, 2 × 20 mins	
3	Wash gel in H ₂ O for 20 mins	
4	Sensitize gel in 0.02% Na ₂ S ₂ O ₃ for 1 min	
5	Wash gel in H ₂ O, 3 × 20 s	
6	Incubate gel in cold 0.1% AgNO ₃ , 20 mins at 4°C	
7	Wash gel in H ₂ O, 3 × 20 s	
8	Change gel chamber	
9	Wash the gel in H ₂ O for 1 min	
10	Develop gel in 3% Na ₂ CO ₃ , 0.05% formalin	Observe the color and change solution when the developer turns yellow. Terminate when the staining is sufficient
11	Wash the gel in H ₂ O for 20 s	
12	Terminate staining in 5% HAc	
13	Wash the gel in H ₂ O, 3 × 10 mins	
14	Leave the gel at 4°C in 1% HAc	

Extraction of genomic DNA from *Bacillus cereus*

The procedure used for extraction of DNA was a modification of a previously described protocol (Chin *et al.*, 1999 Grosskopf *et al.*, 1998). *Bacillus cereus* was cultivated in 5 ml of tryptic soy broth with and without PACs at 30°C with rotary shaking at 180 rpm. 2 ml of cell suspensions were harvested from each condition via centrifugation for 5min at 13,000 rpm at room temperature. 300 µl of sodium phosphate buffer (120mM, pH 8.0) was used to resuspend the cell pellets and 30 µl SDS-solution (10%) was added to the samples. Samples were allowed to incubate at RT for 10 min, followed by the addition of 100 mg of acid washed glass beads (0.1 mm diameter). Sigma tubes were placed in the bead beater for 30 seconds and processed two times at 2500 rpm to lyse the cells. Samples were then centrifuged at 13000 rpm for 10 min at room temperature. After centrifugation, the supernatant was decanted to clean 2 ml microcentrifuge tubes containing 300 µl of chloroform:isoamyl alcohol (24:1). The supernatant and chloroform:isoamyl alcohol were manually shaken for 1 min and centrifuged for 5 min at 13000 rpm twice and then transferred to a clean 2ml tube. Sodium acetate was added at 1/10 the volume, and the remaining space within the tube was filled with 100% ethanol and vortexed. The DNA was allowed to precipitate for 1 hr at room temperature and then was centrifuged for 15 min at 13000 rpm. The supernatant was discarded and 500µl of 70% ethanol was added to the DNA pellet and centrifuged for 15 min at 13000 rpm. Ethanol was then discarded and the DNA pellet was dried using the vacufuge (Eppendorff) for 5 min. The pellet was then resuspended in sterile water. Extracted DNA was visualized by gel electrophoresis on a 1% agarose gel to obtain an estimate of the size and quality of the genomic DNA.

Site-directed mutagenesis of *Bacillus cereus* ATCC 14579

The primers for the site directed mutagenesis of *B. cereus*, Table 3, were designed using the guidelines outlined by the manufacturer (Stratagene). Each primer was between 30-37 bp long, and had overlapping ends to allow for better complementation to target DNA sequence. The primers were designed in accordance with the proteomic results obtained from *B. cereus* cells grown in the presence and absence of PACs. Each PCR reaction contained 1 µl each of a forward and reverse primer (125 ng µl⁻¹), 40.5 µl of UV treated DI water, 1 µl dNTPs, 5 µl 10x solution, 1 µl *Taq* polymerase, and 0.5 µl template DNA (50 ng µl⁻¹). The thermal cycling program was as follows: heat reaction tube to 95°C for 30 sec; this was followed by 18 cycles of 95°C for 30 sec, 55°C for 1 min, and 68°C for 2 min. After thermal cycling, the tubes were placed on ice for 2 minutes to cool the reaction to ≤37°C. The desired amplification was checked by electrophoresis of 10 µl of the product on a 1% agarose gel. After verification, 1 µl of *Dpn* I restriction enzyme was added directly to each amplification reaction, the reaction was mixed via pipetting, and incubated at 37°C for 1 hour to digest the nonmutated supercoiled dsDNA. The mutated DNA was transformed into XL1-Blue supercompetent cells by adding 1 µl of the *Dpn* I-treated DNA to 50 µl of supercompetent cells. The transformation reactions were streaked onto plates containing an appropriate antibiotic, 80 µg/ml X-gal and 20 mM IPTG, incubated at 37°C for >16, and screened for blue colonies. These blue colonies had their plasmid DNA extracted from them and confirmed. After the mutated sequences were confirmed, the plasmid DNA was ligated into a *Bacillus cereus* vector, electroporated into the organism being studied, and incubated in the absence of antibiotic selection for several days to allow reciprocal recombination and subsequent loss of the shuttle vector (Zeibell *et al.*, 2006).

Table 3. Primers used for the PCR site-directed mutagenesis of the formate acetyltransferase and 30S ribosomal protein S4 genes. Primer names are based on: *30s*= 30S ribosomal protein S4; *forA*= Formate acetyltransferase; M1=insertion mutation; M2=deletion mutation; M2=deletion mutation; F=forward primer; R=reverse primer.

Primer	Sequence	Location (Base Pairs)	Reference
30SM1F	GCACGGCGATAAAACTTCATGATCCTTCTTGAAGCTC	243 - 279	This study
30SM1R	GAAGTTTTATCGCCGTGCTTACCAGGCATTTTACC	226 - 260	This study
30SM2F	CACGGCGAACTTCATGATCCTTCTTGAAGCTCGTC	244 - 278	This study
30SM2R	CATGAAGTTCGCCGTGCTTACCAGGCATTTTAC	227 - 259	This study
<i>ForAM1F</i>	GAACGTGATAAAACGGTGGCGTTCTTGATATGG	193 - 225	This study
<i>ForAM1R</i>	GCCACCGTTTTATCACGTTCTTTCGTTGTTAAATC	178 - 213	This study
<i>ForAM2F</i>	GAACGTGAACGGTGGCGTTCTTGATATGGATAC	193 - 225	This study
<i>ForAM2R</i>	GAACGCCACCGTTCACGTTCTTTCGTTGTTAAATC	178 - 212	This study

III. RESULTS

Extraction and quantification of cranberry proanthocyanidins

Upon extraction and concentration of the cranberry PACs via column chromatography as described above, the samples were analyzed via HPLC. Since cranberry proanthocyanidins were not commercially available, vanillin was used as a standard to determine which peaks of the HPLC output corresponds to the A-type proanthocyanidins found in the cranberry fruit (Figure 2). In spite of the lack of evidence for deterioration, following HPLC verification, the cranberry PACs were stored in the dark at -20°C to ensure the phytochemicals were not photodegraded.

Comparative protein analysis of *B. cereus* ATCC 14579 grown in the presence and absence of cranberry PACs

Differential protein expression between *B. cereus* ATCC 14579 cells grown in the presence and absence of cranberry PACs, a SDS-PAGE gel is illustrated in Figure 3. Evidence of up-regulation of four proteins in the presence of PACs was observed, and the bands were excised and sequenced. The data returned from excising and sequencing the bands identified the following proteins, from the highest to lowest molecular weight: formate acetyltransferase, acetate/butyrate kinase, 30S ribosomal protein S4, and hypothetical repetitive exported protein. The protein data obtained from this experiment was utilized to provide a map to target what genes would be knocked out to generate mutants to be used for the various challenge tests.

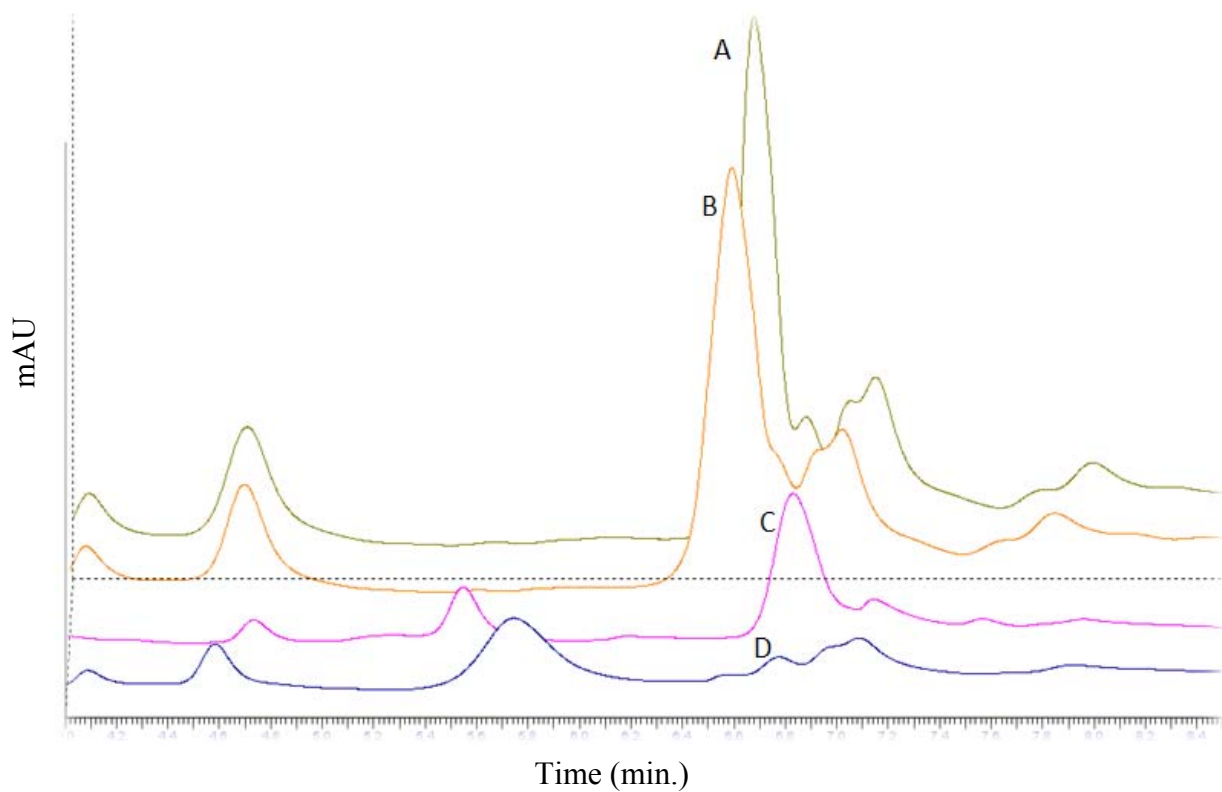


Figure 2. AHPLC analysis of varying concentration of cranberry PACs where (A) is 160 μ g/ml, (B) 80 μ g/ml, (C) 40 μ g/ml, and (D) 20 μ g/ml.

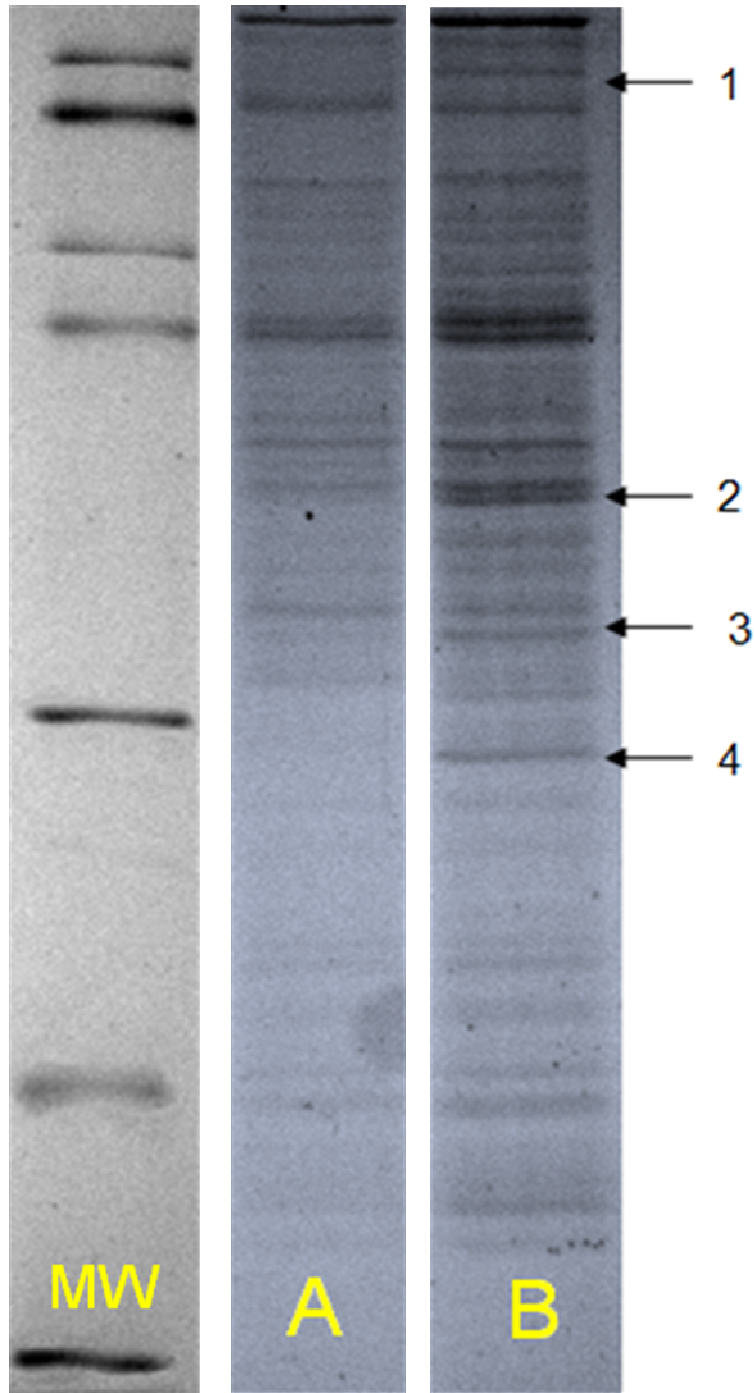


Figure 3. SDS-PAGE analysis of *B. cereus* ATCC 14579 cells grown in the (A) absence and (B) presence of cranberry PACs (200µg/ml). The arrows identify the following proteins: (1) formate acetyltransferase; (2) acetate/butyrate kinase; (3) 30S ribosomal protein S4; (4) hypothetical repetitive exported protein.

Generation of *ΔforA* and *Δ30S* knockout mutants

The protein data on up-regulated proteins was used as a guideline to target two genes to be knocked out for study, one for formate acetyltransferase, and a second 30S ribosomal protein S4. Figures 4 and 5 represent schematics of how the knock-out mutants were derived. Following the final reciprocal recombination, the mutant alleles were probed with specific primers to ensure that the insertion or deletion was achieved (Figure 6).

Cranberry PAC's effect on the growth of *B. cereus*

The effect that an increasing concentration of cranberry PACs has on the growth of *B. cereus* was measured via spectrophotometry (Figure 7). The bar chart shows that growth was not inhibited by the supplementation of increasing concentrations of cranberry PACs, but rather maintained or in some cases bolstered by the presence. At the highest concentration of PACs, the optical density of the WT cells was nearly double that of the cells grown in an environment with the cranberry phytochemical missing. With regards to the mutants, although there was a slight increase in the optical density of the *ΔforA* mutant, the *Δ30S* mutant remained relatively unchanged over the range of concentrations. Overall, the group that responded the most positive to the cranberry treatment was the WT cells of *B. cereus*, followed by the *Δ30S* mutants, and finally the *ΔforA* mutants showed the least amount of growth. ANOVA single factor analysis showed that there is a significant difference between the growth rates of the three test groups.

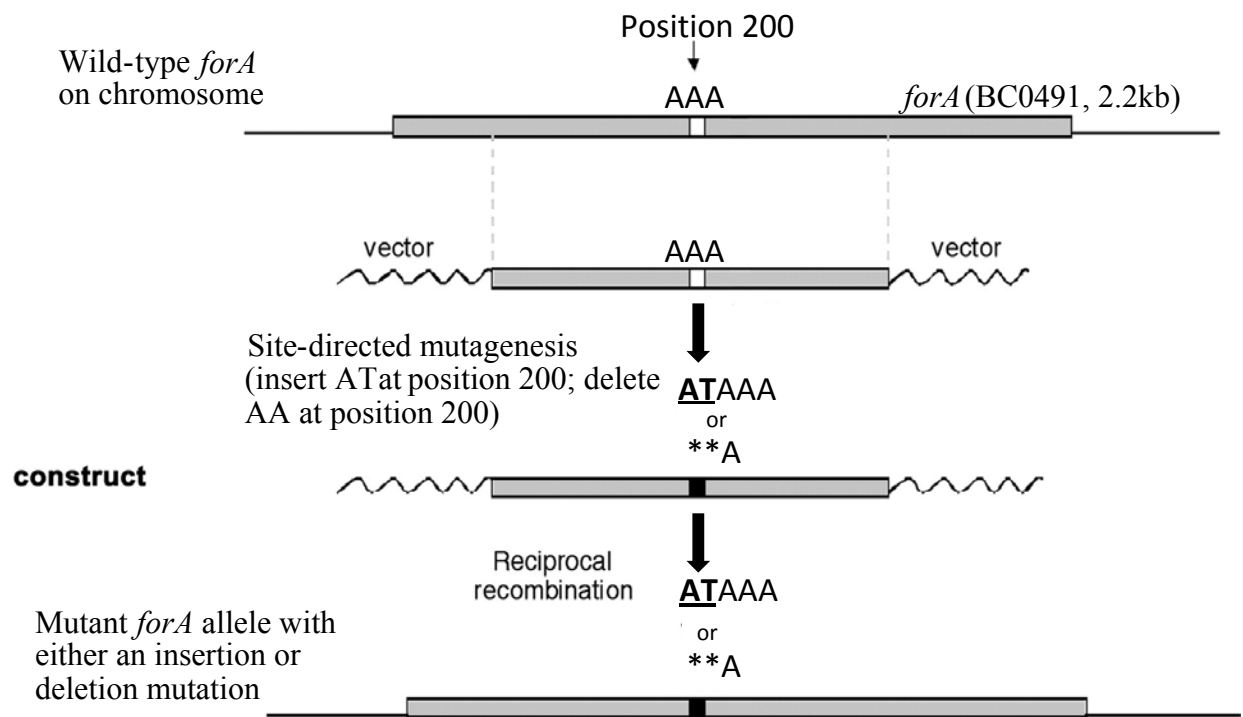


Figure 4. Schematic representation of the procedure utilized to generate an insertion or deletion mutation in the *B. cereus forA* gene.

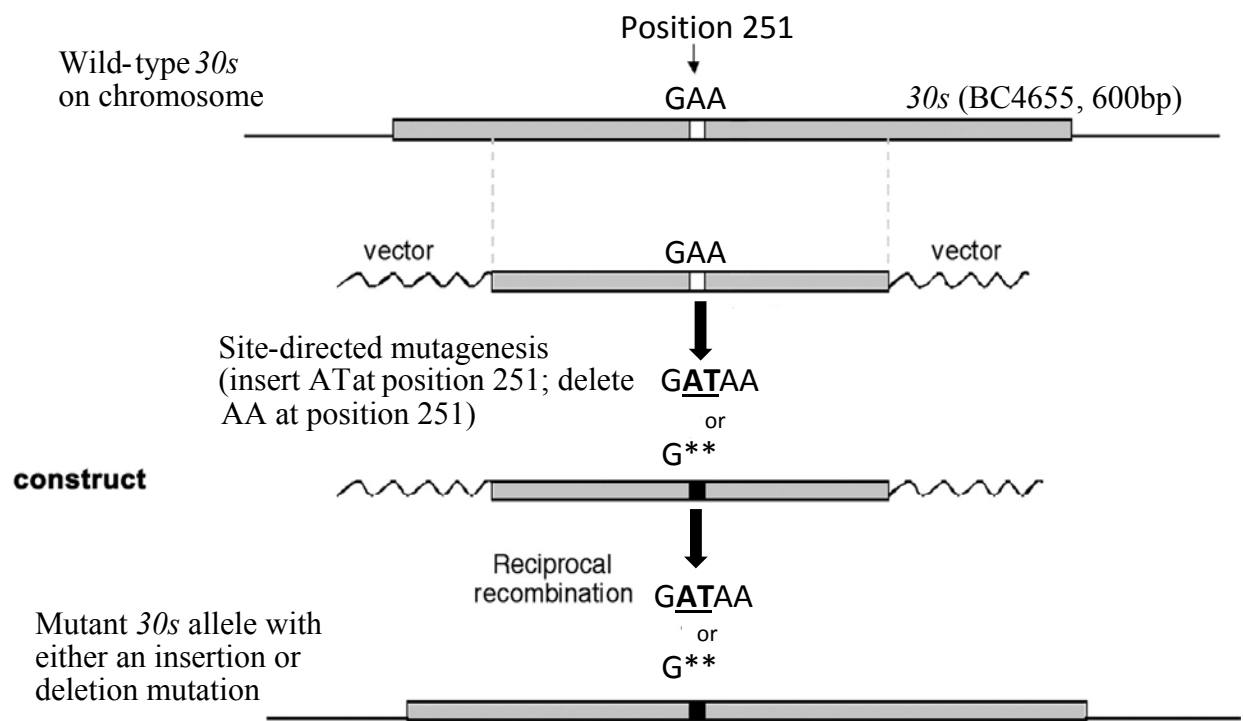


Figure 5. Schematic representation of the procedure utilized to generate an insertion or deletion mutation in the *B. cereus 30s* gene.

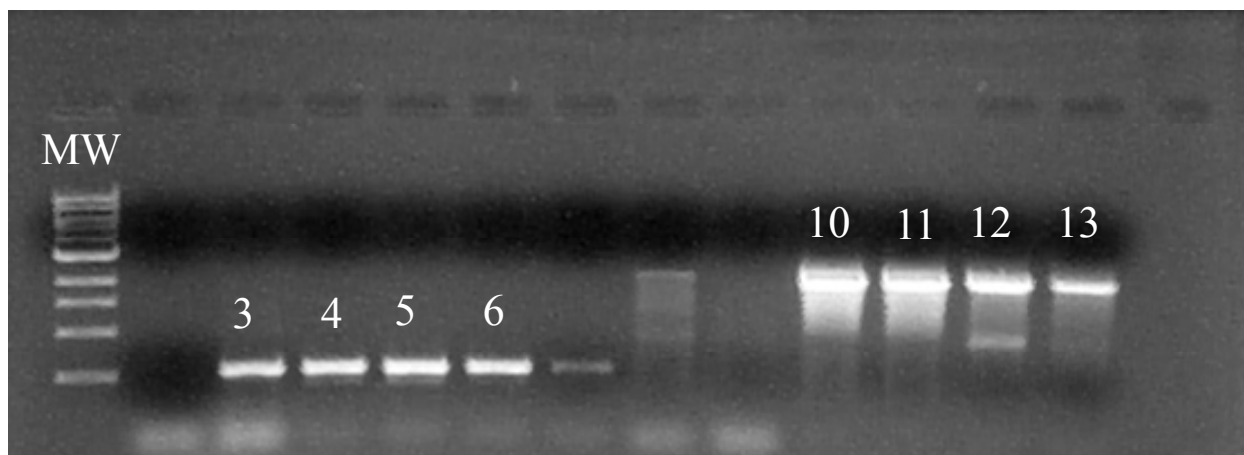


Figure 6. Agarose gel electrophoresis amplification results after site-directed mutagenesis reactions. Lane MW: DNA ladder standard (5 kb); lanes 3&4, PCR amplicon of the *30s* gene using insertion mutagenesis primers; lanes 5&6, PCR amplicon of the *30s* gene using deletion mutagenesis primers; lanes 10&11, PCR amplicon of the *forA* gene using insertion mutagenesis primers; lanes 12 and 13, PCR amplicon of the *forA* gene using deletion mutagenesis primers.

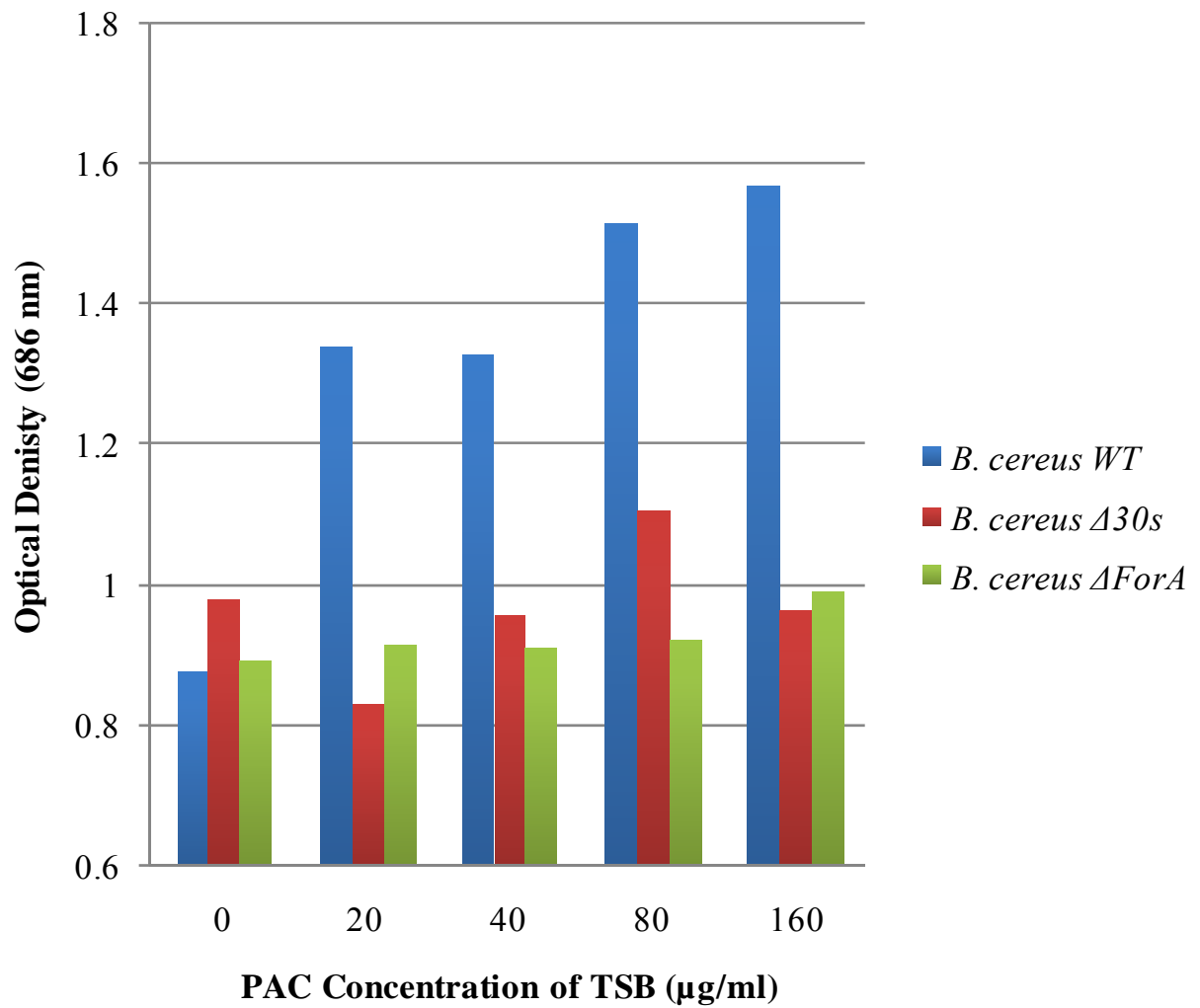


Figure 7. Optical density (686 nm) of *B. cereus* grown at 30°C in TSB with increasing concentrations of cranberry PACs.

Cranberry PAC's effect on the *B. cereus* WT and mutants response to environmental stresses

The *B. cereus* WT and mutant cells, grown up in the preceding experiment, were placed through a series of challenge tests which simulate a possible clean in place regimen. The first test was to heat the cells at 85°C, take samples at 5 minute increments, and do a plate count. The results are conveyed in log reduction which was calculated by taking the LOG₁₀ of the original cell count divided by the viable cell count made on the plates the following day. The results show that after five minutes, the WT *B. cereus* demonstrated increasing resistance to the heat treatment as the cranberry concentration increased, as witnessed by the decreasing log reduction values (Figure 8). Both the *ΔforA* and *Δ30S* mutants showed the same trend, but overall these mutagenized cells were reduced to a greater degree, and more quickly than their WT counterparts. After 10 minutes of exposure, all of the *B. cereus* cells from the control to the 20μg/ml of PACs are completely reduced (Figure 9).

The WT cells along with the mutants do show decreased levels of reduction at both the 80μg/ml and 160μg/ml levels, however both sets of the mutants, *ΔforA* more so than *Δ30S*, are more sensitive to the heat treatments than the WT. After 15 minutes of exposure to the 85°C temperature, all of the cells in all of the variants were completely reduced, except for the WT cells grown in 80 and 160μg/ml of cranberry PACs which had on average 20 colony forming units still viable (Figure 10). Although a difference in survival was observed between the variants, ANOVA single factor analysis suggested that the values are not significantly different from one another.

The second test performed was to expose the variants to varying pHs to include 1, 4, 7, 10, and 13. The pHs of 4, 7, and 10 failed to reduce the cell count in a substantial manner, made evident by the lawn of growth observed after 24 hr of incubation (data not shown). At pH 1, all

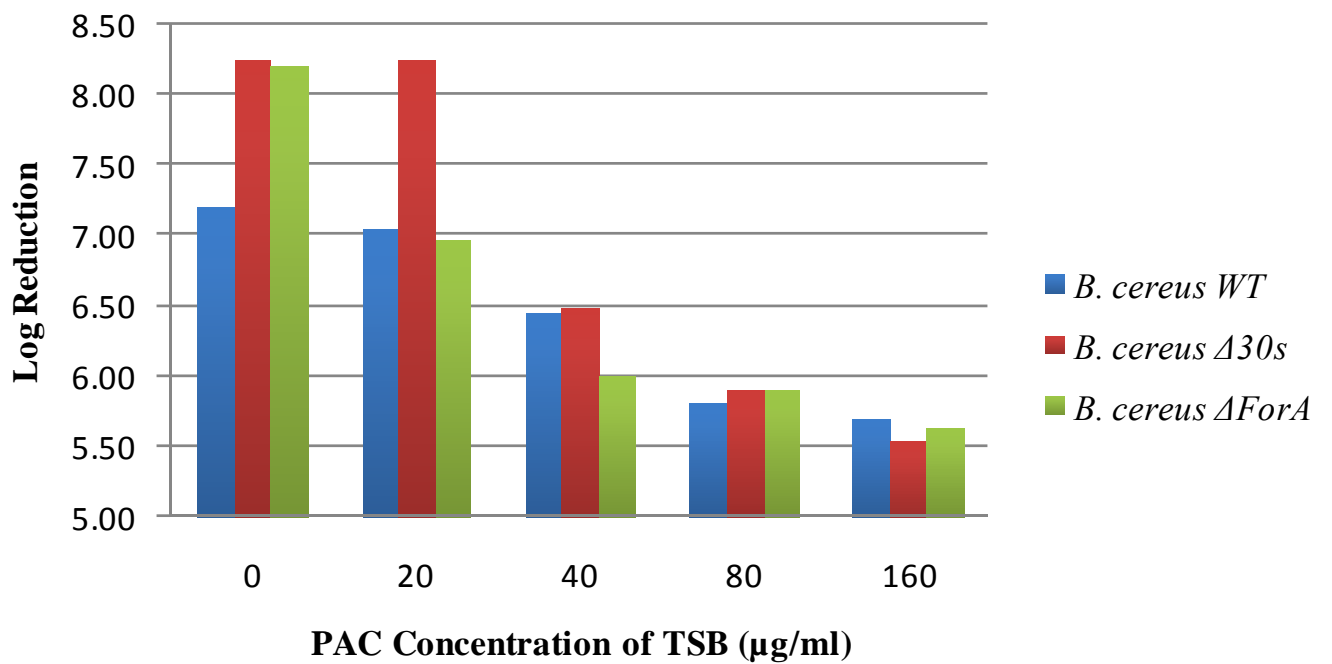


Figure 8. Log reduction of *B. cereus* ATCC 14579, $\Delta forA$, and $\Delta 30S$ mutants grown in TSB supplemented with varying cranberry PAC concentrations, at 85°C (5 minutes of exposure).

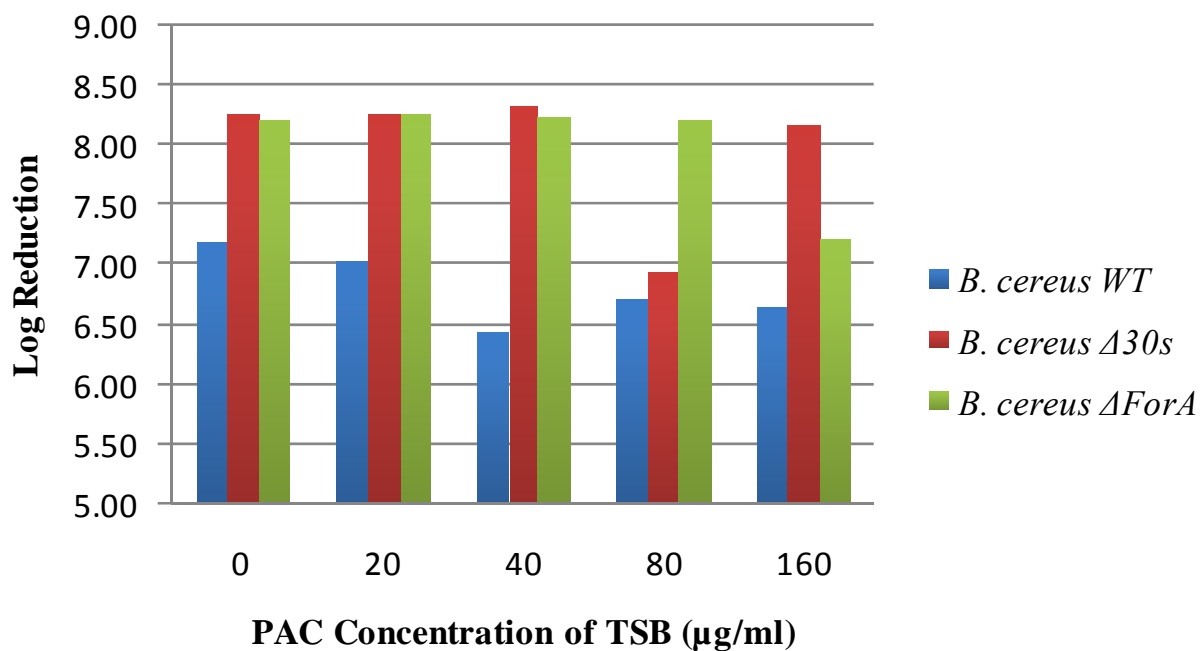


Figure 9. Log reduction of *B. cereus* ATCC 14579, $\Delta forA$, and $\Delta 30S$ mutants grown in TSB supplemented with varying cranberry PAC concentrations, at 85°C (10 minutes of exposure).

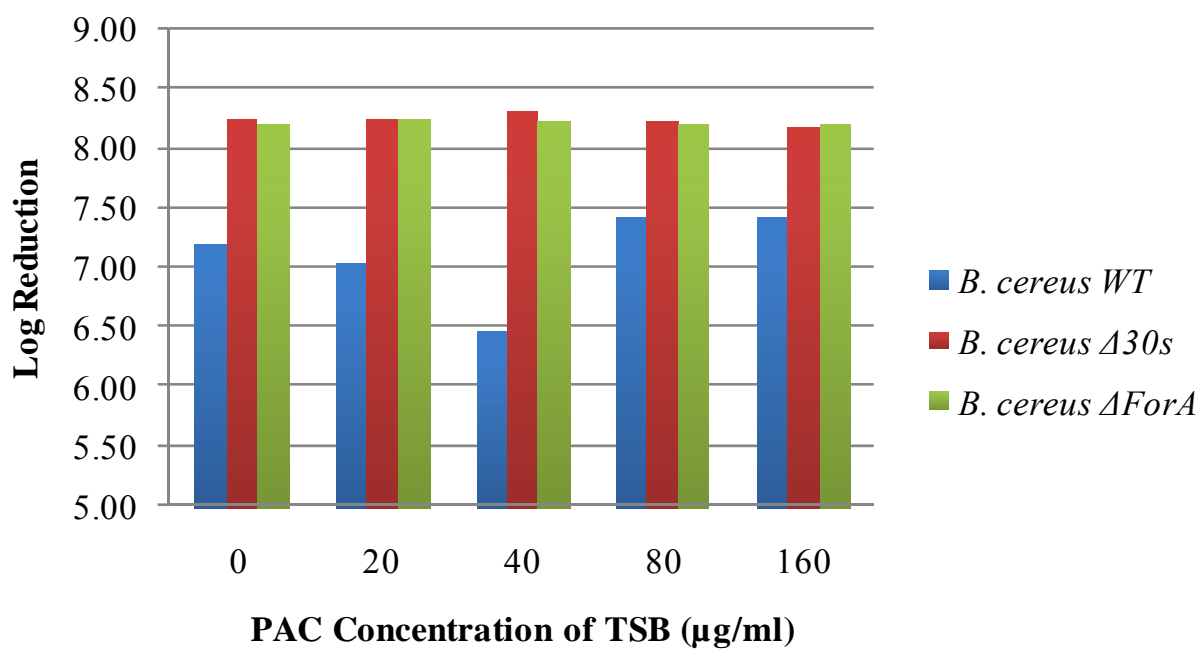


Figure 10. Log reduction of *B. cereus* ATCC 14579, $\Delta forA$, and $\Delta 30S$ mutants grown in TSB supplemented with varying cranberry PAC concentrations, at 85°C (15 minutes of exposure).

variants displayed a similar pattern of an inversely proportional relationship between an increase in PAC concentration and decrease in sensitivity to this challenge test (Figure 11). However, the degree of resistance varied between the cell types. The least sensitive at pH 1 were the WT cells grown in LB supplemented with 160µg/ml of cranberry PACs, whose viability seem to be unaffected by the extremely low pH. Between the *ΔforA* and *Δ30S* mutants, the *Δ30S* mutant seemed to be slightly less sensitive to this condition, although ANOVA analysis interprets these results to be not significantly different.

At pH 13, *B. cereus* WT and mutants, as a whole, were less sensitive to this high pH environment as opposed to the low (Figure 12). Once again the inversely proportional trend of a decrease in sensitivity matched with an increase in cranberry PACs repeats. With regards to the WT *B. cereus* cells, at the 80 and 160µg/ml PAC level, there was no observable reduction of cell numbers. Overall, the WT cells proved to be the least sensitive, followed by the *Δ30S* mutant cells, and finally the *ΔforA* mutants. Once again, although a difference in survival was observed between the variants, ANOVA single factor analysis interpreted the values as not being significantly different from one another.

Effect of cranberry PACs on the cell surface hydrophobicities of *B. cereus* ATCC 14579, *ΔforA* and *Δ30S* mutants

The change in cell surface hydrophobicities of *B. cereus* variants exposed to increasing concentrations of cranberry PACs was measured via the bacterial adherence to hexadecane assay. The hydrophobic values of the cells grown in the absence of the cranberry PACs were relatively similar across the WT and both mutants (Figure 13). The WT cells proved to have the greatest cell surface hydrophobicity, followed by the *Δ30S* and *ΔforA* mutants respectively. *B.*

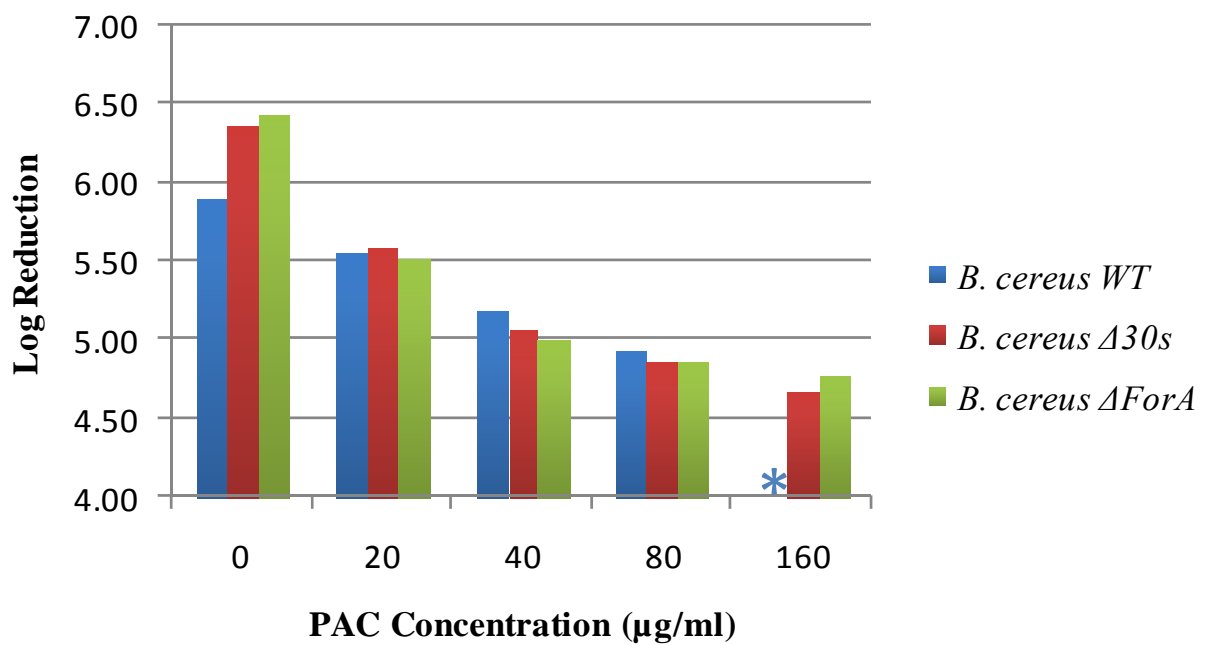


Figure 11. Log reduction of *B. cereus* ATCC 14579, $\Delta forA$, and $\Delta 30S$ mutants grown in TSB supplemented with varying cranberry PAC concentrations, exposed to LB media adjusted to a pH of 1 for 15 minutes. * indicates that log reduction could not be calculated due to cells being too numerous to count.

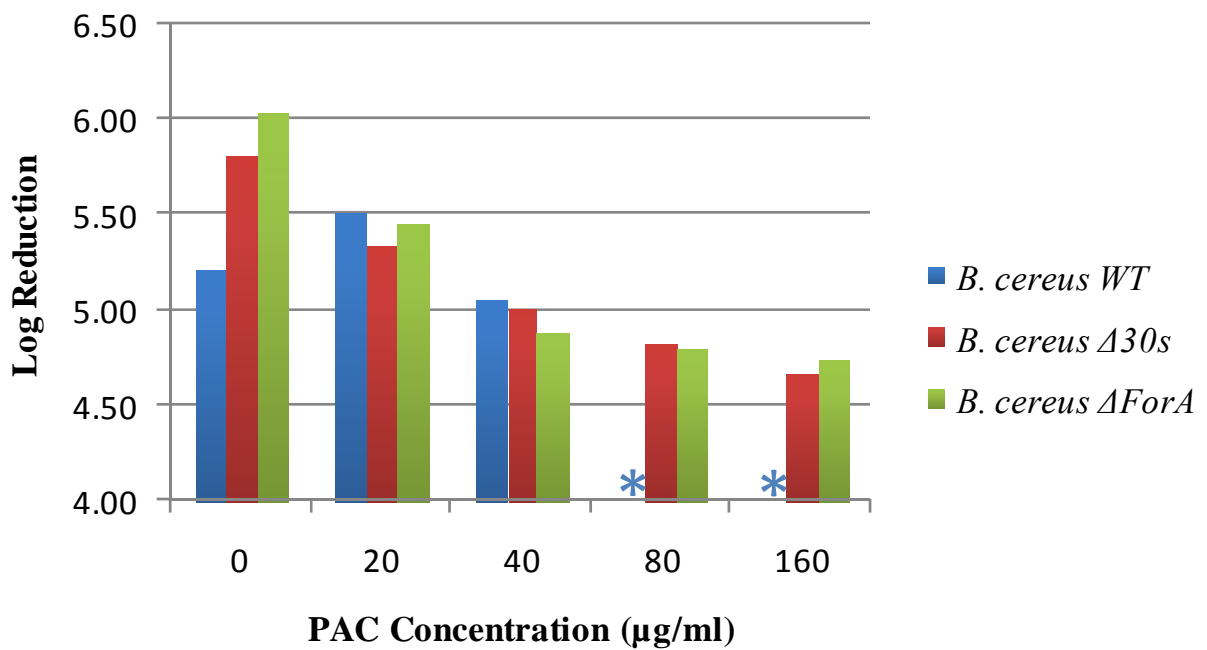


Figure 12. Log reduction of *B. cereus* ATCC 14579, $\Delta forA$, and $\Delta 30S$ mutants grown in TSB supplemented with varying cranberry PAC concentrations, exposed to LB media adjusted to a pH of 13 for 15 minutes. * indicates that log reduction could not be calculated due to cells being too numerous to count.

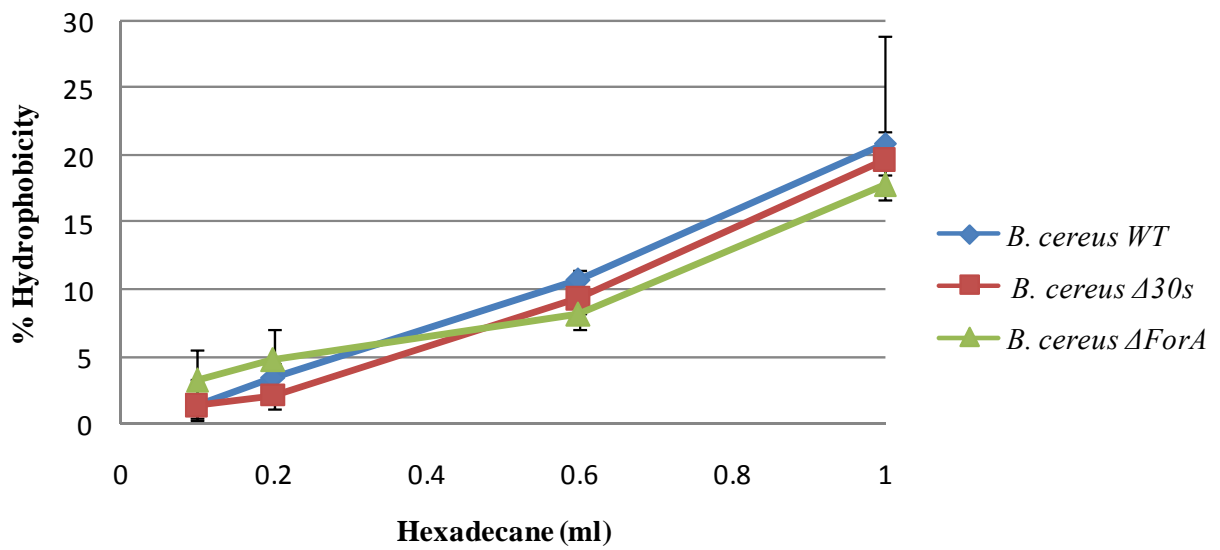


Figure 13. Hydrophobicities measured by the BATH assay of *B. cereus* ATCC 14579, $\Delta 30S$, and $\Delta forA$ mutants grown in TSB with no PAC supplementation. Error bars indicate the standard deviations from triplicate trials.

B. cereus cells grown in TSB supplemented with 20µg/ml of cranberry PACs demonstrated an increase of 9% in the maximum cell surface hydrophobicity relative to the controls with no PACs. This maximum value was observed in the WT cells, with the $\Delta 30S$ mutant almost reaching the same level (Figure 14). The $\Delta forA$ mutant in this experimental variable maintained a relatively constant hydrophobicity value substantially depressed from the *B. cereus* WT and $\Delta 30S$ cells. As the PAC concentration was increased to 40µg/ml, an increase in surface hydrophobicity was again observed (Figure 15). At this concentration, a greater disparity between the hydrophobicity values of the WT cells and the $\Delta 30S$ mutant was observed. Although the $\Delta forA$ mutant was once again relegated to having the lowest cell surface hydrophobicity, these cells had showed a substantial increase in values not seen in any other PAC supplementation condition up to this point. At 80µg/ml, a further separation between WT and mutant hydrophobicity values was observed (Figure 16). At this concentration, there was roughly a 2.5 fold difference between the highest, WT, and lowest, $\Delta forA$, hydrophobicity values. Finally, at the cranberry PAC concentration of 160µg/ml, the highest hydrophobicity values were observed (Figure 17). The difference between the highest and lowest hydrophobicity values is over 3 fold. As previously seen at the 20µg/ml level, not only did the $\Delta forA$ mutant hydrophobicity values remain constant throughout the trials, there values were also depressed compared to the WT and $\Delta 30S$ cells. ANOVA statistical analysis revealed that even though a difference was observed among all test groups, only at the 160µg/ml concentration level were the cell surface hydrophobicities significantly different.

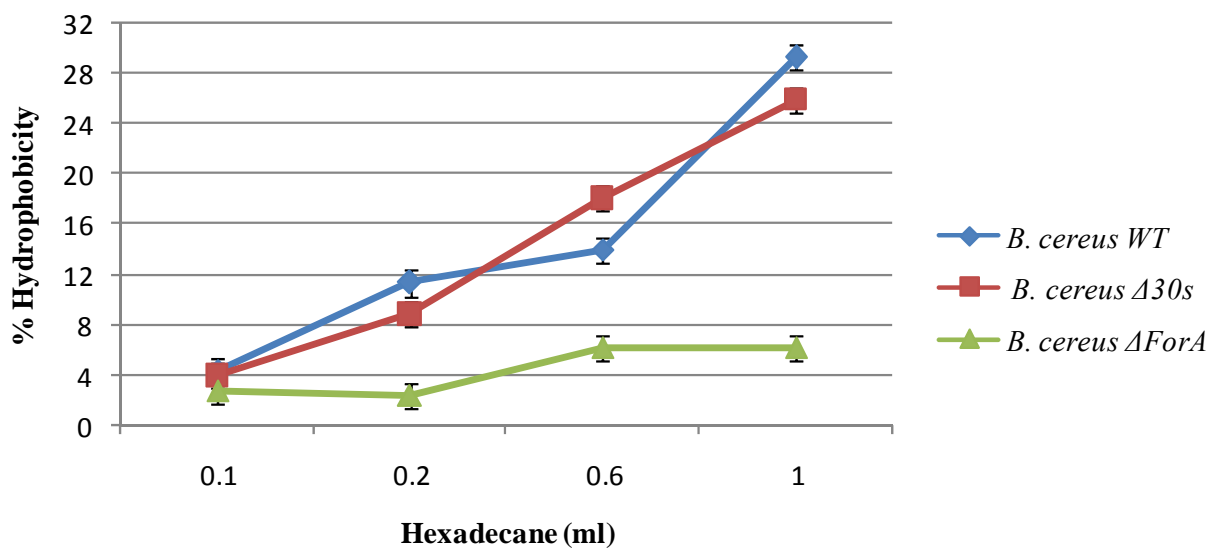


Figure 14. Hydrophobicities measured by the BATH assay of *B. cereus* ATCC 14579, $\Delta 30s$, and $\Delta forA$ mutants grown in TSB supplemented with 20 $\mu\text{g/ml}$ PAC. Error bars indicate the standard deviations from triplicate trials.

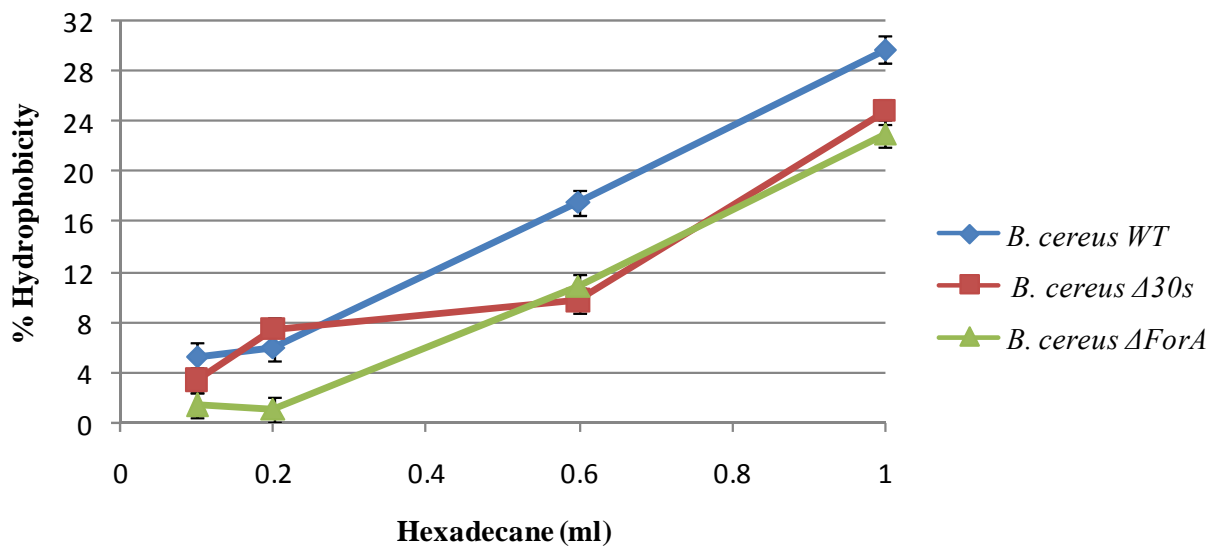


Figure 15. Hydrophobicities measured by the BATH assay of *B. cereus* ATCC 14579, $\Delta 30s$, and $\Delta forA$ mutants grown in TSB supplemented with 40 $\mu\text{g/ml}$ PAC. Error bars indicate the standard deviations from triplicate trials.

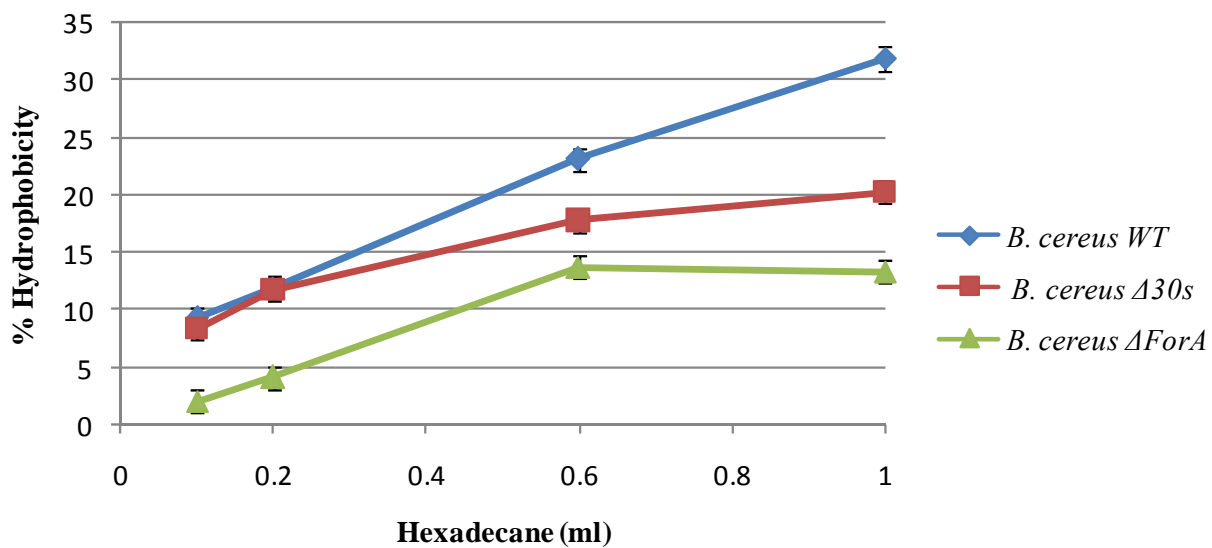


Figure 16. Hydrophobicities measured by the BATH assay of *B. cereus* ATCC 14579, $\Delta 30s$, and $\Delta forA$ mutants grown in TSB supplemented with 80 $\mu\text{g/ml}$ PAC. Error bars indicate the standard deviations from triplicate trials.

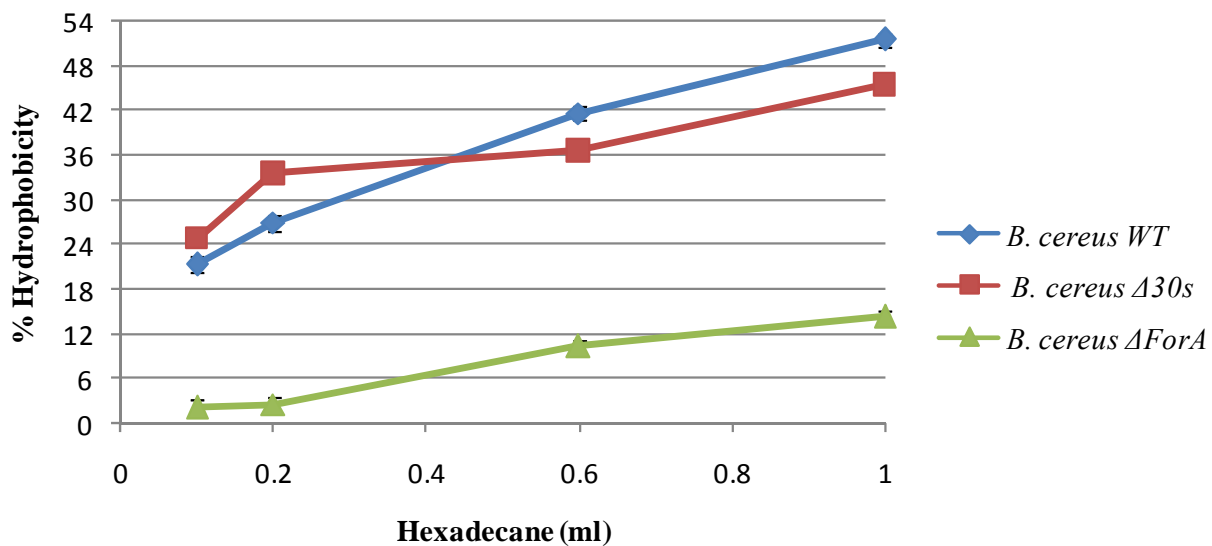


Figure 17. Hydrophobicities measured by the BATH assay of *B. cereus* ATCC 14579, $\Delta 30s$, and $\Delta forA$ mutants grown in TSB supplemented with 160 $\mu\text{g/ml}$ PAC. Error bars indicate the standard deviations from triplicate trials.

Effects of increasing cranberry PACs on the swarming motility of the *B. cereus* WT and mutant cells

To observe how cranberry PACs alter *B. cereus*' community dynamics, their effect on the cells ability to exhibit a swarming response was monitored (Figure 18). As a baseline, cells grown in the presence and absence of cranberry proanthocyanidins are illustrated in Figure 19. The cranberry PAC concentration for this experiment was 200µg/ml. Observations indicate that as the cranberry PAC concentration increases, the *B. cereus* WT and mutant cells decrease in their swarming range. The most drastic change was witnessed with the WT cells, whose range was nearly cut in half from the lowest to the highest concentration. Although a decline in swarming motility was observed with both the $\Delta forA$ and $\Delta 30S$ mutants, their decrease was not as precipitous as the WT condition. Although a difference in response to cranberry PACs was recorded among the *B. cereus* WT and mutant cells, ANOVA single variance testing failed to categorize these results as significantly different.

Effects of increasing cranberry PACs on the swimming motility of the *B. cereus* variants

Modulation of the motility of the *B. cereus* WT and mutant cells by cranberry PACs is shown in Figure 20. As seen for swarming motility, swimming motility is limited as cranberry PAC concentrations are elevated. Again, the most drastic reduction is observed with the WT cells with an initial swimming zone diameter of 15mm, and end up exhibiting a swimming motility of 10mm at the highest cranberry concentration. Once again, the mutants are least affected by the cranberry concentration, as their swimming zone remains relatively constant over the range of cranberry concentrations. Of the two mutants, the $\Delta 30S$ mutant showed the greatest swimming response, whereas the $\Delta forA$ mutant, although greater than the WT, was still second

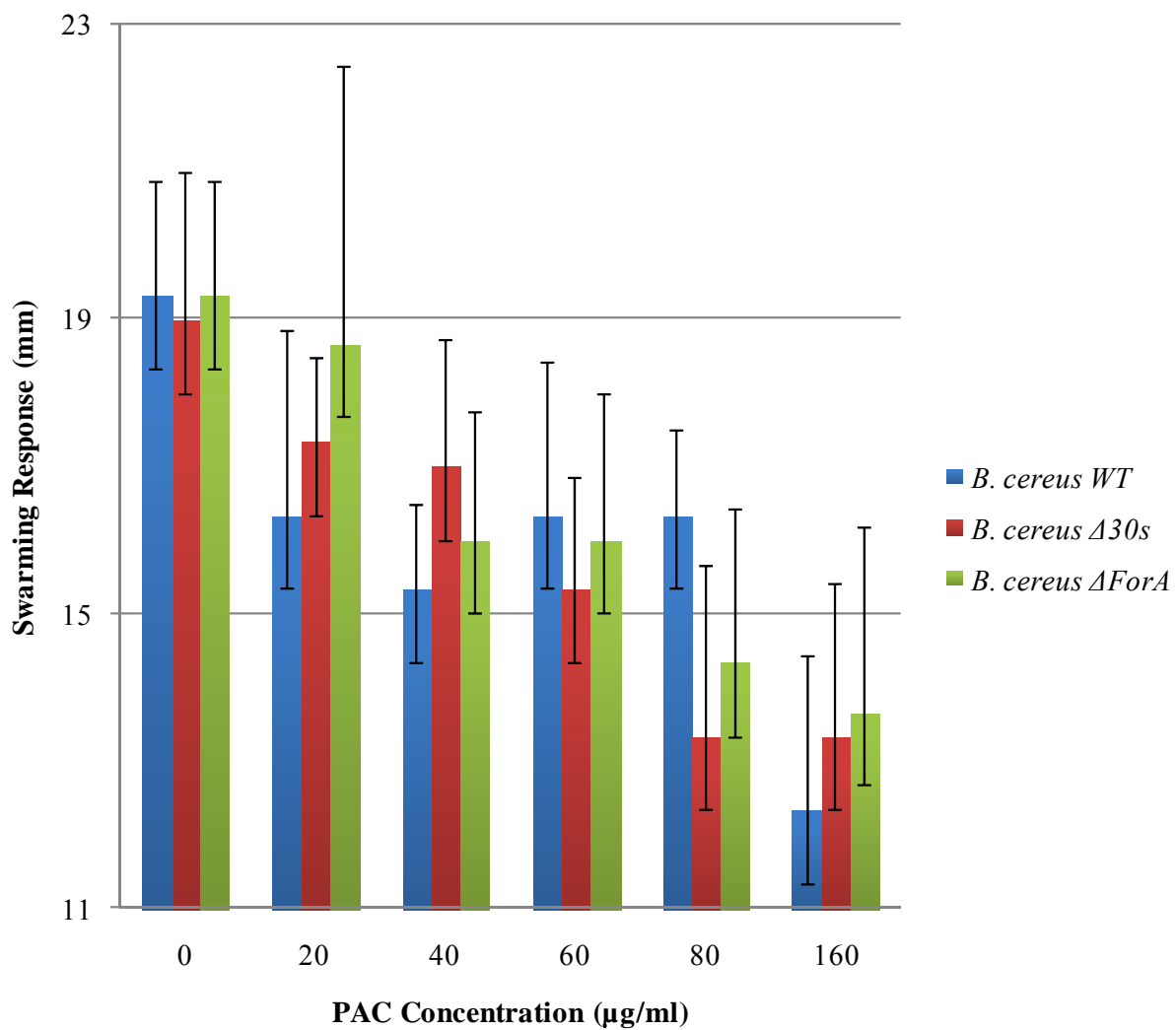


Figure 18. Effect of increasing concentrations of cranberry PACs on the swarming behavior of *B. cereus* ATCC 14579 on TSA (1%, w/v, agar). Error bars indicate the standard deviations from triplicate trials.

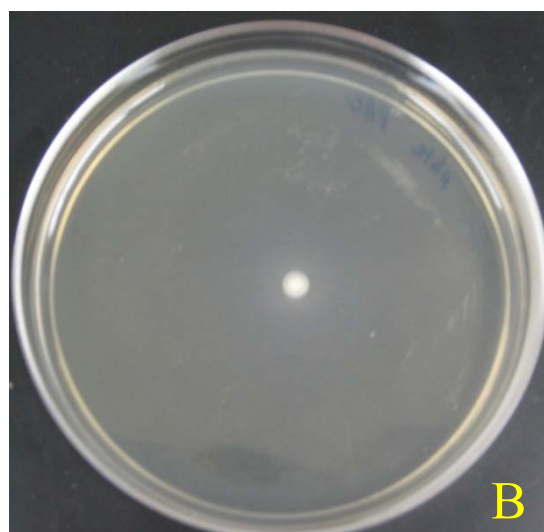


Figure 19. Swarming motility of *B. cereus* ATCC 14579. Cells were inoculated on swarming plates in the absence of (A) and supplemented with 200($\mu\text{g}/\text{ml}$) proanthocyanidins (B). They were photographed after an 18 h incubation period at 30°C.

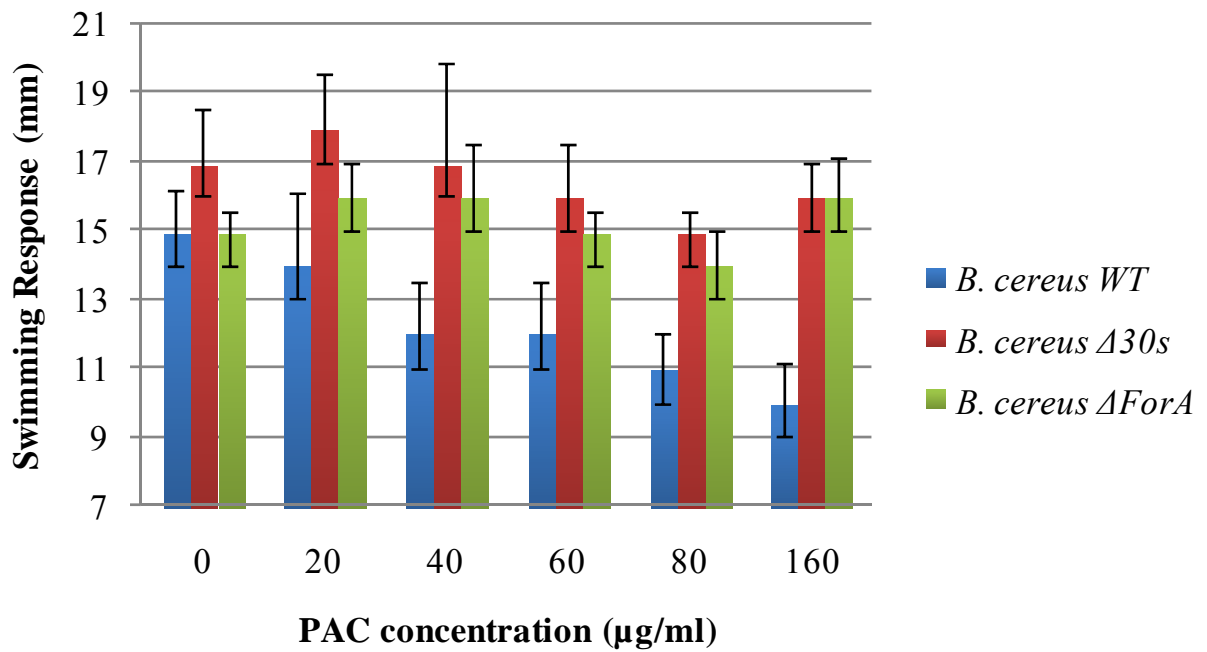


Figure 20. Effect of increasing concentrations of cranberry PACs on the swimming motility of *B. cereus* ATCC 14579 on semisolid TSA (0.25%, w/v, agar). Error bars indicate the standard deviations of triplicate trials.

best. ANOVA single factor analysis reveals that there is a significant difference between the swimming motility values of the *B. cereus* variants.

Effect of increasing cranberry PAC concentration on the ability of *B. cereus* to adhere to a tissue culture plate

A 24 well tissue culture plate was sprayed with increasing PAC concentration levels to measure the ability of the *B. cereus* variants to adhere to this abiotic surface (Figure 21). The crystal violet assay for attachment showed that the WT cells most readily attached to the surface, indicated by the high absorbance numbers. As the cranberry concentrations were increased, the number of cells bound to the surface of the 24 well plate increased. On the other hand, the mutated cells (*Δ30S*, *ΔforA*) showed little response to the change in cranberry concentration. The *Δ30S* mutant, however, seems to adhere more effectively to the abiotic surface of the 24 well tissue culture plate than its *ΔforA* counterpart.

Spore analysis of the *B. cereus* WT and mutants in increasing concentrations of cranberry PACs

Malachite green spore staining was carried out to examine how increasing cranberry concentrations effect the endospore formation in the *B. cereus* WT and mutant cells (Figure 22). There is a visible difference between those cells that were grown without the cranberry phytochemical present, and those grown in TSB supplemented with 160μg/ml of PACs. At the highest level of cranberry supplementation, nearly every other cell stains positive for endospore formation.

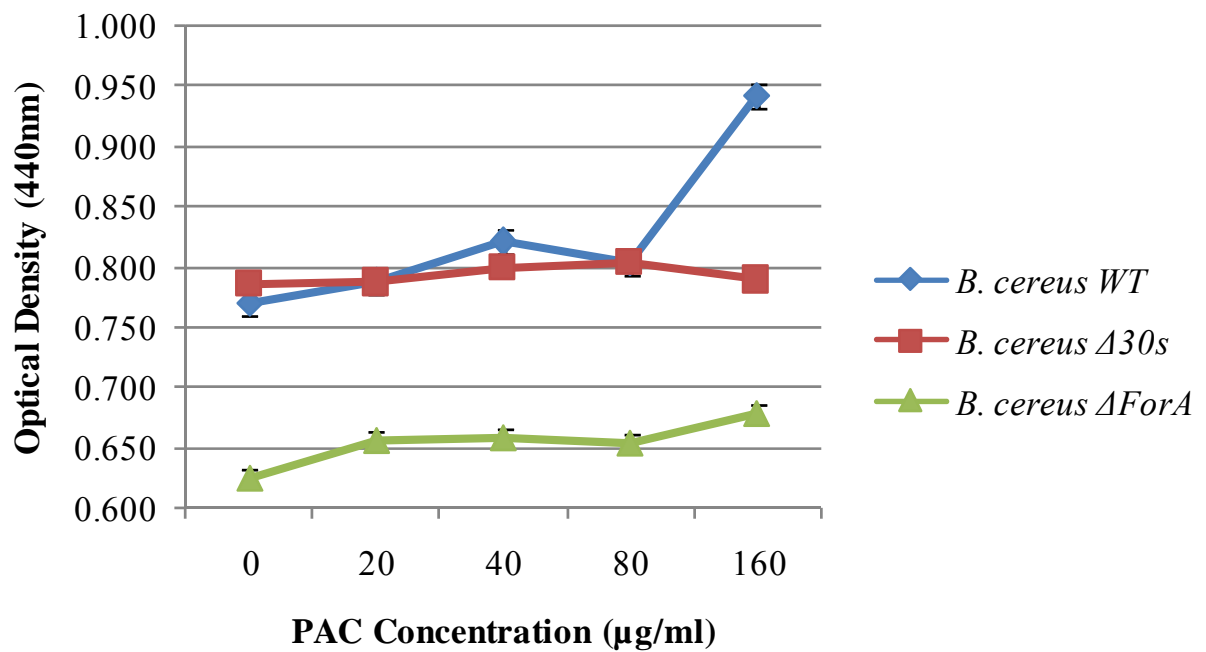


Figure 21. Biofilm formation in a 24 well tissue culture plate by *B. cereus* ATCC 14579 incubated at 30oC for 24 hrs, in varying concentrations of cranberry PACs. Error bars indicate the standard deviations from triplicate trials.

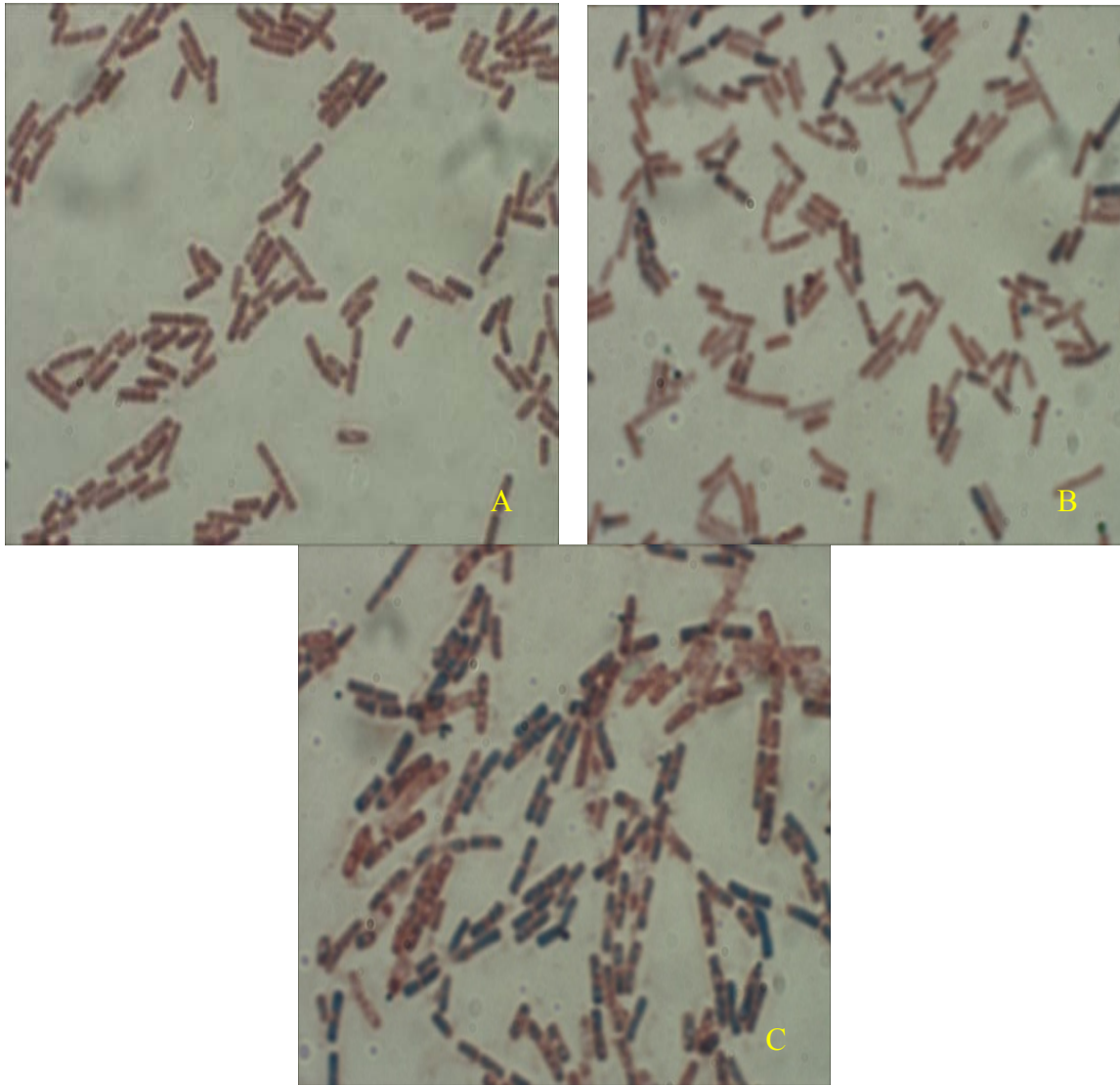


Figure 22. Spore analysis (1000X) of *B. cereus* ATCC 14579 grown in TSB supplemented with varying concentrations of cranberry PACs: (A) no PAC addition, (B) 20µg/ml PAC, and (C) 160µg/ml PAC . Cells were photographed after 24h of incubation at 30°C.

V. DISCUSSION

Microbial contamination responsible for billions of dollars of losses in the food processing industry. Many hospital acquired infections, and in the worst case scenarios death, are a result of environmental contamination. To combat this problem, attachment and subsequent biofilm prevention is often the target of research and therapeutic strategies. One of the least sensitive, most adherent bacteria is *B. cereus*, the spores of which can withstand many commonly used food preservation practices including pasteurization. Upon germination of the spores, the response of the vegetative cells to the stresses imposed on them in the final product, will determine their capacity to grow, causing spoilage and/or food-borne infections (de Vries *et al.*, 2005).

Natural products to treat these problems, when available, are often the remedy chosen. The phytochemicals most often utilized with urinary health are those found in cranberries. Although it was initially believed that the high acidity of cranberry was responsible for anti-adhesion activity, Johnson-White *et al.* (2006) proved otherwise. They found that even after neutralization of the acidity, dialysis against water to remove sugars, benzoic acid, and other small molecules from the cranberry concentrate, reduction in nonspecific binding of *E. coli* and *S. aureus* to borosilicate glass microscope slides was observed. A-type proanthocyanidins were shown to actually reduce adhesion. These A-linked polymers displayed anti-adhesion activity *in vitro* at 60 µg/ml, whereas those proanthocyanidins from grapes exhibited slight activity at 1200 µg/ml, while other B-types were not active (Howell, 2005). In this work, as a logical extension, these A-type compounds were analyzed for potential uses outside of the body.

The original hypothesis was that A-type cranberry PACs would limit the primary attachment of *B. cereus* to an abiotic surface, since the fractions found within cranberry juice have a proven record of preventing the attachment of various microorganisms in vivo. Despite this fact, the above research shows that cranberry PACs actually promote attachment of *B. cereus*, while simultaneously making the cells more sedentary. Thus the enhancement of attachment may be only partially linked to biofilm formation. This marked increase in attachment could be attributed to the fact that the *B. cereus* cells are forming endospores in higher concentrations when grown in the presence of cranberry PACs as seen in Figure 22. When comparing the concentration levels needed in order to bring about the endospore-forming state, it is evident that at and above the 80 µg/ml PAC concentration is the threshold at which the triggering of this physiological event occurs. Although it seems as if the increase in cranberry PAC concentration leads to an increase in the growth of *B. cereus* (Figure 7), this heightened OD₆₈₆ may be the result of the difference in the levels of sporulation.

Upon viewing the shift in hydrophobicity and loss of motility (Figure 19), investigations were made at the proteomic level. A comparison of *B. cereus* cells grown in the presence and absence of cranberry lead to the discovery of the proteins 30S ribosomal protein S4, and formate acetyltransferase (Figure 3). The formate acetyltransferase protein is 98% homologous to a protein found in *E. coli* known as pyruvate formate lyase (PFL1). An anaerobic culture of a facultative bacterium like *Escherichia coli* is limited by ATP. Under anaerobic conditions, the net yield of ATP per glucose is only 2 if pyruvate is reduced to lactate as in a homolactate-producing *pfl* mutant (Zhu, 2004). This yield can be increased by an additional ATP per glucose if two molecules of pyruvate are converted to one each of acetate and ethanol (Neijssel, 1996). The enzyme complex which starts this transformation of pyruvate to acetate and ethanol is

pyruvate formate lyase, which metabolizes pyruvate to formate and acetyl coenzyme A (Kessler, 1996). Thus, a bacterium that can convert glucose to acetate and ethanol will produce three net ATPs while still maintaining the overall redox balance of the carbon. The PFL mechanism involves an unusual radical cleavage of pyruvate in which two cysteines and one glycine form radicals that are required for catalysis (Marchler-Bauer *et al.*, 2007). The 30S ribosomal protein S4 functions as a rho-dependent antiterminator of rRNA transcription, increasing the synthesis of rRNA under conditions of excess protein, allowing a more rapid return to homeostasis (Torres *et al.*, 2001). To investigate the role these proteins play in primary attachment of *B. cereus*, knock out mutants were generated (Figures 4-6) and subjected to the same physical challenges as the WT cells.

As mentioned earlier, results from this research have led to the notion that the cranberry PACs have caused *B. cereus* to form endospores. This reaction to the PACs, have been shown to effectively increase the surface hydrophobicities of the WT *B. cereus* cells (Figures 13-17). The hydrophobicities of the WT cells were consistently higher than those of the mutants probably due to the fact that the cranberry PACs triggered the stress response in the cells causing them to sporulate. On average, the hydrophobicities of the $\Delta 30S$ mutants were the second highest. The lack of the 30S ribosomal protein S4 possibly translated into the loss of efficiency of *B. cereus*' stress response, thus less cells sporulate in the presence of cranberry PACs, ultimately leading to a decrease in overall hydrophobicity. Lastly, the $\Delta forA$ mutants displayed the lowest hydrophobicities in the presence and absence of cranberry PACs. The removal of the formate acetyltransferase enzyme may have led to the inability of *B. cereus* to effectively initiate substrate level phosphorylation, due to the inability to completely utilize pyruvate, which may be a trigger for the cells to undergo sporulation. A similar trigger has been observed by de Vries *et*

al. (2005), who through the use of chemically defined media, found that lactic acid initiated dipicolinic acid production, a known sporulation trigger, much sooner in addition to the release of the spore from the mother cell more rapidly (de Vries *et al.*, 2004).

The observations made in this research on the role surface hydrophobicity plays in the attachment of the *B. cereus* to an abiotic surface was also supported by Faille *et al.* (2002) who witnessed that, regardless of the surface (stainless steel, PVC, glass, Teflon), the cells with the greatest hydrophobicity showed the greatest amount of attachment, which in their case was *B. cereus*. These results parallel those discovered in this research as seen on Figure 21. There was a drastic increase of the attachment of *B. cereus* to the surface of the 24-well tissue culture plate which coincided with an increase in PAC concentration. This phenomenon could be explained by the fact that the *B. cereus* cells sporulate in response to environmental stresses placed upon them by the cranberry PACs, making them more hydrophobic and likely to adhere to this particular abiotic surface.

Not only were *B. cereus* cells in the Faille study the most hydrophobic, they were also the most resistant to CIP procedure which entailed a 5min rinse with softened water, 10 min cleaning with an alkaline detergent at 50°C, followed by a rinse with softened water. Their results fall into line with the above research where it was observed that the *B. cereus* cells grown in the higher concentrations of cranberry PACs were the most resistant to heat killing, and an increase in pH (Figures 8-12). The decrease in sensitivity of the *B. cereus* WT cells to physical and chemical stresses is inversely proportional to the concentration of cranberry PACs. The observed data is contrary to what was seen by the work done by Liu *et al.* (2005). They observed, by atomic force microscopy, cranberry juice altering the hydrophobicity of the P-

fimbriae of *E. coli* making them more hydrophilic thus making them less likely to bind to a surface.

Figures 8 through 12 show that the mutagenized cells became more sensitive to heat and pH treatments, while also regaining swarming and swimming motilities. This again could be attributed to the fact that as the *B. cereus* cells lose the ability to either evoke a stress response ($\Delta 30S$) or undergo efficient substrate level phosphorylation ($\Delta forA$) they are less likely to form endospores, thus become more sensitive to challenging environments. As witnessed by de Vries *et al.* (2004), as *B. cereus* transitioned from using glucose to undergoing homolactic fermentation, the cells entered stationary phase, lost their motility, and became arranged into aggregates. By knocking out the formate acetyltransferase gene, the *B. cereus* cells are less likely to initiate this homolactic fermentation thus retaining their swimming and swarming motilities even amongst cranberry PACs, as seen in Figures 18 and 20. In general, the $\Delta forA$ mutant seems to deviate from the WT state more so than the $\Delta 30S$ mutant with regards to motility, pH resistance, and heat tolerance.

When speculating upon the reason for the observed sporulation in the presence of PACs, one can trace back to the defense mechanism of the plant containing the phytochemical and how this particular microbe may combat it. As mentioned before, the deposition of proanthocyanidins in the endothelial layer of the seed coat in many species would be an example of a protective barrier, which functions by binding metals through complexation involving their o-diphenol groups (Scalbert, 1991). If these PACs are limiting the nutrients available to *B. cereus* as their concentration increases, this could explain why endospores are formed so rapidly and at such high concentrations.

Future research areas with regards to this project could be the examination of how the cranberry PACs alter the quorum sensing protein expression of *B. cereus* cells. Another area that could be investigated could be how mixed microbial communities respond to the exposure to high concentrations of PACs.

V. REFERENCES

- Adamson, G.E., S.A. Lazarus, A.E. Mitchell, R.L. Prior, G. Cao, P.H. Jacobs, B.G. Kremers, J.F. Hammerstone, R.B. Rucker, K.A. Ritter, and H.H. Schmitz.** 1999. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agr. Food Chem.* **47**:4184–4188.
- Bavington, C., and C. Page.** 2005. Stopping Bacterial Adhesion: A Novel Approach to Treating Infections. *Respiration* **72**:335-344.
- Burger, O., E. Weiss, N. Sharon, M. Tabak, I. Neeman, and I. Ofek.** 2002. Inhibition of *Helicobacter pylori* adhesion to human gastric mucus by a high-molecular-weight constituent of cranberry juice. *Crit. Rev. Food Sci. Nutr.* **42**:279–284.
- Chin, K.J., T. Lukow, and R. Conrad.** 1999. Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. *Appl. Environ. Microbiol.* **65**:2341–2349.
- deVries, Y.P., R.D. Atmadja, L.M. Hornstra, W.M. deVos, and T. Abee.** 2005. Influence of glutamate on growth, sporulation, and spore properties of *Bacillus cereus* ATCC 14579 in defined medium. *Appl. Environ. Microbiol.* **71**:3248–3254.
- Dixon, R.A., D.Y. Xie, and S.B. Sharma.** 2005. Proanthocyanidins--a final frontier in flavonoid research? *New Phytol.* **165**:9-28.
- Faille, C., C. Jullien, F. Fontaine, M. N. Bellon-Fontaine, C. Slomianny, and T. Benezech.** 2002. Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: role of surface hydrophobicity. *Can. J. Microbiol.* **48**:728-738.
- Furukawa, S.T., N. Narisawa, T. Watanabe, T. Kawarai, K. Myozen, S. Okazaki, H. Ogihara, and M. Yamasaki.** 2005. Formation of the spore clumps during heat treatment increases the heat resistance of bacterial spores. *International Journal of Food Microbiology* **102**:107-111.
- Grosskopf, R., P.H. Janssen, and W. Liesack.** 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.* **64**:960–969.
- Howell, A.B., N. Vorsa, A. DerMarderosian, and L.Y. Foo.** 1998. Inhibition of adherence of P-fimbriated *Escherichia coli* to uroepithelial-cell surfaces by proanthocyanidin extracts of cranberries. *New England J. Med.* **339**:1085-1086.
- Howell, A.B., J.D. Reed, C.G. Krueger, R. Winterbottom, D.G. Cunningham, and M. Leahy.** 2005. A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. *Phytochemistry* **66**:2281-2291.

- Husmark, U., and U. Rønner.** 1992. The influence of hydrophobic, electrostatic and morphologic properties on the adhesion of *Bacillus* spores. *Biofouling* **5**:335-344.
- Johnson-White, B., L. Buquo, M. Zeinali, and F.S. Ligler.** 2006. Prevention of Nonspecific Bacterial Adhesion In Immunoassays by Use of Cranberry Juice. *Anal. Chem.* **78**:853-857.
- Klavenes, A., T. Stalheim, O. Sjøvold, K. Josefsen, and P.E. Granum.** 2002. Attachment of *Bacillus cereus* spores with and without appendages to stainless steel surfaces. *Trans IchemE* **80**:312-318.
- Kondo, K., M. Kurihawa, K. Fukuhara, T. Tanaka, T. Suzuki, N. Miyata, and M. Toyoda.** 2000. Conversion of procyanidin B-type (catechin dimer) to A-type: evidence for abstraction of C-2 hydrogen in catechin during radical oxidation. *Tetrahedron Letters* **41**:485-488.
- Lindsay, D., M.C. Oosthuizen, V.S. Brözel, and A. von Holy.** 2002. Adaptation of a neutrophilic dairy-associated *Bacillus cereus* isolate to alkaline pH. *Journal of Applied Microbiology* **92**:81-89.
- Liu, Y., M.A. Black, L. Caron, and T.A. Camesano.** 2005. Role of cranberry juice on molecular-scale surface characteristics and adhesion behavior of *Escherichia coli*. *Biotechnology and Bioengineering* **93**:297-305.
- Marchler-Bauer, A., J.B. Anderson, M.K. Derbyshire, C. DeWeese-Scott, N.R. Gonzales, M. Gwadz, L. Hao, S. He, D.I. Hurwitz, J.D. Jackson, Z. Ke, D. Krylov, C.J. Lanczycki, C.A. Liebert, C. Liu, F. Lu, S. Lu, G.H. Marchler, M. Mullokandov, J.S. Song, N. Thanki, R.A. Yamashita, J.J. Yin, D. Zhang, and S.H. Bryant.** 2007. CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Research* **35**:D237-D240.
- Meer, R.R., J. Baker, F.W. Bodyfelt, and M.W. Griffiths.** 1991. Psychrotrophic *Bacillus* spp. in Fluid Milk Products: A Review. *Journal of Food Protection* **54**:969-979.
- Murphy, S.C.** 2005. Shelf-Life of Fluid Milk Products: Microbial Spoilage – Evaluation of Shelf-Life. <http://www.foodscience.cornell.edu/mqip/indexmqip.html#Dairy%20Science%20Factsheets>:
- Neijssel, O. M., M. J. T. de Mattos, and D. W. Tempest.** 1996. Growth yield and energy distribution, p. 1683-1692. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
- Neto, C., C.G. Krueger, T.L. Lamoureaux, M. Kondo, A.J. Vaisberg, R.A.R Hurta, S. Curtis, M.D. Matchett, H. Yeung, M.I. Sweeney, and J.D. Reed.** 2006. MALDI-TOF MS characterization of proanthocyanidins from cranberry fruit (*Vaccinium macrocarpon*) that inhibit tumor cell growth and matrix metalloproteinase expression in vitro. *Journal of the Science of Food and Agriculture* **86**:18-25.

- Oosthuizen, M., B. Steyn, J. Theron, P. Cosette, D. Lindsay, A. von Holy, and V. Brözel.** 2002. Proteomic Analysis Reveals Differential Protein Expression by *Bacillus cereus* during Biofilm Formation. *Appl. Environ. Microbiol.* **68**:2770-2780.
- Parkar, S.G., S.H. Flint, and J.D. Brooks.** 2003. Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *J. Appl. Microbiol.* **96**:110-116.
- Scalbert, A.** 1991. Antimicrobial properties of tannins. *Phytochemistry* **30**:3875–3883.
- Sobota, A.E.** 1984. Inhibition of bacterial adherence by cranberry juice: potential use for the treatment of urinary tract infections. *J. Urol.* **131**:1013-1016.
- Stalheim, T., and P.E. Granum.** 2001. Characterization of spore appendages from *Bacillus cereus* strains. *Journal of Applied Microbiology* **91**:839-845.
- Steinberg, D., M. Feldman, I. Ofek, and E.I. Weiss.** 2004. Effect of a high-molecular-weight component of cranberry on constituents of dental biofilm. *J. Antimicrob. Chemother.* **54**:86–89.
- Tanaka, T., K. Kondou, and I. Kouno.** 2000. Oxidation and epimerization of epigallocatechin in banana fruits. *Phytochemistry* **53**:311-316.
- Torres, M., C. Condon, J.M. Balada, C. Squires, and C.L. Squires.** 2001. Ribosomal protein S4 is a transcription factor with properties remarkably similar to NusA, a protein involved in both non-ribosomal and ribosomal RNA antitermination. *EMBO J.* **20**:3811–3820.
- Weiss, E.I., R. Lev-Dor, Y. Kashamn, J. Goldhar, N. Sharon, and I. Ofek.** 1998. Inhibiting interspecies coaggregation of plaque bacteria with a cranberry juice constituent. *J. Am. Dent. Assoc.* **129**:1719-23.
- Zeibell, K., S. Aguila, V. Yan Shi, A. Chan, H. Yang, and J.H. Miller.** 2007. Mutagenesis and repair in *Bacillus anthracis*: the effect of mutators. *J. Bacteriol.* **189**:2331-8.
- Zhu, J., and K. Shimizu.** 2004. The effect of *pfl* gene knockout on the metabolism for optically pure D-lactate production by *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **64**:367-375.