Casein Induced Changes in Surface Tension Modifies the Mode of Surface Translocation in *Bacillus cereus*

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ABSTRACT

Bacillus cereus is a motile Gram-positive, rod-shaped spore-forming bacterium that dwells in soil. When grown on solidified nutrient-rich media, these bacteria exhibit swarming activity, a type of high-energy motility facilitated by the hyperproduction of flagella. However, on bovine milk agar *B. cereus* exhibits a distinctive, nonflagellar mode of motility known as sliding, which is characterized by elaborate dendritic outgrowths from the central colony mass. Data from our laboratory demonstrated that sliding in *B. cereus* was not suppressed by the addition of 1% glucose to milk agar and is therefore not a result of starvation. This suggests that one or more components of milk stimulate sliding motility in *B. cereus*. Previous data in our laboratory showed that the major milk components calcium, potassium, lactose, vitamin D, and lactoferrin do not induce sliding motility in *B. cereus*. Therefore, *B. cereus* 14579 was grown on tryptone agar supplemented with casein in concentrations equivalent to that in whole milk. Under these conditions, casein successfully induced sliding in *B. cereus* 14579. Casein is a hydrophobic protein, so we hypothesized that the bacteria are responding to a change in the surface tension of the agar. To test this hypothesis, *B. cereus* 14579 was grown on tryptone agar supplemented with the hydrophobic non-ionic detergent NP-40, which also induced sliding in *B. cereus.* We tested whether the addition of casein or NP-40 altered the surface tension of tryptone agar using a simple assay in which we measured the time it took for drops of water and mineral oil to run down a 5 cm distance on agar plates oriented at a 45º angle. Results indicate that these agents do alter the surface tension of the agar, and suggest that *B.cereus* modifies its mode of surface translocation in response to the physical change in its environment. Using transposon mutagenesis methods, we have isolated sixteen strains of *B. cereus* 14579 that do not exhibit sliding when grown on agar supplemented with milk or NP-40. DNA from the mutant strains has been isolated and cloned in an effort to identify the genes involved in the regulation of sliding.

BACKGROUND

Bacillus cereus is a gram-positive rod shaped bacterium that dwells in soil. This spore-forming bacterium is a facultative anaerobe, meaning that it will perform aerobic respiration if oxygen is present and is also capable of fermentation in the absence of oxygen. The organism is also an opportunistic pathogen in humans and is often a contaminant of unpasteurized dairy products (Bottone, 2010).When grown on solidified nutrient rich media these bacteria exhibit apparent swarming activity, a type of high-energy motility employed by *B. cereus* due to the hyperproduction of flagella. Swarming cells typically move on surfaces in a coordinated fashion forming expanding colonies with smooth echinulate edges, and are affected by the regulator PlcR. According to Gohar et al. (2002), PlcR positively regulates flagellar production in *B. cereus* 14579. On the other hand, when the bacteria are introduced into a nutrient-deprived environment, they fail to produce flagella in an effort to conserve energy while searching for nutrients (Hsueh et al., 2007).Sliding has also been observed in *B. subtilis*, induced by potassium ions as well as surfactin, a lipopeptide, resulting in widespread dendritic growth (Kinsinger et al., 2003). On milk agar, *B. cereus* exhibits a distinctive sliding behavior, producing elaborate dendritic outgrowths from the central mass of the colony. Recent data in our laboratory showed that the addition of 1% glucose to milk agar does not suppress the sliding behavior (Avery et al., 2012), contradicting the idea that sliding is the result of a starvation effect. This would suggest that one or more components of milk stimulate sliding motility in *B. cereus*. Consequently, *B. cereus* was grown on tryptone agar supplemented with the major components of milk such as calcium, potassium, lactose, vitamin A, and vitamin D with concentrations equivalent to whole milk as well as relative dilutions. Based on these experiments, it was concluded that these major components of milk do no induce sliding behavior in *B. cereus* (Zeigler at al., 2012). Therefore, tryptone agar will be supplemented with additional components of bovine milk to determine if one or more components of bovine milk induce sliding motility in *B. cereus*. Using transposon mutagenesis methods (Larsen et al., 2002) we have isolated sixteen strains of *B. cereus* 14579 that do not exhibit sliding when grown on agar supplemented with milk , casein, or NP-40. DNA from the mutant strains has been isolated and cloned in an effort to identify the genes involved in the regulation of sliding. PCR reactions have been conducted on both the isolated *B. cereus* mutant DNA samples and the products of transposon mutagenesis in order to amplify mutant DNA sequence. Once these genes have been identified, we will be able to identify their gene products involved in signal transduction or regulation of gene expression.

METHODS

Bacillus cereus strain 14579 was obtained from American Type Culture Collection. Tryptone agar plates were made with 1.5% agar and 3% tryptone. Casein and NP-40 assays used tryptone agar plates supplemented with varying concentrations of the major milk protein casein and the non-ionic detergent NP-40.100% concentrations of casein are those found in whole milk. All inoculations of plates were performed using a spot technique with sterile toothpicks. It was made sure that plates did not have obvious standing condensation on the surface when inoculation occurred so as to prevent swimming or swarming motility and promote sliding. Bacteria were taken from fresh cultures of *B. cereus* on blood agar plates. Plates were examined after 24 and 48 hours of incubation at 30ºC. Plates were incubated in a 30ºC humidified incubator 1 hour prior to inoculation to prevent drying. *B. cereus* cells were observed using an Olympus CX31 microscope. Images of magnified cells were obtained using a Moticam 2500 camera. Surface tension experiments were conducted by altering the hydrophobicity of tryptone agar by adding casein or the nonionic detergent NP-40. Two straight lines 5cm apart were drawn on bottom of plates and designated as start and stop points. A ramp was built at an angle of 45º, confirmed by a goniometer. Plates were set on the ramp and 20 µl of mineral oil was pipetted onto the start line. Simultaneously, a timer was started and the time for the mineral oil droplet to reach the stop line was recorded. The width of the mineral oil streak down the agar was also measured with a ruler and recorded. This procedure was repeated using 20 µl of water. In an effort to identify the genes involved in *B. cereus* sliding, wild-type *B. cereus* 14579 cells that slide on milk, NP-40, and casein supplemented agar were isolated in our lab. These cells were used to conduct transposon mediated mutagenesis (Larsen et al., 2002) from which we identified sixteen mutants that did not slide on milk,NP-40, or casein. DNA was extracted from these mutants according to the methods of Simmon et al. (2004). Polymerase Chain Reaction (PCR) was then performed on the products of transposon mediated mutagenesis using primers tpnRL17–1 and tpnRL13–2 designed for the end of the transposon in order to amplify the sequence flanking the gene disrupted by the transposon (Larsen et al., 2002). Isolated DNA from the sixteen *B. cereus* mutants was also used to conduct a semi random, two-step (ST)- PCR reaction (Stolyar et al., 2007) using PCR primers (tpnRL 17-1,CEKG 1,2,3 and tpnRL 17-2, CEKG 4) and two successive PCR reactions in an effort to amplify mutant DNA sequence.

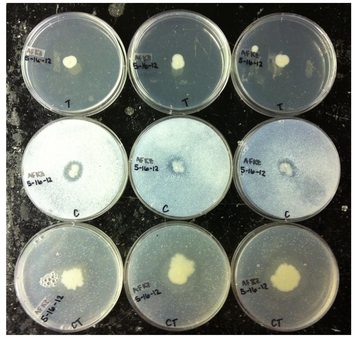


Fig. 1. Casein induces sliding on agar plates. Sliding motility was apparent on 1.5% agar plates containing 3% casein (center row) and 3% casein with 3% tryptone (bottom row) after 48 hr incubation. No sliding is observed on 3% tryptone agar (top row).

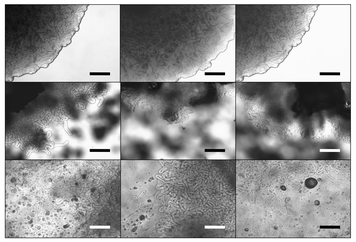


Fig. 2. Microscopic images of casein-induced sliding on agar plates. Dendritic projections were observed at the edge of growth on 1.5% agar plates containing 3% casein (center row) and 3% casein with 3% tryptone (bottom row) after 48 hr incubation. Smooth, echinulate edges were observed on 3% tryptone agar (top row). Bar = 500 µm.

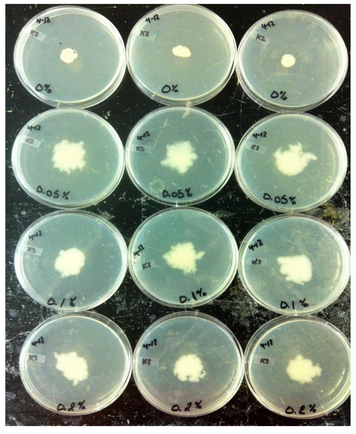


Fig 3. Tryptone agar supplemented with NP-40 induces sliding motility in *B. cereus*. NP-40 concentrations of 0.05%, 0.1%, and 0.2% induced consistent sliding relative to one another. Sliding motility observed on agar with NP-40 compared to the control after 48 hr incubation.

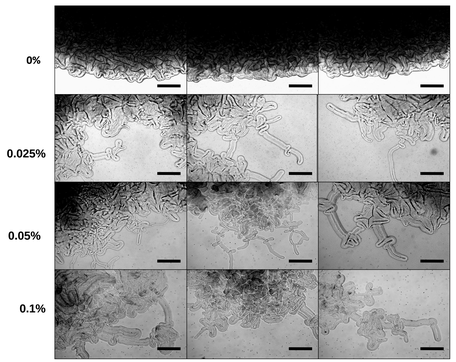
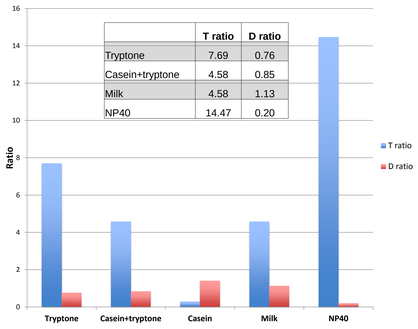


Fig. 4. Microscopic images demonstrate sliding motility induced by NP-40. Sliding motility consistent with increased percentages of NP-40 after 48 hr incubation. Dendritic projections are discernible from echinulate edges characteristic of swarming in the tryptone control. Bar = 500 µm.



	T ratio	D ratio
Tryptone	7.69	0.76
Casein+tryptone	4.58	0.85
Milk	4.58	1.13
NP40	14.47	0.20

Fig. 5. Surface tension assay of NP-40 and Casein. T ratio represents the time elapsed for oil / time elapsed for water. D ratio represents the width of oil / width of water. Oil ran slower on NP40, milk, and casein + tryptone whereas water ran faster. Oil spread out less on NP40 and casein + tryptone. Casein and NP40 alter the surface tension and/or the hydrophobicity of the agar

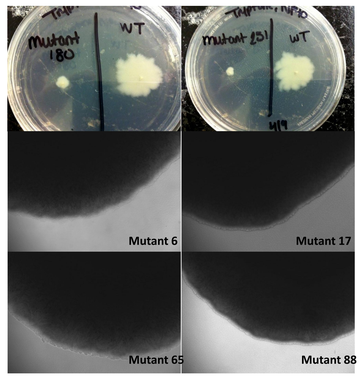


Fig. 6. *B. cereus* mutants that do not slide on agar supplemented with NP-40. A concentration of 0.1% NP-40 did not induce sliding compared to wild-type *B. cereus* after 48 hr incubation.

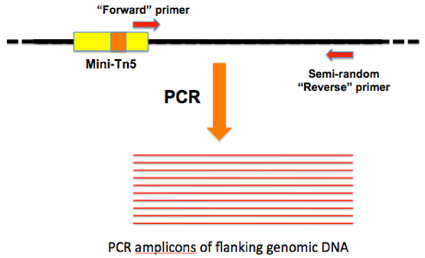


Fig. 7. Schematic of transposon mutagenesis procedure. pRL27-mini Tn5 was electroporated into *B. cereus* cells . Mutants were isolated on NP-40 + tryptone agar supplemented with Kanamycin.

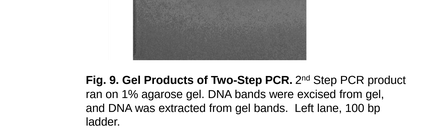


Fig. 8. Schematic of Two Step PCR procedure. Isolated DNA from the sixteen *B. cereus* mutants was also used to conduct a semi random, two-step (ST)- PCR reaction using PCR primers (tpnRL 17-1,CEKG 1,2,3 and tpnRL 17-2, CEKG 4) and two successive PCR reactions.

First Step PCR Primers	Second Step PCR Primers
tpnRL17-1 5-AACAAGCCAGGGATGTAACG-3	tpnRL17-2 5-AGCCCTTAGAGCCTCTCAAAGCAA-3
CEKG2A 5-GGCCACGCGTCGACTAGTACN10AGAG-3	CEKG4 5-GGCCACGCGTCGACTAGTAC-3
CEKG2B 5-GGCCACGCGTCGACTAGTACN10ACGCC-3	
CEKG2C 5-GGCCACGCGTCGACTAGTACN10GATAT-3	

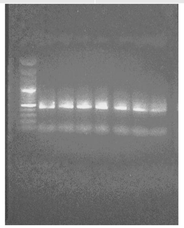


Fig. 9. Gel Products of Two-Step PCR. 2nd Step PCR product ran on 1% agarose gel. DNA bands were excised from gel, and DNA was extracted from gel bands. Left lane, 100 bp ladder.

CONCLUSION

Casein was the only major component of bovine milk that induced sliding motility in *B. cereus*. Casein successfully induced sliding motility in *B. cereus* at concentrations of 10-100%, characterized by dendritic outgrowths from the central mass of the colony. Swarming motility was observed at 0% casein. As a result of these findings we hypothesized that surface tension or perhaps the hydrophobicity of the substrate is playing a role in the sliding motility observed. To test this we supplemented tryptone agar with the nonionic detergent Tergitol NP-40 and observed sliding cells. This confirms that the physical nature of the casein is causing this sliding response.

We are continuing to conduct experiments to test the surface tension of the agar supplemented with tryptone, casein, tryptone and casein, NP40, and milk. We concluded that casein and NP-40 change the surface tension of the media, although possibly not in the same way. Our results indicate that these agents do alter the surface tension of the agar and that the bacteria sense this change and modify their mode of surface translocation

Recently we have isolated mutants that were defective in sliding on milk and exhibited only swarming activity. These mutants also do not exhibit sliding on agar supplemented with NP40 or casein. These mutants have been isolated and cloned in an effort to identify genes that are involved in regulation of sliding motility through the process of transposon mediated mutagenesis. Once these genes have been identified, we will be able to identify their gene products involved in signal transduction or regulation of gene expression.

Literature Cited

Avery, L.E., McCorry, J., Zeigler, K.E., Bruns, V., and Aaronson, L.R. 2012. Milk-induced sliding of *Bacillus cereus* Is not due to nutrient starvation. *Abstracts of the 113th General Meeting of the American Society for Microbiology.*

Bottone, E.J. (2010) *Bacillus cereus*: A volatile human pathogen. *Clin. Microbiol. Rev*. 23: 382-398.

Gohar, M., Okstad, O.A., Gilois, N., Sanchis, V., Kolsto, A.-B., and Lereclus, D. (2002) Two dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon. *Proteomics* 2: 784-791.

Hsueh, Y.-H., Somers, E. B., Lereclus, D., Ghelardi, E. and Wong, A. C. L. (2007) Biosurfactant production and surface translocation are regulated by PlcR in *Bacillus cereus* ATCC 14579 under low-nutrient conditions. *Appl. Env. Microbiol.* 73: 7225-7231.

Kinsinger, R. F., Shirk, M. C., and Fall, R. (2003) Rapid surface motility in *Bacillus subtilis* isdependent on extracellular surfactin and potassium ion. *J. Bacteriol.* 185: 5627-5631.

Larsen, R.A., Wilson, M.M., Guss, A.M., Metcalf, W.W. (2002) Genetic analysis of pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch Microbiol.* 178 :193–201.

Stolyar, S., He, Q., Joachimiak, M.P., He, Z., Yang, Z.K., Borglin, S.E., Joyner, D.C., Huang, K., Alm, E., Hazen, T.C., Zhou, J., Wall, J.D., Arkin, A.P.,Stahl, D.A. (2007) Response of *Desulfovibrio vulgaris* to Alkaline Stress. *J. Bacteriol.* 189: 8944-8952.

Zeigler, K.E.. Avery, L.E. and Aaronson, L.R. 2012. Analysis of the effects of the major components of bovine milk on induction of sliding motility in *Bacillus cereus. Abstracts of the 113th General Meeting of the American Society for Microbiology.*

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