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Substratum attachment location and biofilm formation by *Bacillus cereus* strains isolated from different sources: Effect on total biomass production and sporulation in different growth conditions

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ABSTRACT

Bacillus cereus, an endospore forming human pathogen associated with foodborne diseases, can form biofilms and attach to surfaces of processing equipment in the food industry. It is a consistent source of contamination and/or cross contamination of processed food products. The objective of this study was to understand substratum attachment location and biofilm formation behavior of *B. cereus* strains under different growth conditions. A total of 60 strains isolated from food, human, or farm and a number of reference strains were used in this study. Substratum attachment locations of these strains in 96-well microtiter plates were highly diversified among these strains. Strains isolated from food showed higher preference to attach at the air-liquid interface during early stage of biofilm formation. To the best of our knowledge, this is the first report showing the level of substratum attachment location and biofilm formation of *B. cereus* strains isolated from different sources. Substratum properties did not affect biofilm formation location when a number of selected strains were grown on stainless steel coupon, indicating that biofilm formation location might be independent of the type of substratum. Substratum attachment location and biofilm formation related phenotypes such as total biomass production, number of sessile cells, and sporulation were closely correlated. Substratum attachment location and sporulation behavior were strongly affected during biofilm formation under nutrient stress condition. The number of spores was significantly increased in biofilms grown under nutrient stress condition even though total biomass formation was lower. Our results on substratum attachment location and related biofilm formation behavior are substantially important for food industries where different surfaces are prone to *B. cereus* attachment, particularly for setting up and implementing clean in place (CIP) protocols.

1. Introduction

B. cereus, a Gram-positive, rod shaped, and spore forming bacterium, is widespread in nature. It is commonly isolated from soil and plants. It can also grow in insects and mammals intestine (Stenfors Arnesen, Fagerlund & Granum, 2008; Vilain, Luo, Hildreth, & Brözel, 2006). *B. cereus* can cause gastrointestinal diseases (diarrhea) and emetic syndromes (nausea and vomiting) that can be fatal, particularly in older and debilitated people (Agata, Ohta, Mori, & Isobe, 1995; Fratamico, Bhunia, & Smith, 2005). It is also responsible for opportunistic human pathogenesis such as nosocomial bacteremia, sepsis, pneumonia, and meningitis (Arnaout et al., 1999; Hoffmaster et al., 2006; Richard, Van der Auwera, Snoeck, Daneau, & Meunier, 1988). *B. cereus* is associated with food spoilage, a major threat to the food industry (Pirttijarvi, Andersson, & Salkinoja-Salonen, 2000). For example, it is responsible for the spoilage of pasteurized milk and milk products due to its spore forming property that can survive during pasteurization and refrigeration (Meer, Baker, Bodyfelt, & Griffiths, 1991). *B. cereus* can attach onto different food processing equipment surface and grow biofilm (Ryu & Beuchat, 2005).

A biofilm is a surface attached microbial community enclosed with extracellular polymeric substance (EPS) matrix (Donlan, 2002). Bacterial biofilm formation is a very complex and dynamic process that generally occurs in five consecutive stages - (i) initial reversible attachment (planktonic cells attach to a surface); (ii)

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irreversible attachment (EPS and microcolony formation); (iii) early development of biofilm structure; (iv) biofilm structure maturation, and (v) dispersion of biofilm (Stoodley, Sauer, Davies, & Costerton, 2002). The initial reversible and irreversible stages of biofilm formation are very important to establish a biofilm structure onto a substratum because it is in these two stages that the free floating planktonic cells commit to attach a location of a substratum surface to initiate the biofilm cycle (Karunakaran & Biggs, 2011; Parkar, Flint, Palmer, & Brooks, 2001). Bacterial biofilm growth cycle provides the opportunity for them to adapt and survive in diverse environmental conditions by protecting bacterial cells from predators and the immune system, supporting their division and providing a physical protection against mechanical stimulus (Hall-Stoodley, Costerton, & Stoodley, 2004). Therefore, cells embedded in the biofilm EPS matrix (sessile cells) shows intrinsic resistance to bactericides and antibiotics compared to their free-floating planktonic counterparts (Halstead et al., 2015). Biofilms are very difficult to remove from the attached surfaces due to irreversible and viscous properties of the EPS (Garrett, Bhakoo, & Zhang, 2008). Affluent nutrients and other organic components in food processing environment favors *B. cereus* biofilm formation. *B. cereus* biofilm formation phenotype such as high cell density, spore formation, and resistance to disinfectants making this organism a persistent source of human pathogen and product contamination.

Adhesion of bacteria to a substratum depends on the thermodynamic properties of bacterial surface and the properties of the substratum to be attached (van Loosdrecht, Lyklema, Norde, & Zehnder, 1989). *B. cereus* has been reported to be able to attached onto various surfaces such as stainless steel (Ryu & Beuchat, 2005), glass wool (Oosthuizen et al., 2002), and industrial surfaces such as rubber, conveyer belt, floor, and gasket (Chmielewski & Frank, 2003). It has been reported that *B. cereus* not only can preferentially form biofilms at air and liquid interface, but also can form weak biofilms on submerged substratum under static conditions (Auger, Krin, Aymerich, & Gohar, 2006; Oosthuizen, Steyn, Lindsay, Brözel, & Von Holy, 2001; Wijman, De Leeuw, Moezelaar, Zwietering, & Abee, 2007). However, to the best of our knowledge, the relation of *B. cereus* biofilms attached on different substratum locations to other biofilm-related phenotypes such as total biomass formation and sporulation has not been reported yet.

Therefore, the purpose of this study was to determine *B. cereus* biofilm substratum attachment location and their effect on the properties of total biomass produced, sessile cell numbers, and sporulation during different growth conditions. A total of 60 *B. cereus* strains were chosen to compare their biofilm formation on substratum location and their effect on the number of viable sessile cells density and sporulation. In this study, we focused on the substratum location for biofilm formation with a multifaceted approach to investigate total biomass formation and sporulation behavior. Biofilm formation was investigated by using polystyrene microtiter plates under different growth conditions. Total biomass formation and the number of spores were measured by crystal violet assay and standard plate count, respectively.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A total of 60 *B. cereus* strains isolated from different sources including food, human, and farm as well as a number of reference strains were used in this study to determine their biofilm formation on substratum locations and other biofilm-related phenotypes (Table S1). *B. cereus* strains were confirmed by growing in selective media (Mannitol Egg Yolk Polymyxin agar) and ribosomal RNA sequencing. Characteristics of some of these strains such as toxin production have been described elsewhere (Forghani et al., 2015; Kim et al., 2012). Strains were streaked onto Brain Heart Infusion (BHI) (Becton Dickinson) agar (Daejung) plates using stocks stored in BHI broth containing 15% glycerol (Daejung) at - 80 °C. These streaked plates were incubated at 30 °C for 24 h. A single colony was picked and inoculated into 10 ml BHI broth followed by incubation at 30 °C for 18 h (stationary phase) without shaking.

2.2. Surface attachment of biofilm formation

In vitro static biofilm was formed in 96-well polystyrene microtiter plates (flat bottom) (Spl LifeSciences) using previously published protocols of Merritt, Kadouri, and O'Toole (2005) with slight modifications. Briefly, strains were grown in 96-well microtiter plates filled with 200 μl BHI broth per well and inoculated with 1% (vol/vol) of overnight culture. Plates were wrapped with parafilm to avoid evaporation and incubated at 30 °C for 24, 48, or 72 h. After appropriate incubation, parafilm was removed and biofilm formation location was observed and recorded (Wijman et al., 2007). Biofilm formation locations were also investigated and photographed after staining with crystal violet right before the addition of 70% ethanol (Gao, Foulston, Chai, Wang, & Losick, 2015).

2.3. Biomass quantification and cell enumeration

Strains were grown in 96-well microtiter plates filled with 200 μl BHI broth and inoculated with 1% of overnight culture followed by incubation at 30 °C for 24, 48, or 72 h. Total biomass produced was determine using protocols described previously (Castelijn, van der Veen, Zwietering, Moezelaar, & Abee, 2012). Briefly, after appropriate incubation time, the medium was removed carefully from each well by using a pipette to discard unattached cells. Wells were washed three times with 225 μl of sterile phosphate-buffered saline (PBS) (pH 7.4, Gibco). Biofilms were stained with 200 μl of 0.1% CV in water (Difco) for 30 min. CV that failed to bind to biofilms was discarded and wells were washed again three times with PBS. Subsequently, 200 μl of 70% ethanol was added to each well and incubated at room temperature for 30 min to release the biofilm bound by CV. The absorbance of the resulting CV solution was measured at wavelength of 595 nm on a microplate reader (Molecular Devices).

The number of viable sessile cells were quantified with standard plate counting following published protocols (Castelijn et al., 2012). Briefly, after appropriate incubation, the media was discarded from the 96-well microtiter plates and wells were washed three times with PBS. The attached biofilms were swabbed to detach cells. Swabs and suspended biofilms were subsequently transferred to a tube filled with sterile glass beads (<106 μm) (Sigma, St. Louis, USA) and vortexed with the maximum speed for 1 min to separate cells. The solution was transferred to a new 96-well microtiter plate for appropriate serial dilutions in PBS. A hundred microliter of diluted sample was spread onto BHI agar plates. Agar plates were incubated at 30 °C for 24 h and the number of colonies was enumerated.

The number of spores in biofilms was measured using published protocols (Hayrapetyan, Muller, Tempelaars, Abee, & Groot, 2015). Briefly, cells were swabbed to separate them from the attached biofilms. Swabs and suspended biofilms were vortexed to separate into individual cells as described earlier and 150 μl of suspended biofilm was heated at 80 °C for 10 min in a water bath to inactivate vegetative cells. The number of spores was determined by standard plate counting as described earlier for biofilm and planktonic cell counts.

The number of planktonic cells were measured from the supernatants of 96-well microtiter plates filled with 200 μl BHI broth and inoculated with 1% of overnight culture followed by incubation