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Impact of the Isolation Source on the Biofilm Formation Characteristics of *Bacillus cereus*

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The human pathogen and food spoiler *Bacillus cereus* can form biofilms that act as a persistent source of contamination, which is of public health concern. This study aimed to understand how the source of isolation might affect the behavior of biofilm formation. Biofilm formation abilities of 56 strains of *B. cereus* isolated from different environments, including human food poisoning, farm, and food, were determined. Crystal violet assay results revealed significant ($p < 0.05$) differences in biofilm formation abilities among the strains isolated from different sources only at an early stage of incubation. However, strain origin showed no impact on later stage of biofilm formation. Next, correlation of the group of isolates on the basis of their biofilm-forming abilities with the number of sessile cells, sporulation, and extracellular polymeric substance (EPS) formation was determined. The number of sessile cells and spores in biofilms was greatly influenced by the groups of isolates that formed dense, moderate, and weak biofilms. The contribution of extracellular DNA and/or proteins to EPS formation was also positively correlated with biofilm formation abilities. Our results that the source of isolation had significant impact on biofilm formation might provide important information to develop strategies to control *B. cereus* biofilm formation.

Keywords: *Bacillus cereus*, isolation source, biofilm formation, sporulation, extracellular polymeric substances

Introduction

Biofilms are surface-attached microbial communities enclosed with extracellular polymeric substances (EPS) [1]. Biofilm formation is a very complex and dynamic process [2]. EPS of biofilms are mainly composed of polysaccharides, proteins, and extracellular DNA (eDNA) [3, 4], although the compositions differ largely from species to species [5]. Cells embedded in the biofilm EPS are more resistant to disinfectants and antibiotics compared with free floating planktonic cells [6]. Microorganisms have numerous mechanisms to resist disinfectants. Modification of genetic expression in sessile cells and structure-associated resistance are probably the main mechanisms involved in *Pseudomonas aeruginosa* biofilms [7].

Bacillus cereus is a spore-forming, gram-positive, and facultative anaerobic bacterium. This organism is found ubiquitously in a wide range of environments, including soil, raw and processed foods, water, and feces [8]. It is well known that *B. cereus* is a potential food-poisoning agent that can cause gastrointestinal diseases (diarrhea) and emetic syndromes (vomiting) due to a number of enterotoxins and emetic toxin (cereulide) [9]. According to the US Centers for Disease Control and Prevention, *B. cereus* caused 235 foodborne related outbreaks and 2,050 illness cases with an estimated cost of $ 0.35 million from 1998 to 2008 [10, 11]. In Europe, a total of 291 outbreaks, of which 101 cases were hospitalized, were attributed to *B. cereus* in 2014 [12]. In addition, *B. cereus* is responsible for infections, skin lesions, and meningitis [13]. *B. cereus* causes food spoilage, and thereby can attribute to huge economic losses. For example, it is responsible for off-flavor and structural changes in milk by producing extracellular spoilage enzymes [14].

The environment in food production is affluent in nutrients and other organic components that favor *B. cereus* biofilm formation on different surfaces, including stainless steel, rubber, conveyer belt, floor, and gasket [15]. In addition, *B. cereus* can colonize on plant roots and forms biofilms [16]. So far, the biofilm formation abilities of *B. cereus* strains isolated from different food production-related niches have not been reported yet. However, several comparative studies have been performed on *B. cereus* strains on their biofilm formation abilities [17–19]. Biofilms formed by *B. cereus* have high cell density, with the presence of spores, thus making them resistant to heat, pasteurization, and canning [20]. In our previous work, we have shown that the *B. cereus* isolation source can effect the substratum attachment location [21]. These characteristics make this organism a persistent source of human pathogenicity and product contamination. Therefore, *B. cereus* biofilm formation and its related phenotypes are of serious public health concerns. Recently, a number of studies have revealed diverse biofilm formation abilities of *Listeria monocytogenes* [22, 23], *Salmonella* [24, 25], *Staphylococcus aureus* [26], *Pseudomonas aeruginosa* [26], and *Escherichia coli* [26] depending on their source of isolation. Diversities such as total biomass production, the number of sessile cells, spore formation, and the composition of EPS are very important for understanding the biofilm formation mechanism, virulence, and risk measurement, particularly for the discovery of potential control strategies for ubiquitous *B. cereus*. In this study, our focus was to determine whether biofilm formation properties such as total biomass formation, the number of cultivable sessile cells, and sporulation abilities of *B. cereus* strains isolated from various environmental sources might be different.

Materials and Methods

Bacterial Strains and Culture Conditions

A total of 56 *B. cereus* strains (Table 1) isolated from different environmental sources, including human (*i.e.*, feces and vomit) (n = 13), farm (soil, manure, and water) (n = 18), and food (n = 25), were used in this study to investigate their biofilm formation abilities. These strains were identified by using *B. cereus* selective media and ribosomal RNA sequencing. The substratum attachment location of these strains have been described [21]. The strains were streaked onto brain heart infusion (BHI) (Becton Dickinson, USA) agar plates from stocks stored in BHI broth supplemented with 15% glycerol (v/v) at −80°C. The plates were incubated at 30°C for 24 h. BHI broth was inoculated with a single colony and incubated at 30°C for 18 h without agitation.

Biofilm Formation

Static biofilms were grown according to a protocol described previously [27] with slight modification. Briefly, *B. cereus* was grown in 96-well microtiter plates (Spl LifeSciences, Korea) filled with 200 µl of BHI medium per well and was inoculated with 1% (v/v) of overnight culture. A group of wells filled with 200 µl of BHI medium and without bacterial inoculation was used as a control.

Biofilm Quantification

Crystal violet (CV) assay was used to measure biofilm formation as described previously [25]. Briefly, after incubation, the medium was removed and the wells were washed three times with phosphate-buffered saline (PBS) (Life Technologies, USA). Attached biofilms were stained with 0.1% (w/v) CV for 30 min. CV that did not bind to biofilms was discarded. The wells were washed again three times with PBS. Subsequently, 70% (v/v) ethanol was added to each well and incubated for 30 min to release biofilm bound by CV. The solubilized CV was quantified by measuring the absorbance value at a wavelength of 595 nm (Molecular Devices, UK).

The number of sessile cells was determined by cell enumeration as described previously [17] with some modifications. Briefly, attached biofilms were scraped by swabbing wells to detach cells. Suspended biofilms were vortexed with glass beads (<106 µm) (Sigma, USA). The suspension was transferred to a new 96-well plate for appropriate serial dilutions in PBS. One hundred microliters of the sample was used to spread onto BHI agar plates followed by incubation at 30°C for 24 h. Subsequently, the number of colonies was enumerated.

The number of spores in the biofilms was measured using published methods [17] with slight modification. Briefly, biofilm was grown in BHI broth in 96-well plates at 30°C for 24, 48, or 72 h. The biofilms were washed and scraped as described previously. The suspended biofilm was heated at 80°C for 10 min to inactivate vegetative cells. The number of spores was determined by plate counting. Thirty microliters of the sample was transferred to a 96-well plate for appropriate dilutions. Subsequently, 100 µl of the sample was transferred to a BHI agar plate, followed by incubation at 30°C for 24 h. The number of colonies was enumerated.

Planktonic cells were measured from the supernatants of 96-well plates used for biofilm formation, by plate counting as described previously [28].

DNase I and Proteinase K Treatments

Biofilms were grown in 96-well plate in BHI broth as described in the biofilm formation section. After washing with PBS, attached biofilms were treated with Proteinase K (100 µg/ml), DNase I (5 U/ml), or PBS (control) as described previously [29]. The treated biofilms were quantified by CV assay and cell enumeration as described previously [25].

Statistical Analysis

The averages of separate sets of data from the CV assay, cell

Table 1. *B. cereus* strains used in this study.

Group	Strain[a]	Isolation source	Obtained from
Human (n = 13)	ATCC 4810/72	Human vomit	ATCC
	JIHE 6	Feces	JIHE
	JIHE 15	Feces	JIHE
	JIHE 21	Feces	JIHE
	JIHE 22	Feces	JIHE
	JIHE 23	Feces	JIHE
	JIHE 24	Feces	JIHE
	JIHE 36	Feces	JIHE
	JIHE 54	Feces	JIHE
	JIHE 60	Feces	JIHE
	JIHE 61	Feces	JIHE
	JIHE 78	Feces	JIHE
	JIHE 80	Feces	JIHE
Food (n = 25)	F4433/73	Meat loaf	ATCC
	KCTC 1526	Food	KCTC
	KFDA 219	Food	KFDA
	KFDA 220	Food	KFDA
	KFDA 221	Food	KFDA
	KFDA 222	Food	KFDA
	KFDA 223	Food	KFDA
	KFDA 224	Food	KFDA
	KFDA 225	Food	KFDA
	KFDA 226	Food	KFDA
	KFDA 250	Food	KFDA
	KUGH 85	Rice cake	KUGH
	KUGH 27	Grain	KUGH
	GIHE 61-1	Soybean paste	GIHE
	GIHE 61-3	Soybean paste	GIHE
	GIHE 61-5	Soybean paste	GIHE
	GIHE 62-4	Soybean paste	GIHE
	GIHE 86-1	Soybean paste	GIHE
	GIHE 86-3	Soybean paste	GIHE
	GIHE 86-4	Soybean paste	GIHE
	GIHE 86-5	Soybean paste	GIHE
	GIHE 86-7	Soybean paste	GIHE
	GIHE 86-8	Soybean paste	GIHE
	GIHE 86-9	Soybean paste	GIHE
	GIHE 86-10	Soybean paste	GIHE
Farm (n = 18)	ATCC 14579	Air from a cow shed	ATCC
	ATCC 21366	Soil	ATCC
	ATCC 25621	Animal feces	ATCC
	ATCC 12480	Sheep rumen	ATCC
	ATCC 21768	Chicken manure	ATCC
	KCTC 1094	Japanese soil	KCTC
	Bc-K 4	Farm	KFDA
	Bc-K 6	Farm	KFDA
	Bc-K 7	Farm	KFDA
	Bc-K 8	Farm	KFDA
	Bc-K 10	Farm	KFDA
	Bc-K 11	Farm	KFDA
	Bc-K 14	Farm	KFDA
	Bc-K 16	Farm	KFDA
	Bc-K 17	Farm	KFDA
	Bc-K 18	Farm	KFDA
	Bc-K 19	Farm	KFDA
	Bc-K 20	Farm	KFDA

[a]All strains were obtained from our previous study [21]. ATCC, American Type Culture Collection; JIHE, Jeonbuk Institute of Health and Environment; KCTC, Korean Collection for Type Culture; KFDA, Korea Food & Drug Administration; KUGH, Gyeonggi-do Research Institute of Health and Environment; GIHE, Gangwon Institute of Health and Environment.

counts, and spore counts from each combination of growth conditions through three independent experiments were analyzed. For the effect of DNase I or Proteinase K treatment, average data from two experiments were composited and analyzed. To compare various effects on biofilm formation, one-way analysis of variance (ANOVA) with Tukey's post hoc and multiple linear regression analysis were performed using SPSS ver. 22 (SPSS Statistics; IBM, USA). Statistical significance was considered when the p value was less than 0.05.

Results

Total Biomass Formation of *B. cereus* Isolates

As an initial screening for whether the biofilm formation abilities of *B. cereus* might be dependent on the source of isolation, strains isolated from human, farm, and food samples were tested. Results of CV assay for the three groups of isolates are shown in Fig. 1. Based on OD values, human and farm isolates showed significantly ($p < 0.05$) higher biofilm formation abilities than food isolates after 24 and 48 h of incubation (Figs. 1A and 1B). Meanwhile, human and farm isolates showed similar biofilm formation abilities in these periods (24 and 48 h of incubation). Interestingly, after 72 h of incubation, the origin of isolation failed to show any effect on the ability of biofilm formation (Fig. 1C).

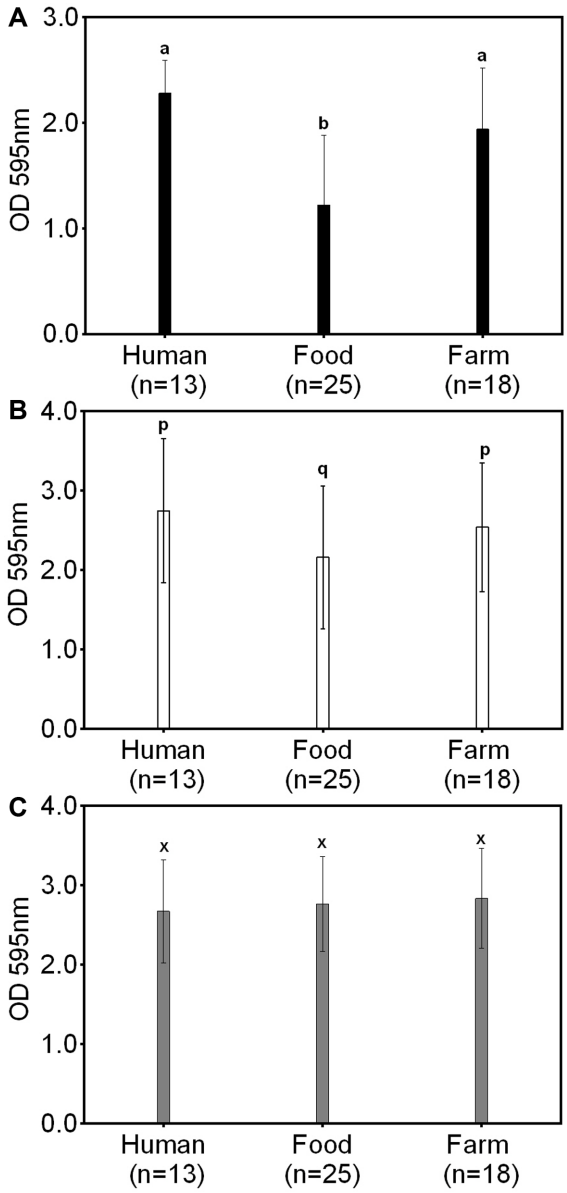

Fig. 1. Overview of biofilm formation by *B. cereus* strains grouped by origin of isolation.

B. cereus strains were grown in 96-well microtiter plates in BHI broth at 30°C. Total biomass production was measured by crystal violet assay at 24 h (**A**), 48 h (**B**), or 72 h (**C**) after incubation. These data represent the averages of three independent biological experiments for each group of isolates. Error bars indicate standard deviation. Groups with different alphabets are significantly different within each parameter and condition (one-way ANOVA and Tukey's post hoc test; $p < 0.05$).

Density of Biofilm Formation by *B. cereus* Isolates

To determine the density of biofilms formed by the three groups of isolates, biofilm formation abilities were categorized as dense (OD ≥ 1.5), moderate (0.3 ≤ OD ≤ 1.5), and weak (OD ≤ 0.3) according to previously published descriptions [19]. The density of biofilm formation was substantially different among the three groups of isolates at a given incubation time as well as among different incubation periods (Fig. 2 and Table 2). Based on the absorbance values at 595 nm, human isolates had the highest biofilm formation capacities. Out of 13 isolates from this group, 9 isolates (69%) had dense biofilm formation capacity and 4 isolates (31%) had moderate biofilm formation capacity. There was no weak biofilm-forming isolate in this group after 24 h of incubation. For 18 farm isolates, 11 (61%) were dense biofilm formers, whereas 7 (39%) were moderate biofilm formers. For the 25 food isolates, 5 (20%) formed dense biofilms, 16 (64%) formed moderate biofilms, and 4 (16%) failed to form any biofilms. After 48 h of incubation, all 13 human isolates were capable of forming dense biofilms. Of the food and farm groups of isolates, 19/25 (76%) and 15/18 (83%) were dense biofilm formers, respectively, whereas 6 (24%) and 3 (17%) were moderate biofilm formers, respectively. Human isolates remained to have the highest biofilm formation capacities. All human isolates (100%) formed dense biofilms after 72 h of incubation. However, the food and farm isolates failed to show any noticeable difference in biofilm formation after 72 h of incubation compared with 48 h of incubation.

According to the CV assay, the biofilm formation abilities among the group of strains isolated from different sources were different only at the initial stage of incubation. Therefore, the tested isolates were regrouped on the basis of their biofilm formation abilities such as dense, moderate, and weak biofilm-forming isolates, for further experiments.

Number of Sessile Cells Yielded by *B. cereus* Isolates

The number of cultivable sessile cells was measured to determine whether it had any correlation with their biofilm formation abilities. The numbers of cells yielded by the three groups of isolates that formed dense, moderate, and weak biofilms are shown in Fig. 2. In line with the results obtained from the CV assay for biofilms after 24 h of incubation, a higher number of cells was found for dense biofilm-forming isolates (8.61 ± 0.44 logCFU/well) than that for moderate (7.71 ± 0.40 logCFU/well) and weak (7.46 ± 0.40 logCFU/well) biofilm-forming isolates. Meanwhile, a significantly ($p < 0.05$) higher number of cells was obtained for moderate than weak biofilm-forming isolates under this growth condition. After 48 and 72 h of incubation, a higher number of cells was found for dense biofilm-forming isolates than that for moderate biofilm-forming isolates.

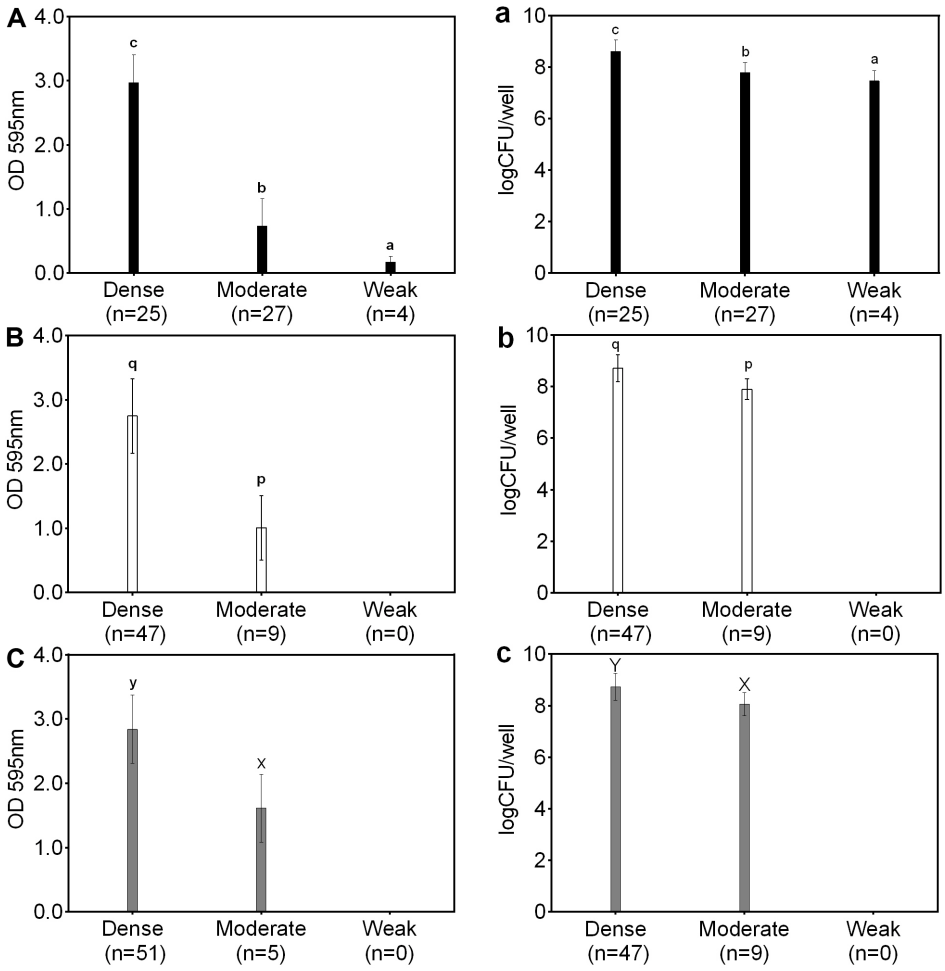

Fig. 2. Total biomass production and cells yielded by *B. cereus* strains grouped by their biofilm formation abilities.

B. cereus strains were grown in 96-well microtiter plates in BHI broth at 30°C. Total biomass production was measured by crystal violet assay at 24 h (**A**), 48 h (**B**), or 72 h (**C**) after incubation. The number of cultivable sessile cells was enumerated by standard plate counting after swabbing biofilms into PBS and subsequently growing in BHI agar plates at 30°C for 24 h (**a**), 48 h (**b**), or 72 h (**c**). These data represent the averages of three independent biological experiments for each group of isolates. Error bars indicate standard deviation. Groups with different alphabets are significantly different within each parameter and condition (one-way ANOVA, Tukey's post hoc test, and Student's *t*-test; $p < 0.05$).

Table 2. Density of biofilms formed by 56 strains of *B. cereus* grouped by isolation origin.

Isolation group	24 h			48 h			72 h		
	Dense	Moderate	Weak	Dense	Moderate	Weak	Dense	Moderate	Weak
Human (n = 13)	9 (69%)	4 (31%)	-	13 (100%)	-	-	13 (100%)	-	-
Food (n = 25)	5 (20%)	16 (64%)	4 (16%)	19 (76%)	6 (24%)	-	22 (88%)	3 (12%)	-
Farm (n = 18)	11 (61%)	7 (39%)	-	15 (83%)	3 (17%)	-	16 (89%)	2 (18%)	-
Total (n = 56)	25 (45%)	27 (48%)	4 (8%)	47 (84%)	9 (16%)	-	51 (91%)	5 (9%)	-

Biofilm formation abilities were categorized as Dense, $OD_{595nm} \geq 1.5$; Moderate, $0.3 \leq OD_{595nm} \leq 1.5$; or Weak, $OD_{595nm} \leq 0.3$. Data are OD values obtained from crystal violet assay.

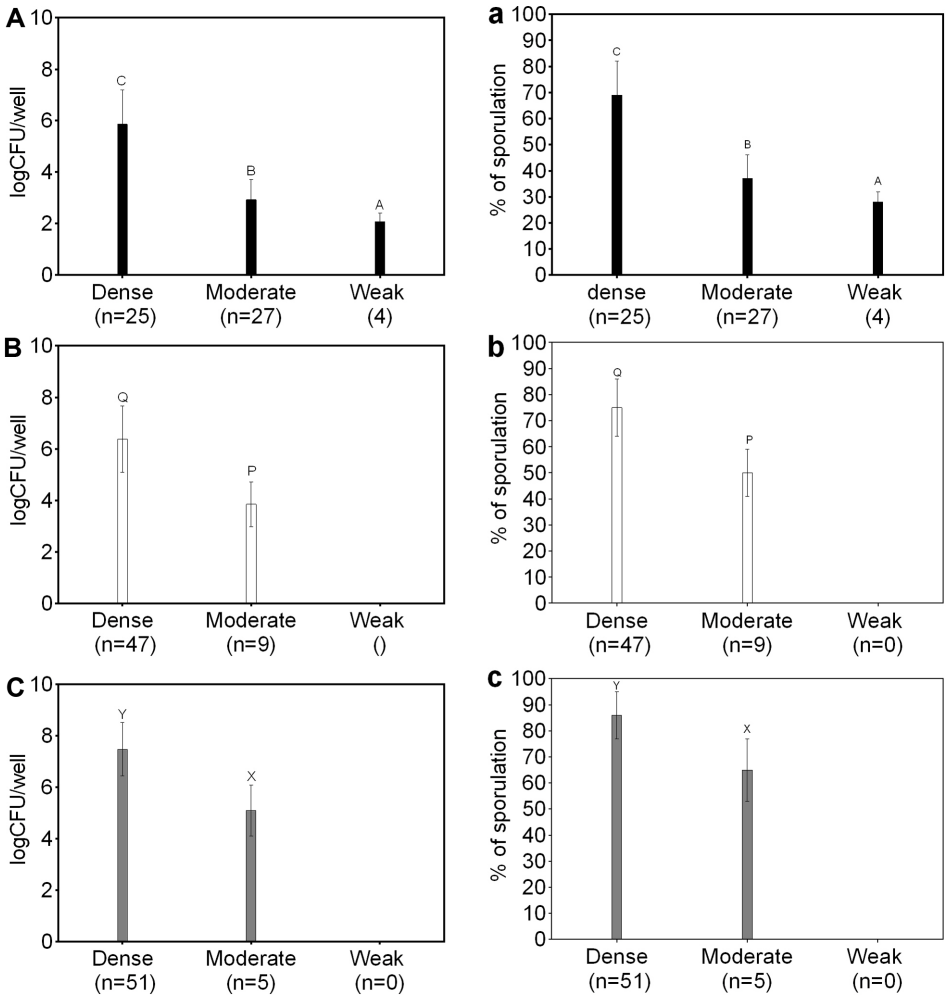

Fig. 3. Sporulation behavior in biofilms of *B. cereus* strains grouped by their biofilm formation abilities.

Biofilms were grown in 96-well polystyrene microtiter plates in BHI broth at 30°C and incubated for 24, 48, or 72 h. The number of spores was quantified by heating suspended biofilms at 80°C for 10 min and subsequently plating onto BHI agar to determine the CFU. The average number of spores yielded after incubation for 24 h (**A**), 48 h (**B**), or 72 h (**C**). Percentage of spore formation ability compared with the number of vegetative cells in biofilms for 24 h (**a**), 48 h (**b**), or 72 h (**c**). Data represent the average of three independent biological experiments for each group of isolates. Error bars represent standard deviation. Groups with different alphabets are significantly different within each incubation time (one-way ANOVA, Tukey's post hoc test, and Student's *t*-test; $p < 0.05$).

Notably, after 48 and 72 h of incubation, there was no weak biofilm-forming isolates among all of the tested strains.

Sporulation Behavior of Biofilms Formed by *B. cereus* Isolates

The sporulation behavior of *B. cereus* biofilms was investigated to determine whether the degree of spore formation was different among the groups of isolates forming dense, moderate, and weak biofilms. The results are shown in Fig. 3. There was significant ($p < 0.05$) difference in the number of spores between dense biofilm-forming isolates (5.86 ± 1.34) and moderate biofilm-forming isolates (2.93 ± 0.78) (Fig. 3A) after 24 h of incubation. However, weak biofilm-forming isolates (2.08 ± 0.32) showed a lower ($p < 0.05$) number of spores compared with the other two groups. The spore formation efficacy (the number of spores formed compared with the number of sessile cells) was assessed and the results were found to be largely varied among the three groups of isolates (dense, moderate, and weak) after 24 h (Fig. 3). The sporulation abilities of the three groups (dense, moderate, and weak) were found to be 69%, 37%, and 28%, respectively (Fig. 3a). Similar

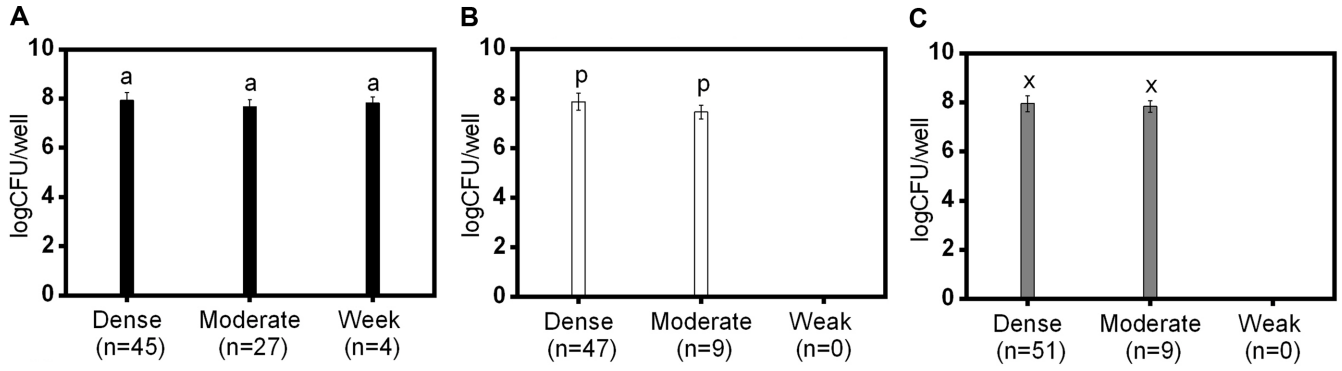

Fig. 4. Planktonic cells yielded by *B. cereus* strains grouped by their biofilm formation abilities.

B. cereus strains were grown in BHI broth in 96-well polystyrene plates at 30°C for 24 h (**A**), 48 h (**B**), and 72h (**C**). Planktonic cells were measured from supernatants of each well by standard plate counting. Each bar represents data of the average number of planktonic cells in logCFU/well for each group of isolates obtained from three independent biological experiments. Error bars represent standard deviation. To compare the yielded planktonic cells among different isolations, one-way analysis of variance with Tukey's post hoc test ($p < 0.05$) was performed.

variations in the number of spores yielded and sporulation ability were found among the three groups of isolates after 48 and 72 h of incubation (Figs. 3B and 3C). Sporulation ability was found to be 75% and 50% for dense biofilm-forming isolates and moderate biofilm-forming isolates, respectively, after 48 h. Moreover, after 72 h of incubation, sporulation ability was increased to 86% and 65% for dense biofilm-forming strains and moderate biofilm-forming isolates, respectively (Figs. 3b and 3c).

Planktonic Cells Yielded by *B. cereus* Isolates

The number of planktonic cells yielded by *B. cereus* isolates was determined to understand whether there was any difference among the three groups of isolates forming dense, moderate, and weak biofilms. However, there was no significant ($p > 0.05$) difference in planktonic cell numbers among the three groups of isolates during the entire incubation period (Fig. 4).

Composition of EPS Matrix Formed by *B. cereus* Isolates

To determine the compositions of the EPS matrix, particularly DNA and proteins, in the different biofilm formation abilities of *B. cereus*, biofilms formed in 96-well microtiter plates in BHI at 30°C for 48 h were assessed. Biofilms were treated with either Proteinase K (100 µg/ml) or DNase I (5 U/ml) as described in the Materials and Methods. The results are shown in Fig. 5. The effects of Proteinase K and DNase I treatments varied greatly between groups of dense and moderate biofilm-forming isolates examined. OD_{595nm} values of the two groups were significantly ($p < 0.05$) decreased after DNase I treatment or Proteinase K treatment (Fig. 5A). A higher reduction in OD_{595nm} values of biofilms after Proteinase K treatment compared with DNase I treatment was found for the dense biofilm-forming group of isolates. After treatment with Proteinase K and DNase I, biofilms for the dense biofilm-forming group of isolates were reduced up to 82% and 58%, respectively, and were significantly ($p < 0.05$) decreased after Proteinase K treatment than after DNase I treatment. However, no significant ($p > 0.05$) difference between Proteinase K (56% reduction) and DNase I treatment (63%) in the biofilms formed by moderate group of isolates was observed.

Cultivable cell numbers in the biofilms also varied between the group of isolates after treatment with Proteinase K and DNase I treatment. After treatment with Proteinase K, the number of sessile cells in the biofilms was significantly ($p <0.05$) decreased for the two groups of isolates (Fig. 5B). However, no significant ($p <0.05$) decrease in number of cells was obtained after treatment with DNase I for both dense and moderate groups of isolates. Biofilms of the dense group of isolates showed significant ($p <0.05$) resistance to DNase I than to Proteinase K treatment in the number of sessile cells. Moreover, there was no significant ($p > 0.05$) difference in the number cells between DNase I and Proteinase K treatment for the moderate group of isolates (Fig. 5B).

Discussion

In this study, the biofilm formation abilities of *B. cereus* strains with different ecological origins were determined. Strains originated from human food-poisoning, farm, and food samples were used to determine the relationship

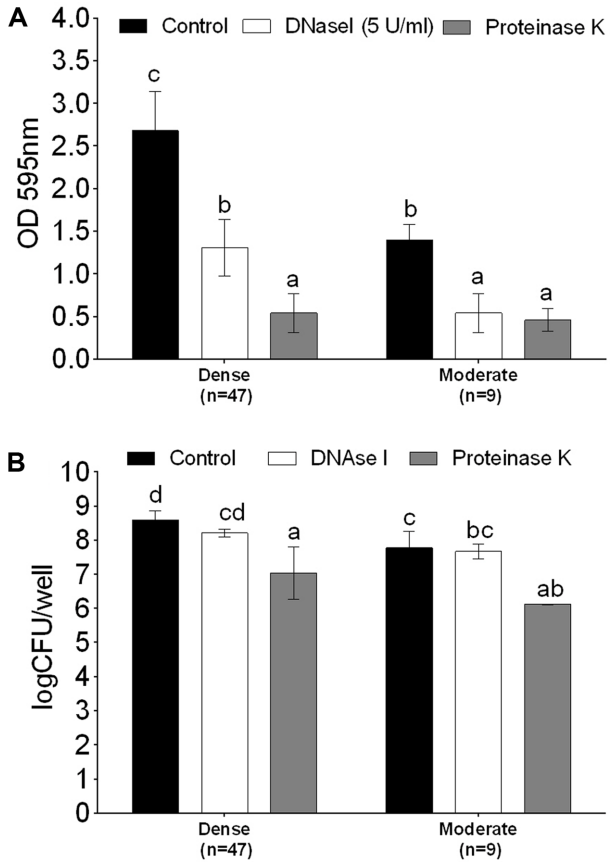

Fig. 5. Effect of DNase I or Proteinase K treatment on biofilms formed by *B. cereus* strains grouped by their biofilm formation abilities.

Biofilms were grown on 96-well plates in BHI broth at 30°C for 48 h. The crystal violet assay (OD values) (**A**) and the number of sessile cells (logCFU/well) (**B**) of Control, DNase I (5 U/ml), and Proteinase K (100 μg/ml) treatment. Data represent the averages of two independent biological experiments. Error bars represent standard deviation. Groups with different alphabets indicate significant difference (one-way ANOVA and Tukey's post hoc test and Student's *t*-test; $p < 0.05$).

between the source of isolation and biofilm formation ability. Results of CV assay revealed that food-poisoning and farm-related isolates had significantly higher biofilm formation abilities compared with food isolates at the early stage of biofilm formation. These results suggest that *B. cereus* biofilm formation could largely be influenced by their source of isolation. Our results showed that *B. cereus* biofilm formation could be influenced by their source of isolation, in agreement with the results of Auger *et al.* [18] showing that *B. cereus* strains from soil or digestive tract infections had significantly higher biofilm formation abilities than strains isolated from other diseases. However, our results were different from the results reported by Kamar *et al.* [31] showing that there was no significant difference in biofilm formation among strains isolated from different sources, such as nonpathogenic, food-poisoning, and clinical-related samples. In a recent review on *B. cereus* biofilms [16], it was indicated that there is no impact of origin on biofilm formation ability of strains isolated from different environmental conditions. In our previous paper, we found that the *B. cereus* surface attachment location can vary according to their source of isolation, and a study on a few number of strains showed that attachment location also correlates to their total biomass and sporulation behavior [21]. Significant difference in biofilm formation abilities depending on sources of origin has been reported for other bacterial species, including *L. monocytogenes* [22, 23], *Salmonella* [25], and *Staphylococcus aureus* [26]. In the present study, CV assay and cell enumeration were combined to assess the biofilm formation behavior of *B. cereus* strains forming dense, moderate, and weak biofilms. Significantly higher number of cultivable sessile cells was found for dense biofilm-forming isolates than that for moderate and weak biofilm-forming isolates at 24 h of biofilm formation, in line with the results obtained from CV assay. Linear correlation between *B. cereus* biofilm formation (CV assay) and the number of cultural cells have been reported previously [17, 32]. In this study, the origin of isolation did not show any significant effect on the level of biofilm formation after incubation for a longer period (72 h). This might be due to differences in the rate of biofilm formation ability among the three groups of isolates based on their source of isolation. However, EPS might have increased, contributing to the higher rate of biofilm formation for these groups of isolates [33]. Extracellular components (*e.g.*, eDNA and protein) might have contributed to the *B. cereus* biofilm. This could be supported by data obtained after rearranging the strains based on their biofilm formation abilities, such as dense, moderate, and weak, and subsequent Proteinase K and DNase I treatments. The presence of eDNA in the biofilm matrix could be supported by the fact that DNase I treatment significantly reduced both the OD values and the number of cells (Fig. 5). In addition, Proteinase K treatment significantly decreased the OD values for both dense and moderate biofilm-forming strains tested. These data suggest that the EPS matrix in *B. cereus* biofilms contains both DNA and proteins. They might play a major role in biofilm formation. These results are in agreement with the findings of previous studies showing that protein and eDNA can contribute to *B. cereus* biofilm matrix formation [34]. Notably, our results showed good

correlation in EPS compositions between the dense and moderate biofilm-forming groups of *B. cereus* isolates (Fig. 5). However, there were no correlations in EPS composition (protein and eDNA contribution) and biofilm formation after treatment with Proteinase K or DNase I for the moderate group of isolates. This could be due to too few components of EPS examined for the biofilm matrix composition experiment. More information about EPS compositions and their contribution to biofilm formation between the dense and moderate biofilm-forming groups of isolates is required. Microscopic observations could provide accurate assessment of the EPS structure in biofilms formed by strains of different biofilm formation abilities [36, 37].

One of the important features of *B. cereus* biofilm formation is the heterogeneity in sporulation behavior [38]. Therefore, we examined the sporulation behavior in biofilm formation of *B. cereus* strains divided into groups based on their biofilm formation abilities in this study. Our results revealed significant differences in the number of spores and sporulation efficiency in the group of isolates with dense, moderate, and weak biofilm-forming abilities. These results are consistent with the results obtained from CV assay and the number of cells in biofilms formed by the three groups of isolates. It has been previously reported that *B. cereus* biofilm formation (the CV assay) is linearly correlated with the number of spores [17, 32].

In conclusion, this study showed that *B. cereus* biofilm formation was influenced by their source of isolation. However, such variability in biofilm formation among different groups of *B. cereus* was only found at the early stage of biofilm formation due to the higher contribution of other EPS components such as eDNA and protein to biofilm formation. However, further study is needed to address this possibility. The examined strains also showed wide diversity in the biofilm formation properties, such as total biomass production, the number of sessile cells, and sporulation behavior. Significant impact of high biofilm formation on EPS compositions was observed for the *B. cereus* groups of isolates based on their biofilm formation abilities. These results indicate that it is important to use more screening to reveal biofilm formation behavior and their related phenotypes among *B. cereus* strains with different environmental origins and biofilm formation abilities. Considering the wide variations in biofilm formation abilities among the tested strains of this study, mutagenesis and/or transcriptome analysis is needed in the future to identify factors involved in biofilm formation and factors associated with phenotypes. Our results on biofilm formation by *B. cereus* strains isolated from different sources provide important information to make niche-specific strategies to control biofilms of *B. cereus*.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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