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Direct-Imaging-Based Quantification of *Bacillus cereus* ATCC 14579 Population Heterogeneity at a Low Incubation Temperature▽

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***Bacillus cereus* ATCC 14579 was cultured in microcolonies on Anopore strips near its minimum growth temperature to directly image and quantify its population heterogeneity at an abusive refrigeration temperature. Eleven percent of the microcolonies failed to grow during low-temperature incubation, and this cold-induced population heterogeneity could be partly attributed to the loss of membrane integrity of individual cells.**

Bacillus cereus is a food poisoning- and food spoilage-causing organism that can be found in a large variety of foods (4, 23). There are two illnesses associated with *B. cereus*, namely, emetic and diarrheal intoxication (17, 24). Most of the strains related to cases or outbreaks of *B. cereus* food-borne poisoning were shown to be unable to grow at 7°C (1, 12). The average temperatures of domestic refrigerators have been investigated in various surveys around the world and often ranged from 5°C to 7°C, but extreme values exceeded 10°C to 12°C (5, 16). Inadequate chilling was indeed reported in various incidents of *B. cereus* food-borne illness (7, 8, 18, 19), pointing to the importance of appropriate refrigeration of foods contaminated with *B. cereus* to control its growth and toxin production in foods (9).

Several studies have demonstrated that microorganisms can show diversity in their population stress response, even in an apparently homogeneous stress environment (6, 11, 21, 22). However, only very limited data describing the heterogeneity in growth performance of individual cells from food-borne pathogens cultured at low temperatures are available (10). Because inadequate chilling of food is one of the factors that contribute to the number of incidents of *B. cereus* food-borne illness, there is a need for better understanding of its growth performance at lowered incubation temperatures. In this study, we used the direct-imaging-based Anopore technology (6, 13–15) to quantitatively describe the population heterogeneity of *B. cereus* ATCC 14579 cells at 12°C. The minimum temperature for the growth of *B. cereus* ATCC 14579 in brain heart infusion (BHI) broth is 7.5°C (personal communication from F. Carlin), but various food-borne-associated *B. cereus* isolates were shown to be unable to grow at 10°C (1). Therefore, in this study, a culturing temperature of 12°C was chosen, to mimic temperature abuse of refrigerated foods. In addition, the membrane integrity of individual cells was assessed using both membrane permeant and impermeant nucleic acid dyes in order to get more insight into cellular characteristics that may contribute to heterogeneity in growth response.

Growth performance in broth at a low incubation temperature. Previous studies have shown that the temperature history of the inoculum culture and its growth conditions have pronounced effects on the growth performance of cells (see, e.g., references 2, 10, 20, and 25). Therefore, an exponentially growing working culture that was already precultured in BHI broth at 12°C, with shaking at 200 rpm, was used in this study to assess the effect of a low incubation temperature rather than the effect of temperature down shock on the growth performance of *B. cereus* ATCC 14579. The cold-adapted, exponentially growing working culture (optical density at 600 nm of 0.4 to 0.5) was inoculated in fresh, precooled (12°C) BHI broth in duplicate and further incubated at 12°C with shaking at 200 rpm. At regular time intervals, appropriately diluted aliquots were plated on BHI agar plates and BHI agar plates supplemented with 2.5% and 5% (wt/vol) sodium chloride to test cell injury. At the initial sampling point ($t = 0$ h), the viable counts on the BHI plates supplemented with 5% salt were lower than the counts on the BHI plates and the BHI plates supplemented with 2.5% salt (Fig. 1a). After this initial time point, the plate counts were similar on the three different plating media. The growth kinetics showed an initial lag period, after which exponential growth resumed at 6 h (Fig. 1a) with a specific growth rate of 0.051 $\log_{10}$ h^{-1} (95% confidence interval, 0.045 to 0.056). To check possible effects of medium conditioning on the observed lag phase, the exponentially growing working culture was also inoculated into culture supernatant, which was prepared by filtering a cold-adapted exponentially growing culture through a membrane filter, and further incubated at 12°C with shaking at 200 rpm. However, the use of culture supernatant did not reduce the observed lag phase (data not shown). Both the lag phase and the lower viable counts on the BHI plates supplemented with 5% salt after inoculation of the inoculum pointed to the susceptibility of the exponentially growing culture at this low incubation temperature.

Quantitative analysis of population heterogeneity on Anopore strips. Cells were cultured in microcolonies on porous Anopore strips to directly image and quantify the heteroge-

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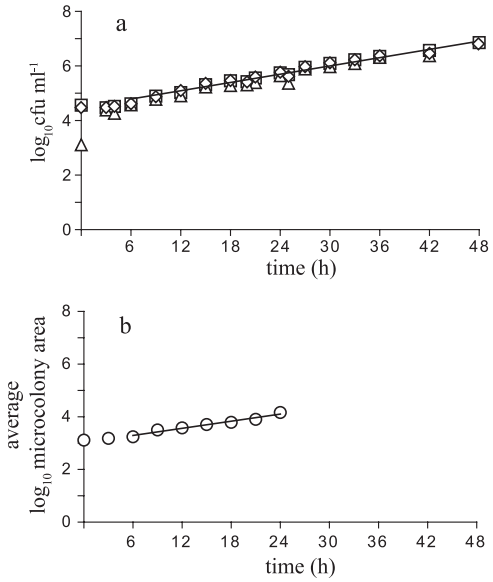

FIG. 1. (a) Growth of *Bacillus cereus* ATCC 14579 in BHI broth at 12°C. Cells were plated on BHI agar plates (◇), BHI agar plates plus 2.5% salt (□), and BHI agar plates plus 5% salt (△). (b) Growth of microcolonies on Anopore strips placed on BHI agar plates at 12°C. The area of each microcolony per imaging time point was measured in pixels and $\log_{10}$ transformed. Data points represent the average microcolony size of the grown population of microcolonies per imaging time point (○). The specific growth rates in broth and on Anopore strips were estimated by linear regression using the time points that represented exponential growth (continuous lines).

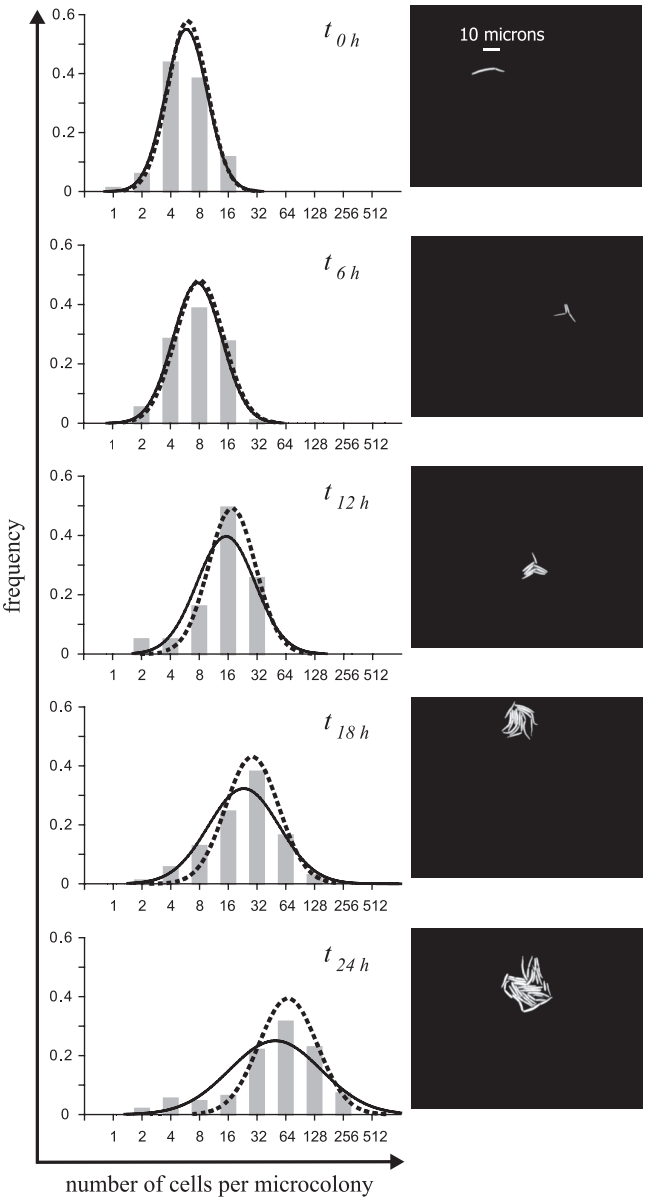

FIG. 2. Examples of images of *Bacillus cereus* ATCC 14579 cultured on Anopore strips which were placed upon BHI agar plates at 12°C. Observed and fitted frequency distributions of the number of cells per microcolony for the imaging time points (t) 0, 6, 12, 18, and 24 h are shown. Histogram shows observed frequencies of number of cells per microcolony. Continuous curves show fitted normal distributions of the $\log_{10}$-transformed microcolony areas, solid lines represent the distribution of all microcolonies, and dashed lines represent the distribution of grown microcolonies only.

neous growth response. Anopore strips (96% ethanol sterilized) were placed on precooled (12°C) BHI agar plates, and the cold-adapted exponentially growing working culture was diluted in precooled (12°C) BHI broth and inoculated in 2-μl aliquots onto the upper surface of each Anopore strip (8 by 36 mm by 60 μm, 0.2-μm-diameter pore size, pore density of up to 50%; Whatman International, Maidstone, United Kingdom). The plates were sealed with Parafilm to prevent dehydration of the agar surface. A thin layer of medium fluid on top of the Anopore strip, drawn up by the capillary action of the pores, allows daughter cells to slide along the planar surface of the Anopore strip to form an expanding one-cell-layer-thick microcolony during growth. The plates containing the Anopore strips were incubated at 12°C. At regular time intervals, an Anopore strip was transferred to a microscope slide (50 by 76 by 1 mm) covered with a 1-mm-thick film of 1% (wt/vol) solidified low-melting-point agarose. The agarose was dissolved in peptone saline solution containing 1.7 μM SYTO-9 dye to stain the microcolonies on the Anopore strip. The term microcolony was used for colonies that consisted of a single cell or a few cells at the start of the incubation, as well as for the expanded microcolonies later on in time after incubation. The Anopore strips were imaged using a Zeiss Axioplan 2 Imaging microscope equipped with an LD Plan-Neofluar 63×/0.75 Corr Ph2 objective lens and a single-band fluorescein isothiocyanate (FITC) filter. A charge-coupled-device (CCD) camera (Quantix; Photometrics, United States) controlled by Imaging-Pro Plus 5.0 software was used to capture images of the microcolonies. The saved images of the microcolonies were used to measure the area of each microcolony in pixels, excluding the intercell area, using ImageJ software (version 1.34s). An average number of 103 microcolony images per imaging time point were analyzed to determine the distribution of microcolony size for each imaging time point, following the procedure described previously (6).

Figure 2 shows examples of images of *B. cereus* ATCC 14579 cells grown on Anopore strips. The distribution at the first imaging time point shows that the sizes of the microcolonies

ranged from 1 cell per microcolony up to 16 cells per microcolony. Phase-contrast microscopic imaging of the cold-adapted inoculum confirmed this size range. The growth kinetics of the microcolonies also showed an initial lag phase before exponential growth resumed at 6 h (Fig. 2). The growth of the microcolonies was monitored up to 24 h, as a second layer of cells within the microcolonies was observed after this imaging time point. The resumption of microbial growth resulted in expansion of microcolony size because the number of cells forming the microcolony increased over time, and the frequency distributions of microcolony size shifted to the right (Fig. 2). The reproducibility of the frequency distribution at the final imaging time point ($t = 24$ h) was confirmed in an independent reproduction of cell culturing on Anopore strips. The data in Fig. 3 show that the variability of microcolony sizes increased after the resumption of growth. To quantitatively assess the observed heterogeneity of microcolony sizes, the frequency distributions per imaging time point were statistically tested for normality using the Anderson-Darling test and the Kolmogorov-Smirnov test as described earlier (6). The continuous curves in Fig. 2 show the fitted normal distributions. The observed frequency distributions were normally distributed until 6 h according to the two statistical tests ($P > 0.05$). After this imaging time point, the normal distribution could not be acceptably fitted according to both tests ($P < 0.05$). The deviation from normality was maximal at the final imaging time point ($t = 24$ h). Both the images and the observed frequency distribution at this time point indicated that some microcolonies, represented by the first three bins of the frequency distribution, failed to continue growing at 12°C. In order to quantitatively describe the population heterogeneity, this nongrown population, 11% of the microcolonies, was separated from the grown population of microcolonies for each imaging time point. This mathematical procedure resulted in both a nongrown population and a grown population of microcolonies per imaging time point. The frequency distribution of the grown population for each imaging time point was tested for normality, and this was confirmed by both the A-D test and the K-S test ($P > 0.05$). The fitted normal distributions of the grown population are also shown in Fig. 2. The discrimination of the two populations per time point resulted in a rather stable variance of microcolony sizes of the grown population over time (Fig. 3). In our previous study, the Anopore technology was used for quantification of the population heterogeneity of *B. cereus* ATCC 14579 grown at 30°C in the absence and presence of osmotic stress (6). Culturing of cells at 30°C resulted in a homogeneous growth response expressed in a stable variance of microcolony sizes over time (Fig. 3) (see reference 6). Exposure to osmotic stress (5% [wt/vol] sodium chloride) at 30°C (6) induced a heterogeneous growth response similar to that observed in the present study at 12°C.

In order to estimate the specific growth rate of cells grown on Anopore strips, the average microcolony sizes of the grown population of microcolonies from imaging time point 6 h until 24 h were used for linear regression (Fig. 1b). The estimated specific growth rate, 0.045 $\log_{10}$ h^{-1} (95% confidence interval, 0.035 to 0.055), was not significantly different from the estimated specific growth rate of cells cultured in broth ($P > 0.05$). This correspondence of the growth kinetics of cells grown on Anopore strips and in broth was previously demonstrated

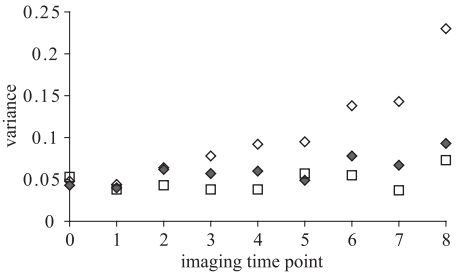

FIG. 3. The variances of the observed frequency distributions of the $\log_{10}$-transformed microcolony areas versus the imaging time points at 12°C and 30°C. The intervals of imaging were 3 h at 12°C and 15 min at 30°C (see reference 6). ◇, Distribution at 12°C of all microcolonies; ◆, distribution at 12°C of grown microcolonies only; □, distribution of microcolonies at 30°C.

for *B. cereus* ATCC 14579 at 30°C and during exposure to additional osmotic stress conditions (2.5% and 5% [wt/vol] sodium chloride) at 30°C (6). The Anopore growth system's characteristics might have contributed to this remarkable similarity of the initial growth kinetics of cells grown on Anopore strips and in broth compared to those of other surface growth systems (3).

Membrane integrity of individual cells grown in microcolonies. An advantage of the Anopore technology is that growth kinetics, as well as characteristics of individual cells, can be studied directly using different fluorescent dyes. Therefore, extra Anopore strips were used at each imaging time point to examine the membrane integrity of the individual cells within the microcolonies by double staining these microcolonies using 1.7 μM SYTO-9 dye and 10 μM propidium iodide (PI) dye. When SYTO-9 dye and PI are used in combination, intact cells are labeled green and cells with damaged membranes are labeled red. The double-stained microcolonies were imaged using a dual-band FITC/TxRed filter, and a CCD camera (Axiocam MRc; Zeiss, the Netherlands) controlled by Axiovision 4.2 software was used to capture images of the double-stained microcolonies. The results of double staining of the exponentially growing inoculum at the initial imaging time point ($t = 0$ h) demonstrated that about 3 to 5% of the microcolonies consisted of cells that had lost their membrane integrity during the low-temperature preincubation. This percentage is higher than the 0.5 to 1% found for exponentially growing cultures of *B. cereus* ATCC 14579 cultured at 30°C (data not shown).

Representative images of the nongrown population and the grown population of microcolonies at the final imaging time point ($t = 24$ h) are shown in Fig. 4a and b, respectively. The nongrown population showed green-labeled microcolonies, red-labeled microcolonies, and microcolonies of which the individual cells were either green or red labeled (Fig. 4a). The observation of green-labeled, nongrown microcolonies suggested that the loss of culturability of the nongrown microcolonies was not only due to loss of membrane integrity. This is in agreement with the finding that the percentage of nongrown microcolonies (11%) was higher than the percentage of red-labeled microcolonies at the initial imaging time point.

Most of the grown microcolonies were either fully green labeled or mainly green labeled (Fig. 4b). Interestingly, though, some grown microcolonies consisted of a large number of red-labeled cells, indicating that these cells' membranes were com-

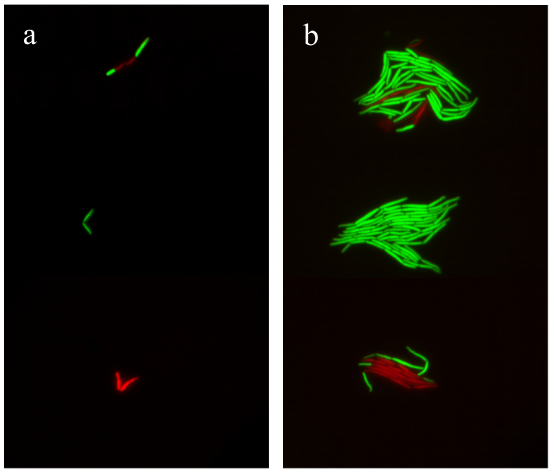

FIG. 4. Labeling of *Bacillus cereus* ATCC 14579 microcolonies with SYTO-9 and propidium iodide after 24 h of culturing on Anopore strips which were placed upon BHI agar plates at 12°C. At this imaging time point, two populations of microcolonies were observed, a nongrown population (a) and a grown population (b).

promised at some point during growth at 12°C (Fig. 4b). This finding pointed also to the heterogeneous growth response of *B. cereus* ATCC 14579 at a low incubation temperature.

Concluding remarks. The results of this study demonstrate that one of the most commonly used methods to control bacterial growth, chilling, results in a heterogeneous growth performance of *B. cereus* ATCC 14579. The direct-imaging-based Anopore technology was shown to be well suited to quantify in detail the cold-induced population heterogeneity for this strain. This study provides prospects for quantifying the population heterogeneity at lower incubation temperatures for other spore-forming and non-spore-forming food-borne pathogens, including strains which are able to produce toxins at abusive temperatures, such as emetic *B. cereus* (9). Our study showed that 11% of the microcolonies of *B. cereus* ATCC 14579 failed to continue growing at 12°C. Translating this to foods, in the case of initial low levels of contaminants, i.e., 1 to 5 cells per product unit, this observed population heterogeneity has an impact on the variability in microbial outgrowth. However, at higher contamination levels in foods, such a fraction of nongrowing cells will be overgrown by the larger fraction of growing cells. This indicates that the observed chill-induced population heterogeneity is too limited to have a major impact on the total exposure assessment when foods are contaminated with high concentrations of *B. cereus* and stored at an abusive refrigeration temperature.

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