

Quantitative Analysis of Population Heterogeneity of the Adaptive Salt Stress Response and Growth Capacity of *Bacillus cereus* ATCC 14579[∇]

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Bacterial populations can display heterogeneity with respect to both the adaptive stress response and growth capacity of individual cells. The growth dynamics of *Bacillus cereus* ATCC 14579 during mild and severe salt stress exposure were investigated for the population as a whole in liquid culture. To quantitatively assess the population heterogeneity of the stress response and growth capacity at a single-cell level, a direct imaging method was applied to monitor cells from the initial inoculum to the microcolony stage. Highly porous Anopore strips were used as a support for the culturing and imaging of microcolonies at different time points. The growth kinetics of cells grown in liquid culture were comparable to those of microcolonies grown upon Anopore strips, even in the presence of mild and severe salt stress. Exposure to mild salt stress resulted in growth that was characterized by a remarkably low variability of microcolony sizes, and the distributions of the log₁₀-transformed microcolony areas could be fitted by the normal distribution. Under severe salt stress conditions, the microcolony sizes were highly heterogeneous, and this was apparently caused by the presence of both a nongrowing and growing population. After discriminating these two subpopulations, it was shown that the variability of microcolony sizes of the growing population was comparable to that of non-salt-stressed and mildly salt-stressed populations. Quantification of population heterogeneity during stress exposure may contribute to an optimized application of preservation factors for controlling growth of spoilage and pathogenic bacteria to ensure the quality and safety of minimally processed foods.

Bacillus cereus is a common contaminant of various foods (2, 9), causing both spoilage and food poisoning. Vegetative cells of *B. cereus* can produce two types of toxins that can harm the consumer (11). The heat-stable emetic toxin is formed in the food itself, while the diarrheal toxins, called enterotoxins, are produced during intestinal growth of *B. cereus*.

In the last decades, there has been a substantial increase in production and sales of mildly processed ready-to-use foods (9). The microbial stability and safety of minimally processed foods are based on the use of various mild preservation factors. However, successive application of mild preservation factors can influence the ability of the organism to survive and grow in the food. *B. cereus* gains increased resistance during exposure to mild stresses by triggering of the so-called adaptive stress response (7, 8, 10, 29). Furthermore, it is becoming apparent that there is heterogeneity within a population in the way individual cells deal with stress, even in a homogeneous environment. A fraction of a microbial population may be more stress resistant, resulting in a biphasic nature of the stress response, and such heterogeneous resistance can be confirmed to be genotypic (1, 20, 27) or nongenotypic (5, 10, 16). The adaptive stress response and population heterogeneity might affect safety margin settings for food processing conditions

and, therefore, should be addressed to optimally apply mild preservation factors in minimally processed foods.

Indirect and direct methods have been developed to study the variability of responses of individual cells. A method that is frequently used is an indirect approach based on turbidity measurements (for example, references 13, 14, 22, and 25). Detection times or optical density growth curves of single-cell-generated cultures are used to estimate the variability of the lag times of individual cells, assuming that the specific growth rate is constant after the first division for each population engendered by a single cell. Direct methods make use of imaging of individual cells (12, 26, 32, 35) or spores (31) and can provide growth kinetics of individual cells and spores without extrapolation over time.

In this study, we used a direct method to observe the growth of individual vegetative cells from the initial inoculum stage to the microcolony stage on a planar, ceramic material, sold under the trade name of Anopore. Anopore is a rigid aluminum oxide that is extremely porous (pore density is up to 50%) compared to other membranes (19). Recently, strips of Anopore were shown to be an effective support for culturing and imaging of microorganisms (17, 18). Microbial growth is possible on Anopore strips when these are placed upon agar, which supplies the nutrients to the cells from beneath, through the pores. A thin layer of water on top of the Anopore strips, drawn up by capillary action of the pores, enables the cells to slide along a surface rather than being layered in one location. This might influence the specific growth rate of the cells, as it was shown that the growth rate of cells growing as surface

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colonies on a gelatin layer was smaller than the growth rate of cells in broth (6). The estimated doubling times of *Escherichia coli* 2613 and *Enterobacter aerogenes* grown on Anopore strips were, however, found to be comparable to the estimated doubling times in broth under nonstressing conditions (17, 18).

To date, quantitative, direct observations of the first generations of single cells have been focused on the growth of cells after stress exposure. Further knowledge about the behavior of individual cells during the early phase of stress exposure is necessary when the population heterogeneity of the stress response has to be quantified. In this study, the growth parameters of the bacterial population as a whole during mild and severe salt stress exposure were investigated for *B. cereus* ATCC 14579 and compared to the growth kinetics of individual microcolonies. Moreover, the heterogeneity of the adaptive stress response and growth capacity of individual microcolonies were quantitatively assessed.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *Bacillus cereus* ATCC 14579 was stored frozen (-80°C) in brain heart infusion (BHI) broth (Becton Dickinson, France) supplemented with 25% (vol/vol) glycerol (Sigma, The Netherlands). The concentration of sodium chloride in BHI broth without extra supplementation of sodium chloride was 0.5%. The bacteria were cultivated before each experiment in 10 ml BHI broth and incubated at 30°C with shaking at 200 rpm (SW20; Julabo Labortechnik GmbH, Germany) for 12 to 18 h. To produce exponentially growing cells, this culture was diluted 1:200 (vol/vol) in an Erlenmeyer flask (250 ml) containing 50 ml fresh BHI broth. The flask was incubated at 30°C with shaking at 200 rpm until the optical density was 0.4 to 0.5 at 600 nm (Novaspac II spectrophotometer; Pharmacia Biotech, United Kingdom).

Growth in broth. To determine the growth kinetics of the population as a whole in liquid culture, an exponential-phase inoculum was added to 50 ml BHI broth, 1:100 (vol/vol), in duplicate, after which the initial sample was taken ($t = 0$). To expose the cells to non-salt, mild salt, and severe salt stress conditions, BHI broth without and with supplementation of sodium chloride (VWR-International, France) was added (the supplementary concentrations of sodium chloride were 2.5% and 5% [wt/vol], respectively), after which the suspensions were incubated at 30°C with shaking at 200 rpm. At constant intervals, 1-ml aliquots were taken and serial dilutions were made in 9 ml of peptone saline solution (1 g neutralized bacteriological peptone [Oxoid, United Kingdom] supplemented with 8.5 g sodium chloride per liter). Fifty- μl aliquots of the appropriate dilutions were surface plated on BHI agar plates (BHI broth supplemented with 12 g agar [Oxoid, United Kingdom] per liter) by using a spiral plater (Eddy Jet; IUL Instruments, Spain). Appropriately diluted aliquots of the mildly and severely salt-stressed cells were also plated on BHI agar plates supplemented with 2.5% and 5% (wt/vol) sodium chloride. The plates were incubated at 30°C for 16 to 48 h, depending on the sodium chloride concentration of the BHI agar plates. The results are expressed in \log_{10} CFU ml^{-1} .

Growth on Anopore strips. Anopore strips (8 by 36 mm; 60 μm thick; 3×10^9 pores per cm^2 ; pore density up to 50%; a gift from PamGene International, 's-Hertogenbosch, The Netherlands) were manufactured, washed, and sterilized as previously described (17). The strips were placed upon BHI agar plates and prewarmed for a minimum of 30 min at 30°C in a moist chamber. To inoculate the Anopore strips with cells, exponentially growing cells were diluted 1:10, 1:100, and 1:1,000 (vol/vol) in prewarmed BHI broth at 30°C . The dilutions were inoculated in 2- μl aliquots on the upper surface of each Anopore strip. The plates were closed with Parafilm and then incubated at 30°C in a moist chamber. To expose the cells to mild salt stress and severe salt stress conditions, exponentially growing cells were inoculated on Anopore strips which were placed upon BHI agar plates supplemented with 2.5% and 5% (wt/vol) sodium chloride, respectively.

For each imaging time point, one Anopore strip was transferred right side up 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 (Invitrogen, The Netherlands). The agarose was dissolved in peptone saline solution, and 5 μM SYTO-9 dye (Invitrogen, The Netherlands) was added to stain the microcolonies on the Anopore strip. Staining was for 20 min at room temperature in the dark. For the sake of simplicity, the term microcolony is used for colonies that consisted of a single cell

or a few cells at the start of the incubation as well as for microcolonies later on in time after expansion.

Microscopy and image analysis. Strips were imaged directly (without the use of a coverslip or immersion oil) using an Olympus BX-41 fluorescence microscope equipped with 10 \times and 50 \times UmPlanF1 objective lenses and U-MWIBA and U-M41007 filters (17). A Kappa charge-coupled device camera controlled by Kappa Image Base software was used to capture the images. The saved images were analyzed quantitatively using ImageJ software to implement background correction, conversion to a binary image, and calculation of microcolony area. The total cell area of each microcolony was measured two-dimensionally in pixels, excluding the intercell area. It was assumed that the ratio between cell volume and cell area was constant over time, supposing both that the increase of cell number corresponded with the measured increase of cellular surface and that the bacterial cell shape was constant during the experimental time.

The intervals of imaging were 15, 20, and 30 min for the reference condition (nonsalt), mild salt (2.5%), and severe salt (5%) stress conditions, respectively. Images of the microcolonies were obtained until layers of cells within the microcolonies were observed. An average number of 85 (between 47 and 199) microcolony images per imaging time point were processed.

Quantification of heterogeneity. Microsoft Excel was used to calculate the distribution of microcolony areas per imaging time point for each experimental condition, and the observed frequency distributions were presented in histograms. To optimally envisage the increase of microcolony area over time, the binning of the histograms was based on the number of cells per microcolony. In order to estimate the number of cells per microcolony, the areas of the individual microcolonies were divided by the area of an average single cell measured at the first imaging time point of (non)stress exposure.

The extreme value, gamma, lognormal, and Weibull distributions were fitted to the observed frequency distributions of microcolony areas using @RISK 4.5.5 (professional edition; Palisade Europe, Middlesex, United Kingdom), which is an add-in program for Microsoft Excel. The goodness of fit of the four distributions was evaluated using the chi-square (χ^2) test, the Anderson-Darling (A-D) test, and the Kolmogorov-Smirnov (K-S) test. After that, the individual microcolony areas were \log_{10} transformed, and the variance and the mean of the \log_{10} -transformed microcolony areas were calculated for each imaging time point. The observed frequency distributions of the \log_{10} -transformed microcolony areas were tested for normality using the χ^2 test, the A-D test, and the K-S test, and SPSS software (version 12.0.1) was used to verify the K-S test.

Determination and comparison of growth kinetics in broth and on Anopore strips. The specific growth rate in broth was estimated by linear regression using the average viable counts per time point obtained with BHI agar plates (in \log_{10} CFU ml^{-1}). A straight line was fitted to the mean values that appeared to represent exponential growth. The data points of an initial lag phase or decline phase preceding the exponential growth phase were excluded from the linear regression analysis. To improve the objectivity of the exclusion of points from the linear regression analysis, the lag-exponential growth model (33) was fitted to the data points, and the upper 90% of the data points of the exponential growth phase were included in the analysis. The lag-exponential growth model was extended with a stationary phase when the data points showed three phases. Consequently, 80% of the data points of the exponential growth phase were included in the linear regression analysis. Slopes were derived from the linear regression analysis, and the specific growth rate μ (\log_{10} h^{-1}) was determined from the formula $\mu = \text{slope}$.

The specific growth rate of the microcolonies on Anopore strips was estimated by using a procedure similar to the one used to estimate the specific growth rate in broth but using the mean microcolony area per time point after \log_{10} transformation of each microcolony area.

Comparison of the estimated specific growth rate in broth and on Anopore strips was made with Student's t test (two-sided).

RESULTS

Population dynamics in broth. The growth response of *B. cereus* ATCC 14579 was investigated after abrupt shifts to non-salt stress (reference), mild salt stress (2.5%), and severe salt stress (5%) conditions. The growth dynamics of the three experimental conditions are shown in Fig. 1. Compared to the reference condition (Fig. 1a), exposure to 2.5% salt (Fig. 1b) resulted in an initial period of reduced growth before exponential growth started. An initial decline in viable counts was

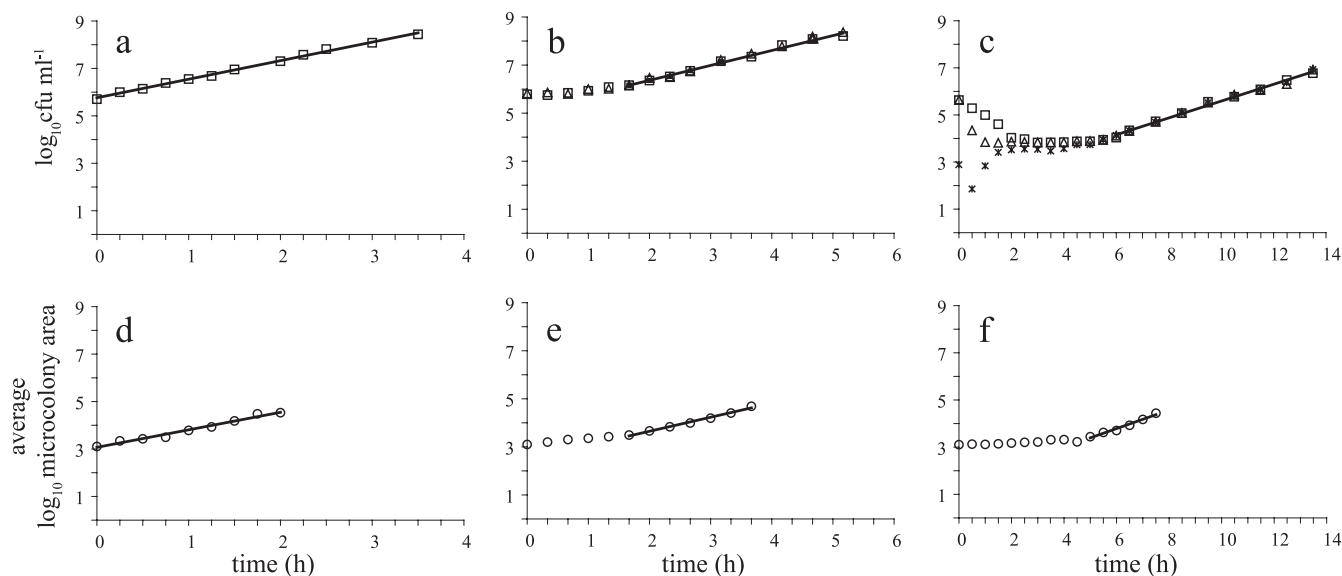


FIG. 1. Growth of *Bacillus cereus* ATCC 14579 in BHI broth without addition of salt (a) or with addition of 2.5% salt (b) or 5% salt (c) at 30°C. Viable cells were plated on BHI agar plates (□), BHI agar plates with the addition of 2.5% salt (△), and BHI agar plates with the addition of 5% salt (*), and growth of individual microcolonies on Anopore strips placed upon BHI agar plates was determined without addition of salt (d) or with the addition of 2.5% salt (e) or 5% salt (f) at 30°C. The areas of individual microcolonies per imaging time point were measured two-dimensionally in pixels and log₁₀ transformed. Data points represent the average microcolony size per imaging time point (○). For the 5% salt stress condition, the data points represent the average microcolony sizes of the growing population only. The specific growth rates were estimated by linear regression using the time points that represented exponential growth (continuous line).

observed after the abrupt shift to 5% salt, and exponential growth was resumed after a lag period (Fig. 1c). The viable counts obtained with BHI agar plates were used to estimate the specific growth rate for the three experimental conditions. As expected, the specific growth rate in broth decreased with increasing salt concentration (Table 1).

To further assess the population growth response, cells were plated both on nonselective plates (BHI agar plates) and on selective plates (BHI agar plates supplemented with 2.5% and 5% salt). Figure 1b shows that the viable counts on nonselective and selective media were comparable after the abrupt exposure to 2.5% salt. However, the osmotic shift to 5% salt resulted in a remarkable initial difference in viable counts on nonselective and on selective plates (Fig. 1c). After this initial stage, the viable counts on both nonselective plates and plates supplemented with 2.5% salt became comparable but were still higher than the viable counts on plates supplemented with 5% salt. A viable count stasis was reached on nonselective and both selective media before exponential growth resumed.

Viable count data describe the culturability of the popula-

tion as a whole. To examine the effect of an abrupt severe osmotic shift on the viability of the individual cells, the membrane integrity of the individual cells was tested during exposure to 5% salt, using both SYTO-9 and propidium iodide (PI) nucleic acid dyes. The permeant nucleic acid dye SYTO-9 can generally enter all bacteria in a population, while the impermeant nucleic acid dye PI penetrates only bacteria with damaged membranes (LIVE/DEAD *BacLight* bacterial viability kit) (15). The loss of culturability of a fraction of the population after the severe osmotic shift was contributory to the loss of membrane integrity, as the percent reduction in viable counts was found to correlate closely with the percentage of PI-labeled cells at the time that exponential growth resumed (data not shown).

Population dynamics on Anopore strips. To determine the growth dynamics of individual cells after osmotic shifts in more detail, growth of individual microcolonies was monitored using Anopore strips, which were placed upon BHI agar plates without the addition of salt (Fig. 2), with addition of 2.5% salt (Fig. 3), and with addition of 5% salt (Fig. 4). The 100-fold dilution of the inoculated culture resulted in a suitable density of microcolonies on the Anopore strips and, therefore, this dilution factor was used to determine the increase of microcolony area over time for each of the three experimental conditions. The observed frequency distributions of the number of cells per microcolony and example images of microcolonies of *B. cereus* ATCC 14579 are shown in Fig. 2, 3, and 4, respectively, for the non-salt, 2.5% salt, and 5% salt stress conditions. The frequency distributions and the images illustrate that the size of the microcolonies expanded over time. The monitoring times were 2 h, 3.7 h, and 7.5 h for the three different growth conditions. At the initial imaging time point, the maximum bin

TABLE 1. Estimated specific growth rate of *Bacillus cereus* ATCC 14579 grown on Anopore strips placed upon BHI agar plates and in BHI broth without addition of salt or with addition of 2.5% or 5% salt at 30°C

% NaCl (wt/vol)	Growth (log ₁₀ h ⁻¹) (95% CI) ^a	
	On Anopore strip	In broth
0	0.74 (0.64–0.84)	0.78 (0.75–0.81)
2.5	0.58 (0.52–0.65)	0.62 (0.57–0.67)
5	0.39 (0.31–0.48)	0.36 (0.33–0.38)

^a CI, confidence interval.

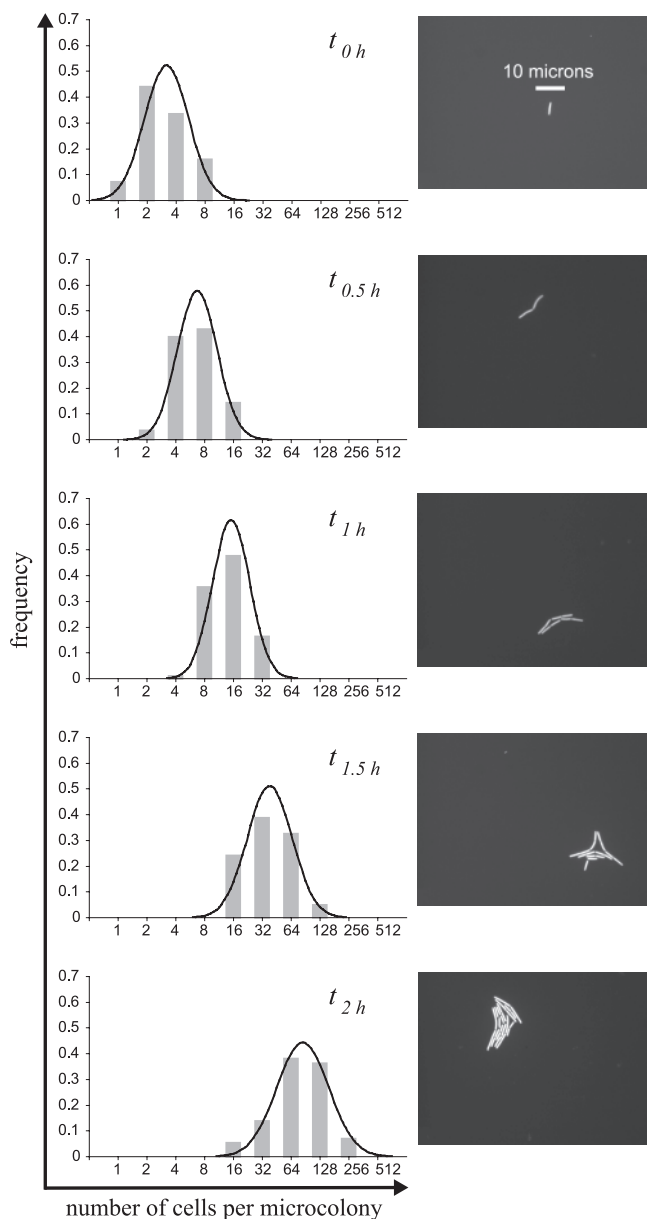


FIG. 2. Example images of *Bacillus cereus* ATCC 14579 cultured on Anopore strips, which were placed upon BHI agar plates without addition of salt at 30°C. Observed and fitted frequency distributions of the number of cells per microcolony are shown for imaging time points (t) at 0, 0.5, 1, 1.5, and 2 h. The histograms show observed frequencies of numbers of cells per microcolony. Continuous curves show fitted normal distributions of the \log_{10} -transformed microcolony areas.

number of the histograms was up to 8 cells per microcolony. Microscopic observations of exponentially growing cells in suspension confirmed this range of cells per microcolony. The first imaging time point of stress exposure showed that the average single cell area was comparable for the non-salt stress and 2.5% salt and 5% salt stress conditions. Moreover, single cells that could be individually discriminated later on during the experimental time did not show a difference in measured cellular surfaces for the three experimental conditions. As the microcolony size expanded over time, the number of cells per

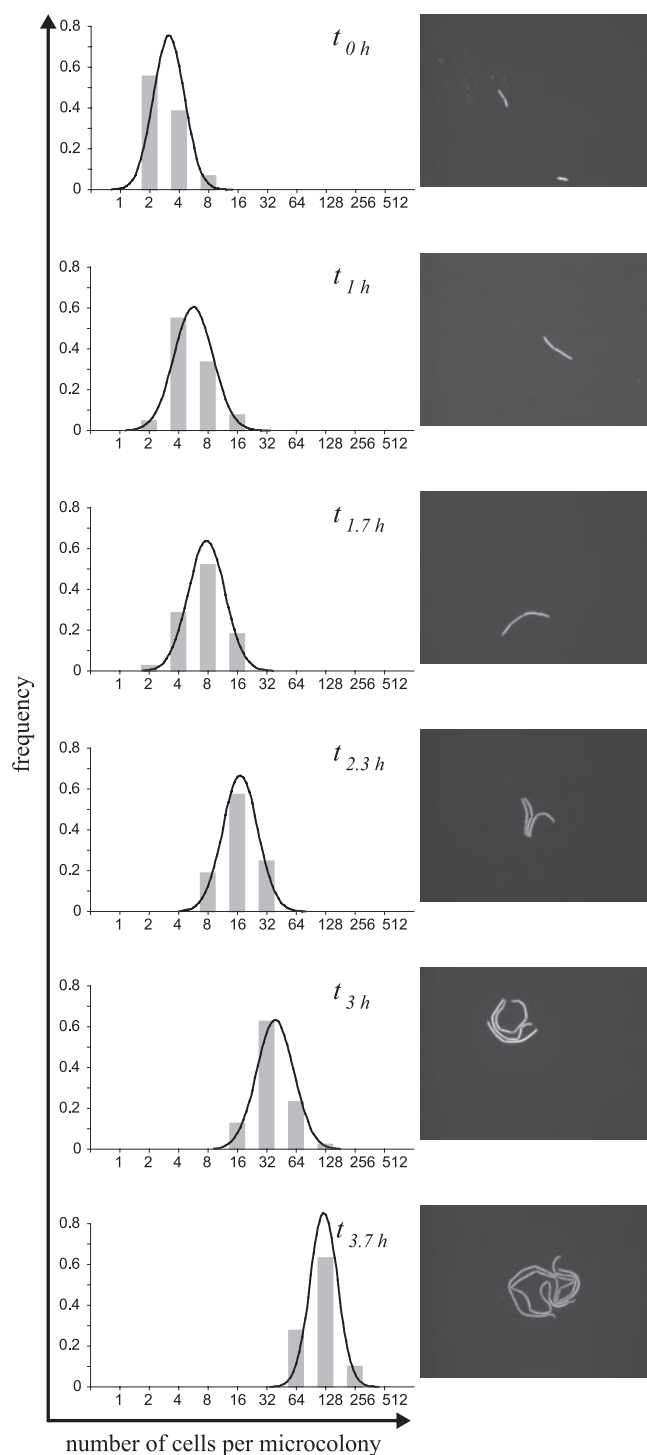


FIG. 3. Example images of *Bacillus cereus* ATCC 14579 cultured on Anopore strips, which were placed upon BHI agar plates with the addition of 2.5% salt (wt/vol) at 30°C. Observed and fitted frequency distributions of the number of cells per microcolony are shown for imaging time points (t) at 0, 1, 1.7, 2.3, 3, and 3.7 h. Histograms show observed frequencies of numbers of cells per microcolony. Continuous curves show fitted normal distributions of the \log_{10} -transformed microcolony areas.

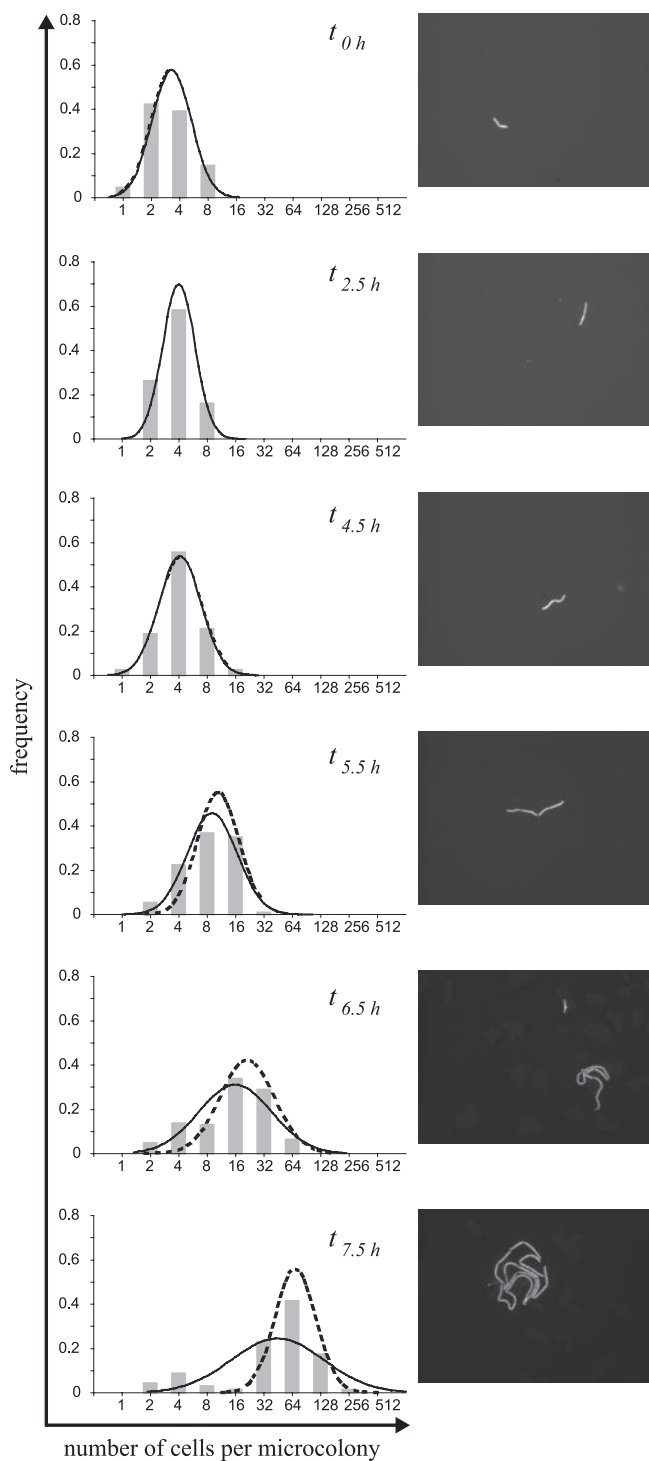


FIG. 4. Example images of *Bacillus cereus* ATCC 14579 cultured on Anopore strips, which were placed upon BHI agar plates with the addition of 5% salt (wt/vol) at 30°C. Observed and fitted frequency distributions of the number of cells per microcolony are shown for imaging time points (t) at 0, 2.5, 4.5, 5.5, 6.5, and 7.5 h. Histograms show observed frequencies of numbers of cells per microcolony. Continuous curves show fitted normal distribution of the \log_{10} -transformed microcolony areas; solid lines represent the distribution of all microcolonies, and dashed lines represent the distribution of the growing microcolonies only.

microcolony increased, and the histograms shifted to the right (Fig. 2, 3, and 4). A maximum of five generation times was monitored until the cells formed a second layer within the microcolony, resulting in a maximum bin number of 256 cells per microcolony at the final imaging time point. Exposure to 2.5% salt and 5% salt showed a period of reduced or no growth. Furthermore, the number of bins per histogram notably increased over time when the microcolonies were exposed to 5% salt.

Quantification of population heterogeneity. To quantitatively describe the frequency distribution of the microcolony areas for each imaging time point, four commonly used statistical distributions were fitted per data set: the extreme value, gamma, lognormal, and Weibull distributions. The numbers of imaging time points for the three experimental conditions were 9, 12, and 16, respectively, for the non-salt stress condition and 2.5% and 5% salt stress conditions. Based on the χ^2 , the A-D, and the K-S test statistics, the four distributions were ranked for each data set in order to choose the best overall distribution. The A-D and the K-S statistics gave quite similar results and ranked the lognormal distribution as the best distribution. Based on the χ^2 test, both the extreme value and the lognormal distribution were selected as appropriate distributions, but the distribution ranking was influenced by the number and location of the bins of the distribution. Neither the A-D test nor the K-S test requires binning, and they may therefore be less arbitrary (36). The advantage of the lognormal distribution is that the distribution parameters themselves have a specific meaning, parameter a being the mean and parameter b describing the spread of the data (23). Moreover, when a variable is lognormally distributed, the logarithm of the variable is normally distributed (34).

To examine the distributions of the microcolony areas in more detail, the normal distribution was fitted to the \log_{10} -transformed microcolony areas for each imaging time point, and the χ^2 , the A-D, and the K-S tests were applied to test the normality of the data sets. The continuous curves in Fig. 2, 3, and 4 show the frequencies generated by the normal distribution. For the reference condition (Fig. 2) and the 2.5% salt stress condition (Fig. 3) the \log_{10} -transformed microcolony areas were normally distributed according to both the χ^2 test and the K-S test ($P > 0.05$), but the A-D test was not accepted for all the data sets (5 out of 21). The A-D statistic highlights differences between the two tails of the fitted distribution and the input data (3), and the data sets which were not accepted according to the A-D test were indeed less symmetric at the two tails. In addition, the normal distribution could not be applied to all data sets for the 5% salt stress condition (Fig. 4). After 5 h, the distributions were significantly different from the normal distribution ($P < 0.05$) according to both the A-D test and the K-S test, but the χ^2 test did not confirm this finding. The histograms in Fig. 4 supported the findings of the A-D and the K-S tests, as the symmetry of the observed frequency distributions decreased over time, resulting in a negatively skewed distribution.

The spread of each data set was illustrated by the number of bins and the width of the frequency distribution and could be expressed in the variance. The variances of the observed frequency distributions for the nonsalt stress condition and the 2.5% salt stress condition were similar and rather stable over

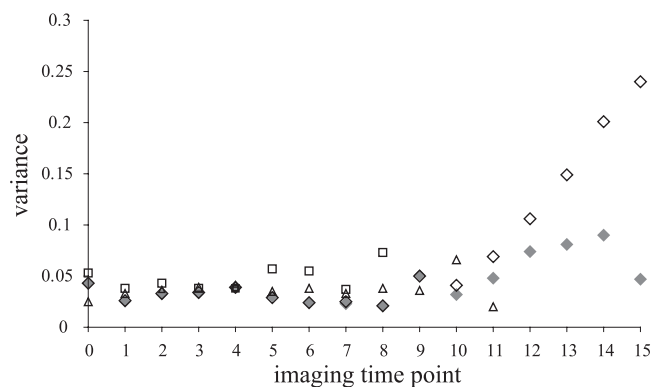


FIG. 5. Variances of the observed frequency distributions of the \log_{10} -transformed microcolony areas versus the imaging time points per salt stress condition. The intervals of imaging were 15, 20, and 30 min for conditions of non-salt stress (0%; \square), mild salt stress (2.5%; \triangle), and severe salt stress (5%; \diamond , distribution of all microcolonies, and \blacklozenge , distribution of the growing microcolonies only).

time (Fig. 5). This indicated that exposure to mild salt stress resulted in growth that was characterized by a noticeably low variability of the individual microcolony sizes over time, and this variability was comparable to that during non-salt stress exposure. By contrast, the variances of the distributions during exposure to 5% salt clearly increased for the last imaging time points. The final imaging time point for the 5% salt stress condition showed that most of the microcolonies were able to resume growth during 5% salt stress exposure (85% of the microcolonies), while a subpopulation of microcolonies failed to do so during the time scale of the experiment. To investigate this finding further, the distribution of the growing subpopulation at the final imaging time point was tested for normality using the three statistical tests, and this was confirmed ($P > 0.05$). Subsequently, the nongrowing population as observed at the final imaging time point was also marked for the other imaging time points. The frequency distribution of the nongrowing population was separated from the total population for each imaging time point, showing two subpopulations, a growing and nongrowing population per time point. Figure 4 shows the fitted normal distribution of the growing population for each data set. The distributions of the growing population were tested for normality, and this was confirmed for all imaging time points using both the χ^2 and the K-S tests ($P > 0.05$). The A-D test was not accepted for these data sets (3 out of 16), of which the two tails of the observed frequency distribution were less symmetric. Figure 5 illustrates that the discrimination of two subpopulations resulted in a remarkable decrease of the variance for the last imaging time points. The variances of the observed frequency distributions of the growing population were comparable to the variances of the distributions for the nonsalt and 2.5% salt stress conditions.

Finally, the average microcolony size was calculated for each imaging time point and used to estimate the specific growth rate of the microcolonies on Anopore strips. The calculation of the average per time point for the 5% salt stress condition was restricted to the growing subpopulation. Figure 1d, e, and f show the growth of *B. cereus* ATCC 14579 on Anopore strips placed upon BHI agar plates without addition of salt (Fig. 1d)

and with the addition of 2.5% salt (Fig. 1e) and 5% salt (Fig. 1f). The non-salt stress condition showed no initial lag phase. The onset of the exponential growth phase for the 2.5% and 5% salt stress conditions was, respectively, after 1.7 h and 5 h, preceding a phase of reduced growth or a lag phase. The estimated specific growth rates of the microcolonies on Anopore strips decreased with increasing hypertonic conditions (Table 1).

Comparison of growth kinetics in broth and on Anopore strips. It has been shown in other studies that lag time and initiation of growth under stressful conditions can be affected by the size of the inoculum (28, 30) and, therefore, a similar inoculum size was used in both the broth and the Anopore experimental setups. Comparison of the growth kinetics obtained in broth and on Anopore strips suggested that the onsets of the exponential phases were comparable (Fig. 1a versus d and b versus e), except for the 5% salt stress condition. In the latter situation, the exponential growth phase started after 6 h and 5 h, respectively, in broth (Fig. 1c) and on Anopore strips (Fig. 1f). The specific growth rates in broth and on Anopore strips for each experimental condition were statistically compared, and confidence intervals are presented in Table 1. The specific growth rates in broth were not significantly different from the specific growth rates on Anopore strips for the three tested experimental conditions ($P > 0.05$). Moreover, the linear regression lines of both the viable count data and the microcolony data were found to be parallel for the non-salt, 2.5%, and 5% salt stress conditions (Fig. 1a versus d, b versus e, and c versus f), as the differences between the viable counts and the mean values of the \log_{10} -transformed microcolony areas remained constant over time for each experimental condition.

DISCUSSION

The plate count method was used to investigate the effects of mild and severe salt stress exposure on the population dynamics of *B. cereus* ATCC 14579 as a whole. It is becoming increasingly known that single cells that have originated from the same population exhibit marked variability in stress resistance (1, 5, 10, 16, 20, 27). Therefore, in this study the effects of abrupt shifts to hypertonic environments were further examined at the single-cell level. Direct imaging methods are well suited to reveal the heterogeneity within a microbial population, and in the present study Anopore strips were used to quantitatively assess the heterogeneity of the stress exposure response and growth capacity of individual *B. cereus* ATCC 14579 microcolonies over time.

Growth of a bacterial population is determined by the proliferation of individual cells and results in an increase in cell number and total cell volume over time. Using direct imaging, the growth of microcolony area was determined by measuring the individual microcolony areas two-dimensionally in pixels at regular intervals. In order to estimate the proliferation of cells using these data, it was assumed that the ratio between cell volume (three dimensions) and cell area (two dimensions) was constant during the experimental time scale. Consequently, it was assumed both that the increase in cell number corresponded with the measured increase of cellular surface and that the bacterial cell shape did not change over time. Our

study showed that the specific growth rate of cells in liquid culture was similar to that of cells grown in microcolonies, both in the absence and the presence of salt stress. Moreover, the onset of the exponential growth phase was comparable in both experimental setups. These findings illustrated the remarkable similarity of growth kinetics of cells grown in broth and on Anopore strips and supported the acceptability of the assumption.

Previous studies have shown that several statistical distributions can be used to adequately describe the variability of the lag times and turbidity detection times of unstressed and stressed single-cell-generated bacterial populations (for example, references 13, 14, and 22) and the variability of interdivision times of cells after stress exposure (21). Our study confirmed the findings of others (36), that the statistic used to test the acceptance of distribution fitting influences the conclusions of acceptance. McKellar and Hawke proposed to choose a distribution of which the parameters are interpretable (23). In this study, the \log_{10} -transformed microcolony areas were normally distributed during non-salt stress and mild salt stress exposure, and the normal distribution could also be applied to the distributions of the growing subpopulation under severe salt stress conditions. The range of the variances of the distributions was rather small, and the use of exponentially growing cells might have affected this remarkably low variability of individual microcolony sizes over time.

Both the viable count growth curves and the direct imaging approach showed that after an abrupt osmotic shift to 5% salt, a fraction of cells within the initial population was able to resume exponential growth, indicating heterogeneity in the population's response to severe salt stress. It should be noted that the initial reduction of viable counts after the severe osmotic shift differed from the fraction of the nongrowing population on Anopore strips, but this phenomenon was not investigated any further. Labeling of cells which were grown in liquid culture using the fluorogenic nucleic acid stain propidium iodide demonstrated that loss of membrane integrity coincided with the loss of culturability. Loss of culturability of a subpopulation of cells by abrupt severe osmotic shifts within the growth-permissive range was previously observed for *Salmonella enterica* serovar Typhimurium (24) and *Listeria monocytogenes* (28). It can be hypothesized that the resumption of growth after an initial lag period was due either to the recovery of a fraction of the population or was caused by a very small highly resistant subpopulation which was able to grow exponentially, directly after the osmotic shift without or with having an initial period of recovery and adaptation. Moreover, it may be the case that the resumption of growth was the net result of different responses by subpopulations, as hypothesized by Mellefont et al. (24). A loss of viability as well as recovery, stress adaptation, and exponential growth might occur simultaneously in different subpopulations. Considering the microcolony data as well as the viable count growth curves, the most likely scenario seems that the resumption of growth was the result of the recovery and adaptation of a fraction of the population. The microcolony data suggested that a large fraction of the population was able to initiate growth after an initial lag period. In addition, the growth experiment in broth demonstrated that a viable count stasis was reached on non-selective and selective media before exponential growth was

resumed. This may indicate that this fraction of the population had adapted to the stressful environment and could resuscitate and proliferate even under severe stress conditions. However, the possible presence of a very small highly resistant subpopulation, which was able to initiate exponential growth immediately after the osmotic shift without having a recovery and adaptation period, could not be dismissed either, as this fraction of the population can be rather small and may not be differentiated from the recovering cells.

Anopore is a highly inert and resistant material and is unlikely to be damaged by most stress applications, including heat. The rigidity of the Anopore strips facilitates the transfer of strips from one condition to another in order to successively expose cells to various stresses. Therefore, for further research, the Anopore approach may provide possibilities to assess the first responses of cells to a succession of environmental changes.

The recent interest in the underlying sources of population heterogeneity emphasizes that it is a well-recognized problem within the field of quality and safety of minimally processed foods (4, 5). This study showed that Anopore strips were well suited to monitor individual microcolonies at the early stage of stress exposure. Furthermore, this direct imaging approach could be used to quantitatively describe the population heterogeneity of the adaptive stress response and growth capacity of individual microcolonies. Quantification of population heterogeneity during stress exposure might contribute to the optimal assessment of safety margin settings for food processing conditions in order to guarantee the quality and safety of minimally processed foods.

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