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# Stochasticity in Colonial Growth Dynamics of Individual Bacterial Cells

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Conventional bacterial growth studies rely on large bacterial populations without considering the individual cells. Individual cells, however, can exhibit marked behavioral heterogeneity. Here, we present experimental observations on the colonial growth of 220 individual cells of *Salmonella enterica* serotype Typhimurium using time-lapse microscopy videos. We found a highly heterogeneous behavior. Some cells did not grow, showing filamentation or lysis before division. Cells that were able to grow and form microcolonies showed highly diverse growth dynamics. The quality of the videos allowed for counting the cells over time and estimating the kinetic parameters lag time ( $\lambda$ ) and maximum specific growth rate ( $\mu_{\max}$ ) for each microcolony originating from a single cell. To interpret the observations, the variability of the kinetic parameters was characterized using appropriate probability distributions and introduced to a stochastic model that allows for taking into account heterogeneity using Monte Carlo simulation. The model provides stochastic growth curves demonstrating that growth of single cells or small microbial populations is a pool of events each one of which has its own probability to occur. Simulations of the model illustrated how the apparent variability in population growth gradually decreases with increasing initial population size ( $N_0$ ). For bacterial populations with  $N_0$  of  $>100$  cells, the variability is almost eliminated and the system seems to behave deterministically, even though the underlying law is stochastic. We also used the model to demonstrate the effect of the presence and extent of a nongrowing population fraction on the stochastic growth of bacterial populations.

**All the physical and chemical laws that are known to play an important part in the life of organisms are of statistical kind.**

—Erwin Schrödinger, *What Is Life? The Physical Aspect of the Living Cell* (1)

Erwin Schrödinger, with his outstanding book, *What is Life?*, published in 1944 (1), provided the spark that ignited an explosion in biology research at the single-cell level. Much of the focus of this research has been to investigate the diversity at the molecular level also known as “molecular noise” (2). The noise at the molecular level refers to stochastic variations associated with each of the processes along the path of genomic information flow such as gene activation, transcription, and translation (3–6). Some studies have further extrapolated heterogeneity from the underlying molecular source right up to the cellular phenotype, reporting a significant phenotypic variability among single cells, including cell division and growth behavior (2, 7, 8).

The findings on the variability in single-cell growth responses received the attention of researchers working in the field of predictive microbiology. Traditional predictive microbiology uses deterministic mathematical models that describe the growth of large microbial populations as a whole without considering the individual cells. The importance of single-cell variability was raised after the recent developments in quantitative microbial risk assessment (9). Deterministic models that provide point estimates are generally not sufficient to satisfactorily manage microbial safety risks (10–12). Indeed, if, for instance, the consequences of unacceptable levels of pathogenic microorganisms in a food are grave, knowledge of only the mean population growth is unlikely to be a sufficient basis for management decisions on the safety risk. Since contamination with pathogens usually occurs with very low numbers, the development of stochastic approaches that can describe the

variability of single-cell behavior is necessary for realistic estimations of safety risks (13).

The available information on the heterogeneity in the growth behavior of single cells is in general limited, due mainly to the technical difficulties in monitoring the growth of individual cells. Recently, some studies focused on the development of specific devices for the observation of individual cell division. Elfving et al. (14) designed a flow chamber mounted on a microscope equipped with a digital camera in which the cells attached to a transparent solid surface. The shear force of the flow removed the daughter cells, making it possible to monitor the consecutive divisions of a single cell. Wakamoto et al. (15) and Siegal-Gaskins and Crosson (16) developed microfluidic devices to monitor elongation rates and interdivision times of single cells. All the above methods allow for monitoring one cell alone, with the daughter cell being removed after division. On solid substrates (i.e., solid foods), however, bacterial growth is colonial, in which case interactions among cells of a forming colony may occur (17–20).

In this study, we developed a quantitative experimental investigation using an automated time-lapse microscopy method to monitor the colonial growth of single cells of *Salmonella enterica* serotype Typhimurium. The method allows for the evaluation of

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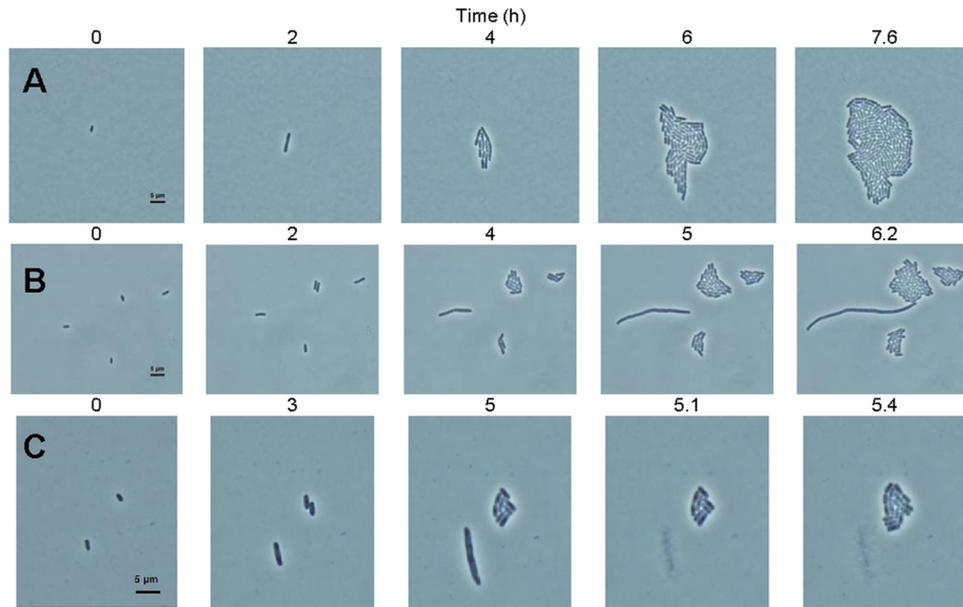


FIG 1 Time-lapse microscopy images of the behavior of *Salmonella* Typhimurium individual cells over time. (A) Cell able to grow and form a colony; (B) cell filamentation; (C) cell lysis before division.

the heterogeneity in the growth dynamics of microcolonies originating from single cells and for the quantitative description of stochasticity in bacterial growth using Monte Carlo simulation.

## MATERIALS AND METHODS

**Bacterial strain and growth media.** The bacterial strain used in the study was *Salmonella enterica* serotype Typhimurium FSL S5-520 (bovine isolate), kindly provided by Martin Wiedmann (Cornell University, Ithaca, NY). A stock culture of the strain was stored frozen ( $-70^{\circ}\text{C}$ ) onto Microbank porous beads (Pro-Lab Diagnostics, Ontario, Canada). A working culture of the strain was stored refrigerated ( $5^{\circ}\text{C}$ ) on tryptone soy agar (TSA; Lab M Limited, Lancashire, United Kingdom) slants and was renewed bimonthly. The strain was activated by transferring a loopful from the TSA slant into 10 ml of tryptone soy broth (TSB; Lab M Limited) and incubating it at  $37^{\circ}\text{C}$  for 24 h. Twenty microliters of a 24-h culture of the strain, after two 10-fold serial dilutions in one-quarter-strength Ringer's solution (Lab M Limited), was added to 500  $\mu\text{l}$  of TSA solidified on a glass slide, and the 20- $\mu\text{l}$  volume was left to dry in a biological safety cabinet for 5 min. The inoculated agar was covered by a coverslip and sealed with silicone to avoid dehydration. The inoculum size was approximately  $10^6$  to  $10^7$  CFU/ml.

**Time-lapse microscopy.** The colonial growth of single cells was monitored by phase-contrast time-lapse microscopy using a z-motorized microscope (Olympus BX61; Olympus, Tokyo, Japan) equipped with a  $100\times$  objective (Olympus) and a high-resolution device camera (Olympus DP71). The sample was maintained at  $25^{\circ}\text{C}$  using a temperature-controlled stage (Linkam PE60; Linkam Scientific Instruments, Surrey, United Kingdom). An in-house program was developed with the ScopePro module of the ImageProPlus image analysis software version 6.3 (MediaCybernetics Inc., Bethesda, MD), which allows the system to be automatically turned on and off before and after the capture of an image. Images of the field of view were acquired every 5 min for 6 to 8 h. The quality of the images was improved by developing an autofocus procedure with an extended depth of focus (EDF) system. The above procedure allows for multiple (20 to 30) serial images in different z-axis planes to be captured and then combines the best focal areas of the serial images into a single in-focus image (z-stack). Individual final images were compiled to give a sequence of frames for the field of view, which was further trans-

formed into a video showing the behavior of the same cell over time throughout the experiment.

**Data analysis and modeling.** The behavior of a total of 220 *S. Typhimurium* single cells on TSA at  $25^{\circ}\text{C}$  was monitored. The high quality of the images allowed for monitoring the cell size, the division times, and the number of cells in each microcolony with time using the ImageProPlus image analysis software. Cell counting was performed for up to 100 cells per microcolony using the manual tag of ImageProPlus. After counting, data were transformed to the respective growth curves showing the exact number of cells in each microcolony originating from a single cell over time. The obtained growth curves were then fitted to the primary model of Baranyi and Roberts (21) for the estimation of lag time ( $\lambda$ ) and maximum specific growth rate ( $\mu_{\text{max}}$ ). In order to describe the abrupt transition from the lag to the exponential phase characterizing the observed growth, the values of the parameters  $m$  and  $n$  of the model were fixed to 0 and 20, respectively.

The data of  $\lambda$  and  $\mu_{\text{max}}$  were fitted to various distributions using the @Risk 4.5 for Excel software (Palisade Corporation, Newfield, NY). The goodness of fit was compared using three different methods:  $\chi^2$ , Anderson-Darling (A-D), and Kolmogorov-Smirnov (K-S). The best-fitted distributions based on the above criteria were further introduced into an exponential model with lag (see equation 1 below) to describe the growth of individual cells using Monte Carlo simulations.

## RESULTS AND DISCUSSION

### Heterogeneity in the colonial growth dynamics of single cells.

We present a detailed quantitative experimental investigation on the behavior of 220 *S. Typhimurium* single cells on TSA at  $25^{\circ}\text{C}$  using automated time-lapse microscopy. In Fig. 1, we show representative examples of the observed behavior of single cells including (i) cell division and formation of a microcolony, (ii) filamentation, and (iii) cell lysis before division. In 213 cells (96.8%), an increase in cell length followed by division and colony formation was observed (Fig. 1A; see Movie S1 in the supplemental material). Seven cells (3.2%) were not able to divide. Among those, six cells showed a filamentous phenotype with an extensive increase in cell length leading to the formation of unusually long

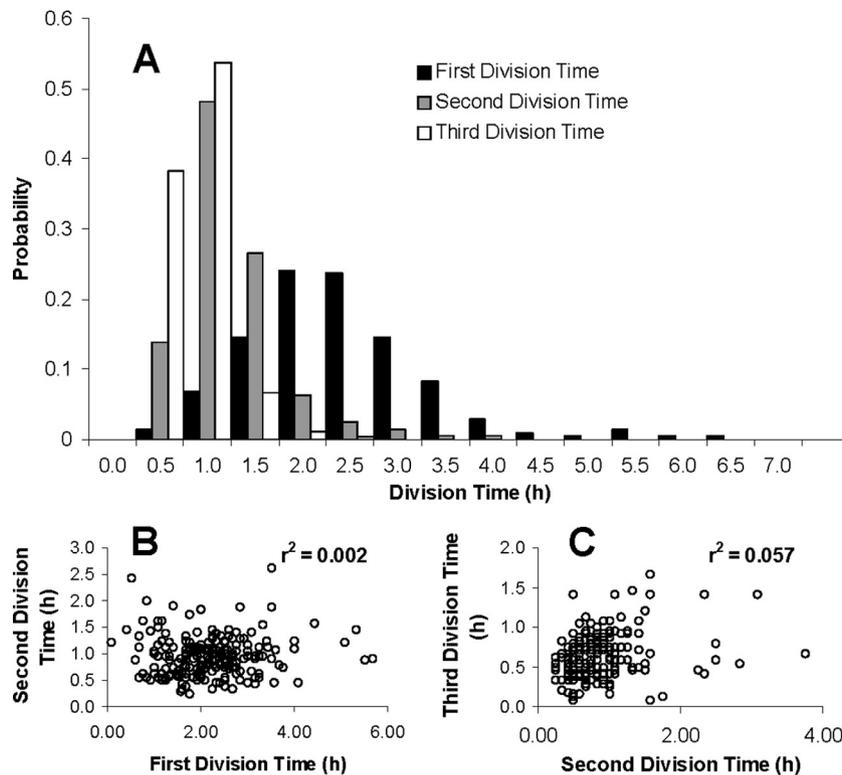


FIG 2 Variability in the division times of *Salmonella* Typhimurium individual cells. (A) Distribution of first, second, and third division times. (B, C) Relation between successive division times.

cells, up to 20 times the initial length (Fig. 1B; see Movie S2 in the supplemental material), while in one cell, lysis before division was observed (Fig. 1C; see Movie S3 in the supplemental material). Filamentation and cell lysis were also observed, at a very low frequency, in the daughter cells during colony formation (see Movie S4 in the supplemental material). Previous studies have reported that the latter “behavioral noise” is the observable consequence of the noise in gene expression related to stochastic fluctuations during transcription and translation that lead to differences in protein levels (4, 22, 23). For example, the observed difference among the cells related to the filamentous phenotype could be attributed to the noise in the production of protein FtsZ or other proteins involved in altering cell division such as ZipA and SulA (24–26). Similarly, cell lysis of *Salmonella* is possibly linked to the bacterial suicide systems (27) such as the toxin-antitoxin system PezAT (also known as the epsilon/zeta system), which is expressed in many pathogenic bacteria to program their own individual death (28).

The quality of the videos obtained from the time-lapse microscopy method allowed us to analyze the behavior of individual cells through monitoring characteristics such as the division times, the cell length, and the growth kinetics of a microcolony over time. The distributions of the first three division times are shown in Fig. 2A. Both the mean and the spread of division time distributions decreased with generations. The time to first division, which includes the lag time, is significantly longer and ranges from 0.1 to 6.2 h with a mean and standard deviation of 2.21 h and 0.96 h, respectively. For the second and third divisions, the mean decreased to 1.10 and 0.65 h and the standard deviation to 0.54 and

0.31 h, respectively. These results are in accordance to the findings of the studies of Métris et al. (29) and Pin and Baranyi (30), who reported a similar trend for the generation times of *Escherichia coli* cells. In order to investigate if division-related information is inherited, the relation between successive division times was evaluated. The poor correlation shown in Fig. 2B indicates no intergenerational “memory” related to the time required for division.

The actual source of division time variability is not known. Some studies have reported cell length as a critical factor for division (31, 32). Our results do not support this. The initial length of the 220 tested cells at time zero ranges from 0.86 to 3.75  $\mu\text{m}$ , with a mean of 2.21  $\mu\text{m}$  and a standard deviation of 0.57  $\mu\text{m}$  (Fig. 3A). The correlation coefficient between the initial cell length and the first generation time is very low (Fig. 3B). Very low correlation coefficients ( $r = 0.003$  to  $0.020$ ) were also observed between the initial cell length and the second or the third division time, indicating that longer cells do not systematically provide shorter division time and vice versa. This can be attributed to the increased variability of the cell length at the time of division, which can range from about 2 to 5 times the initial length (see Movie S5 in the supplemental material).

The limited available studies on individual cell behavioral noise focus on the division times of a single cell, with the daughter cells being removed (14–16). The distributions of these division times can be used to describe the growth of microbial populations through a birth model (30). This approach, however, does not take into account potential microbial interactions within a microcolony during colonial growth (17–20). The main obstacle in describing the variability in the colonial growth dynamics of single

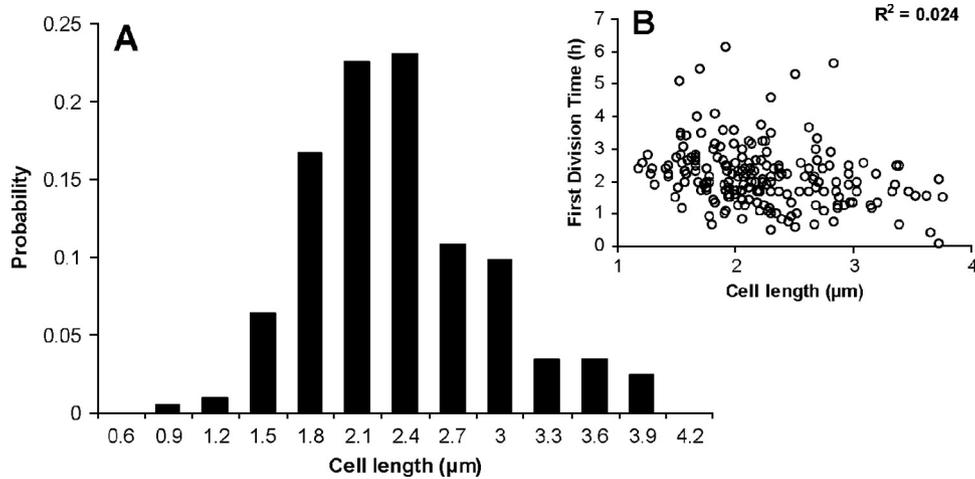


FIG 3 Variability in the initial cell length (at time  $t = 0$ ) of *Salmonella* Typhimurium individual cells. (A) Probability distribution of the initial cell length; (B) relation between initial cell length and first division time.

cells is the experimental error related to the methods applied for monitoring growth. The time-lapse microscopy method applied in this study allows for counting the actual cells with time in each microcolony originating from a single cell and thus for eliminating the experimental error of other indirect methods used for monitoring microbial growth. Cell counting was performed for up to 100 cells per microcolony when the growth of the colony was still two-dimensional. In Fig. 4, we present the growth curves of representative single cells of *S. Typhimurium*, demonstrating the high variability in their growth dynamics. In order to describe this variability, we fitted the growth data of each microcolony to the Baranyi and Roberts (21) primary model for the estimation of the growth kinetic parameters (i.e.,  $\lambda$  and  $\mu_{\max}$ ). The kinetic parameters were analyzed as a function of the final number of cells in a microcolony. For this purpose, the number of cells over time in each microcolony was fitted to the primary model and the kinetic parameters were estimated for different final cell numbers. The analysis showed that when the number of cells in a microcolony exceeded 20 to 25, both kinetic parameters reached constant values, which varied significantly among microcolonies (Fig. 5). This

can be attributed to the variability in the generation time of single cells, which decreases with generations (Fig. 2), as well as to potential differences in the so-called “community effect” including the colony’s microenvironmental factors such as pH (19, 20) and cell-to-cell communication (quorum sensing) (17, 18, 33).

The distributions of  $\lambda$  and  $\mu_{\max}$  values, estimated by the primary model for 213 microcolonies originating from single cells, are shown in Fig. 6A. The  $\lambda$  values range from 0 to 5.72 h, with an average value of 1.72 h and a standard deviation of 0.92 h. For  $\mu_{\max}$ , the range is from 0.30 to 1.33  $\text{h}^{-1}$  and the average value and standard deviation are 0.77  $\text{h}^{-1}$  and 0.16  $\text{h}^{-1}$ , respectively. The estimated coefficients of variation (%CV = standard deviation/mean  $\times$  100) are 53.7% for  $\lambda$  and 20.5% for  $\mu_{\max}$ . A poor relation between  $\lambda$  and  $\mu_{\max}$  is found with a correlation coefficient ( $r^2$ ) of 0.022 (Fig. 6B).

**A stochastic approach in microbial growth.** The need for stochastic bacterial growth models has increased since the establishment of quantitative microbial risk assessment (QMRA) as the basis of food safety management (34). The use of bacterial growth models in QMRA has different demands from those of “traditional” deterministic models (13). Deterministic models are developed and validated to produce point estimates of microbial

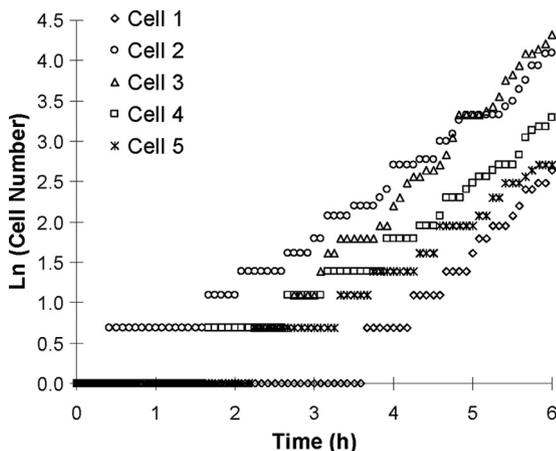


FIG 4 Representative colonial growth curves of *Salmonella* Typhimurium individual cells.

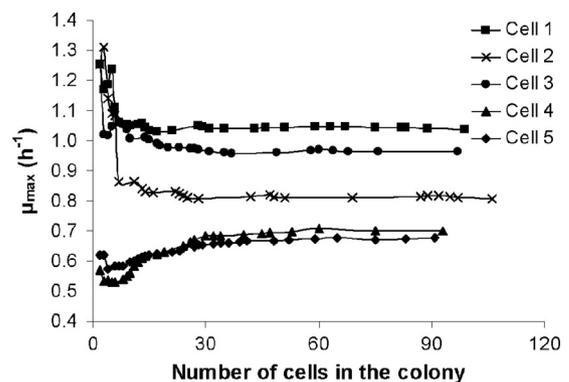
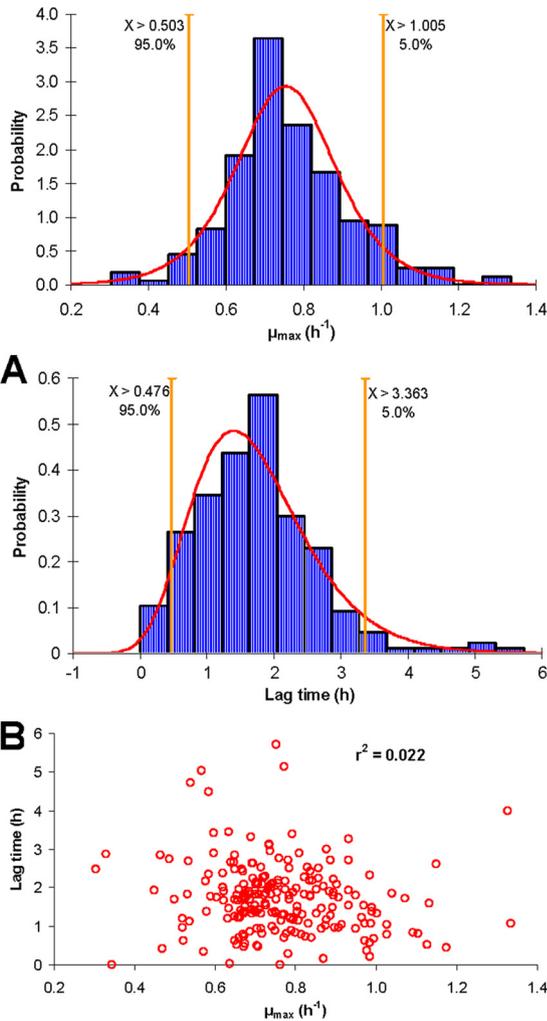


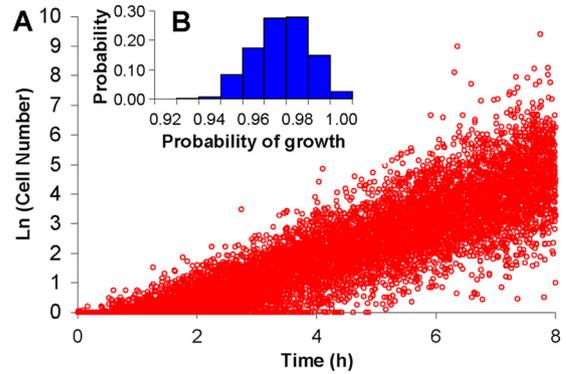
FIG 5 Maximum specific growth rate ( $\mu_{\max}$ ) as affected by the increasing number of cells in the microcolony over time for five representative individual cells.



**FIG 6** Variability in the kinetic parameters, lag time ( $\lambda$ ), and maximum specific growth rate ( $\mu_{\max}$ ) for 213 microcolonies originating from a single cell. (A) Probability histograms of  $\mu_{\max}$  (top) and  $\lambda$  (bottom). Red lines represent the best-fitted distributions to the data (logistic for  $\mu_{\max}$  and log normal for  $\lambda$ ). (B) Relation between  $\lambda$  and  $\mu_{\max}$ .

population levels. In QMRA, however, microbial populations should be expressed in terms of probability (i.e., to predict the probability distribution of the microbial concentration at the time of consumption). Considering that food poisoning outbreaks are usually triggered by contamination of foods with a very low number of pathogenic cells, the description of the variability of single-cell behavior has a key role in QMRA.

To interpret the observations, we devised a stochastic model for microbial growth. For this, we described quantitatively the variability of the kinetic parameters of single-cell colonial growth. The distributions with the best fit were the logistic distribution for  $\mu_{\max}$  and the log normal distribution for  $\lambda$ . In Fig. 7A, we present the fitted distributions together with the density data for  $\lambda$  and  $\mu_{\max}$ . We also described the variability in the probability of growth for a single cell using the bootstrap method (35). Based on the results, which showed that 213 of 220 cells were able to grow and form colonies, the probability of growth,  $P_g$ , for an individual cell was estimated as  $\text{Binomial}(220, 213/220)/220$  and ranged from



**FIG 7** Simulation output of the stochastic model for the colonial growth of an individual cell. (A) Model prediction for the growth of a single cell using Monte Carlo simulation with 10,000 iterations and a uniform distribution for time  $t$  [ $t \sim \text{Uniform}(0, 8)$ ]. (B) Description of probability of growth for an individual cell using Monte Carlo simulation with 10,000 iterations.

0.914 to 1 with a mean value of 0.968 and a standard deviation of 0.0119 (Fig. 7B).

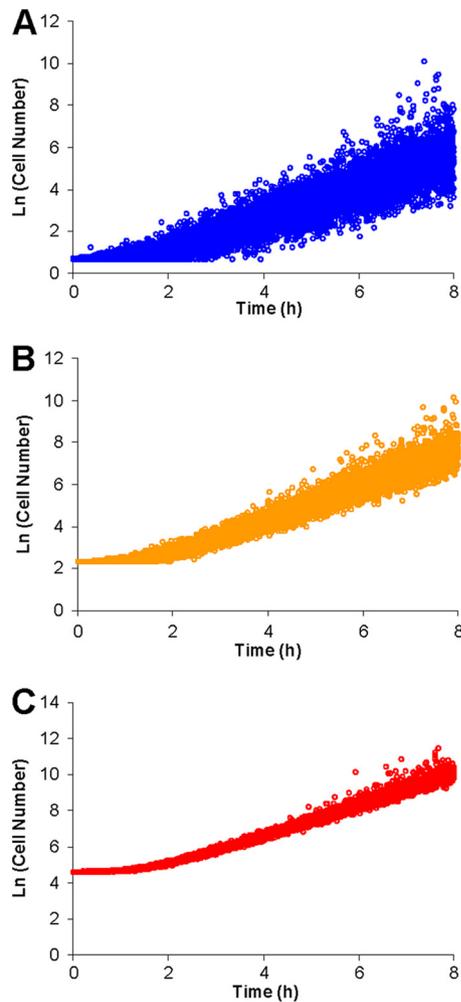
The stochastic model was devised by introducing the distributions of the kinetic parameters into a simple exponential growth with lag model as follows:

$$N_t = (N_0 - N_g) + \sum_{i=1}^{N_g} \begin{cases} 1 & \text{for } t \leq \lambda_i \\ e^{\mu_{\max i} (t - \lambda_i)} & \text{for } t > \lambda_i \end{cases} \quad (1)$$

where  $N_t$  is the total number of cells in a population at time  $t$ ,  $N_0$  is the initial number of cells in the population at  $t = 0$ ,  $N_g \sim \text{Binomial}(N_0, P_g)$  is the initial number of cells in the population at  $t = 0$  that are able to grow and form a colony,  $P_g$  is the mean probability of growth ( $P_g = 0.968$ ), and  $\mu_{\max} \sim \text{Logistic}(0.754, 0.085)$  and  $\lambda \sim \text{LogNormal}(3.355, 0.896, \text{shift}(-1.628))$  are the probability distributions of  $\mu_{\max}$  and  $\lambda$ , respectively, of a microcolony originating from a single cell.

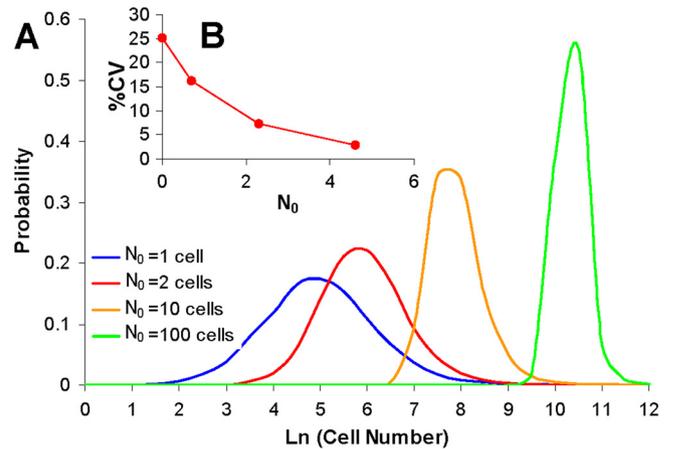
The model describes the growth of a bacterial population, initially consisting of  $N_0$  cells, over time as the sum of cells in each of the  $N_0$  imminent microcolonies originating from a single cell. The above approach allows for taking into account the heterogeneity in the growth dynamics of single cells by introducing the kinetic parameters in the model as probability distributions using Monte Carlo simulation. In addition to the growth kinetics, the model also describes the probability of growth for single cells. This is an important component of the model, since previous studies have shown that the growth limits of individual cells vary significantly and this variation can affect the growth behavior of microbial populations, especially under conditions close to the boundary of growth (36).

In Fig. 7A, we present the output of the model for  $N_0$  of 1 using Monte Carlo simulation with 10,000 iterations and with a uniform distribution for  $t$  [ $t \sim \text{Uniform}(0, 8)$ ]. The output of the model is a stochastic growth curve in which the number of cells in the population at any time is a probability distribution. For example, in Fig. 7A the number of cells in a microcolony originating from a single cell after 8 h of growth can be either 7 (1st percentile) or 2,324 (99th percentile), with a most probable number of 314 (mode). This information is important, since it allows for the estimation of the risk (probability) that one cell multiplies to reach an infective dose.



**FIG 8** Effect of the initial number of cells,  $N_0$ , on the stochastic growth of a microbial population. (A) Model predictions for the growth of microbial populations initially consisting of 2 cells (A), 10 cells (B), and 100 cells (C). Growth is predicted by the stochastic model using Monte Carlo simulation with 10,000 iterations and a uniform distribution for time  $t$  [ $t \sim \text{Uniform}(0, 8)$ ].

The proposed model can be used for evaluating the effect of the initial number of cells ( $N_0$ ) on the variability of population growth. **Figure 8** presents the results of Monte Carlo simulations with  $N_0$  equal to 2, 10, and 100 cells. The simulations show that as  $N_0$  increases, the apparent variability in the growth of the total population decreases significantly, and it is almost eliminated for  $N_0$  of 100 cells. In addition, an increase in  $N_0$  results in a shorter lag phase of the population. The latter was also observed for *E. coli* by Pin and Baranyi (30), who reported that the lag phase decreased significantly as the inoculum size increased from 1 to 100 cells. The effect of  $N_0$  can be clearly seen in **Fig. 9**, which shows the distribution of the number of cells after 8 h of growth for bacterial populations with different  $N_0$  values. As  $N_0$  increases from 1 to 100 cells, the distribution of the number of cells in the population becomes narrower, with the %CV of the number of cells decreasing from 25.1% to 2.78% in a logarithmic scale. The latter simulations explain why variability in microbial growth is usually not observed in laboratory growth experiments in which large microbial populations, often of hundreds or thousands of cells, are used.



**FIG 9** Effect of the initial population size ( $N_0$ ) on the variability of the number of cells in the population after 8 h of growth. (A) Predicted probability distributions of the number of cells in a microbial population initially consisting of 1, 2, 10, and 100 cells after 8 h of growth. (B) Effect of  $N_0$  on the coefficient of variation (%CV) of the number of cells in the population after 8 h of growth. Growth is predicted by the stochastic model using Monte Carlo simulation with 10,000 iterations for  $t$  of 8 h.

In the case of large microbial populations, variability becomes negligible through the law of large numbers, and the system seems to behave in a deterministic manner even though the underlying laws are probabilistic. This has been reported previously by several studies, with emphasis on the variability in the lag time of individual cells (37–40). In this study, we show the overall variability in the colonial growth of individual cells, including the growth rate, and how it is affected by  $N_0$ . In general, analysis of the  $N_0$  effect demonstrates that deterministic models that provide point estimates can describe satisfactorily the growth of microbial populations consisting of 100 cells or more. For smaller populations, however, stochastic models are required since a deterministic approach that misses the information on variability can lead to erroneous estimations of safety risks.

The majority of the available growth models focus on the kinetics of growth without taking into account the probability of growth of the cells, assuming that all cells in the population are able to grow. The results of the present work showed that under optimum conditions, 213 of 220 single *S. Typhimurium* cells were able to grow and form colonies. However, for stressed cells or under less favorable conditions, the probability of growth may be significantly lower (36). We used the stochastic model to evaluate the effect of  $P_g$  on stochastic growth. **Figure 10** demonstrates a comparison in the growth of two microbial populations with  $N_0$  of 100 and  $P_g$  equal to 1.0 (all 100 cells are able to grow) and 0.1 (10 of 100 cells are able to grow). Although the two simulations presented in **Fig. 10** are based on the same probability distributions for the kinetic parameters  $\lambda$  and  $\mu_{\max}$ , a decrease in  $P_g$  resulted in a longer  $\lambda$  and an increased variability in the population growth. As previously reported by Koutsoumanis (36), the longer lag phase is due to an additional “pseudolag” caused by the decreased ratio between the growing and nongrowing fractions of the population, while the higher variability is attributed to the smaller number of cells that are able to grow (**Fig. 8**).

New insight may come from the investigation and the stochastic mathematical description of single-cell behavior. In addition,

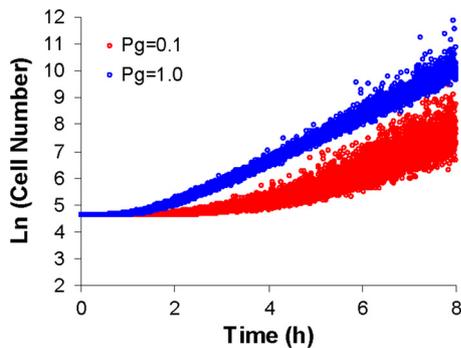


FIG 10 Comparison of the stochastic growth of two microbial populations with  $N_0$  of 100 cells and probability of growth of the individual cells ( $P_g$ ) equal to 1.0 (all 100 cells are able to grow) and 0.1 (about 10 of 100 cells are able to grow). Growth is predicted by the stochastic model using Monte Carlo simulation with 10,000 iterations and a uniform distribution for time  $t$  [ $t \sim \text{Uniform}(0, 8)$ ].

stochastic models can improve significantly the credibility of risk assessment studies. The next steps in this research are to describe the effects of factors such as cell physiological state (39), strain variability (41, 42), and growth environment on single-cell behavior and to develop effective stochastic models for microbial growth in foods.

#### ACKNOWLEDGMENTS

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