

Computer Simulation of *Clostridium botulinum* Strain 56A Behavior at Low Spore Concentrations

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It is generally assumed that spore behavior is independent of spore concentration, but recently published mathematical models indicate that this is not the case. A Monte Carlo simulation was employed in this study to further examine the independence assumption by evaluating the inherent variance in spore germination data. All simulations were carried out with @Risk software. A total of 500 to 4,000 iterations were needed for each simulation to reach convergence. Lag time and doubling time from a higher inoculum concentration were used to simulate the time to detection (TTD) at a lower inoculum concentration under otherwise identical environmental conditions. The point summaries of the simulated and observed TTDs were recorded for the 26 simulations, with kinetic data at the target inoculum concentration. The ratios of the median ($R_m = \text{median}_{\text{obs}} / \text{median}_{\text{sim}}$) and 90% range ($R_r = 90\% \text{ range}_{\text{obs}} / 90\% \text{ range}_{\text{sim}}$) were calculated. Most R_m and R_r values were greater than one, indicating that the simulated TTDs were smaller and more homogeneous than the observed ones. R_r values departed farther from one than R_m values. Ratios obtained when simulating 1 spore with 10,000 spores deviated the farthest from one. Neither ratio was significantly different from the other when simulating 1 spore with 100 spores or simulating 100 spores with 10,000 spores. When kinetic data were not available, the percent positive observed at the 95th percentile of the simulated TTDs was obtained. These simulation results confirmed that the assumption of independence between spores is not valid.

Mathematical modeling is an analytical approach using calculus, algebra, and basic probability theories to obtain precise numerical values for the variables in question (11, 19). Under certain conditions, when the predictor variables are known and the relationship between the predictor and response variables is simple, analytical models are an ideal solution. However, this ideal approach quickly breaks down when faced with the complexity of the real world. It is rare to be able to accurately describe a biological procedure by mathematical equations. In addition, even if it is possible to construct equations, more often than not, these equations are not solvable without using numerical iterations or making considerable approximations of the real problem. Finally, every measurement in the real world is associated with a variance and a particular degree of uncertainty (5, 18) that cannot be described by an analytical model.

A simulation approach is much more suitable than an analytical approach when dealing with complicated problems with an inherent variance. Instead of using a model to calculate an exact result for a variable, a simulation calculates a value many times over, each time assuming new values for each input, to give an estimation of the real value (15). Monte Carlo simulation is the most widely used simulation method in biology (4, 16) and has been used to study the mechanism in such biochemical procedures as cell cycle control (9) and plasmid replication (10). While many plasmid replication models have been exploited using the molecular, modeling, and simulation methods (3, 12, 13, 20), Kuo and Keasling (10) found that the

use of a Monte Carlo simulation to compare the current theories was advantageous, since the possible combinations of replication and partition theories make laboratory experiments cumbersome and time consuming.

Monte Carlo simulation was used in this research to investigate whether *Clostridium botulinum* strain 56A spores communicate with each other during the process of germination, outgrowth, and multiplication. Independence between the spores was assumed for all the simulations. Each simulation used a higher inoculum to simulate the time to detection (TTD) of a lower one, keeping all the environmental conditions the same. For example, results from 10,000 spores (at pH 6.5, 0.5% salt, and 30°C) were used to simulate the results for 1 spore (at the same pH, salt level, and temperatures). If the independence assumption is true, then the simulated results should resemble the observed results. If communication exists between spores or between cells and spores, however, then the simulated TTDs, by using data from a higher inoculum, should be shorter and more homogeneous than the observed TTDs.

MATERIALS AND METHODS

Calculation of the doubling time and lag time from OD data. Optical density (OD) data were collected for *C. botulinum* strain 56A germination and growth under 81 different environmental conditions, and each condition was replicated in 44 microtiter wells (22). A calibration curve relating observations at an OD at 620 nm to log (CFU/ml) was generated, and the detection limit of the microtiter plate reader was determined experimentally.

A series of equations was used to convert the maximum growth rates into doubling times at the inflection point of the Gompertz equation by using the calibration curve. Microbial lag times were also calculated from TTDs by subtracting the time used by germinated spores to reach the detection limit of the machine. These calculations were carried out for each growth curve and for all conditions.

Distributions for lag doubling times, TTD, and spore number. The lag times, doubling times, and TTDs were input into BestFit version 4.0 (Palisade Corpo-

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ration, Newfield, N.Y.). All continuous distributions were fit to the data, and the choice of the most suitable distribution was based on goodness-of-fit statistics, visual observations, and parsimony of the parameters.

Plate counts from ~ 1 spore/well were pooled. A total of 1,032 plate counts were included, ranging from 0 to 10 CFU/well. All discrete distributions available in BestFit version 4.0 were fit to the data and evaluated using the chi-square statistic.

Plate counts from ~ 100 spores/well were pooled. A total of 931 plate counts were included, ranging from 14 to 184 CFU/well. Both discrete and continuous distributions were fit to the data.

Simulation configuration. All simulations were conducted with @Risk (Palisade Corporation), which is an add-in program for Excel (Microsoft Corporation, Seattle, Wash.).

Each condition was represented by a separate spreadsheet. Every recalculation of the spreadsheet represented one replicate. The number of spores per iteration was generated from the spore number distribution mentioned above. All the spores were assigned distinct lag times and multiplication rates. Each spore was represented by one column, and each time increment was represented by one row.

The simulated TTD, doubling and lag times, and total cell concentration were set as outputs. Simulations were monitored for the change in percentiles, means, and standard deviations (SDs) of each output. Simulation was halted when the results converged (i.e., all changes were lower than 1.5%). The doubling time, lag time, and spore number outputs were checked to ensure biological sensibility (i.e., doubling time and lag time were greater than zero) and computational accuracy (spore number did not surpass the maximum number of columns). The output for simulated and observed TTDs was saved in separate files and analyzed as described below.

Simulation procedure and data analysis. Simulation mimics the practice of applying results at high inoculum sizes to systems with lower initial load. Both the lag time and the doubling time were from a higher inoculum. Three types of simulations were carried out: 10,000 spores simulating 1 spore, 10,000 spores simulating 100 spores, and 100 spores simulating 1 spore.

An array of spores was generated, each with a different lag time and doubling time. For each spore, the time increased until it was larger than its lag time and the spore then became a cell and the number of cells doubled according to an assigned doubling rate. The simulated number of cells in a well increased until it reached a value representing a minimum detection limit. The time when the detection limit was first reached was recorded as the simulated TTD. The observed TTD for the condition being simulated was represented as a distribution in the same spreadsheet, so the same number of observed and simulated TTDs was generated.

Point summaries for both simulated and observed TTDs were recorded, and ratios of the location and spread in simulated and observed data were calculated. Location indicators included ratios for the mean ($R_{\text{mean}} = \text{mean}_{\text{obs}}/\text{mean}_{\text{sim}}$) and median ($R_m = \text{median}_{\text{obs}}/\text{median}_{\text{sim}}$), with the latter one being a robust estimate. Spread indicators included ratios for the SD ($R_{\text{SD}} = \text{SD}_{\text{obs}}/\text{SD}_{\text{sim}}$) and 90% range ($R_r = 90\% \text{ range}_{\text{obs}}/90\% \text{ range}_{\text{sim}}$), the latter one also being robust. Models were developed to describe the effect of the factors (inoculum difference, temperature, pH, and salt concentration) on the robust ratios since these estimations are more conservative, i.e., closer to one.

When kinetic data were not available at a lower inoculum for comparison, the simulated TTDs were summarized and the time to reach 95% growth was obtained (the 95th percentile of the simulated TTDs), defined as $\text{Day}_{95\% \text{ Sim}}$. The observed percentage of positive wells at this time was obtained experimentally.

RESULTS

Spore number distributions. Plate counts for target 1 spore/sample were pooled (a total of 1,032 counts) and fit to all the discrete distributions (negative binomial, Poisson, geometric, and integer uniform) available in BestFit version 4.0 (Palisade Corporation). The best distribution was the negative binomial, as judged by the chi-square statistics, with the Poisson distribution coming in a close second. There was no significant difference in these two fits by visual observation, and the Poisson distribution had only one parameter while the negative binomial had two. Based on its acceptable fit, its simplicity, its ease of biological interpretation, and its wide use in the mi-

crobial field of study, we chose Poisson ($\lambda = 2.08$ spores/well) as the distribution of target 1 spore/well. Both the discrete and continuous distributions were fit for target 100 spores/well. The distribution chosen for target 100 spores/well was normal ($\mu = 67.02$, $\sigma = 24.44$).

Maximum number of simulated columns in the simulation template. Each spore was represented by one separate column. The maximum number of columns in the simulation was set up such that the maximum number of spores generated in all iterations did not exceed this number. Each simulation achieved convergence in fewer than 5,000 iterations. The 1 to 1/5,000 = 0.9998 = 99.98th percentile of the spore number distribution gives the value such that the maximum spore number would exceed it only 1 out of 5,000 times. These values were calculated in Splus 2000 (MathSoft, Inc., Seattle, Wash.) to be 153.5568 and 9 for 100 and 1 spore/sample, respectively. The maximum numbers of spreadsheet columns were set at 160 and 12 for slight additional safety factors, and none of the simulations produced spores numbers exceeding these values.

Simulation. There were 50 simulations total, 26 with kinetic data at the target inoculum and 24 without. For some conditions, the simulated TTD agreed well with the observed TTDs,

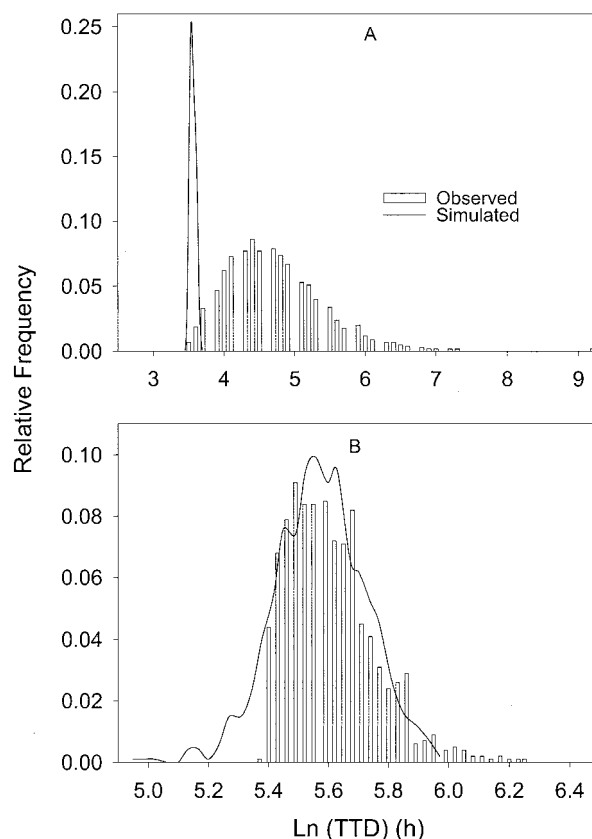


FIG. 1. The simulated TTDs were smaller and more homogeneous than the observed TTDs under some conditions (A), while they resembled each other under other conditions (B). (A) Total of 10,000 spores simulating 1 spore at 30°C, pH 6.5, and 2% salt. (B) Total of 100 spores simulating 1 spore at 15°C, pH 6.5, and 0.5% salt. The simulated TTDs were obtained by using lag times and doubling times from a higher inoculum size to predict the results at a lower one, keeping the environmental factors the same.

while in other cases, the two TTDs were very different from each other (Fig. 1). Spread ratios (R_r and R_{SD}) were generally larger than the corresponding location ratios (R_m and R_{mean}) for the same environmental condition and same spore simulation scenario (Table 1). R_m and R_r were more conservative estimators than R_{mean} and R_{SD} , respectively (Table 1); therefore, discussions will only be made on R_m and R_r from this point on. In all but four conditions, both ratios were greater than one (Fig. 2). The median of the observed and simulated TTDs were not dramatically different from each other, so the data points fell closely to the 45° line, although mainly below the line (Fig. 2A). The spread of the observed TTDs was much larger, however, as evidenced by the far-dispersed data points below the 45° line, even on the natural log scale in Fig. 2B. A general trend could be observed that, when 10,000 spores/well was used to simulate 100 spores/well, both ratios of the median and range were smaller than those in simulations that used 10,000 spores to simulate 1 spore. Using 100 spores to simulate 1 spore had a ratio of median and range similar to that when 10,000 spores were used to simulate 100 spores (Fig. 3).

To quantify the effect of different spore scenarios (10,000 spores simulating 100 and 1 spore, 100 spores simulating 1 spore) and the effect of environmental factors, regression models were developed for R_m and R_r Splus 2000 (MathSoft, Inc.). The simplified polynomial equations are presented in equations 1 and 2 for R_m ($R^2 = 0.729$) and R_r ($R^2 = 0.878$), respectively.

$$\begin{aligned}
 R_m = & -98.182 \\
 & + 0.197\text{Dummy}_1 + 0.119\text{Dummy}_2 \\
 & + 0.748\text{Temp} - 4.967\text{NaCl} + 31.244\text{pH} \\
 & - 0.135\text{NaCl}^2 - 2.477\text{pH}^2 \\
 & - 0.110\text{Temp} \times \text{pH} + 0.865\text{NaCl} \times \text{pH} \quad (1)
 \end{aligned}$$

$$\begin{aligned}
 \ln(R_r) = & -348.108 \\
 & + 1.020\text{Dummy}_1 + 0.118\text{Dummy}_2 \\
 & - 0.547\text{Temp} - 6.819\text{NaCl} + 119.991\text{pH} \\
 & + 0.016\text{Temp}^2 - 10.130\text{pH}^2 \\
 & - 0.106\text{Temp} \times \text{NaCl} + 1.560\text{NaCl} \times \text{pH} \quad (2)
 \end{aligned}$$

In both equations, the categorical variables (Dummy_1 and Dummy_2) were indicators for spore scenarios. Dummy_1 represents those simulations in which 10,000 spores simulated 1 spore. Dummy_2 represents those simulations in which 100 spores simulated 1 spore. Using 10,000 spores to simulate 100 spores served as the baseline for comparison. Each of these two categorical variables had two possible values, 1 when the spore simulation scenario was the one it represented and 0 when it was not. Therefore, Dummy_1 equaled Dummy_2 equaled 0 when 10,000 spores were used to simulate 100 spores, Dummy_1 equaled 1 and Dummy_2 equaled 0 when 10,000 spores were used to simulate 1 spore, and Dummy_1 equaled 0 and Dummy_2 equaled 1 when 100 spores were used to simulate 1 spore. Simulating 1 spore with 10,000 spores resulted in a larger difference in the median of observed TTDs

and that of simulated TTDs than the result obtained from using 10,000 spores to simulate 100 spores (equation 1). Using 100 spores to simulate 1 spore also resulted in a larger difference in R_m , but not one as large as the difference obtained when using 10,000 spores to simulate 1 spore. The P values observed for both dummy variables, however, were larger than 0.05 (0.109 for Dummy_1 and 0.103 for Dummy_2). Therefore, the difference in R_m between the three different spore scenarios was only marginally significant statistically. The effect of Dummy_1 on R_r was very prominent (equation 2): $e^{1.02}$ was ≈ 2.78 times larger when simulating 1 spore with 10,000 spores than that with simulating 100 spores with 10,000 spores. This term was also very significant ($P < 0.00001$). The increase over baseline in $\ln(R_r)$ when simulating 1 spore with 100 spores was about 0.12, but with a nonsignificant P value (0.282). The effect from the environmental factors was quite complicated and not the major focus in our research.

When kinetic growth was not observed for the target condition, the simulated TTDs were compared with the percent positive over time data (Table 2). The percent positive observed at the time of the 95th percentile of the simulated TTD (i.e., at this time, 95% of the simulated wells had shown growth) is presented. Only 2 of 24 conditions ever resulted in detectable turbidity changes in more than 10% of the wells. Thirteen conditions had no observed growth.

DISCUSSION

Why use simulation? Statistical equivalence (i.e., the result from 1 experiment with 1,000 spores being identical to the results from 1,000 experiments with 1 spore each) has been assumed for many years; however, it is valid only when spores or cells are independent from each other. If spores and cells interact with their neighbors through quorum sensing (7, 8), the independence requirement for statistical equivalence is violated. We have shown previously by mathematical modeling that inoculum size effect exists in the *C. botulinum* spore system (21, 22). The modeling approach, however, is not an efficient tool to directly test the hypothesis of communication between spores.

By using data collected for the growth of *C. botulinum* spores at different inoculum concentrations, pH, temperature, and salt levels, a computer simulation was carried out, assuming independence between spores. In all 50 simulations, the results from a higher inoculum size were used to simulate the TTD at a lower inoculum size while all environmental factors were kept the same. If spores do not interact with each other, the simulated results from high spore concentrations should resemble the results at lower spore concentrations. On the other hand, if spores do communicate with each other, it should result that the simulated TTDs are smaller and more homogeneous than the observed TTDs. Therefore, by comparing the simulated and observed TTDs, conclusions can be made on the validity of the independence assumption.

This independence assumption can be realized best with simulation. In a real microbial experiment, it is almost impossible to construct a system where spores stay reasonably close but certain physical barriers in between prevent them from signaling each other. Even if the theoretical design is possible, the complexity of the system makes it very expensive to build

TABLE 1. Observed and simulated TTDs when lag times and doubling times from a higher inoculum size were used to simulate the results at a lower inoculum size with all other environmental factors being the same

| Spore scenario ^c | Variable | | | | TTD (h) | | | | | | | | O ^a /S ^b ratio | | | | |
|-----------------------------|-----------|-----|----------|--------|---------|-------|-------------------------|-----|-----|-------|--------|-------|--------------------------------------|-------|------------|------|------|
| | Temp (°C) | pH | NaCl (%) | O or S | Mean | SD | Percentile ^d | | | | | | Mean | SD | Percentile | | |
| | | | | | | | Min | 5 | 50 | 95 | Max | 95-5 | | | 50 | 95-5 | |
| 4-2 | 30 | 6.5 | 0.5 | O | 23 | 2 | 18 | 21 | 23 | 27 | 42 | 6 | 1.2 | 2.6 | 1.2 | 3.2 | |
| | | | | S | 19 | 1 | 17 | 18 | 19 | 20 | 22 | 2 | | | | | |
| | 30 | 6.5 | 2.0 | O | 29 | 2 | 27 | 27 | 29 | 33 | 33 | 5 | 1.1 | 1.7 | 1.1 | 1.8 | |
| | | | | S | 27 | 1 | 25 | 26 | 27 | 29 | 34 | 3 | | | | | |
| | 30 | 6.5 | 4.0 | O | 72 | 12 | 49 | 56 | 70 | 93 | 139 | 37 | 1.2 | 3.0 | 1.2 | 3.1 | |
| | | | | S | 61 | 4 | 53 | 56 | 60 | 68 | 94 | 12 | | | | | |
| | 30 | 6.0 | 0.5 | O | 93 | 69 | 24 | 32 | 72 | 222 | 563 | 190 | 3.5 | 70.6 | 2.8 | 63.3 | |
| | | | | S | 27 | 1 | 25 | 25 | 26 | 28 | 30 | 3 | | | | | |
| | 30 | 6.0 | 2.0 | O | 131 | 83 | 3 | 28 | 112 | 288 | 603 | 259 | 2.4 | 8.7 | 2.1 | 8.1 | |
| | | | | S | 54 | 10 | 27 | 37 | 54 | 69 | 87 | 32 | | | | | |
| | 30 | 6.0 | 4.0 | O | 138 | 89 | 40 | 66 | 119 | 265 | 1,158 | 199 | 1.4 | 14.8 | 1.2 | 10.0 | |
| | | | | S | 98 | 6 | 72 | 88 | 98 | 108 | 120 | 20 | | | | | |
| | 30 | 5.5 | 0.5 | O | 125 | 52 | 21 | 55 | 119 | 221 | 368 | 166 | 2.6 | 6.0 | 2.5 | 6.1 | |
| | | | | S | 47 | 9 | 20 | 34 | 47 | 61 | 105 | 27 | | | | | |
| | 22 | 6.5 | 0.5 | O | 48 | 1 | 45 | 47 | 48 | 50 | 51 | 3 | 0.9 | 0.3 | 0.9 | 0.3 | |
| | | | | S | 53 | 3 | 47 | 49 | 52 | 57 | 69 | 8 | | | | | |
| 22 | 6.5 | 2.0 | O | 71 | 8 | 45 | 60 | 71 | 84 | 127 | 24 | 1.4 | 3.1 | 1.4 | 3.0 | | |
| | | | S | 53 | 2 | 47 | 49 | 52 | 57 | 64 | 8 | | | | | | |
| 22 | 6.0 | 0.5 | O | 84 | 22 | 48 | 54 | 82 | 123 | 199 | 69 | 1.2 | 7.9 | 1.2 | 8.7 | | |
| | | | S | 69 | 3 | 59 | 65 | 69 | 73 | 85 | 8 | | | | | | |
| 22 | 6.0 | 2.0 | O | 141 | 49 | 54 | 80 | 132 | 236 | 551 | 156 | 1.6 | 6.7 | 1.5 | 6.5 | | |
| | | | S | 89 | 7 | 55 | 76 | 89 | 100 | 117 | 24 | | | | | | |
| 15 | 6.5 | 0.5 | O | 190 | 4 | 180 | 184 | 190 | 198 | 208 | 14 | 0.9 | 0.5 | 0.9 | 0.6 | | |
| | | | S | 217 | 8 | 202 | 208 | 216 | 233 | 256 | 25 | | | | | | |
| 15 | 6.5 | 2.0 | O | 468 | 106 | 208 | 316 | 458 | 671 | 1,032 | 355 | 1.6 | 5.4 | 1.5 | 5.7 | | |
| | | | S | 300 | 20 | 171 | 265 | 302 | 327 | 357 | 62 | | | | | | |
| 15 | 6.0 | 0.5 | O | 430 | 131 | 232 | 261 | 409 | 691 | 1,073 | 430 | 1.6 | 9.8 | 1.5 | 10.0 | | |
| | | | S | 275 | 13 | 221 | 256 | 273 | 299 | 334 | 43 | | | | | | |
| 4-0 | 30 | 6.5 | 0.5 | O | 55 | 60 | 20 | 22 | 37 | 137 | 953 | 115 | 2.2 | 25.9 | 1.5 | 19.1 | |
| | | | | S | 25 | 2 | 22 | 23 | 25 | 29 | 67 | 6 | | | | | |
| | 30 | 6.5 | 2.0 | O | 152 | 1,021 | 26 | 37 | 89 | 347 | 58,301 | 310 | 4.4 | 670.8 | 2.5 | 77.4 | |
| | | | | S | 35 | 2 | 31 | 33 | 35 | 37 | 40 | 4 | | | | | |
| | 30 | 6.5 | 4.0 | O | 164 | 71 | 41 | 77 | 150 | 300 | 592 | 223 | 1.7 | 3.4 | 1.7 | 3.4 | |
| | | | | S | 94 | 21 | 58 | 69 | 90 | 135 | 225 | 66 | | | | | |
| | 15 | 6.5 | 0.5 | O | 269 | 44 | 211 | 219 | 259 | 351 | 461 | 132 | 1.0 | 1.8 | 0.9 | 1.7 | |
| | | | | S | 281 | 25 | 233 | 250 | 276 | 328 | 413 | 78 | | | | | |
| | 22 | 6.5 | 0.5 | O | 108 | 24 | 72 | 77 | 102 | 156 | 184 | 79 | 1.5 | 2.2 | 1.4 | 2.4 | |
| | | | | S | 74 | 11 | 54 | 61 | 72 | 94 | 130 | 33 | | | | | |
| | 22 | 6.5 | 2.0 | O | 170 | 114 | 57 | 83 | 142 | 347 | 1,841 | 265 | 1.6 | 12.1 | 1.4 | 11.5 | |
| | | | | S | 104 | 9 | 90 | 95 | 102 | 118 | 264 | 23 | | | | | |
| | 2-0 | 30 | 6.5 | 0.5 | O | 57 | 76 | 20 | 22 | 38 | 142 | 1,738 | 119 | 2.0 | 18.7 | 1.4 | 9.9 |
| | | | | | S | 28 | 4 | 19 | 23 | 27 | 35 | 67 | 12 | | | | |
| | | 30 | 6.5 | 2.0 | O | 146 | 279 | 27 | 38 | 90 | 354 | 5,386 | 317 | 4.1 | 48.2 | 2.7 | 17.6 |
| | | | | | S | 35 | 6 | 22 | 28 | 34 | 46 | 79 | 18 | | | | |
| 30 | | 6.5 | 4.0 | O | 162 | 71 | 60 | 79 | 142 | 302 | 553 | 223 | 1.9 | 3.9 | 1.7 | 4.3 | |
| | | | | S | 87 | 18 | 45 | 64 | 84 | 116 | 302 | 52 | | | | | |
| 15 | | 6.5 | 0.5 | O | 268 | 44 | 211 | 218 | 258 | 345 | 517 | 126 | 1.0 | 1.1 | 1.0 | 1.0 | |
| | | | | S | 257 | 39 | 139 | 198 | 254 | 325 | 392 | 127 | | | | | |
| 22 | | 6.5 | 0.5 | O | 108 | 24 | 73 | 77 | 104 | 150 | 202 | 73 | 1.7 | 2.6 | 1.7 | 2.5 | |
| | | | | S | 63 | 9 | 37 | 49 | 63 | 78 | 94 | 29 | | | | | |
| 22 | | 6.5 | 2.0 | O | 170 | 135 | 61 | 84 | 142 | 344 | 3,692 | 260 | 2.0 | 8.9 | 1.7 | 5.4 | |
| | | | | S | 104 | 9 | 90 | 95 | 102 | 118 | 264 | 23 | | | | | |

^a Observed TTD.

^b Simulated TTD.

^c 4-2, using 10,000 spores/well to simulate 100 spores/well; 4-0, using 10,000 spores/well to simulate 1 spore/well; 2-0, using 100 spores/well to simulate 1 spore/well.

^d Min, minimum; Max, maximum.

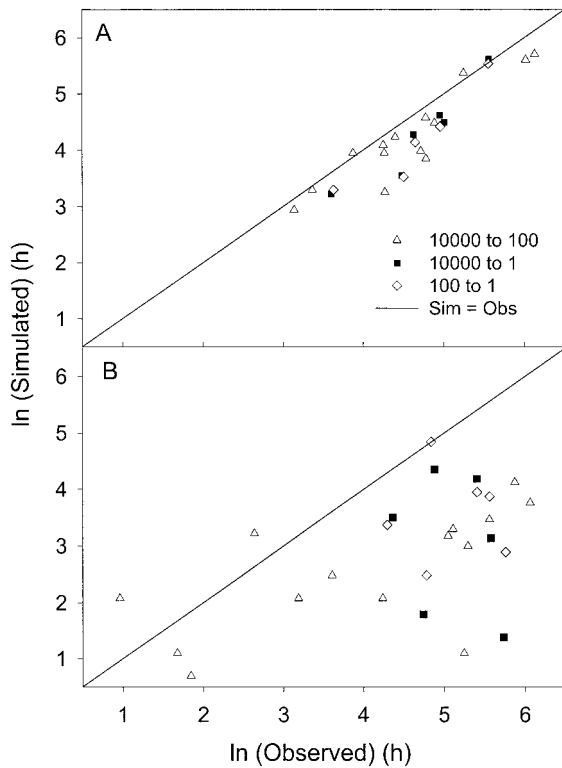


FIG. 2. Plot of the median (A) and 90% range (B) of the simulated and observed TTDs at various conditions.

and operate. Running such a system requires a considerable amount of financial support and, in many cases, a very long time. On the other hand, simulation is the natural approach to carry out this methodology. Independence between spores and cells on the spreadsheet is the default setting. A recalculation of the spreadsheet (a replicate) takes less than 1 s. Any one simulation can be finished in 1 h when the simulation settings are optimized. Therefore, computer simulation is the best (and probably only) technique for realizing the assumption of independence.

Choice of the best distribution. The choice of the best distributions was based on both the fitness of statistics and visual observations. For continuous data, the Anderson-Darling (A-D) and Kolmogorov-Smirnov (K-S) tests were given more weight than the chi-square test since these two statistics were more powerful and less arbitrary (17). The chi-square statistic is probably the oldest (14) and most widely used of all goodness-of-fit statistics. Sometimes the chi-square test can result in completely different conclusions depending on the bin number chosen. Presently, no prescription is guaranteed to produce a valid and unbiased result. When setting up the program parameters in BestFit, the equal probability option instead of the equal bin option was chosen for chi-square binning. With this specification, bins in chi-square test have equal probability intervals for each fitted distribution instead of equal lengths for the input data. To some extent, this method can remove the arbitrariness of bin selection in a chi-square test, which can be a major drawback (2, 11). While the chi-square test is essentially a comparison between a histogram of the data and the

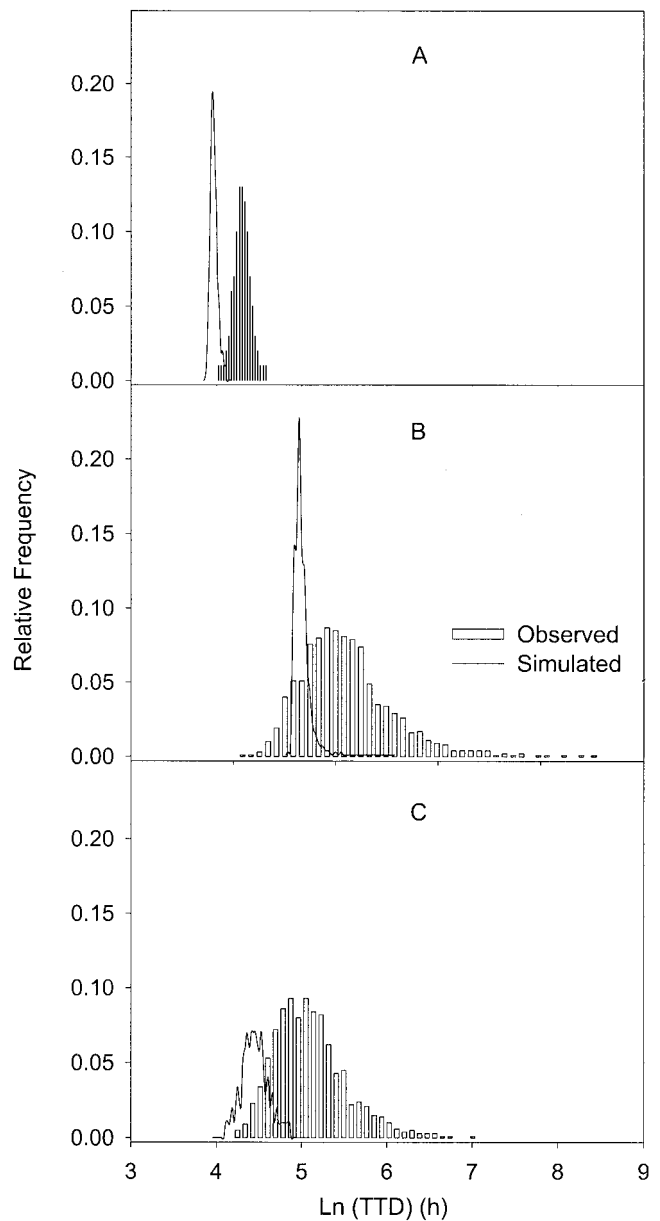


FIG. 3. The observed and simulated TTDs at 22°C, pH 6.5, and 2% salt. Three different spore scenarios are presented in different panels: (A) 10,000 spores simulating 100 spores; (B) 10,000 spores simulating 1 spore; (C) 100 spores simulating 1 spore.

density function of the fitted distribution, the K-S method compares the empirical cumulative distribution derived from the data and the cumulative distribution of the fitted function. Therefore, no grouping of the data or bin size specification was needed. The K-S statistics find the largest difference between the two distributions, and thus, it focuses in the middle of the distribution. The A-D statistics are a modification of the K-S statistics obtained by putting more weight at the two tails (probability tends to 0 or 1); therefore, it amplifies the discrepancies at the two ends (1). In most situations, the A-D and K-S statistics gave quite similar results while the chi-square statistics resulted in very different findings. Visual observations were

TABLE 2. Simulated TTDs when lag times and doubling times from a higher inoculum size were used to simulate the TTDs at a lower inoculum size, keeping the environmental factors the same^a

| Spore scenario ^b | Variable | | | Simulated TTD (h) | | | | | | | | Comparison | |
|-----------------------------|-----------|-----|----------|-------------------|-----|-------------------------|-----|-----|-----|-------|-------------------------------------|------------------|------|
| | Temp (°C) | pH | NaCl (%) | Mean | SD | Percentile ^c | | | | | Day _{95%} Sim ^d | Obs ^e | |
| | | | | | | Min | 5 | 50 | 95 | Max | | | 95-5 |
| 4-2 | 30 | 5.5 | 2.0 | 87 | 10 | 60 | 73 | 87 | 103 | 133 | 30 | 4 | 0 |
| | 22 | 6.5 | 4.0 | 159 | 16 | 102 | 131 | 159 | 183 | 215 | 52 | 8 | 0 |
| | 22 | 5.5 | 0.5 | 66 | 11 | 39 | 48 | 65 | 84 | 138 | 36 | 4 | 0 |
| | 22 | 5.5 | 2.0 | 136 | 5 | 124 | 128 | 135 | 145 | 153 | 17 | 6 | 0 |
| 4-0 | 30 | 6.0 | 0.5 | 34 | 2 | 31 | 32 | 34 | 37 | 40 | 5 | 2 | 2 |
| | 30 | 6.0 | 2.0 | 90 | 18 | 41 | 61 | 89 | 120 | 161 | 59 | 5 | 7 |
| | 30 | 6.0 | 4.0 | 138 | 23 | 85 | 109 | 134 | 183 | 236 | 74 | 8 | 2 |
| | 30 | 5.5 | 0.5 | 86 | 22 | 35 | 54 | 84 | 124 | 183 | 70 | 5 | 9 |
| | 30 | 5.5 | 2.0 | 136 | 26 | 78 | 95 | 135 | 182 | 221 | 87 | 8 | 5 |
| | 22 | 6.5 | 4.0 | 254 | 74 | 130 | 173 | 237 | 386 | 735 | 213 | 16 | 0 |
| | 22 | 6.0 | 0.5 | 90 | 14 | 62 | 74 | 87 | 116 | 210 | 42 | 5 | 0 |
| | 22 | 6.0 | 2.0 | 129 | 29 | 56 | 96 | 124 | 180 | 437 | 84 | 8 | 0 |
| | 22 | 5.5 | 0.5 | 139 | 58 | 52 | 76 | 127 | 250 | 498 | 174 | 10 | 0 |
| | 22 | 5.5 | 2.0 | 204 | 68 | 129 | 143 | 184 | 340 | 669 | 197 | 14 | 0 |
| | 15 | 6.5 | 2.0 | 388 | 37 | 251 | 329 | 386 | 450 | 552 | 121 | 19 | 0 |
| | 15 | 6.0 | 0.5 | 378 | 61 | 241 | 308 | 365 | 486 | 802 | 178 | 20 | 7 |
| 2-0 | 30 | 6.0 | 0.5 | 77 | 47 | 16 | 31 | 65 | 168 | 358 | 137 | 7 | 16 |
| | 30 | 6.0 | 2.0 | 105 | 63 | 26 | 40 | 89 | 227 | 472 | 187 | 9 | 11 |
| | 30 | 6.0 | 4.0 | 134 | 71 | 65 | 77 | 112 | 278 | 546 | 201 | 12 | 7 |
| | 30 | 5.5 | 0.5 | 116 | 53 | 19 | 47 | 107 | 215 | 524 | 168 | 9 | 9 |
| | 22 | 6.0 | 0.5 | 88 | 31 | 47 | 56 | 79 | 146 | 347 | 90 | 6 | 0 |
| | 22 | 6.0 | 2.0 | 141 | 45 | 57 | 86 | 132 | 222 | 469 | 136 | 9 | 0 |
| | 15 | 6.5 | 2.0 | 485 | 120 | 214 | 306 | 464 | 716 | 716 | 410 | 30 | 0 |
| | 15 | 6.0 | 0.5 | 433 | 130 | 128 | 258 | 414 | 674 | 1,289 | 416 | 28 | 10 |

^a Kinetic data at the target inoculum size are not available; therefore, the observed percent growth positive at the 95th percentile of the simulated TTDs is presented instead.

^b 4-2, using 10,000 spores/well to simulate 100 spores/well; 4-0, using 10,000 spores/well to simulate 1 spore/well; 2-0, using 100 spores/well to simulate 1 spore/well.

^c Min, minimum; Max, maximum.

^d Time in days when 95% of the simulated wells showed growth.

^e The actual percentage of growth observed at day_{95%} Sim.

made with the histogram of the data overlaid with the fitted distribution. Special attention was paid to the mode of the fitted distribution compared to that of the histogram and the percentage covered by the two tails. Linearity of the quantile-quantile plot and probability-probability plot was also checked. The quantile-quantile plot highlights the difference at the tails of the data and the fitted distribution. The probability-probability plot, on the other hand, amplifies the difference in the middle (11). The distribution with fewer parameters was chosen when both fit the data very well.

Some conditions have less than 80% growth observed at the end of the continuous observation period. A distribution was found for these conditions, although the resulting distribution was seriously biased for the fast-germinating spores. Whenever used in the simulation or in a comparison of the observed and simulated TTDs, these conditions were given special attention.

Spore number distributions. The Poisson parameter λ is the mean of the distribution (also the variance). The fitted Poisson distribution with a λ of 2.08 indicates that the average number of spores/well is 2 when the target inoculum size is 1 spore/well. This is a direct result of the modification in the experimental procedure to reduce the chance of a well getting no spore (21, 22).

When a variable is specified as discrete, each distinctive value of this variable is treated separately. Plate counts of target 100 spores/well have more than 100 distinctive values,

resulting in a large number of categories and rendering all discrete distributions unacceptable. The central-limit theorem states that when the number of observations is very large, the Poisson distribution approaches the normal distribution (15). More than 900 plate count observations are included for a target of ~100 spores/well; therefore, the normal distribution can be used in place of the Poisson distribution. While the normal distribution was not chosen as best by any fit statistics, when observed graphically, however, the normal distribution appeared to fit the histogram equally as well as the best choice (data not shown). Because of its universal application in many fields and its ease of use, the normal distribution was chosen for target 100 spores/well. In a recent publication, Corradini et al. studied the applicability of eight different probability distributions to bacterial plate counts in three different food products by using maximum likelihood estimation (6). They also assumed that a continuous distribution could be used to describe the plate count as long as the number of observations was large. Theoretically, the distribution for a nonnegative variable like plate count should be skewed to the right, but Corradini et al. found that the actual distributions are not extremely asymmetric. Occasionally, the normal distribution fit the observed values even better than those right-skewed distributions.

Meaning of the simulation results. The lag times and doubling times calculated from turbidometric measurement at a

higher spore concentration were used to simulate the TTDs of a lower spore concentration. The results show that simulated TTDs are smaller and more homogeneous than the observed TTDs, indicating that interactions exist between the spores or between cells and spores. Additionally, there are differences between the three spore scenarios: 10,000 spores simulating 1 or 100 spores and 100 spores simulating 1 spore. The largest difference between the variance of the simulated and observed TTDs occurs when 10,000 spores are used to simulate 1 spore, which is also the largest difference between the simulating inoculum and the target inoculum. The other two spore scenarios have the same ratio of simulating inoculum and target inoculum (simulating inoculum/target inoculum = 100 in both cases), and the difference between the variance of the simulated and observed TTDs is also similar (equation 2). R_m was the largest again when using 10,000 spores to simulate 1 spore, followed by that when using 100 spores to simulate 1 spore, and the smallest was obtained when using 10,000 spores to simulate 100 spores. The effect of the categorical variable (Dummy₁ and Dummy₂) on the ratio of median, however, is only marginally significant at 0.1 levels. It is nonetheless interesting that the ratios of the median and 90% range are larger when using 100 spores to simulate 1 spore than those when using 10,000 spores to simulate 100 spores, since both use results from an inoculum level 100 times higher than that of the target inoculum. This suggests that the possible cutoff point of the low and high inoculum concentrations is a number lower than 100 spores. Since the 100:1 spore simulation crosses the boundary, the difference between the simulated and observed TTDs is larger. The cutoff point implied here is different from that by the modeling approach, which suggests a level between 100 and 1,000 spores (21).

Although this research disapproves the independence assumption between spores, it is still premature to conclude at this point that signaling exists between spores during germination without any physical evidence of the putative signaling molecule. More research using the microbiological and biochemical approaches is required to further the investigation before a mechanistic explanation of the germination process can be reached.

Conclusions. The simulation approach was used to investigate the hypothesis that communication exists between spores or between spores and cells during germination, outgrowth, and growth. Growth parameters at a higher inoculum were used to simulate the TTD at a lower inoculum size, assuming spores were independent from each other. The simulated TTDs were smaller and more homogenous than the observed TTDs. When the difference between the simulating and target inoculum was the largest, the difference in the variance of the simulated and observed TTDs was also the largest. Results from this approach agree with our previous results by using

mathematical models that spores are not independent from each other during germination, outgrowth, and growth.

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