

***Bacillus megaterium* spore germination is influenced by inoculum size**

M.L. Caipo*, S. Duffy, L. Zhao and D.W. Schaffner

Food Risk Analysis Initiative, Rutgers, the State University of New Jersey, New Brunswick, NJ, USA

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Aims: The effect of spore density on the germination (time-to-germination, percent germination) of *Bacillus megaterium* spores on tryptic soy agar was determined using direct microscopic observation.

Methods and Results: Inoculum size varied from approximately 10^3 to 10^8 cfu ml⁻¹ in a medium where pH = 7 and the sodium chloride concentration was 0.5% w/v. Inoculum size was measured by global inoculum size (the concentration of spores on a microscope slide) and local inoculum size (the number of spores observed in a given microscope field of observation). Both global and local inoculum sizes had a significant effect on time-to-germination (TTG), but only the global inoculum size influenced the percentage germination of the observed spores.

Conclusions: These results show that higher concentrations of *Bacillus megaterium* spores encourage more rapid germination and more spores to germinate, indicating that low spore populations do not behave similarly to high spore populations.

Significance and Impact of the Study: A likely explanation for the inoculum size-dependency of germination would be chemical signalling or quorum sensing between *Bacillus* spores.

INTRODUCTION

Predictive microbiology is based on the notion that bacteria behave reproducibly, and this behaviour can be described as a function of environmental factors (e.g. pH, organic acid concentration) (McMeekin *et al.* 1993). Since foods can contain low numbers of some bacteria (Dodds 1993), the effect of inoculum size is important in predictive food microbiology. An inverse effect of inoculum size has been observed on the lag time of *Listeria monocytogenes* (Augustin *et al.* 2000), the time to detection of *Clostridium botulinum* (Zhao *et al.* 2000) and the germination rate of *Bacillus stearothermophilus* (Llaudes *et al.* 2001). These studies used indirect methods of observation (turbidimetric measurements, colour change) to examine the effect of varying inoculum sizes, but no

one has examined this effect using direct microscopic observation.

All spore populations contain a small number of spores that appear to be resistant to germination (Gould 1970). Researchers have proposed models for spore germination in an attempt to explain the heterogeneous distribution of dormancy in spore populations. Woese *et al.* suggested that spores may contain a varying number of 'germination molecules' in low numbers per spore (Woese *et al.* 1968). In this sense, the apparent spore germination of a population may be related to inoculum size, since at higher spore densities a greater percentage of rapidly-germinating spores will be present.

The rate of spore germination, and therefore time-to-germination, is known to be influenced by factors such as pH, temperature, heat treatment, germinant concentration and medium composition (Moir 1992). In this study, those factors were held constant while the inoculum size of *Bacillus megaterium* spores was varied to assess the effect of spore numbers on the resulting germination behaviour. The extent of germination and time-to-germination were measured through direct microscopy and mathematically modelled to gain further insight into the relationship between inoculum size and germination.

Correspondence to: D.W. Schaffner, Food Risk Analysis Initiative, 65 Dudley Road, Rutgers, the State University of New Jersey, New Brunswick, NJ 08901-8520, USA (e-mail: schaffner@aesop.rutgers.edu).

*Present address: Facultad de Ingeniería Agroindustrial, Universidad San Ignacio de Loyola, Av. La Fontana 550, La Molina, Lima 12, Peru.

MATERIALS AND METHODS

Micro-organism

Bacillus megaterium ATCC 14581 was chosen for this research based on its ability to grow at ambient temperature and because its large size facilitates direct microscopic observation.

Spore preparation

Spores were prepared by using $2 \times$ SG sporulation agar because it is reported to give high spore densities (Nicholson and Setlow 1990). All ingredients were obtained from Sigma unless otherwise noted. The medium consisted of: 16.0 g nutrient broth (Difco), 2.0 g KCl, 0.5 g MgSO₄ and 17.0 g agar in 1 litre of water. The medium pH was adjusted to 7, then autoclaved and cooled. The following components were added after cooling: 1.0 ml 1 mol l⁻¹ Ca(NO₃)₂; 1.0 ml 0.1 mol l⁻¹ MnCl₂·4H₂O, 1.0 ml 0.001 mol l⁻¹ FeSO₄ and 2.0 ml 50% glucose (w/v). Aliquots (30 ml) of the sporulation medium were poured into disposable Roux bottles (Corning Glass, Corning, NY, USA) equipped with a 0.2 µm membrane to allow air in but keep contaminants out. When the sporulation agar solidified and dried, 3 ml of fresh *Bacillus megaterium* cells in tryptic soy broth (Difco) in exponential phase were added and spread over the agar surface inside each container. The containers were incubated at 31°C and approximately 5 days later, the spores were harvested by agitation with glass beads in sterile water. This mixture of spores and cells was centrifuged at 1300 g for 20 min, washed with ¼ volume 1 mol l⁻¹ KCl/0.5 mol l⁻¹ NaCl, rinsed with sterile deionized water, then washed with 1 mol l⁻¹ NaCl, and rinsed with sterile deionized water again. Lysozyme (50 µg ml⁻¹) was added in the presence of buffer (¼ volume TrisCl, 0.05 mol l⁻¹, pH 7.2), and incubated with constant stirring at 4°C overnight. Lysozyme was removed by centrifuging 8 times (at 1300 g) and washing with sterile deionized water. The resulting spore slurry was stored at 4°C.

Microscopy method

The inoculum sizes varied from about 10 to 1 000 000 spores per microscope slide. Sodium chloride concentration (0.5% w/v) and pH (7) were kept constant. Phase contrast optics were used to observe the change from dormant spore to vegetative cell to calculate spore germination rates. A 10 µl sample of heat-activated (80°C × 20 min) spores (approximately 10³ to 10⁸ cfu ml⁻¹) was smeared on a Probe Clip imaging chamber (Sigma Chemical Co., St. Louis, MO, USA) and air-dried to form a thin film. A 50 µl aliquot of warm tryptic soy agar (TSA, Difco) was added to this spore film, and a clean glass slide was used to

mount the imaging chamber for observation at room temperature under the microscope (Olympus BH-2, Tokyo, Japan) at 500× total magnification. Selected microscope fields (containing from one to 54 spores) were photographed at appropriate time intervals. Spore germination results were calculated from the photographs by counting the number of phase bright spores and phase dark spores over time.

Statistical analysis

Global inoculum size ranged from about 10¹ to about 10⁶ cfu 10 µl sample⁻¹ as determined by plate count, while local inoculum size was obtained by counting the number of spores in a given microscope field. The correlation between global and local inoculum sizes was determined with the 'correlation' function in Excel (Microsoft, Redmond, WA, USA).

Time-to-germination (TTG) was calculated for each spore in minutes. The final time was recorded, and a notation was made that this data point was right-censored for the spores that did not germinate in the observed time frame. The SAS Lifereg procedure (SAS Institute Inc., Cary, NC, USA) was used to model the data with both linear and quadratic terms for each inoculum size. The Weibull distribution was used for the error term.

Percent germination was calculated for each microscope field observed and modelled using logistic regression with Splus 2000 software (MathSoft, Inc., Seattle, WA, USA). The full equation used for the logistic regression is shown in Equation 1, and stepwise regression eliminated any insignificant terms. Percent germination is expressed as a proportion of spores between 0 and 1.

RESULTS

The phase change (bright to dark) indicating germination was observed for the smaller inoculum sizes (10¹ to 10³ spores per 10 µl sample⁻¹, global inoculum sizes of 1 to 3) and the larger inoculum sizes (global inoculum sizes of 4 to 6), and the results are shown in Figs 1 and 2. The smaller inoculum sizes (Fig. 1) exhibited more random germination behaviour than the larger inoculum sizes (Fig. 2). The lowest inoculum size, 10 spores slide⁻¹, did not show the appearance of phase dark spores in any field after 24 h (data not shown). At 100 and 1000 spores slide⁻¹ (global inoculum sizes of 2 and 3, respectively), some fields exhibited spore germination, as evidenced by phase change, whereas others did not. In none of the fields at these inoculum sizes did all of the spores germinate in the time course of these experiments.

The larger inoculum sizes, shown in Fig. 2, show at least some germination in all observed fields. When the global

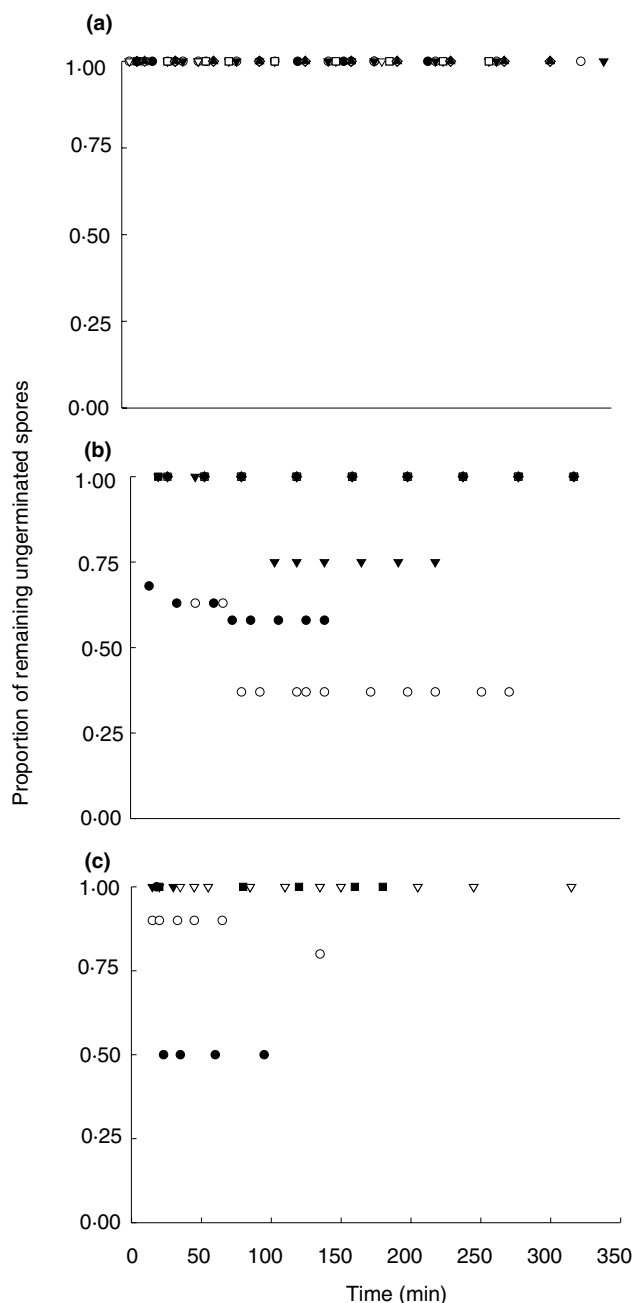


Fig. 1 The proportion of ungerminated spores observed in samples inoculated with about (a) 10^1 , (b) 10^2 and (c) 10^3 spores $10 \mu\text{l}^{-1}$ *Bacillus megaterium*. The number of spores observed in each field (local inoculum size) for global inoculum size = 1 were: (●) 1; (○) 9; (▼) 6; (▽) 3; (■) 2; (□) 3; (◆) 2; (◇) 9; (△) 1. The local inoculum sizes for global inoculum size = 2 were: (●) 19; (○) 8; (▲) 4; (▽) 1; (■) 1; (□) 1; (◆) 2. The local inoculum sizes for global inoculum size = 3 were: (●) 6; (○) 10; (▼) 6; (▽) 10; (■) 2

inoculum size was 6, nearly all of the spores germinated within 2 h. A trend can clearly be observed in Fig. 2; as the global inoculum size increases, the extent of germination

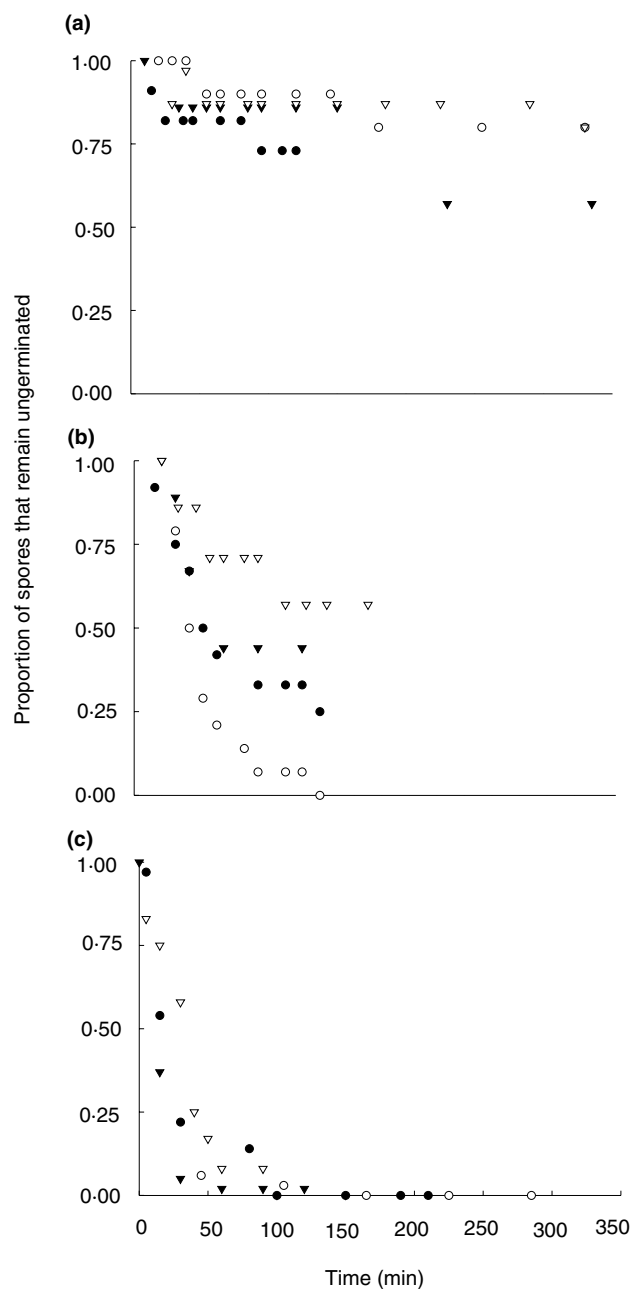


Fig. 2 The proportion of ungerminated spores observed in samples inoculated with about (a) 10^4 , (b) 10^5 and (c) 10^6 spores $10 \mu\text{l}^{-1}$ *Bacillus megaterium*. The number of spores observed in each field (local inoculum size) for global inoculum size = 4 were: (●) 11; (○) 10; (▼) 7; (▽) 15. The local inoculum sizes for global inoculum size = 5 were: (●) 12; (○) 14; (▼) 9; (▽) 7. The local inoculum sizes for global inoculum size = 6 were: (●) 37; (○) 36; (▽) 54; (▼) 12

increases and the time required for germination decreases. At the larger global inoculum sizes, the local inoculum sizes also tend to be larger. The correlation between the global and local inoculum sizes was determined to be 0.56205, but

the Lifereg analysis was not unduly affected by this correlation.

Accurate TTG data were obtained for the 200 spores that germinated in the time course of these experiments, but TTG was right-censored for the 130 spores that did not germinate (39.4%). The best-fitting Lifereg model for the TTG data (log likelihood = -203.4) was obtained when TTGs were log-transformed. The resulting model, shown in Equation 2, includes significant linear terms for both global ($P < 0.0001$) and local ($P = 0.0001$) inoculum sizes.

This model is shown graphically in Fig. 3, and the logarithmic relationship between the inoculum sizes and TTG is clear. Local inoculum size has a greater effect on the TTG at low global inoculum sizes than at the higher global inoculum sizes, where local crowding has less of an impact on TTG.

The extent of germination in the time course of these experiments was modelled using logistic regression. Local inoculum size was found to have an insignificant impact ($P = 0.3482$) on the percentage of spores that germinated by the end of observation, and was thus removed by backward stepwise regression to yield the model shown in Equation 3.

The logistic distribution is sigmoidal, so the effect of global inoculum size is non-linear (Fig. 4). It is important to note that the model does predict some germination at very low inoculum sizes (the y -intercept is positive) and does not predict 100% germination, even at a global inoculum size of 6 (10^8 cfu ml⁻¹). The model appeared to have no systematic bias in its predictions.

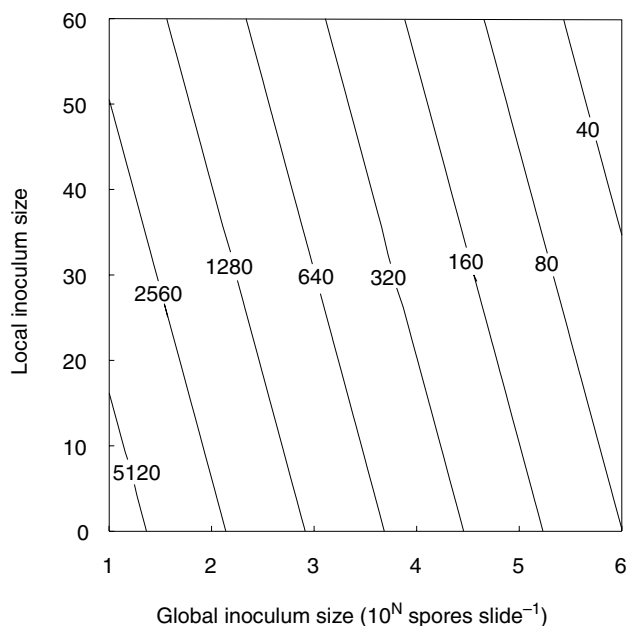


Fig. 3 Time-to-germination (TTG) of *Bacillus megaterium* as determined by global and local inoculum sizes. TTG is listed in minutes

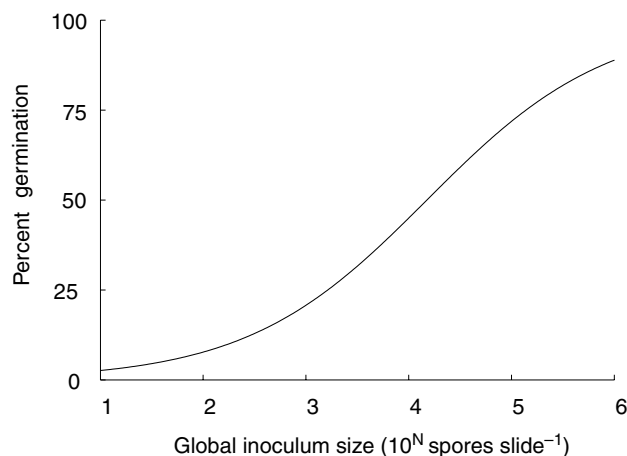


Fig. 4 Predicted extent of germination of *Bacillus megaterium* as a function of global inoculum size

DISCUSSION

Germination is usually studied as part of germination, outgrowth and lag by indirect methods such as observing turbidity (Leblanc and Lefebvre 1984; Zhao *et al.* 2000) or colour change (Llaudes *et al.* 2001). Direct microscopic observation has been used when trying to assess the efficacy of germinant molecules and environmental conditions (temperature) on time-to-germination (Billon *et al.* 1997; Thrane *et al.* 2000). Several authors have tried to model germination, either through theory or experiments (Woese *et al.* 1968; Lefebvre and Leblanc 1988). The present study combined the technique of direct microscopic observation with empirical mathematical modelling to examine the effect of inoculum size on germination.

Previously developed mathematical models for germination include an exponential distribution as a function of time (Vary and McCormick 1965), an exponential distribution dependent on the number of theoretical germinative molecules in a spore (Woese *et al.* 1968), a sigmoidal distribution based on the theoretical range of TTG ('microlag and microgermination') of a population of spores (Leblanc and Lefebvre 1984), and a biphasic sigmoidal function (Hashimoto *et al.* 1969) that could not be validated by others (Gould 1970). Our models describe germination as a function of the number of spores, rather than the presence of theoretical or added germinant molecules. Lifereg analysis was used to model TTG because of its ability to handle time-censored data, and logistic regression was chosen to model percentage of germination because logistic models are both more accurate than linear regression models in modelling percentage data and never predict outside of the 0–100% range (Zhao *et al.* 2001). The extent of germination was solely a function of global inoculum size in this study, meaning that

the proximity of spores to one another was far less important than the population density of spores. The logistic model is in agreement with the literature; using high but unspecified inoculum sizes, Billon *et al.* (1997) found that 75–95% of observed spores in a microscope field germinated, similar to the model's predictions at global inoculum sizes of 5 and 6. Both the population density and the local proximity of the spores affected the speed with which spores germinate, indicating that some aspect of local crowding encourages faster germination.

One of the limitations of our dataset is that the conditions were followed for differing amounts of time (for instance, the global = 0 slides were followed for 24 h), and this led to different right-censored TTGs for different inoculum sizes. To verify that this bias in the dataset did not harm the validity of the model or the significance of both local and global inoculum sizes, the dataset was truncated to 150 min, when most of the germination had already occurred (Figs 1 and 2). This truncated dataset was analysed using SAS Lifereg, and both variables were still highly significant (both $P < 0.0001$) in a good fitting model (data not shown). This increased our confidence in the significance of both the effects of global inoculum size and local crowding.

The present work does not conflict with the established notion that certain spores are faster germinators than others, and that there exists a distribution of TTG within a spore population (Woese *et al.* 1968; Stewart *et al.* 1981; Leblanc and Lefebvre 1984). The inherent variability of TTG in spores could offer a partial explanation to our results; the faster TTG of spores at a higher inoculum size may be due to the greater amount of fast-germinating spores at higher inoculum sizes. However, this theory does not account for the significance of the local inoculum size. The present results indicate that spore populations respond to both global population density and local crowding.

There is no doubt that spores have the ability to sense and respond to their environments (Johnstone 1994). Spores can respond to the presence of germinants (i.e. L-alanine) by germinating more rapidly (Moir 1992). However, the phenomenon of quorum sensing has not been previously associated with germination, though quorum sensing has been linked to several other phenomena in *Bacillus* spp. (Lazzazera *et al.* 1999; Prego 1999). Other work in this laboratory involving indirect observation of germination has also indicated that each spore's germination is not necessarily independent of the presence of other spores (Llaudes *et al.* 2001).

It is not a difficult conceptual leap from exogenous molecules affecting time-to-germination to some kind of intercellular communication, such as quorum sensing, where molecules are secreted by one spore (or cell) and sensed by another spore. Adapting current theories about germination to include intercellular signalling does not change their basic

premises. Woese *et al.* suggested that a critical concentration of a 'germination substance' must be reached before the irreversible process of germination begins (Woese *et al.* 1968). This theory allowed for a small number of germinant molecules per spore to help build up a sufficient supply of the germination substance, as well as for extracellular L-alanine to be an activator of an enzyme that produces the germination substance. Therefore, secreted germinant signals from other spores could serve a similar role to L-alanine in this theory, or could be the germination substance themselves. It is known that *B. megaterium* spores release significant quantities of calcium ions and dipicolinic acid (DPA) during germination, as well as free amino acids and sugars due to breakdown of the spore coat (Foster and Johnstone 1990). Since both the nutrients and chemicals are good triggers of *Bacillus* germination (Paidhungat and Setlow 2000), it is possible that one or more of these molecules is responsible for the apparent communication between spores.

Intercellular communication provides an explanation for the significant effect of local crowding on time-to-germination. A spore would be more likely to be affected by signals sent from a spore nearby than far away, as a signal molecule diffusing through the medium would be most successful at reaching spores close by. An increased local population would increase the number of germinative molecules that a spore could respond to, thus speeding up germination. Global inoculum size affects the number of spores on a slide (the population density) and therefore, the total density of germinative molecules. This would account for the greater extent of germination (and even faster TTG) at higher global inoculum sizes. It should be noted, though, that global inoculum size has a fairly strong correlation with local inoculum size for obvious reasons and therefore, it is difficult to be certain what effects each parameter has on the TTG. This hypothesis could be tested using cell sorting or other micromanipulation techniques where single spores could be placed in specific locations on a microscope slide.

Most models for spore germination involve only extracellular chemicals like L-alanine (Johnstone 1994), and do not consider germination triggers produced by the spore itself. The results of the present study indicate that there could be intercellular communication encouraging germination at higher inoculum sizes. This phenomenon should be explored at a physiological level, as modelling can only offer indirect evidence for biological phenomena.

Studies in predictive food microbiology usually use very large inoculum sizes for ease of experimentation and to reduce the effect of inherent biological variability on the results. Several studies with spore-forming bacteria have shown that at lower inoculum sizes, there is increased variability and inconsistency in time-to-toxicity (Graham *et al.* 1996), time-to-turbidity (Whiting and Oriente 1997),

time-to-detection (Zhao *et al.* 2000) and time-to-spoilage (Llaudes *et al.* 2001). The results presented here concur with the literature; *Bacillus* acts more synchronously (narrower range of TTG) at higher inoculum sizes (Fig. 2), and more randomly at lower inoculum sizes (Fig. 1). Because foods can be contaminated with low numbers of spores (Dodds 1993), predictive microbiologists may be sacrificing accuracy for ease when using large inoculum sizes.

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REFERENCES

- Augustin, J.C., Brouillaud-Delattre, A., Rosso, L. and Carlier, V. (2000) Significance of inoculum size in the lag time of *Listeria monocytogenes*. *Applied and Environmental Microbiology* **66**, 1706–1710.
- Billon, C.M.P., McKirgan, C.J., McClure, P.J. and Adair, C. (1997) The effect of temperature on the germination of single spores of *Clostridium botulinum* 62A. *Journal of Applied Microbiology* **82**, 48–56.
- Dodds, K.L. (1993) *Clostridium botulinum* in foods. In *Clostridium botulinum Ecology and Control in Foods* ed. Hauschild, A.H.W. and Dodds, K.L. pp. 53–68. New York, NY: Marcel Dekker.
- Foster, S.J. and Johnstone, K. (1990) Pulling the trigger: the mechanism of bacterial spore germination. *Molecular Microbiology* **4**, 137–141.
- Gould, G.W. (1970) Germination and the problem of dormancy. *Journal of Applied Bacteriology* **33**, 34–49.
- Graham, A.F., Mason, D.R. and Peck, M.W. (1996) Predictive model of the effect of temperature, pH and sodium chloride on growth from spores of non-proteolytic *Clostridium botulinum*. *International Journal of Food Microbiology* **31**, 69–85.
- Hashimoto, T., Frieben, W.R. and Conti, S.F. (1969) Germination of single bacterial spores. *Journal of Bacteriology* **98**, 1011–1020.
- Johnstone, K. (1994) The trigger mechanism of spore germination: current concepts. *Journal of Applied Bacteriology Symposium Supplement* **76**, 17S–24S.
- Lazzazera, B., Palmer, T., Quisel, J. and Grossman, A.D. (1999) Cell-density control of gene expression and development in *Bacillus subtilis*. In *Cell-Cell Signaling in Bacteria* ed. Dunny, G.M. and Winans, S.C. pp. 27–46. Washington D.C.: ASM Press.
- Leblanc, R. and Lefebvre, G.M. (1984) A stochastic model of bacterial spore germination. *Bulletin of Mathematical Biology* **46**, 447–460.
- Lefebvre, G.M. and Leblanc, R. (1988) The kinetics of change in bacterial spore germination. In *Physiological Models in Microbiology* ed. Bazin, M.J. and Prosser, J.I. pp. 45–71. Boca Raton, FL: CRC Press.
- Llaudes, M., Zhao, L., Duffy, S. and Schaffner, D.W. (2001) Simulation and modeling of the effect of small inoculum size on the time to spoilage by *Bacillus stearothermophilus*. *Food Microbiology* **18**, 395–405.
- McMeekin, T.A., Olley, J.N., Ross, T. and Ratkowsky, D.A. (1993) *Predictive Microbiology*. Taunton: Research Studies Press Ltd.
- Moir, A. (1992) Spore germination. In *Biology of Bacilli: Applications to Industry* ed. Doi, R.H. and McGloughlin, M. pp. 23–38. Boston: Butterworth-Heinemann.
- Nicholson, W.L. and Setlow, P. (1990) Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus* ed. Harwood, C.R. and Cutting, S.M. pp. 391–450. Chichester: John Wiley & Sons.
- Paidhungat, M. and Setlow, P. (2000) Role of ger proteins in nutrient and non nutrient triggering of spore germination in *Bacillus subtilis*. *Journal of Bacteriology* **182**, 2513–2519.
- Perego, M. (1999) Self-signaling by Phr peptides modulates *Bacillus subtilis* development. In *Cell-Cell Signaling in Bacteria* ed. Dunny, G.M. and Winans, S.C. pp. 243–258. Washington D.C.: ASM Press.
- Stewart, G.S.A.B., Johnstone, K., Hagelberg, E. and Ellar, D.J. (1981) Commitment of bacterial spores to germinate. *Biochemistry Journal* **198**, 101–106.
- Thrane, C., Olsson, S., Wolstrup, J. and Sorensen, J. (2000) Direct microscopy of *Bacillus* endospore germination in soil microcosms. *Journal of Applied Microbiology* **89**, 595–598.
- Vary, J.C. and McCormick, N.G. (1965) Kinetics of germination of aerobic *Bacillus* spores. In *Spores III* ed. Campbell, L.L. and Halvorson, H.O. pp. 188–199. Ann Arbor, MI: American Society for Microbiology.
- Whiting, R.C. and Oriente, J.C. (1997) Time-to-turbidity model for non-proteolytic type B *Clostridium botulinum*. *International Journal of Food Microbiology* **36**, 49–60.
- Woese, C.R., Vary, J.C. and Halvorson, H.O. (1968) A kinetic model for bacterial spore germination. *Proceedings of the National Academy of Sciences USA* **59**, 869–875.
- Zhao, L., Chen, Y. and Schaffner, D.W. (2001) Comparison of logistic regression and linear regression in modeling percentage data. *Applied and Environmental Microbiology* **67**, 2129–2135.
- Zhao, L., Montville, T.J. and Schaffner, D.W. (2000) Inoculum size of *Clostridium botulinum* 56A spores influence time-to-detection and percent growth-positive sample. *Journal of Food Science* **65**, 1369–1375.