Integrated Kinetic and Probabilistic Modeling of the Growth Potential of Bacterial Populations

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When bacteria are exposed to osmotic stress, some cells recover and grow, while others die or are unculturable. This leads to a viable count growth curve where the cell number decreases before the onset of the exponential growth phase. From such curves, it is impossible to estimate what proportion of the initial cells generates the growth because it leads to an ill-conditioned numerical problem. Here, we applied a combination of experimental and statistical methods, based on optical density measurements, to infer both the probability of growth and the maximum specific growth rate of the culture. We quantified the growth potential of a bacterial population as a quantity composed from the probability of growth and the “suitability” of the growing subpopulation to the new environment. We found that, for all three laboratory media studied, the probability of growth decreased while the “work to be done” by the growing subpopulation (defined as the negative logarithm of their suitability parameter) increased with NaCl concentration. The results suggest that the effect of medium on the probability of growth could be described by a simple shift parameter, a differential NaCl concentration that can be accounted for by the change in the medium composition. Finally, we highlighted the need for further understanding of the effect of the osmoprotectant glycine betaine on metabolism.
probability of growth of Salmonella enterica serovar Typhimurium under osmotic stress.

MATERIALS AND METHODS

Strain. Salmonella enterica serovar Typhimurium strain SL1344 was maintained in basic minimal medium (BMM) (10) with 40% glycerol or in tryptone soy broth (TSB) with 40% glycerol, stored at −80°C. Before each experiment, Salmonella was subcultured twice in BMM or TSB and incubated at 37°C for 7 h and 17 h.

Growth conditions. The osmotic stress of three media, Luria-Bertani medium (LB; 10 g liter−1 tryptone, 5 g liter−1 yeast extract, 10 g liter−1 NaCl), BMM, and BMM with glycine betaine (150 μM glycine betaine [Sigma Chemical Co., United Kingdom] added as an osmoprotectant), was increased by adding NaCl; LB with up to 55 g liter−1 NaCl added (equivalent to 6.5% total NaCl), BMM with up to 35 g liter−1 NaCl added, and BMM plus betaine with up to 55 g liter−1 NaCl added.

Growth curves. Cultures (17 h at 37°C) in TSB and BMM were diluted and inoculated into 100 ml of prewarmed (37°C) growth medium, from TSB to LB and from BMM to BMM both with and without glycine betaine. Sampling was carried out at appropriate intervals up to 15 days according to the growth conditions. The growth curves were measured by plate counts on tryptone soy agar (TSA) (CM0131; Oxoid). The experiments were repeated, and the results reported are averages for replicate samples.

Bioscreen experiments. Salmonella Typhimurium SL1344 was grown from frozen stock in BMM or TSB plus 40% glycerol for 7 h at 37°C. An inoculum of 25 μl was placed in 10 ml fresh BMM or TSB and incubated at 37°C for 17 h. Based on plate counts of test cultures in TSB or BMM incubated at 37°C for 17 h, a dilution series was devised to ultimately give a concentration of 1 cell in 50 μl. For the Bioscreen experiments, cultures were diluted in the test medium, e.g., BMM plus NaCl and glycine betaine or LB plus NaCl, by serial 10-fold dilutions, and then these were further diluted 1 in 2 and, for the highest 10-fold dilution, up to 1 in 64. Wells of a pair of Bioscreen plates (100 wells on each plate) were then filled with 50 μl diluted cells and 350 μl test medium. Each well of the first plate was inoculated with a target concentration of 1 cell per well. This was obtained by a series of binary dilutions. On the other plate, 50 wells were inoculated with ca. 2 cells, 25 wells with 4 cells, 12 with 8 cells, and 5 with 16 cells. These cultures were suitable for studying the stochastic birth/death process of single (or at least few) cells. The remaining 8 wells of this second plate represented population kinetics: they were inoculated with ca. 2 cells, 25 wells with 4 cells, 12 with 8 cells, and 5 with 16 cells. Plates were incubated in the Bioscreen at 37°C for up to 10 days. The number of cells inoculated into each well was estimated from plate counts of the highest 10-fold dilution.

Estimation of the probability of growth for a single cell (p0). The total number of cells, p0, inoculated in the 100 wells of the first microtiter plate, was estimated by plate counts. The aim was to obtain ca. 1-cell/well via dilutions, so we made sure that p0 was around 100. The relative standard deviation (RSD) of the plate count method is usually ca. 10 to 15% (1). As shown in Table A1, our plate count accuracy was 10% or less, so we took RSD[p0] to be ~<0.01.

After incubation in the Bioscreen, the number of negative wells (i.e., where no growth was detected) was recorded. Negative wells could appear only if the number of initial cells or the probability of growth was low. The number of wells in a well follows the Poisson distribution with the parameter p = p0/n. The expected total number of growing cells on the plate is

$$n \ln \left( \frac{n}{w_0} \right)$$

where w0 is the number of negative wells (where no growth was observed). This formula is meaningful only for 0 < w0 < n.

An estimator for the probability of growth of a single cell is

$$p_0 = \min \left( n \ln \left( \frac{n}{w_0} \right) \right) \left( \frac{1}{p_0} \right)$$

The method of C. Dupont and J. C. Augustin (15) was based on the same formula. Note that, at our p0 ~100-cell/plate target concentration, p0 < 1 holds only if w0 > n/e, where e equals exp(1) = 2.718. Therefore, we included only experiments where 37 < w0 < n = 100.

In Appendix, we provide a formula for the RSD2 of the numerator of p0. Using the result that the RSD2 of the ratio of independent variables can be estimated by the sum of the RSD2 values of the numerator and denominator (16), the accuracy of the above p0 statistic can be approximated by

$$\frac{1}{\mu} = \frac{RSD(p_0)^2}{\ln (n - \ln w_0)}$$

The error bars on Fig. 1 are calculated from the above estimation. A simulation study was also carried out to show that this is an accurate estimation of the expected relative error of p0 (see Fig. A1).

Estimation of the maximum specific growth rate, μ, and the physiological state, α0, from OD detection time data. From an optical density (OD) curve of growth in a well, let Tdet denote the time necessary to reach a detection level, which we set as an ODdet of 0.25. The Tdet detection times were estimated for wells with high inocula. The maximum specific growth rate was estimated by the negative reciprocal of the differential quotient

$$-\frac{1}{\mu} = \frac{dT_{det}}{d \ln \text{inoc}}$$

using only the last 8 wells where the expected number of inoculated cells was more than 30 (14). The physiological state of the growing subpopulation, α0, was determined by averaging the transformed Tdet values of the other 192 wells, as in reference 14.

RESULTS

Primary model: growth response parameters obtained from Bioscreen experiments. Table 1 shows the probability of growth for single cells of Salmonella, the physiological state, and the max-
TABLE 1 Estimations of growth response parameters obtained from Bioscreen experiments at different NaCl concentrations in three different media

| Medium         | s (%) | $p_0$  | $\alpha$ | $\ln \mu$ (h$^{-1}$) |
|----------------|-------|--------|----------|----------------------|
| LB             | 4.0   | 1.00   | 0.0468   | 1.100                |
|                | 5.0   | 0.98   | 0.0185   | 0.740                |
|                | 5.5   | 0.88   | 0.0165   | 0.590                |
|                | 6.5   | 0.20   | 0.0013   | 0.330                |
| BMM + betaine  | 3.0   | 0.93   | 0.0418   | 0.474                |
|                | 3.5   | 0.98   | 0.0365   | 0.384                |
|                | 4.0   | 0.69   | 0.3208   | 0.350                |
|                | 4.5   | 0.35   | 0.187    | 0.270                |
| BMM            | 2.0   | 0.89   | 0.0199   | 0.445                |
|                | 2.5   | 0.34   | 0.0342   | 0.290                |
|                | 3.0   | 0.60   | 0.0036   | 0.232                |
|                | 3.5   | 0.03   | ND$^d$   | 0.200                |
|                | 4.0   | 0.01   | ND$^d$   | 0.140                |

$^a$ Physiological state of the growing subpopulation (17).

$^b$ Probability of growth.

$^c$ Natural log of the specific growth rate.

$^d$ ND, not determined. The physiological state could not be evaluated in these conditions because there were not enough data due to the low probability of growth.

Comparison of the response parameters obtained in the Bioscreen and by plate counts. From the secondary models described above, growth curves were generated assuming that the growing subpopulation grows according to the model of Baranyi and Roberts (17) and that the nongrowing one follows linear death kinetics. The maximum population density was considered a constant, $5 \times 10^6$ CFU ml$^{-1}$ in all cases. The predictions, based on Bioscreen data, were compared to growth curves generated by plate counts.
The results are shown in Fig. 4. The predictions were mostly in agreement with the plate count measurements except for the 2.5% NaCl concentration in BMM, where the lag time predicted was longer than that measured by plate count. The plate count was almost identical for 3 and 3.5% NaCl, though the Bioscreen results predicted ca. 20% less growth. At high salt concentrations, 5 and 5.25% NaCl in BMM with glycine betaine, we could not generate Bioscreen data, presumably due to the very small probability of growth. Therefore, the prediction at 5.25% NaCl is an extrapolation and, not surprisingly, differs from the data measured by plate counts (Fig. 4d).

**DISCUSSION**

**Growth rate determined in the Bioscreen and by plate count.** The measurement of bacterial kinetics by optical density has been widely studied. To estimate the specific growth rate by our method using Bioscreen data, an important condition is that the detection level be reached in the exponential phase and that the OD curves from different inocula be parallel. In this case, the method is robust, as shown by references 14, 18, and 19. Our validation data corroborate these findings; as shown in Fig. 5, there is no significant difference in the specific growth rate determined by viable counts and that determined by the Bioscreen, when the cells grow in BMM and in LB. However, with glycine betaine added to BMM, there is a systematic bias of more than 20%.

We observed the same difference (20) between the specific growth rate estimations, when *Escherichia coli* was grown under osmotic stress with and without glycine betaine or choline in the minimal medium. In a previous publication (20), we showed that the metabolic response of *E. coli* to osmotic stress in the presence of these osmoprotectants switched at a threshold NaCl concentra-
The procedure elaborated in reference 14 was valid for an environment where all cells grew ($p_0 = 1$), based on the mathematically proved theorem that, under certain homogeneity conditions, the average, $\alpha_0$, is independent of the inoculum. This parameter is convenient to quantify the physiological state, a kind of quantification of how suitable the growing cells are to the environment.

The expression $p_0 = \alpha_0$, $p_0$ is a parameter quantifying the growth potential of the population. The natural logarithm of its first component, $h_0 = -\ln(p_0)$, has been used by several authors to quantify the "work to be done" by the growing cells. The second part takes into account that in fact only a fraction of the initial cell population is producing the exponential growth.

The $h_0$ work-to-be-done parameter has been shown to be independent of the growth conditions if the cells are pretreated in a systematic way for stresses like temperature (24) but to increase with osmotic stress (10, 25). Figure 3 suggests that both $h_0$ and $-\ln(p_0)$ increase, so both $\alpha_0$ and $p_0$ decrease with stress, but the correlation was weak ($P > 0.1$), and for the sake of simplicity, $h_0$ was taken as a constant in the predictions. More data would be necessary for a decisive analysis.

**Effect of growth medium on the growth response parameters.** A quantification of bias is often used to compare the effect of the growth medium on the growth rate (8, 26). Here, we observed a systematic bias between the different media, although it depended on the measurement method; it was not the same when measured in the Bioscreen as when measured by plate count. A similar systematic shift could be observed for the probability of growth between the different media. In fact, this may be reformulated, based on a generalized z value concept, proposed by Pin et al. (27), as follows: in terms of probability of growth, the addition of glycine betaine to minimal medium is equivalent, at least in the studied region of NaCl, to a $z$ value of $-1.6\%$ NaCl, or a change from minimal medium to LB corresponds to a $z$ value of $-3.5\%$ NaCl. In terms of growth rate, the $z$ values as measured in the Bioscreen would be $-1.6\%$ and $-4.2\%$ NaCl, respectively. These concepts could help to develop useful interpretations for the food industry, where NaCl, a widely used preservative, needs to be reduced because of health issues.

**Conclusions.** In this study, we have shown that the introduced quantification of the growth potential of a bacterial culture, consisting of the proportion of the growing subpopulation (identified by the probability of growth for a single cell) and the suitability of these growing cells, is measurable by optical density experiments, in parallel with the specific growth rate of the population. These experiments lead to a robust description of the effect of rich and minimal media, with and without osmoprotectant, on the probability of growth of Salmonella under osmotic stress. A simple shift parameter of the added NaCl concentration answered the question of what amount of NaCl can be accounted for by adding an osmoprotectant. We also highlighted a gap in our biological understanding of the mechanism by which the osmoprotectant glycine betaine works, a typical area where systems biology approaches should be integrated in predictive modeling in food (28, 29).

It is important to see that, unlike the time to division for a single cell, the growth potential of an inoculated cell can be inferred retrospectively only, from the time when its isogenic descendants are growing exponentially. It can be envisaged as an environment- and history-dependent parameter encoded in the
biochemical network of that initial single cell. The situation is similar to the specific growth rate that can be observed at the population level only; it is in fact a parameter derivable from the probability distribution of single-cell generation times.

The quantification, measurement, and modeling of growth potential of food-borne pathogens have special importance in quantitative microbial risk assessment, to which we hope to contribute with the present study.

APPENDIX

It can be readily seen that the number of growing cells follow the Poisson distribution with the \( p_0 \) mean value, where \( p \) is the average number of initial cells in a well and \( p_0 \) is the probability of growth for a cell.

The number of negative wells on a plate, \( w_p \), can be conceived as the number of failures in a Bernoulli experiment of \( n \) trials, with individual probabilities of \( e^{-n p} \). For the variance of their ratio,

\[
\text{Var} \left[ \frac{w_p}{n} \right] = \frac{e^{-n p}(1 - e^{-n p})}{n}
\]  

(A1)

From the first-order approximation of the natural log function around \( e^{-n p} \):

\[
\text{Var} \left[ \ln \frac{n}{w_0} \right] = \text{Var} \left[ \ln \frac{w_p}{n} \right] \approx e^{p_0} \text{Var} \left[ \frac{w_p}{n} \right] \approx \frac{e^{p_0} - 1}{n}
\]

(A2)

RSD\(^2\) \( \left[ \ln \frac{w_p}{n} \right] \approx \frac{1}{w_0} \frac{1}{n} \frac{1}{\text{Var} \left[ \frac{w_p}{n} \right]} \) \( \approx \frac{1}{w_0} \frac{1}{n} \left( \frac{\text{Var} \left[ \frac{w_p}{n} \right]}{\text{Var} \left[ \frac{w_p}{n} \right]} \right) \) \( \approx \frac{1}{w_0} \frac{1}{n} \) \( \approx \frac{1}{w_0} \frac{1}{n} \) \( \frac{1}{(n - \ln w_0)^2} \)

(A3)

Using \( p \cdot p_0 = \ln n - \ln w_0 \), a practical approximation can be derived:

\[
\text{RSD}^2 \left[ \ln \frac{w_p}{n} \right] \approx \frac{1}{w_0} \frac{1}{n} \left( \frac{\text{Var} \left[ \frac{w_p}{n} \right]}{\text{Var} \left[ \frac{w_p}{n} \right]} \right)
\]

(A4)

an accuracy estimation can be obtained as

\[
\text{RSD}^2 \left[ \frac{w_p}{n} \right] = 0.01 + \frac{1}{w_0} \frac{1}{n} \left( \frac{\text{Var} \left[ \frac{w_p}{n} \right]}{\text{Var} \left[ \frac{w_p}{n} \right]} \right)
\]

(A5)

This estimation was validated by a simulation study (Fig. A1).

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TABLE A1 Three replicate plate counts to estimate cell concentration

| Plate count (colonies/100 μl) in replicate | Medium | s (%) | 1   | 2   | 3   | SD  | RSD (%) |
|--------------------------------------------|--------|-------|-----|-----|-----|------|---------|
| BMM                                        | 2      | 133   | 141 | 132 |     | 4.93 | 3.64    |
|                                            | 2.5    | 119   | 135 | 138 |     | 10.21| 7.82    |
|                                            | 3      | 102   | 126 | 123 |     | 13.08| 11.18   |
|                                            | 3.5    | 129   | 116 | 147 |     | 15.57| 11.91   |
| BMM + betaine                              | 3      | 131   | 137 | 143 |     | 6.00 | 4.38    |
|                                            | 4      | 116   | 118 | 134 |     | 9.87 | 8.04    |

\( ^a \) The relative error (RSD) is around or less than 10%. The RSD remains the same when the culture is diluted, so the estimated number of cells on a plate of 100 wells has the same relative error.

FIG A1 Simulation study where \( p_0 \) (the number of cells on a plate of 100 wells) has an average value of 100 and its relative error is 10% (due to dilutions when the cells were inoculated into wells). The number of cells per well follows the Poisson distribution with the \( p_0 \) mean value, where \( p \) is the average number of initial cells in a well and \( p_0 \) is the probability of growth for a cell.
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