

A comparison of the Bioscreen method and microscopy for the determination of lag times of individual cells of *Listeria monocytogenes*

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Y. WU, M.W. GRIFFITHS AND R.C. MCKELLAR. 2000. Lag phase durations (t_{Lag}) of individual *Listeria monocytogenes* cells were analysed using the NightOwl Molecular Imaging System, and results were compared with mean individual cell lag times (t_L) obtained from the detection time (t_d) method using Bioscreen. With Bioscreen, an average t_L of 6.39 ± 0.89 h was obtained from five separate experiments. With the NightOwl method, an average t_{Lag} of 2.73 ± 0.06 h was obtained from three experiments consisting of eight total replicates. Lag values from the NightOwl and Bioscreen are related by the equation: $t_{Lag} = t_L + DT$, where DT is the doubling time. The equivalent t_{Lag} mean value for the Bioscreen method was 7.11 ± 0.84 h. Individual lag times measured by both methods were normally distributed (r^2 for Bioscreen and NightOwl ranged from 0.951 to 0.999 and from 0.884 to 0.982, respectively). The results suggest that the NightOwl method can provide accurate estimates of individual cell lag times, which will facilitate the development of combined discrete continuous models for bacterial growth.

INTRODUCTION

Predictive microbiology, the use of mathematical models to describe the growth and death of foodborne micro-organisms, has been an area of considerable activity over the last decade. It is based upon the premise that the responses of populations of micro-organisms to environmental factors are reproducible, and that it is possible, from past observations, to predict the responses of micro-organisms by considering environments in terms of identifiable dominating constraints. Proponents claim that predictive microbiology offers many benefits to the practice of food microbiology, and there is growing international interest in its use (Ross and McMeekin 1994).

Kinetic models are perhaps the most useful, since they can be used to predict changes in microbial numbers with time, even if one (or more) of the controlling factors affecting growth is changing e.g. during a chilled distribution chain (McClure *et al.* 1993). Many kinetic models have been developed by fitting growth curves of viable count data obtained from cultures grown in liquid media (Buchanan and Phillips 1990; Wijtzes *et al.* 1993). Viable

count is a traditional, sensitive method for estimating the microbial growth curve, but it is time-consuming and labour-intensive (McClure *et al.* 1993; Dalgaard *et al.* 1994). The amount of data required to generate reliable models has led some researchers to use simple, and often indirect, methods of data collection, such as turbidimetry in laboratory media, rather than the viable count method. Determining bacterial growth rates in broth systems using turbidimetric methods provides a rapid and inexpensive means of modelling; however, the use of indirect methods for growth curve generation may result in generation times different from those determined by using viable counts (Baranyi *et al.* 1993; Dalgaard *et al.* 1994).

Turbidimetric methods such as the Bioscreen method have been used to generate kinetic data for modelling by fitting non-linear regression functions to optical density (OD) data (McClure *et al.* 1993; Stephens *et al.* 1997). Determinations of the specific growth rate (μ) and population lag phase duration (λ) are often difficult with this approach. Thus attempts have been made to use detection times (t_d) (Cuppers and Smelt 1993). The t_d for a turbidimetric instrument can be defined as the time required for an initial measurable increase in OD. When t_d values are plotted against the corresponding inoculum size μ can be calculated as the negative reciprocal of the slope of the

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regression line (Cuppers and Smelt 1993). The mean individual cell lag times (t_L) can be calculated subsequently as the difference between the predicted t_d based on the μ , and the observed t_d . This approach has been used recently to determine t_L for *L. monocytogenes* (McKellar 1998; McKellar and Knight 2000).

With the combination of microscopy and imaging, direct observation of single cell growth has become possible, providing a direct observation of the growth kinetics of individual bacterial cells. Lag phase can be obtained by determining when the first doubling occurs, and growth rate can be estimated by counting the doubling time of the cells. Few studies have attempted to derive kinetic models using microscopy techniques. The behaviour of individual cells in foods is poorly understood. In spoilage situations this may not be important; however, some food-borne pathogens can cause illness from only a few cells (Mackey and Gibson 1997). Thus, determining the lag time of single cells would provide valuable information for risk analysis. Therefore, the purpose of this study was to determine the lag phase of individual cells using microscopy, and to compare the distribution of individual cell lag times with that obtained using the Bioscreen method.

MATERIALS AND METHODS

Strains and culture conditions

Listeria monocytogenes Scott A (human clinical isolate) was obtained from the culture collection of the Food Research Program (Guelph, Ont. Canada). API *Listeria* spp. identification strip (BioMérieux Canada Inc., St Laurent, PQ, Canada) was used to confirm the identity of the culture. The culture was grown for 24 h at 30 °C in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA). Stock cultures were prepared in TSB plus 15% glycerol (BDH Inc., Toronto, Ont., Canada) and 0.3 ml subsamples were frozen in cryovials at -25 °C.

The contents of one cryovial were transferred to 10 ml of TSB, and incubated for 24 h at 30 °C in a shaking water bath (Model 3100, New Brunswick Scientific, Edison, NJ, USA) at 1500 rev min⁻¹. The culture (0.1 ml) was transferred to 10 ml fresh TSB and incubated at 30 °C for 24 h when the cell density was approximately 10⁸ cfu ml⁻¹. For the Bioscreen, the resulting culture was used as the inoculum for experiments. For microscopy, the resulting culture was diluted 1:10 in TSB, and the diluted suspension (approximately 10⁷ cfu ml⁻¹) was used to prepare the slide.

Bioscreen technique

Growth experiments. Serial twofold dilutions of the inoculum were made using fresh TSB to obtain a range of dilu-

tions representing approximately 0–10⁵ cfu ml⁻¹. From each of the twofold dilutions, 350 µl was transferred to wells (40 wells per dilution) of a Bioscreen plate (Labsystems, Helsinki, Finland). The filled plates were placed in the Bioscreen for analysis. Measurements were taken using a wide band filter, with preshaking at medium intensity for 10 s prior to OD reading, at an incubation temperature of 30 °C. Measurements were taken every 4 min for 25 h. Results were reported as t_d (h) and defined as the time required for the Bioscreen to record a 0.05 increase in OD from the initial value.

Viable cells were enumerated for each twofold TSB dilution by spread plating appropriate serial dilutions (0.1 ml) in duplicate onto tryptic soy agar plates (TSA, Difco Laboratories). The plates were incubated at 30 °C for 48 h and counts were determined using a Quebec Counter (American Optical Co., Model 15, Buffalo, NY, USA).

Modelling. Calculation of t_L (mean individual cell lag times) was performed as described previously (McKellar 1998; McKellar and Knight 2000). Briefly, plots of t_d (detection time) obtained from serial dilutions of the original inoculum against \ln cfu ml⁻¹ were used to calculate μ from the slope using the equation:

$$\mu = -1/\text{slope} \quad (1)$$

The calculated μ was then used in the heterogeneous population model (McKellar 1997) to predict the time required to detect growth from a defined number of cells. It was assumed that the dilution giving the largest t_d was equal to 1 cfu well⁻¹ (or \ln cfu well⁻¹ = 0). Simulated values for t_d underestimated the actual t_d by an amount equivalent to t_L . Replicate values of t_L and the standard deviation (S.D._L) were calculated from five independent 40-well trials by subtracting the simulated value for t_d from the experimental t_d values.

For each of the Bioscreen experiments, frequency distributions of individual t_L values from replicate wells were calculated using the non-linear regression function of Prism Version 3.0 (GraphPad Software for Intuitive Science, San Diego, CA, USA). Bin widths were set at 0.7 h intervals, and the number of wells corresponding to each bin was determined. A normal distribution was then fitted to the resulting frequency distribution using Prism.

Microscopic technique

Slide preparation. Glass double cavity slides (75 × 25 mm, 18 mm diameter cavities, 1.75 mm thickness) and coverslips (22 cm²) (VWR Canlab, Mississauga, Ont., Canada) were autoclaved before use.

Two hundred and fifty microlitres of molten TSA were pipetted into each cavity of the slide. The agar in each cavity was covered with a coverslip, and pressure was applied to obtain a flat, smooth surface. The slide was left for 5 min to allow the agar to solidify. The coverslip was removed, 2 µl of sample suspension was pipetted onto the surface of the agar, and another coverslip was applied. The slide was placed in a sealed Petri dish and incubated at 30 °C.

Image capture and analysis. Images were acquired and evaluated using the NightOwl LB 981 Molecular Imaging System (EG & G Berthold, Bad Wildbad, Germany). Microscopic images were obtained using the CCD camera of the NightOwl system mounted on a microscope (Olympus BH2, Capsen Ltd, Markham, Ont., Canada). The growth of the target cells in two or three different fields of view for each slide was visualized using phase-contrast microscopy. Images of each area were captured at time intervals of 0, 1, 1.5, 2, 2.25, 2.5, 2.75, 3, 3.25 and 3.5 h. After each observation, the slide was replaced in a sealed Petri dish and incubated at 30 °C. The number of newly divided cells was counted and these cells were not counted again in later captured images.

Three experiments were conducted; in experiment 1, two replicate areas were viewed per slide; in experiments 2 and 3, three replicate areas were viewed.

Modelling. For each microscopy experiment, the number of cells that had newly divided during each 0.25 h time interval was determined and used as the frequency distribution. A normal distribution was then fitted to the resulting frequency distribution using Prism.

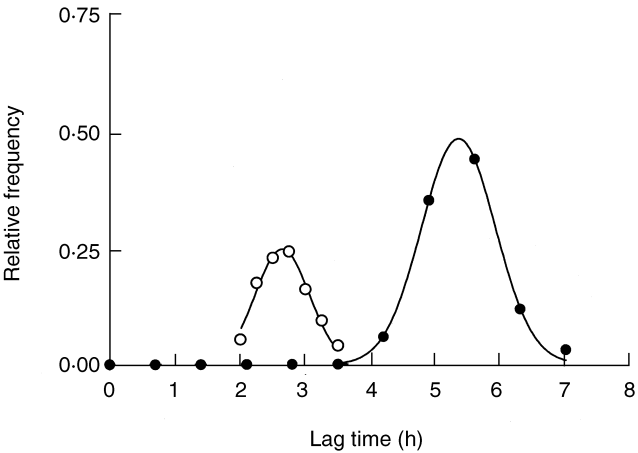


Fig. 1 Examples of normal distribution curves from microscopy. (○) replicate 2 of experiment 2 ($r^2 = 0.984$) and the (●) Bioscreen experiment 1 ($r^2 = 0.997$)

RESULTS

The average t_L from the Bioscreen was 6.39 ± 0.89 h (Table 1). All the data fit a normal distribution well; r^2 values ranged from 0.951 to 0.999 (Fig. 1). Individual values for S.D. from each experiment ranged from 0.567 to 0.991.

With the microscopic method, eight replicates were obtained from three experiments. The total cell number in each field of view was highly variable (from 24 to 166 cells) among replicates because cells were not evenly distributed on the agar (Table 2). The average lag phase duration observed microscopically ($t_{L,ag}$) was 2.73 ± 0.06 h. Non-linear regression analysis indicated that all the replicates were normally distributed; r^2 values ranged from 0.884 to 0.984

Table 1 Results from Bioscreen

	Experiment number					Average
	1	2	3	4	5	
t_L	3.74	6.21	7.19	6.81	6.99	6.39 ± 0.887
S.D.	0.567	0.637	0.646	0.991	0.793	0.727
R^2	0.997	0.999	0.993	0.964	0.951	—
DT	0.677	0.672	0.680	0.680	0.680	—
$t_{L,ag}$	5.57	6.86	7.82	7.48	7.80	7.11 ± 0.843

t_L – mean individual lag times from Bioscreen data (h).
S.D. – standard deviation.
DT – doubling time (h).
 $t_{L,ag}$ – $t_L + DT$.

(Fig. 1). From the images it was observed that not all cells divided within the time period of the experiment although the number of such cells was less than 5% of the total cell population.

The t_L derived using the Bioscreen method assumes that individual cells start growing at maximal growth rate immediately after adaptation (McKellar 1998; McKellar and Knight 2000). The time at which the first cell was observed microscopically to be dividing ($t_{L,ag}$) includes the adaptation period (t_L) and the time required for the cell to double (DT). Thus $t_{L,ag}$ is related to t_L by equation 2.

$$t_{L,ag} = t_L + DT \quad (2)$$

DT was calculated from μ using equation 3:

$$DT = \ln 2 / \mu \quad (3)$$

Calculations for $t_{L,ag}$ from the Bioscreen data are given in Table 1.

DISCUSSION

The results of the present study show that mean individual cell lag times obtained either by the Bioscreen method or by microscopy follow normal distributions (Fig. 1). There have been few studies published on the distribution of bacterial cell lag times. Baranyi (1998) and Baranyi and Pin (1999) proposed that lag times were exponentially distributed. However, other important physiological parameters, such as growth rate, were found to be normally distributed (Kelley and Rahn 1932; Kubitschek 1966; Rubinow 1980). In previous studies employing the Bioscreen, it was assumed that t_L values were normally distributed, and, by using this assumption, the decreased variability in t_L between replicate wells with > 1 cell well⁻¹ initial count was correctly predicted (McKellar 1998; McKellar and

Knight 2000). These same studies showed that the assumption of exponential distributions for t_L led to predictions which did not agree with experimental findings (McKellar 1998; McKellar and Knight 2000). Further work is required to more completely classify the distribution of cell physiological properties.

The lag times determined by the Bioscreen method in this study were slightly greater than were observed previously (McKellar 1998; McKellar and Knight 2000); however, the S.D. values are comparable. The t_L values obtained by the Bioscreen method were also longer than those found microscopically. The reason for this difference is unknown. However, Baranyi *et al.* (1993) and Dalggaard *et al.* (1994) have suggested that the use of indirect methods for growth curve generation might result in generation times different from those determined using viable counts. There may also be differences between growth of microorganisms in solid media on slides and in liquid media due to O₂ availability and other factors. Further research is needed to resolve this issue.

Using microscopy to determine the lag, the zero time point was counted when the first image was captured and there was a slight delay between this first capturing time and the actual inoculation time. This would result in the actual $t_{L,ag}$ being slightly longer than the calculated $t_{L,ag}$. Since this slight time difference was about 5 min in each replicate and was constant for each experiment, it was ignored as a system error.

From the microscopic images, it was observed that some cells did not divide within the time period of the experiment. These cells can be considered dead, metabolically inactive or injured. This is one advantage of the microscopic method since dead, injured or living cells can be distinguished based on their ability to divide. Thus, cells under stress can be directly observed and their recovery can be studied *in vivo*. Furthermore, data on the kinetics of growth at other points in the growth cycle can be obtained.

Table 2 Results from microscopy

	Experiment 1		Experiment 2			Experiment 3			Average
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	
$t_{L,ag}$	2.80	2.84	2.71	2.79	2.65	2.76	2.63	2.67	2.73 ± 0.06
S.D.	0.383	0.306	0.366	0.499	0.414	0.429	0.408	0.361	0.396
R^2	0.884	0.932	0.961	0.963	0.984	0.982	0.962	0.977	—
Total cells*	58	45	24	37	74	166	81	71	69.5 ± 43.6

*Number of total divided cells counted in each area $t_{L,ag}$, mean individual lag times from microscopy data (h).

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