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A novel method for measuring lag times in division of individual bacterial cells using image analysis

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Abstract

A method is presented for determining the time to first division of individual bacterial cells growing on agar media. Bacteria were inoculated onto agar-coated slides and viewed by phase-contrast microscopy. Digital images of the growing bacteria were captured at intervals and the time to first division estimated by calculating the "box area ratio". This is the area of the smallest rectangle that can be drawn around an object, divided by the area of the object itself. The box area ratios of cells were found to increase suddenly during growth at a time that correlated with cell division as estimated by visual inspection of the digital images. This was caused by a change in the orientation of the two daughter cells that occurred when sufficient flexibility arose at their point of attachment. This method was used successfully to generate lag time distributions for populations of *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*, but did not work with the coccoid organism *Staphylococcus aureus*. This method provides an objective measure of the time to first cell division, whilst automation of the data processing allows a large number of cells to be examined per experiment.
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1. Introduction

The modelling of bacterial growth under various environmental conditions is of particular interest to the food industry for predicting shelf life or the possible growth of pathogens in food products. Indeed, several predictive models that incorporate databases describing the growth of common food-borne pathogens are now available including Growth Predictor (www.ifr.ac.uk/Safety/Growth), Pathogen Modelling Program (www.arserrc.gov/mfs/pathogen.htm) and ComBase (www.combase.cc). In this context the key growth parameters are the duration of the lag phase, the maximum specific growth rate and the maximum cell concentration in stationary phase. The duration of the lag phase depends on the previous history of the cells as well as the current environment and is therefore more difficult to predict than the maximum growth rate which, for a given species, depends only on the growth environment. Lag time is however an essential element of predictive modelling and much effort has been directed towards understanding the factors that determine lag (McMeekin et al., 1993; McKellar and Lu, 2003).

Studies of bacterial lag time have traditionally involved measuring the time from inoculation to the start

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of exponential phase growth using relatively large inocula (>1000 cells). However, this method favours those cells with the shortest lag times with the result that the population lag time measured in this way is less than the mean lag time of the individual cells in the inoculum (Baranyi, 1998). This distinction becomes important when considering low levels of contamination when the distribution of individual cell properties such as lag time becomes important in quantitative risk analysis. Baranyi (2002) demonstrated that it is possible to compute population lag from knowledge of the distribution of individual lag times, but that the reverse operation is not possible in practice. Measurement of properties at the single cell level is therefore required to determine lag time distributions within populations.

Although light microscopy has been used for studying growth and division of single cells (Kelly and Rahn, 1932; Powell, 1956; Baldwin and Wegner, 1986; Postgate et al., 1961) the method is subject to uncertainty when applied to determining lag times because it is difficult to decide exactly when cells have divided. Examination of large numbers of cell images in a timed sequence is also very labour intensive, and this limits the number of cells that can be examined per experiment and hence restricts statistical analysis of the results.

The aim of this work was to develop an objective means by which the times of the first cell division of individual members of a bacterial population can be identified and measured. The strategy was to capture digital images of cells growing on agar and to use changes in the dimensions of each cell to identify a parameter that could be used to estimate the division time. This paper will demonstrate that a parameter describing the geometry of a cell, the 'box area ratio' (BAR), can be used for this purpose.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The following strains were stored at −70 °C in Microbank vials (Pro-Lab Diagnostics, Neston, United Kingdom): *Escherichia coli* K-12 strain W3110, *Listeria monocytogenes* NCTC 7973, *Pseudomonas aeruginosa* NCTC 10332, *Bacillus megaterium* NCIMB 9521 and *Staphylococcus aureus* (laboratory strain). Cultures were prepared by inoculating a frozen bead into 10 ml Tryptone Soya Broth (TSB, Oxoid, Basingstoke, United Kingdom) and incubating for 6 h at 37 °C. To produce stationary phase cells, this culture was diluted 1 : 100 (v/v) into fresh TSB and incubated for 18 h with shaking at 37 °C.

2.2. Growth of organisms in slide culture

Microscope slides coated with a thin layer of agar were prepared using Coverwell Press-Seal Imaging Chambers (Z 365866, Sigma-Aldrich Company, Gillingham, United Kingdom). These consist of a thin optical-grade polycarbonate microscope slide bonded to a silicone spacer gasket. The gasket contained a 20 mm diameter well of 0.8 mm depth. Molten Tryptone Soya Agar (TSA, 280 μL) was added to the well and left to solidify and dry for 20 min. A drop of stationary phase culture (*ca* 5 μL was spread over the agar surface and a coverslip placed over the well. The combined depth of the agar and microscope slide was <1 mm, which was found to be sufficient to achieve optical clarity. Slides were incubated at room temperature (20–25 °C) during observation of bacterial growth under the microscope.

2.3. Capturing digital images

Cells were viewed under phase contrast optics using a Nikon Microphot-SA microscope equipped with ×20, ×40 and ×100 objective lenses. Digital images were captured using a CoolSNAP-Pro cf monochrome camera incorporating a 1392 × 1040 pixel CCD and 12-bit 20 MHz digitisation. Images were grabbed at 10–20 min intervals, saved in tif format and analysed using Image-Pro Plus V4.5 image analysis software (Media Cybernetics, Maryland, USA). The software identified the individual cells in each field of view and automatically measured their lengths, widths and thresholded areas. Data were then downloaded to Microsoft Excel for further analysis.

2.4. Automated processing of image analysis data

To automate the BAR method for estimating division times, two Visual Basic programs were required to process the Excel files that were created using Image-Pro Plus. The Excel files contained the relevant cell size parameters (length, width and area) for each cell in each digital image. The image analysis software identified each cell in an image by an object number, but the same cell was rarely given the same identifier in successive images. The Visual Basic program *ObjectTracker*, written in-house, overcame this problem by using the *x*- and *y*-coordinates to track individual cells throughout the sequence of images and to generate coherent time courses for each cell and each measured parameter. If a cell crossed the boundary of the image, or when two cells touched

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