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On the evolution of cell size distribution during bacterial growth cycle: Experimental observations and individual-based model simulations

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Individual-based modelling (IbM) has become a fully incorporated part of predictive microbiology methodologies in the last decade. Previous studies of bacterial culture growth cycle with the IbM simulator INDISIM analysed the evolution of bacterial biomass distribution during the different phases of growth. The predicted forward shift during lag, stability during exponential and backward shift when entering the stationary phase have been experimentally observed in an *Escherichia coli* batch culture by means of flow cytometry and particle size analysis measurements. In addition, the experimental results were analysed using the product distance, a mathematical tool developed to assess the evolution of cell size distribution. These results confirmed the assumptions about the bacterial lag phase made by INDISIM. Moreover, flow cytometry and particle analysis methods were shown to be useful experimental techniques in combination with IbM simulations when studying the evolution of individual properties during the bacterial growth cycle. This is essential in order to provide a new and consistent interpretation of the dynamics and heterogeneity of cell biomass during the growth cycle.

Key words: Bacterial cell cycle phases, biomass distribution, individual-based modelling, flow cytometry, particle size analysis.

INTRODUCTION

Predictive microbiology consists of models that attempt to understand and predict the behaviour of microbial systems. These models are built according to the specific phenomena to be tackled and to the required level of description (Ferrer et al., 2008). One of the open subjects in predictive microbiology is to explain how the intrinsic variability and heterogeneity of axenic bacterial cultures (Julià and Vives-Rego, 2005; Vives-Rego et al., 2003) influences the different phases of their growth (Prats et al., 2008). The full integration of Individual-based modelling (IbM) in the framework of predictive microbiology in the last decade provides a mesoscopic connection between cellular descriptions and the dynamics of bacterial populations (Ferrer et al., 2009).

Individual-based models of populations and communities were defined by Grimm (1999) as 'simulation models that treat individuals as unique and discrete entities which have at least one property in addition to age that changes during the life cycle'. In microbiology, Individual-based models are bottom-up approaches that tackle the links between cells and population; they model the bacteria as individual entities that are subject to a set of biological and physical rules. The collective behaviour of the population emerges from the simulation of a large number of individuals and from their interactions with their neighbouring cells and local environment. Stochasticity, introduced in the rules governing the individual behaviour and interactions, plays a central role in the emergence behaviour observed at the system level.

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Abbreviations: IbM, Individual-based modelling; **FS,** forward scattering; **TFS,** transformed forward scattering; **MSZ,** multisizer.

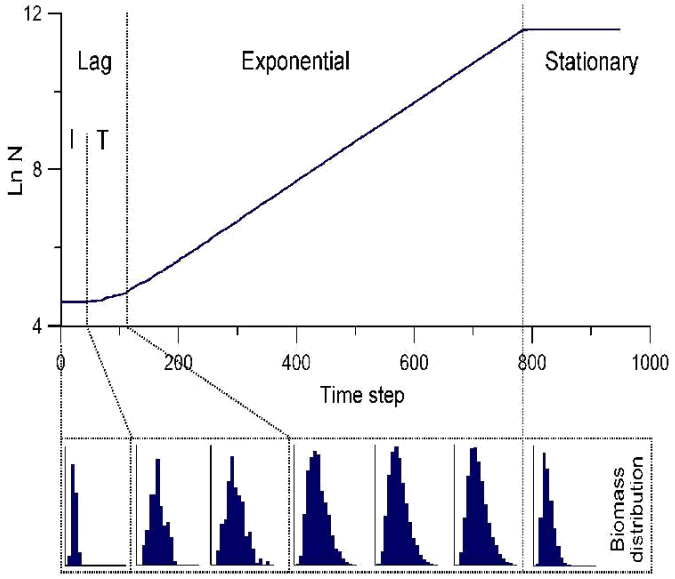

Figure 1. Example of an INDISIM simulation that summarizes some of the results published in Prats et al. (2006): overview of the growth curve (top) and the corresponding evolution of the biomass distribution (bottom, in simulation units).

The use of IbM in the framework of microbiology has produced a pool of interesting results. Two of the available IbM simulators are BacSim (Kreft et al., 1998) and INDISIM (Ginovart et al., 2002a). Both were designed to model and simulate the growth of bacteria under particular conditions, and they have been used to study various specific microbial processes such as those occurring in soil or in food (Ginovart et al., 2002c, 2005). IbMs have proved especially useful in the study of systems with spatial complexity, such as biofilms (Kreft et al., 2001) and bacterial colonies in agar plates (Ginovart et al., 2002b), and for tackling transient stages of growth such as initial or intermediate lag phases (Dens et al., 2004a, 2004b).

Prats et al. (2006) presented a detailed study of the evolution of the biomass distribution of a bacterial culture during the growth cycle. INDISIM simulations were used to reproduce and analyse this evolution during the lag phase in order to extract general underlying principles. The growth of a 100-cell inoculum in a homogeneous batch culture under different conditions was simulated, and the evolution of the biomass distribution was monitored through the different phases of growth. No lag was explicitly included in the model of bacteria, yet a lag phase in the population growth still emerged as a consequence of the inoculated population structure.

Figure 1 shows a summarized set of the INDISIM simulation results presented by Prats et al. (2006). It depicts the evolution of the population biomass distribution (in simulation units) together with the cell density growth curve for a batch bacterial culture. The three phases, lag (initial -I- and transition -T-), exponential and stationary are clearly observed in both plots (Figure 1). The evoution of the system observed in these simulations can be outlined as follows:

1. The inoculated bacteria were sampled from a previous simulation of a culture in the stationary phase. Therefore, the initial mean mass of the population was small, and the biomass distribution differed from that characteristic of the exponential phase.
2. During the lag phase, mean mass increased. At the same time, the biomass distribution changed its shape and showed a forward shift. The lag phase ended when both the biomass distribution shape and the mean mass reached their exponential characteristic values.
3. The exponential phase was characterized by the stability of the biomass distribution, which was maintained as long as there was enough nutrient available to allow unrestricted cell growth without intercellular competition.
4. When the nutrient ran short, the culture entered the stationary phase, denoted by a change in the slope of the growth curve. As this occurred, both a backward shift in the biomass distribution and a decrease in mean mass were observed.

These results showed that, unlike classic continuous models, IbM approaches may provide detailed information of the structure of a microbial population. Thus, experimental techniques that assess cell density such as microscopy count, optical density and plate count, among others (Rasch, 2004), take only partial advantage of the predictions of IbM simulations. Techniques like flow cytometry and particle size analysis, which assess the distribution of individual cell properties in the population of real systems, are more suitable to complement IbM. Specifically, flow cytometry can be used to measure several individual parameters, identify sub-populations or count microorganisms (Vives -Rego et al., 2000), and also to monitor their evolution during the growth cycle (Åkerlund et al., 1995). Particle size analysis allow the simultaneous measurement of cell density and cell size distribution.

This paper has two main objectives. First, we want to show the usefulness and soundness of cytometry techniques in order to experimentally observe IbM predictions at the mesoscopic level (that is, regarding population structure and changes in it). Second, we want to focus particularly on the INDISIM predictions reported by Prats et al. (2006) that have been summarized in this introduction, and to look for the predicted behaviours in a real bacterial culture. In addition, we want to validate the suitability of a mathematical tool named product distance (D(t)), which was defined by Prats et al. (2006) as a measurement of the evolution of biomass distribution during the stages of growth. Product distance is here redefined, adapted and used to assess the evolution of experimental measurements.

MATERIALS AND METHODS

Experimental setup

Experiments were performed using *Escherichia coli CECT 101* (*Colección Española de Cultivos Tipo*, Spain). Bacteria were grown in M9 medium consisting of part A (per liter, Na_2HPO_4, 6 g, KH_2PO_4, 3 g, NaCl, 5 g, NH_4Cl, 1 g); part B(a 1 M solution of $MgSO_4 \cdot 7H_2O$); and part C(a 0.01 M solution of $CaCl_2$). Parts A, B and C were autoclaved separately. 1 ml of part B and 10 ml of part C were added to 1 l of part A. Glucose sterilized by filtration through 0.2 μm was added at 0.5 g per litre. Final pH was 7.5. Cultures were incubated at 35°C with shaking at 150 r.p.m. After inoculation with 0.5% of an equivalent overnight culture obtained under the same culture conditions, growth was monitored by flow cytometry and electric particle size analysis. We measured the cell density and the size distribution of the cultures at different stages of the growth cycle.

Flow cytometry was carried out using a Cytomics FC 500 MPL instrument (Beckman Coulter, Fullerton, CA, USA), operating in the standard configuration. The optical alignment was based on an optimized signal from 10 nm fluorescent beads (Flowcheck, Coulter Corporation, Miami, Florida, USA). Forward scattering (FS) values were measured, stored and analysed. Experimental data were loaded with Summit ® V3.1 software (Cytomation, Inc.), and exported for analysis with Microsoft Excel 2002 (© Microsoft Corporation 1985-2001) and Matlab ® R2006b (© The Mathworks, Inc. 1984-2006).

Particle size analysis was carried out using a Multisizer II (MSZ) electronic particle analyzer (Coulter Corporation ®) with the capacity to process 100 μl of the cell suspension in 0.9% NaCl previously filtered through 0.2 μm. Cell sizes were given in terms of equivalent spheres. Data were analysed with Coulter Multisizer AccuComp software version 1.15 (Coulter Corporation). Files generated by the MSZ were exported in an ASCII (tab delimited) format and analysed with Microsoft Excel 2002 (© Microsoft Corporation 1985-2001) and Matlab ® R2006b (© The Mathworks, Inc. 1984-2006).

Three independent experiments were performed under the same conditions: T35-061121 (A), T35-070123 (B) and T35-070130 (C). After the first one (A), we decided to filter the medium in order to reduce the background noise (0.22 μm). The three trials (A, B and C) provided equivalent results.

Mathematical distances

A mathematical distance between two biomass distributions was introduced by López (1992) to characterize the dynamics of bacterial cultures, and it was then adapted by Prats et al. (2006) to study the lag phase. The starting premise is to assume that the biomass distribution of a bacterial culture remains fixed all throughout the balanced exponential growth. This distance measures the difference between the instantaneous biomass distribution of a bacterial population and its characteristic lognormal distribution during the exponential phase under the given conditions: the closer the distributions, the lower the mathematical distance. These distances were used to analyze INDISIM simulation results: they typically decreased during lag phase, remained close to zero during the exponential phase and increased again when the culture entered the stationary phase.

In order to use them to analyse experimental results, the distances (originally defined to deal with biomasses) were re-defined in terms of the measured diameters. These are called mean diameter distance (Equation 1), diameter distribution distance (Equation 2) and product distance (Equation 3) as follows:

$$D_{\bar{d}}(t) = \frac{\left| \bar{d}(t) - \bar{d}_{exp} \right|}{\bar{d}_{exp}} \quad (1)$$

$$D_{p_k d}(t) = \sum_{k_d=1}^{N} \left| p_{k\,d}(t) - p_{k\,d,exp} \right| \quad (2)$$

$$D(t) = D_{\bar{m}}(t) \cdot D_{pk}(t) = \frac{\left| \bar{m}(t) - \bar{m}_{exp} \right|}{m_{exp}} \cdot \sum_{k_m=1}^{25} \left| p_{km}(t) - \bar{p}_{k_m,exp} \right| \quad (3)$$

where $\bar{d}(t)$ is the mean observed diameter of a sample at the time of measurement *t,* and p_{kd} is the standardized distribution of diameters among the population of the sample. These *distances* are evaluated from a reference measured mean diameter ($\bar{d}_{exp}$) and diameter distribution ($p_{kd,exp}$), which are set as the mean of the exponential phase measurements. The number of diameter classes within the diameter distribution, *N*, is equal to 256 for both the FS and the MSZ measurements (it is the number of channels of both instruments).

RESULTS

Transformation of FS measurements into cell diameter distributions

The relation between FS measurements and cell diameter is not a monotonic function, although there is a correlation between them (Shapiro, 2003; Julià et al., 2000) . Julià et al. (2000) proposed a specific second-order function for different bacterial species that relates FS measurements and equivalent cell diameters, *d*. For an *E. coli* culture, this is (Equation 4):

$$d(\mu m) = \left(8.54 \cdot (fs)^2 - 6.71 \cdot (fs) + 2.08\right) \cdot 10^{-5} \quad (4)$$

where *fs* referred to as the label of the channel (from 1 to 256).

We used this relationship to convert FS measurements into diameters, which we called transformed forward scattering (TFS). Previously, we checked the soundness of Equation 4 by comparing the MSZ measurements with the corresponding TFS values in the first experiment. We assessed the likelihood between MSZ and TFS using the relative differences of three characteristic parameters: mean diameter (Mean), standard deviation (Std Dev), and the median (Median). Table 1 shows this comparison for experiment A. The discrepancy between MSZ and TFS measurements is under 5% in all the evaluated indicators except for the Std Dev of the last sample. Therefore, TFS are consistent with the diameter distributions obtained with the multisizer.

Table 1. Discrepancy (Disc) between diameter distributions obtained with the multisizer (MSZ) and the diameter distributions obtained by transforming forward scatter (FS) distributions with Equation 4 (TFS) in the experiment A (samples taken at T1: 53 min, T2: 131 min, T3: 218 min, T4: 266 min, T5: 328 min).

		Mean	Std. Dev.	Median
	TFS (μm)	0.853	0.11	0.83
T1	MSZ (μm)	0.856	0.113	0.838
	Disc (%)	0.350	2.655	0.955
	TFS (μm)	0.863	0.118	0.847
T2	MSZ (μm)	0.881	0.124	0.861
	Disc (%)	2.043	4.839	1.626
	TFS (μm)	0.863	0.116	0.847
T3	MSZ (μm)	0.862	0.118	0.838
	Disc (%)	0.116	1.695	1.074
	TFS (μm)	0.877	0.117	0.864
T4	MSZ (μm)	0.879	0.122	0.861
	Disc (%)	0.228	4.098	0.348
	TFS (μm)	0.884	0.115	0.864
T5	MSZ (μm)	0.902	0.127	0.886
	Disc (%)	1.996	9.449	2.483

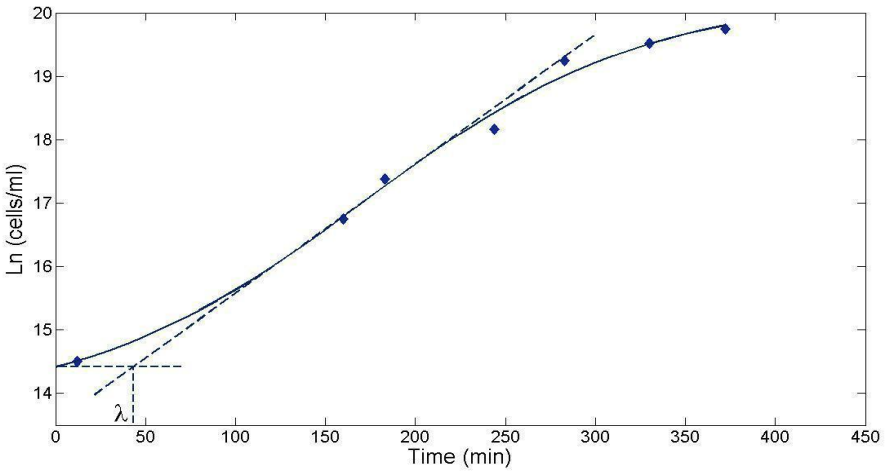

Figure 2. Cell concentration (experiment C), measured with the flow cytometer using an internal calibrator. A sigmoid curve has been fitted ($r^2 = 0.993$). The lag phase calculated by means of the geometrical definition is $\lambda = 43 min$.

Evolution of cell diameter distribution during the growth cycle

The evolution of the cell diameter distribution of all three experimental trials showed the same behaviour. We present only the results of the FS measurements of experiment C to serve as an exemplary result.

Three phases of growth were clearly observed in the experimental evolution of the cell density (Figure 2): lag, exponential and transition to stationary phases. A

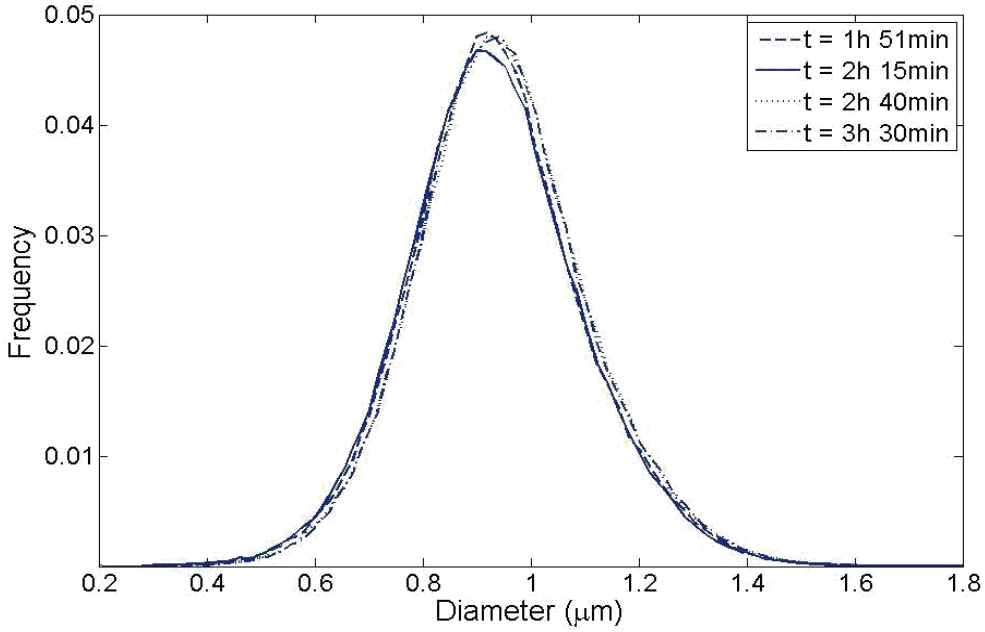

Figure 3. Four FS measurements taken from the exponential phase (experiment C). The FS values have been transformed into diameters with Equation 4, and show the stability of the diameter distribution during exponential balanced growth.

sigmoidal curve was fitted to the growth curve ($r^2 = 0.993$), and the culture lag parameter (λ), using the geometrical definition (Lodge and Hinshellwood, 1943), was determined: $\lambda = 43\ min$.

Stability of the diameter distribution during exponential phase

According to theoretical predictions (Wagensberg et al., 1988), empirical data (Åkerlund et al., 1995) and INDISIM simulations results (Prats et al., 2006), the biomass distribution remains fixed under balanced growth conditions and its shape depends solely on the bacterial strain and the medium conditions. The flow cytometry measurements corroborated this predicted behaviour for the cell diameter distribution. Figure 3 shows the stability of the distribution at four different moments of the exponential phase in experiment C.

Evolution of diameter distribution and the mean diameter

The inoculum was sampled from a previous culture which was in the stationary phase. Since bacteria had undergone a decrease in their biomass caused by starvation conditions, the inoculum showed a shrunken and left-biased diameter distribution different from the exponential one. As a result, a forward shift of this distribution during the lag phase and a backward shift when the culture started starving as it entered the stationary phase were observed (Figure 4). INDISIM simulations showed exactly the same behaviour (Figure 1) and an interpretation of it: when the cells of the inoculum are added to the new fresh medium, they must increase their biomass before starting the reproduction cycle (Prats et al., 2006).

The evolution of the mean diameter was also consistent with INDISIM results. Three different phases could be clearly distinguished: the mean diameter increased during the lag phase, remained approximately constant during the exponential phase and decreased when the culture entered the stationary phase (Figure 5).

Analysing the experimental results with the product distance

The evolution of the measured diameter distribution was analysed with the distances introduced. The evolution of the product distance during experiment C is shown in Figure 6a and compared to the one predicted by INDISIM simulations (Figure 6b). The experimental behaviour follows INDISIM predictions: after an initial decrease during the lag phase, the product distance remained close to zero during the exponential phase and finally increased as the culture entered the stationary phase.

DISCUSSION

INDISIM simulation results provided a detailed explanation of the changes ocurring in the population

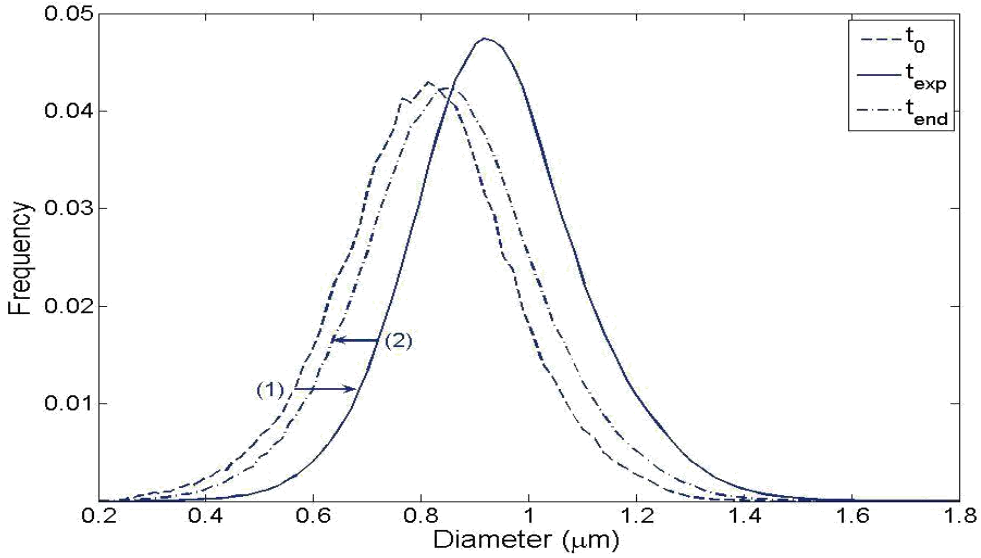

Figure 4. FS-measured distribution at inoculation time (t_0), mean of the measured distributions during exponential phase (t_{exp}) and last FS-measured distribution at the end of growth (t_{end}) (experiment C) . The arrows indicate the displacements (1) from lag to exponential and (2) from exponential to stationary phases. The FS vaules have been transformed into diameters with Equation 4.

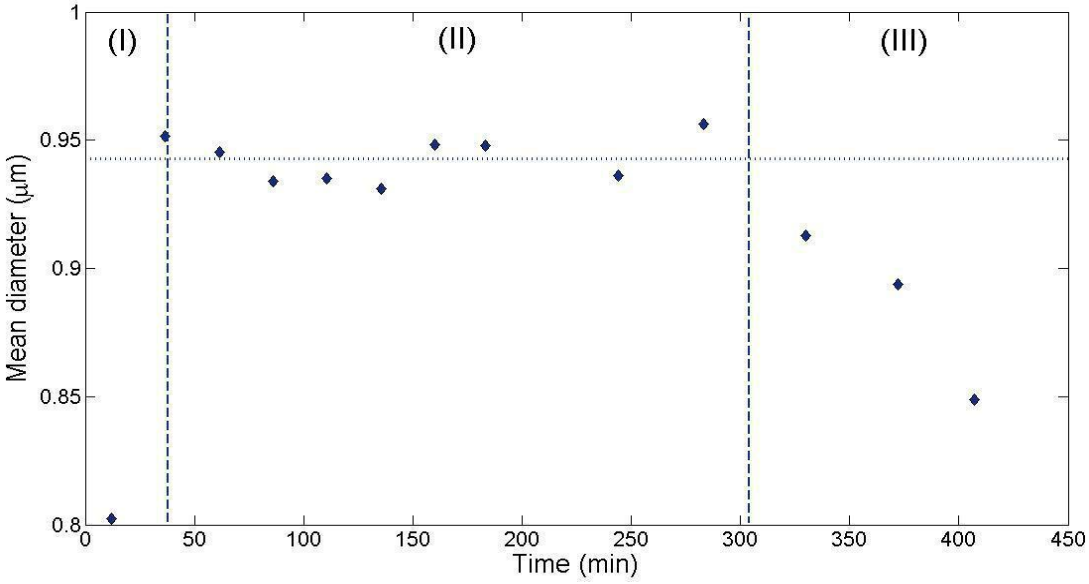

Figure 5. Mean diameter evolution of the culture (experiment C), from FS measurements that have been transformed into diameters with Equation 4. Dashed lines indicate the lag (I), exponential (II) and stationary (III) phases. Dotted line indicates the mean value during exponential phase.

structure during bacterial growth cycle. They focused on the evolution of biomass distribution in the different phases of growth. Simulations showed that during the lag phase there are important changes in this variable. They offered the conclusion that the small biomasses of inoculated bacteria alone can give rise to an individual lag phase, although other factors (such as as metabolic adaptation to a new nutrient) can accompany or prolong it. The lag phase observed for the whole population may be produced by many factors, but the adaptation of the (relatively small) initial mass distribution irrevocably contributes to this interim, since adverse environmental conditions found in the stationary phase produces a shrinkage of bacteria.

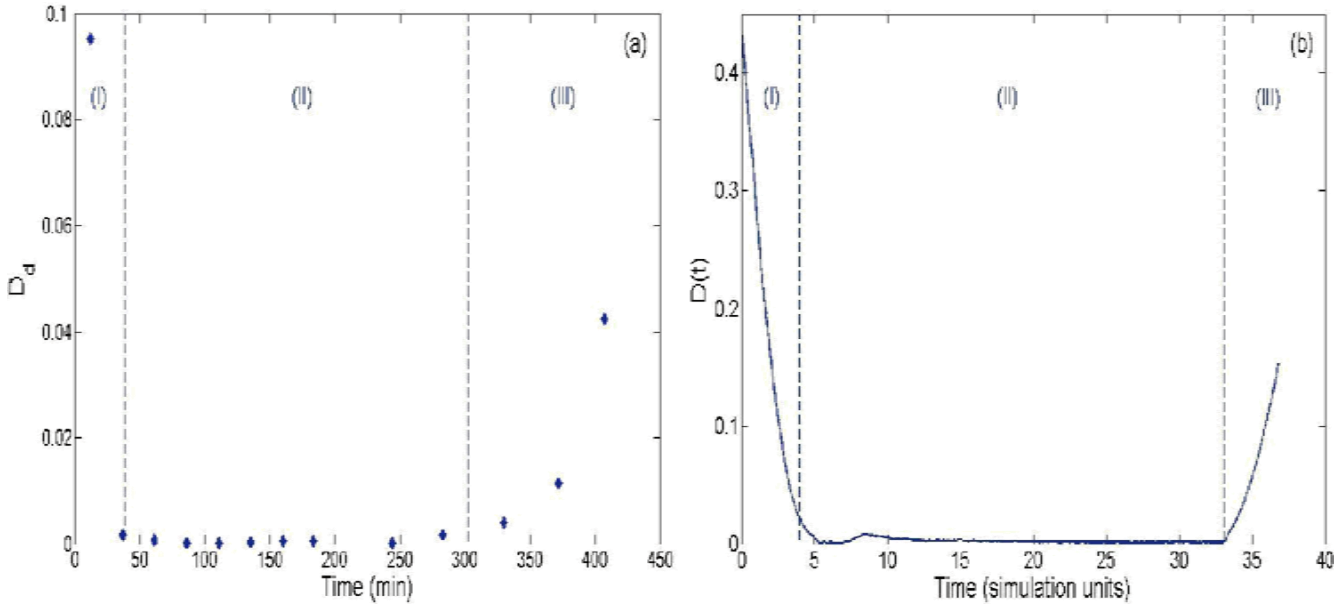

Figure 6. (a) *Product distance* values (Equation 3) for FS measurements previously transformed into diameters with Equation 4 (experiment C), and (b) INDISIM simulation prediction of the evolution of the product distance (Prats et al., 2006). Dashed lines indicate the lag (I), exponential (II) and stationary (III) phases.

These predictions of INDISIM had not been specifically checked until now, since there is a lack of experimental information from this mesoscopic approach. In this paper, we have seen that flow cytometry and electric particlesize analysis are suitable methods for obtaining experimental data at this level of description that are useful to assess IbM predictions. They provide an instantaneous picture of the structure of the studied population, and offer the possibility of studying its evolution from a dynamic perspective. These kinds of measurements are necessary to account for the high heterogeneity of bacteria. This feature is frequently neglected when modelling microbial communities, and it might be responsible for some crucial but not yet understood phenomena.

The experimental results are in agreement with INDISIM predictions. The expected initial forward shift in diameter size distribution during the lag phase, its stability during the exponential phase and its backward shift when the culture enters the stationay phase were reproduced by the real system. We saw, experimentally, an increase in cellular mean mass during the lag phase. This reinforces the INDISIM model which closely links the lag phase of a culture with the adaptation of bacterial sizes. It was a very simple hypothesis, but it is probably present in most bacterial cultures. The observed decrease in the mean size observed at the end of the exponential growth also supports the response modelled by INDISIM and suggests a kind of cyclic behaviour of the size distribution, moving forward when the conditions are proper until balanced growth is achieved, and backward when the conditions become adverse. Such dynamic behaviour of the size distribution strengthens the need for mathematical methods to evaluate it. The product distance enables quantitative description of the evolution of a bacterial population in terms of the distribution of individual properties (diameters) and allows distinction of the different phases of bacterial growth. The product distance is a conceptually simple and precise tool to quantitatively characterize the ongoing adaptation processes undergone by real bacterial communities, often reflected as changes in probably any distribution of individual properties.

To sum up, the combination of flow cytometry, particle analysis and Individual-based Modelling proved useful for the study of lag, exponential and stationary phases of bacterial cultures. To begin, cell- by- cell size analysing techniques provide measurements at both individual and population levels. In addition, INDISIM simulations provide appropriate tools to analyse and explain these observations, and therefore to interpret individual cell size variability in axenic cultures. The connection between the structure observed at a population level and the expected behaviour of bacterial cultures is not yet fully understood, but INDISIM showed confirmed predictive capability. Its application in combination with flow cytometry and particle size analysis provides a promising pathway to gain insight into microbial ecosystems.

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