International Journal of Food Microbiology 285 (2018) 103–109

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Simultaneous growth, survival and death: The trimodal behavior of *Salmonella* cells under osmotic stress giving rise to "Phoenix phenomenon"

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ARTICLE INFO

Keywords:
Salmonella
Osmotic stress
Growth
Death
Phoenix phenomenon

ABSTRACT

Time-lapse microscopy methods were used to monitor growth, survival and death of *Salmonella enterica* serotype Agona individual cells on solid laboratory medium (tryptone soy agar) in the presence of various salt concentrations (0.5%, 3.5%, 4.5% and 5.7% NaCl). The results showed a highly heterogeneous behavior. As NaCl concentration increased, the distribution of the first division time was shifted to higher values and became wider. The mean first division time increased from 1.8 h at 0.5% NaCl to 5.48 h, 16.2 h, and 35.9 h at 3.5%, 4.5% and 5.7% NaCl, respectively. The concentration of NaCl in the growth medium also affected the ability of the cells to divide. The percentage of cells able to grow decreased from 88.9% at 0.5% NaCl to 66.5%, 32.8%, and 6.9% at 3.5%, 4.5% and 5.7% NaCl, respectively. In the latter case (5.7% NaCl), 74 cells out of 406 cells tested (18%) died with mean time to death 5.03 h and standard deviation 6.70 h. To investigate the effect of the behavior of individual cells on the dynamics of the whole population, simulation analysis was used. The simulation results showed that the simultaneous growth, survival and death of cells observed under osmotic stress can lead to a total population behavior known as the "Phoenix" phenomenon. The simulation findings were confirmed by validation experiments using both viable counts and time lapse microscopy. The results of the present study show the high heterogeneity of individual cell responses and the complexity in the behavior of microbial populations at conditions approaching the boundaries of growth.

1. Introduction

Most of the models in Predictive Microbiology are based on simplicity and reductionism describing the behavioral output of microbial populations as a whole in response to environment ignoring microbial individuality. Extensive validation studies in various foods have shown that, although deterministic population models are generally valid for predicting microbial behavior at optimal or suboptimal environmental conditions, they present a very low performance in the case of more stressful and complex environments close to the boundary of growth (Koutsoumanis et al., 2016; Koutsoumanis and Aspridou, 2016). This can be attributed to the highly heterogeneous behavior of bacterial cells at such conditions due to the stochastic variations associated with the genomic information flow including gene activation, transcription and translation (Avery, 2006).

Recent studies on microbial behavior at conditions close to the boundaries of growth have shown a significant heterogeneity in the ability of individual cells to initiate growth (Aguirre and Koutsoumanis, 2016; Koutsoumanis, 2008). Koutsoumanis (2008) studied the growth limits of *Salmonella* Enteritidis individual cells exposed to osmotic stress and reported that as the NaCl concentration increases, the number of the cells in the population able to grow gradually decreases. Aguirre and Koutsoumanis (2016) confirmed the above findings for heat treated and non-treated cells of *Listeria monocytogenes*. Both studies showed that the bistable behavior of individual cells (growth/no growth) under osmotic stress leads to an additional lag in the population growth (called "pseudo-lag") with the total apparent lag of the population being the sum of the physiological lag of the growing cells (time required to adjust to the new environment) and the pseudo-lag induced by the presence of the non-growing cells.

Other studies on microbial behavior at more severe osmotic conditions showed that cells can initially display some loss of viability, but some survivors eventually initiate growth (Mellefont et al., 2005; Zhou et al., 2011). In this case the viable count growth curve exhibits a decrease after inoculation followed by a period in which numbers remain unchanged and, thereafter, exponential growth ensues. The latter has been termed as "Phoenix phenomenon" firstly reported by Collee et al. (1961). Later, Shoemaker and Pierson (1976) studied the "Phoenix

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https://doi.org/10.1016/j.ijfoodmicro.2018.07.012
Received 6 February 2018; Received in revised form 18 June 2018; Accepted 10 July 2018

phenomenon" in the growth of *Clostridium perfringens* and came to the conclusion that it involves three phases: injury, recovery and growth. In contrast, Mellefont et al. (2005) stated that the phenomenon is due to an initial inactivation of a portion of the population followed by growth, rather than injury and recovery of the ability to form colonies on the enumeration medium. However, the above studies as well as all available data about the "Phoenix phenomenon" are based on population level experimentations without studying the behavior of individual cells. To determine whether the subsequent increase in viable counts is due to recovery of culturability of a relatively large fraction of the population, or due to growth within a relatively small fraction of survivors, would require clear discrimination and monitoring of the individual cells within the population.

Recent technological advances enable the study of microbial behavior at the single cell level. Methods based on microscopy and image analysis have been developed and employed for monitoring multiple single cell generations and colonial growth of single cells (Elfwing et al., 2004; Koutsoumanis and Lianou, 2013). Such technologies are expected to be able to differentiate individual cell responses within a population and unravel the underlying mechanisms of microbial behavior under stressful conditions.

For *Salmonella* cells, several studies have reported that as the concentration of sodium chloride increases over 0.5% towards 7–8%, the environmental conditions start to deviate from the optimum towards the growth boundaries (Koutsoumanis, 2008; Zhou et al., 2011). The objectives of the present work were a) to use time lapse microscopy methodologies in order to monitor division and death of *Salmonella* single cells during exposure under various indicative concentrations of sodium chloride within the range of 0.5 to 7% and b) to investigate the role of individual cell heterogeneity in the behavior of microbial populations at conditions close to the boundary of growth.

2. Materials and methods

2.1. Bacterial strain

The bacterial strain used in this study was a *Salmonella enterica* serotype Agona animal isolate, kindly provided by Dr. Martin Wiedmann (Cornell University, New York, United States of America). Stock cultures of the strain were kept frozen (−70 °C) onto Microbank TM porous beads (Pro-Lab Diagnostics, Ontario, Canada), while working cultures were stored refrigerated (5 °C) on tryptone soy agar (TSA; Lab M Limited, Lancashire, United Kingdom) slants and were renewed bimonthly.

2.2. Study of individual cell behavior

The behavior of the pathogen in the presence of various concentrations of sodium chloride was studied at the single cell level through time lapse microscopy and at the population level using plate count. Single cell division times and individual cell times of inactivation were monitored by phase-contrast and confocal laser scanning microscopy, respectively. Due to the microscope setup, the optimum temperature of 37 °C for the long duration of the experiments was a limiting factor. The selected temperature, close to the environmental one, is favorable for *Salmonella* growth and slightly longer division times than at optimum conditions are expected.

2.2.1. Monitoring single cell division times

Single cell growth behavior was studied, under various concentrations of sodium chloride (NaCl 0.5, 3.5, 4.5 and 5.7% w/w), on solid laboratory medium (TSA) at 27 °C. These concentrations were achieved by adding appropriate amounts of salt to the medium which already contains 5 g/l of sodium chloride. The water activity values of the media were measured with an Aqualab water activity meter (Model series 3; Decagon Devices, Inc., Pullman, United States of America) and were the following: 0.995 (0.5% NaCl), 0.977 (3.5% NaCl), 0.973 (4.5% NaCl) and 0.957 (5.7% NaCl).

The strain was activated by transferring a loopful from TSA slants into 10 ml of tryptone soy broth without dextrose (TSB-G; Lab M Limited) and incubating at 37 °C for 18 h. An aliquot of 100 μl of the 18-h culture, after appropriate serial decimal dilutions in one-quarter-strength Ringer's solution (Lab M Limited), with a target population of 10^7 cfu was surface plated on 8 ml TSA (with appropriate amount of NaCl added) solidified in a 90 mm Petri dish. The inoculated agar was left to dry in a biological safety cabinet for 3 min and, then, a piece (approximately 20 ∗ 20 mm) was removed with a scalpel and placed on a glass slide (25 ∗ 75 mm, thickness 1–1.2 mm). The piece of agar was covered by a coverslip and sealed with silicone to avoid dehydration.

Cell division and growth was monitored by phase–contrast time lapse microscopy using a z-motorized microscope (Olympus BX61, Olympus, Tokyo, Japan) equipped with a 100× objective (Olympus) and a high-resolution device camera (Olympus DP71). The sample was maintained at 27 °C using a temperature controlled stage (Linkam PE60, Linkam Scientific Instruments, Surrey, United Kingdom). Images of the field of view were acquired every 5 min for 24 to 72 h. Three to five independent experiments were conducted in order to collect data for approximately 400 cells under each NaCl concentration. An in-house program with the ScopePro module of the ImageProPlus image analysis software version 6.3 (MediaCybernetics Inc. Bethesda, United States of America) was used, which allows the system to be automatically turned on and off before and after the capture of an image. The quality of the images was improved by the autofocus procedure with an extended depth of focus (EDF) system. This procedure allows for multiple (20 to 30) serial images in different z-axis planes to be captured and, then, combines the best focal areas of the serial images into a single in-focus image (z-stack). Individual stack images were compiled and transformed into a time-lapse video showing the behavior of the single cells of the pathogen. Image analysis was performed using the ImageProPlus image analysis software. The high quality of the images allowed the monitoring of division times and the number of cells in the micro-colonies. The manual tag of the software was employed for cell counting. Data about the percentage of dividing and non-dividing cells, first and second division times, cell lysis and filamentation were collected.

2.2.2. Monitoring individual cell times of inactivation

Single cell inactivation behavior was studied in the presence of 5.1 and 5.7% (w/w) sodium chloride on solid laboratory medium (TSA) at 27 °C. For this, the strain was activated by transferring a loopful from TSA slants into 10 ml of tryptone soy broth without dextrose and incubating at 37 °C for 18 h.

In the case of 5.7% NaCl, 100 μl of the 18-h culture, after appropriate serial decimal dilutions in one-quarter-strength Ringer's solution, was surface plated on 14 ml TSA (NaCl supplemented) solidified in a 90 mm Petri dish. The inoculated agar was left to dry in a biological safety cabinet for 10 min and, then, a disc (of 22 mm diameter) was removed with a scalpel, inverted, placed in a glass bottom dish (0.17 mm thickness; WillCo Wells BV, Amsterdam, The Netherlands) and led for microscopic observation. Appropriate amount of gelatin solution 5% w/w (Gelatin from porcine skin, Sigma-Aldrich, United States of America) containing 300 μl Propidium Iodide (PI; 1.0 mg/ml in water – filter sterilized, Sigma-Aldrich, Hannover, Germany) and sodium chloride was added with care to fully cover the sample. Appropriate amount of sodium chloride was added in the gelatin solution to avoid the reduction of the final concentration of NaCl faced by the cells of the pathogen. Three independent experiments were conducted in order to collect data for approximately 400 cells.

In the case of 5.1% NaCl, 100 μl of the 18-h culture, after appropriate serial decimal dilutions in one-quarter-strength Ringer's solution, was surface plated on 14 ml TSA (no NaCl supplemented) solidified in a 90 mm Petri dish. The inoculated agar was left to dry in a biological