Food Microbiology 28 (2011) 648–655

Contents lists available at ScienceDirect

Food Microbiology

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

Individual cell lag time distributions of *Cronobacter* (*Enterobacter sakazakii*) and impact of pooling samples on its detection in powdered infant formula

Rabeb Bennour Miled [a], Laurent Guillier [a], Sandra Neves [a], Jean-Christophe Augustin [b], Pierre Colin [c], Nathalie Gnanou Besse [a,*]

[a] Agence nationale de sécurité sanitaire Anses Laboratoire de sécurité des aliments, Anses 23 Avenue du Général de Gaulle, 94706 Maisons Alfort cedex, France
[b] Unité MASQ, Ecole Nationale Vétérinaire d'Alfort, 7 Avenue du Général de Gaulle, 94704 Maisons Alfort cedex, France
[c] Ecole Supérieure de Microbiologie et de Sécurité Alimentaire, ESMISAB, Plouzane, France

ARTICLE INFO

Article history:
Received 8 January 2010
Received in revised form
6 August 2010
Accepted 10 August 2010
Available online 18 August 2010

Keywords:
Enterobacter sakazakii
Cronobacter
Powdered infant food formula
Lag time
Pooling
Detection
Growth

ABSTRACT

Cells of six strains of Cronobacter were subjected to dry stress and stored for 2.5 months at ambient temperature. The individual cell lag time distributions of recovered cells were characterized at 25 °C and 37 °C in non-selective broth. The individual cell lag times were deduced from the times taken by cultures from individual cells to reach an optical density threshold. In parallel, growth curves for each strain at high contamination levels were determined in the same growth conditions. In general, the extreme value type II distribution with a shape parameter fixed to 5 (EVIIb) was the most effective at describing the 12 observed distributions of individual cell lag times. Recently, a model for characterizing individual cell lag time distribution from population growth parameters was developed for other food-borne pathogenic bacteria such as *Listeria monocytogenes*. We confirmed this model's applicability to *Cronobacter* by comparing the mean and the standard deviation of individual cell lag times to populational lag times observed with high initial concentration experiments. We also validated the model in realistic conditions by studying growth in powdered infant formula decimally diluted in Buffered Peptone Water, which represents the first enrichment step of the standard detection method for *Cronobacter*. Individual lag times and the pooling of samples significantly affect detection performances.

1. Introduction

Enterobacter sakazakii is a Gram-negative bacillus, a member of the *Enterobacteriaceae* family, which has recently been further investigated to clarify its taxonomy. The proposed reclassification of this organism as species (*Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter dublinensis*, *Cronobacter turicensis*, *Cronobacter muytjensii* and *Cronobacter genomospecies* 1) and subspecies (*C. dublinensis subsp. dublinensis*, *C. dublinensis subsp. lausannensis*, *C. dublinensis subsp. lactaridi*) in a new genus, *Cronobacter*, is based on the results of independent molecular methods and biochemical markers (Iversen et al., 2007, 2008a). Cronobacter is considered to be an emerging food-borne pathogen, and has been identified as the causative agent of several outbreaks or sporadic cases of very serious neonatal infections, causing meningitis, septicaemia or necrotising enterocolitis in infants (Arseni et al., 1987; Nazarowec-White and Farber, 1997; Bar-Oz et al., 2001; Van Acker et al., 2001; Iversen and Forsythe, 2003; Lehner and Stephan, 2004). Disease frequency is very low, but the mortality rate has been reported to be as high as 20–50% (Anonymous, 2004; Lehner and Stephan, 2004). In most cases, the source of infection has been powdered infant formula (PIF) fed to the individuals (Simmons et al., 1989; Clark et al., 1990; Van Acker et al., 2001; Lehner and Stephan, 2004; Anonymous, 2005a). Cronobacter's high resistance to osmotic and dry stresses explains its presence and survival in PIF factory products and environments (Breeuwer et al., 2003). In PIF, contamination levels range from 0.36 to 66 cfu per 100 g, but in most cases are lower than 1 cfu per 100 g (Muytjens et al., 1988; Anonymous, 2006a, 2008). Errors in feeding bottle-preparation practices, such as improper holding temperatures, may lead to growth of the pathogen to a critical cell level, and the occurrence of the infection (Bar-Oz et al., 2001; Lehner and Stephan, 2004; Anonymous, 2005a). Iversen and Forsythe (2003) speculated that a reasonable estimate for infection might be close to that postulated for *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b, or *Neisseria meningitidis*, i.e., ca.1000 cfu.

The International Dairy Federation (IDF) and the International Organization for Standardization (ISO) have established

* Corresponding author. Tel.: +33 1 49 77 11 10; fax: +33 1 49 77 11 02.
E-mail address: n.besse@afssa.fr (N.G. Besse).

0740-0020/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2010.08.005

a reference method for the detection of Cronobacter in PIF (ISO/TS 22964, Anonymous, 2006b). The method is based on pre-enrichment in non-selective buffered peptone water (BPW), followed by a selective enrichment procedure (Guillaume-Gentil et al., 2005) in modified lauryl sulphate tryptose (mLST) broth and plating on the chromogenic selective isolation agar "*Enterobacter sakazakii* Isolation Agar" (ESIA). The standard method is currently being revised, with mLST being replaced by the Cronobacter Screening Broth (CSB) used in conjunction with chromogenic media only in the case of a presumptive positive reaction (change in its color). Indeed, *Cronobacter* Screening Broth (CSB) was specifically designed by Iversen et al. (2008b) to be used in conjunction with chromogenic media. It allows presumptive *Cronobacter*-positive samples to be identified before the selective plating step, and also allows *Cronobacter*-negative products to be released at least 24 h earlier than with other standard methods. This is achieved by the inclusion of sucrose and the pH indicator bromocresol purple. Whilst *Cronobacter* spp. can ferment sucrose, most other *Enterobacteriaceae*, which grow as false positives on chromogenic agars, cannot. Consequently, a yellow/orange coloration in the broth after incubation (due to sucrose fermentation) indicates presumptive *Cronobacter*. If the broth is still purple in color after incubation, the sample is presumed to be negative for *Cronobacter*. One must note that for a slow strain, a minimum of 10^4 cfu/ml in BPW may be required after pre-enrichment to ferment CSB (Joosten and Iversen, 2009). From a regulatory point of view, the European Community (EC) Regulation 2073/2005 on microbiological criteria for foodstuffs (Anonymous, 2005b) amended by the recent European Community (EC) Regulation 1441/2007 (Anonymous, 2007) defines a semi-quantitative food safety criteria for *Cronobacter* (quoted as *E. sakazakii*): absence in 10 g, for PIF and dried dietary foods for special medical purposes, intended for infants below 6 months of age. The sampling plan requires 30 sample units to be tested in a batch. For greater convenience and in order to reduce analysis cost, a common practice in the PIF industry is to pool samples at a constant dilution rate, in order to perform a single first pre-enrichment and subsequent analysis. For example the analysis of one 100 g pooled sample diluted in 900 g of BPW, instead of ten samples of 10 g diluted each in 90 g of BPW is quite common. Consequences on *Cronobacter* growth and detection have not been established (Anonymous, 2008).

In conditions of very low contamination levels, individual cell variability can have an important impact on pathogen bacteria growth (Guillier and Augustin, 2006). Knowing how their long-term presence in PIF, and subsequent stress, affect the variability of single-cell lag times is important in assessing the risk of cell recovery and growth in reconstituted milk, where low numbers of stressed cells of pathogenic bacteria may be distributed among PIF samples. Moreover, the impact of individual cell variability on *Cronobacter* growth in the pre-enrichment broth of the standard detection method should also be taken into account, in order to better estimate detection performance. Recently, a model for characterizing individual cell lag time distribution from populational growth parameters was developed for *L. monocytogenes* (Guillier and Augustin, 2006, 2008). The first objective of the present work was to verify this model's applicability to *Cronobacter* subjected to dry stress for different regrowth conditions and strains. The second objective was to validate this model in a realistic condition such as the first enrichment step of the standard detection method. The log count distribution, or vertical distribution (D'Arrigo et al., 2006) was applied to estimate the distribution of the single-cell lag times in BPW. This study also made it possible to evaluate the impact of pooling samples on *Cronobacter* growth and detection.

2. Materials and methods

2.1. Bacterial strains and preparation of the inoculums

To ensure diversity, seven strains of *Cronobacter* were selected from a collection of 64 other *Cronobacter* strains, described and characterized by Miled-Bennour et al. (2010). Characterization of these seven isolates by the pulsed-field gel electrophoresis (PFGE) technique (Miled-Bennour et al., 2010) revealed large variability in molecular fingerprints. Three strains (05 CHPL 40, 05 CHPL 63 and 08 HMPA 02) belonged to different species (*C. malonaticus*, *C. muytjensii* and *C. turicensis* respectively) and four (05 CHPL 18, 05 CHPL 59, 05 CHPL 01 and 05 CHPL 101bis) belonged to the same species (*C. sakazakii*) but had different growth rates (Miled-Bennour et al., 2010) or biofilm formation abilities (results not shown). Strains 05 CHPL 01 (ATCC 29544) and 08 HMPA 02 (DSMZ 18703) are type strains with clinical origin. Strain 05 CHPL 18 was isolated from a PIF product implicated in neonatal infections in 2004 in France, and has the same PFGE profile as a clinical strain isolated a child's cerebrospinal fluid. Strains 05 CHPL 40 and 05 CHPL 101bis were isolated from the PIF production environment and strain 05 CHPL 63 was isolated from food. CHPL and HMPA correspond to the successive names of the same Anses unit.

Stock cultures were maintained at −80 °C using Cryobank tubes (AES Laboratoires, Combourg, France). Prior to each experiment, cultures were revived by plating onto Trypticase Soya Agar with Yeast Extract (TSAYE). To simulate realistic conditions, strains were subjected to desiccation: *Cronobacter* strains were grown in an equal mixture of Brain–Heart Infusion (BHI) broth and sterile infant milk formula (follow-on formula from retailer) for 24 h at 37 °C and then freeze-dried using the CHRIST LOC-2M apparatus (Bioblock Scientific, Ile de France, Vanves cedex, France). For practical reasons this treatment was chosen as it was a simple and reproducible way to create a dry stress. Contaminated powder was further diluted 1 in 100 in PIF intended for infants below 6 months of age (previously tested negative for Cronobacter contamination using the ISO/TS 22964 method, and with a low level of total microflora), and stored for 2.5 months at ambient temperature in the PIF tin before use. Prior to freeze-drying, the culture usually attained 10^9 cfu/ml, and just after freeze-drying and dilution in PIF, the powder contamination level was between 10^6 and 10^8 cfu/g. Before use, the powder contamination level was measured by direct enumeration on TSAYE and ESIA agar (AES Laboratoires, Combourg, France), incubated for 24 h, respectively, at 37 °C and 44 °C. All decimal dilutions were prepared in Tryptone Salt (TS) diluent. Strain 05 CHPL 101bis, whose concentration decreased dramatically over time, could not be used.

2.2. Estimation of population growth parameters

The maximum specific growth rate and the population lag time were estimated for all strains in BHI at 37 °C and 25 °C. From the contaminated powder obtained previously, serial dilutions were performed in BHI in order to obtain 10^2–10^3 cfu/ml in the final dilution. Samples from each broth culture were removed periodically to enumerate cells (cfu/ml) on ESIA agar. The resulting growth curves were fitted to the Baranyi model (Baranyi and Roberts, 1994) using MicroFit software (Institute of Food Research, Norwich, United Kingdom). Curves were fitted by minimizing the sum of squared errors between the data points. Population growth parameters were estimated from at least three growth curve repetitions.

2.3. Determination of individual cell lag times

Individual cell lag times were estimated from turbidity growth curves generated by an automated spectrophotometer, the