International Journal of Food Microbiology 160 (2012) 171–178

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

Strain variability of the biofilm-forming ability of *Salmonella enterica* under various environmental conditions

Alexandra Lianou, Konstantinos P. Koutsoumanis *

Laboratory of Food Microbiology and Hygiene, Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece

ARTICLE INFO

Article history:
Received 1 August 2012
Received in revised form 14 September 2012
Accepted 1 October 2012
Available online 7 October 2012

Keywords:
Salmonella enterica
Strain variability
Biofilm
Environmental conditions

ABSTRACT

The biofilm-forming ability of 60 *Salmonella enterica* strains was assessed at different pH values (3.8–7.0), NaCl concentrations (0.5–8.0%) and temperatures (4–37 °C). A total of 4320 biofilm formation tests (60 strains × 12 different environmental conditions × 6 replicates) were carried out. Biofilm formation was evaluated in tryptone soy broth after 48 h of incubation in polystyrene microtiter plates using crystal violet staining, and its quantification was based on the difference between the optical density measurements of the test and negative control (uninoculated) samples ($\Delta OD_{580\ nm}$). The tested strains formed biofilms under a wide range of environmental conditions, while extensive strain variability was observed with the mean $\Delta OD_{580\ nm}$ values ranging from 0 to 2.388 depending on the strain and the condition evaluated. The strain variability of biofilm formation was affected by all three of the environmental parameters tested, and appeared to increase as the environmental conditions became less favorable for the organism. In addition, the increase in the strain variability caused by pH was found to be much greater than that caused by NaCl or temperature. For example, the coefficient of variation (CV = standard deviation/mean * 100) of $\Delta OD_{580\ nm}$ among the tested strains at pH 7.0–0.5% NaCl-37 °C was 104%, while at pH 3.8–0.5% NaCl-37 °C, pH 7.0–8.0% NaCl-37 °C and pH 7.0–0.5% NaCl-8 °C was 351.5%, 204.1% and 175.6%, respectively. The optimum conditions for biofilm formation, providing the maximum $\Delta OD_{580\ nm}$, varied significantly among the tested strains. Among the evaluated conditions, most of the *S. enterica* strains were clustered as forming their highest amount of biofilm at pH 5.5 (35 strains; 58.3%), at 0.5% NaCl (29 strains; 48.3%) and at 25 °C (32 strains; 53.3%). No relationships were observed between the biofilm-forming ability of the strains and their serotype or their growth kinetic behavior as this was evaluated in a previous study. The findings of this study provide useful information in advancing the current understanding of strain variability, as well as in strain selection for the evaluation of the efficacy of disinfection/sanitation procedures against biofilm formation.

1. Introduction

The architecturally complex multicellular communities termed biofilms provide bacteria with the ability to grow adhered to both biotic and abiotic surfaces (López et al., 2010; Steenackers et al., 2012). Such communities are cell aggregates enclosed in a self-produced extracellular polymeric matrix and constitute a fundamental aspect of bacterial ecology and biology (López et al., 2010; Wong and O'Toole, 2011), representing in a way "the natural stationary phase of bacterial growth" (López et al., 2010). It has been generally acknowledged that biofilm formation is an environmental adaptation strategy, and research findings on the genetic and proteomic basis of such a strategy clearly demonstrate that bacterial cell physiology within a biofilm is markedly different from that in the planktonic mode of growth (Karunakaran et al., 2011). Surface association and biofilm formation provide protection to bacterial cells against a wide range of environmental challenges including UV light radiation, metal toxicity, pH and osmotic changes, dehydration, host immune responses, antimicrobial agents and disinfectants (Hall-Stoodley et al., 2004; Nilsson et al., 2011; Smirnova et al., 2010; Van Houdt and Michiels, 2010).

The developmental process of biofilms involves both cell–surface and cell–cell interactions which determine their structure, function and composition (Karunakaran et al., 2011; Wong and O'Toole, 2011). Such interactions are affected by the chemical and physical environment to which the bacterial cells and the surface are exposed, and take place in the context of an intricate regulatory network (Karatan and Watnick, 2009; Palmer et al., 2007). Due to the complex interactions governing biofilms, their study has drawn the attention of scientists in various disciplines (materials scientists, physicists, chemists, microbiologists and nanotechnology experts), and the role of multidisciplinarity has been recognized as critical for advancing biofilm research (Karunakaran et al., 2011; Wong and O'Toole, 2011). Despite the considerable amount of research that has been conducted in the last 40 years, with several imaging and molecular techniques being developed and used for this purpose, the processes involved in biofilm formation have not been completely

* Corresponding author. Tel./fax: +30 2310991647.
E-mail address: kkoutsou@agro.auth.gr (K.P. Koutsoumanis).

0168-1605/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2012.10.002

elucidated and characterized (Hall-Stoodley et al., 2004; Karunakaran et al., 2011; Smirnova et al., 2010). Indeed, it has been opined that the study of biofilms is about to evolve into a discipline in its own right, referred to as "biofilmology", with significant research potential within the next decade (Karunakaran et al., 2011).

The enteric bacterial pathogen *Salmonella enterica* has been associated with numerous cases of foodborne infections worldwide, with its control constituting an ongoing challenge for food safety and public health authorities (CDC, 2011; EFSA-ECDC, 2012; Scallan et al., 2011). *S. enterica* is capable of forming biofilms on various inert (e.g., plastic, rubber, glass, stainless steel) or living (e.g., plants, animal epithelial cells, gallstones) surfaces, an ability that contributes to its resistance and persistence in both host and non-host environments (Steenackers et al., 2012). Biofilm formation on materials commonly encountered in food-processing environments (e.g., plastic and stainless steel) has major ramifications for the food industry. Biofilms formed by bacterial pathogens, such as *S. enterica*, on food-processing equipment and other food contact surfaces are very likely to serve as a persistent reservoir of contamination, compromising food safety and human health (Iibuchi et al., 2010; Van Houdt and Michiels, 2010). Information regarding the different types of surfaces on which *S. enterica* biofilms are formed, their structural components and the laboratory setups commonly used for their study has been recently reviewed by Steenackers et al. (2012).

Clarification of the network of environmental signals regulating the biofilm formation process of *S. enterica* has been the objective of extensive research (Steenackers et al., 2012). Various environmental parameters with relevance for the food industry have been studied and demonstrated to affect the biofilm formation capability of *S. enterica*, including growth medium composition, incubation temperature, osmolarity, pH and atmosphere (Giaouris et al., 2005; Speranza et al., 2011; Stepanović et al., 2003, 2004; Xu et al., 2010). Furthermore, research data indicate that biofilm formation by *S. enterica* (Agarwal et al., 2011; Díez-García et al., 2012; Oliveira et al., 2006, 2007; Stepanović et al., 2004) and other foodborne pathogens (Nilsson et al., 2011; Reisner et al., 2006; Rode et al., 2007) is strongly strain-dependent. Nevertheless, only few studies have assessed the biofilm-forming ability of multiple *S. enterica* strains, while even more limited are the available research data regarding biofilm production by different strains of the pathogen under various environmental conditions. Therefore, the main objective of the present study was to evaluate the variability in biofilm formation among *S. enterica* strains as affected by environmental conditions, and more specifically by pH, osmolarity (i.e., NaCl concentration) and temperature. Potential associations of the biofilm-forming ability of the tested strains with their serotype and their growth kinetic behavior (Lianou and Koutsoumanis, 2011) also were assessed.

2. Materials and methods

2.1. S. enterica strains

Sixty strains of *S. enterica*, primarily isolates of human or animal (bovine) origin, were evaluated in this study. The tested strains were kindly provided by Dr. Martin Wiedmann (Cornell University, Ithaca NY, USA), Dr. Constantin Genigeorgis (Aristotle University of Thessaloniki, Thessaloniki, Greece) and Dr. Daniil Sergelidis (Aristotle University of Thessaloniki), and represented various serotypes of the pathogen: *S. enterica* serotype Typhimurium (18 strains), *S.* Enteritidis (10), *S.* Newport (9), *S.* Heidelberg (8), *S.* Montevideo (4), *S.* Seftenberg (4), *S.* Infantis (3), *S.* Agona (3) and *S.* Derby (1). A table providing a list of the tested strains along with information regarding their serotype and exact origin and source has been previously published (Lianou and Koutsoumanis, 2011).

Stock cultures of the strains were stored frozen (−70 °C) onto Microbank™ porous beads (Pro-Lab Diagnostics, Ontario, Canada). Working cultures were stored refrigerated (5 °C) on tryptone soy agar (TSA; Lab M Limited, Lancashire, United Kingdom) slants and were renewed bimonthly. Strains were activated by transferring a loopful from the TSA slants into 10 ml of tryptone soy broth (TSB; Lab M Limited) and incubating at 37 °C for 24 h.

2.2. Biofilm formation

2.2.1. Environmental conditions

The ability of the 60 *S. enterica* strains to form biofilms was evaluated in TSB and at different (i) pH values (3.8, 4.5, 5.5 and 7.0), (ii) NaCl concentrations (0.5%, 3.5%, 4.5%, 6.0% and 8.0% w/v) and (iii) temperatures (4 °C, 8 °C, 15 °C, 25 °C and 37 °C). The effects of the environmental parameters (i.e., pH, NaCl concentration and temperature) on biofilm formation were evaluated singly, meaning that the abovementioned values of each parameter were assessed with the other two parameters taking values representing their optimum levels (i.e., pH 7.0, 0.5% NaCl and 37 °C, respectively). Furthermore, the above experimentations were selected so that the biofilm formation of the *S. enterica* strains is evaluated both under favorable and unfavorable (suboptimal or limiting) conditions for the growth of the pathogen. The pH of TSB (with an initial value of 7.3 ± 0.2) was adjusted to the above values with HCl (min. 37%; Sigma-Aldrich, Seelze, Germany) using a digital pH meter with an epoxy refillable pH probe (Orion 3-Star pH Benchtop; Thermo Electron Corporation, Beverly, MA, USA). The above salt concentrations were attained by adding, when needed, to the medium (which has a NaCl concentration (w/v) of 0.5% as part of its basal composition) appropriate amounts of NaCl (Merck, Darmstadt, Germany); the corresponding values of water activity (a_w), measured with an AquaLab water activity meter (Model series 3; Decagon Devices, Inc., Pullman, WA, USA), were the following: 0.997 (0.5% NaCl), 0.982 (3.5% NaCl), 0.976 (4.5% NaCl), 0.970 (6.0% NaCl) and 0.959 (8.0% NaCl). The pH and a_w values of the media were also measured after autoclaving to assure that they were not considerably changed by the sterilization process. With regard to the abovementioned temperatures, these were attained in high-precision incubators (model MIR 153, Sanyo Electric Co., Ora-Gun, Gunma, Japan), and were monitored during incubation using electronic temperature-monitoring devices (Cox Tracer data logger; Cox Technologies, Belmont, NC, USA).

2.2.2. Biofilm formation assay

Biofilm formation was quantified using a colorimetric microtiter plate method. Such a method is generally based on the measurement of the optical density of biofilm mass in microtiter plate wells after crystal violet staining, and has been frequently utilized, in various versions, for the *in vitro* evaluation of biofilm production (Agarwal et al., 2011; Díez-García et al., 2012; Naves et al., 2008; Stepanović et al., 2000, 2003, 2004). The biofilm formation assay applied in the present study was based on previously described procedures, with some modifications, and involved the use of 100-well polystyrene microtiter plates (Oy Growth Curves Ab Ltd., Raisio, Finland) and the automated turbidimetric system Bioscreen C (Oy Growth Curves Ab Ltd.).

More specifically, 20-μl aliquots of each 24-h culture of each one of the *S. enterica* strains were added to 180 μl of TSB (with the characteristics described in 2.2.1) dispensed in microtiter plates. Each strain was tested in six replicate wells, while negative control wells (10 in each microtiter plate), containing broth only, also were included. The microtiter plates were incubated statically at the aforementioned temperatures (depending on the conditions tested) for 48 h. The content of the plates' wells was then discarded and, in order for non-adherent (or reversibly attached) bacterial cells to be removed, the wells were washed with 200 μl of sterile quarter strength Ringer's solution (Lab M Limited); during this rinsing step, the microtiter plates were agitated for 5 min at the medium amplitude setting of the Bioscreen C system. The adherent bacterial cells were fixed with 200 μl of methanol (min. 99.8%; Scharlau Chemie S.A., Barcelona, Spain) per well for 15 min. The microtiter plates were then emptied by