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Cross-protection between controlled acid-adaptation and thermal inactivation for 48 *Escherichia coli* strains

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ABSTRACT

Given the importance of pH reduction and thermal treatment in food processing and food preservation strategies, the cross-protection between acid adaptation and subsequent thermal inactivation for 48 *Escherichia coli* strains was investigated. Those strains were selected among 188 *E. coli* strains according to their odds of growth under low pH conditions as determined by Haberbeck et al. (2015) [Haberbeck, L.U., Oliveira, R.C., Vivijs, B., Wenseleers, T., Aertsen, A., Michiels, C., Geeraerd, A.H., 2015. Variability in growth/no growth boundaries of 188 different *Escherichia coli* strains reveals that approximately 75% have a higher growth probability under low pH conditions than *E. coli* O157:H7 strain ATCC 43888. Food Microbiol. 45, 222–230]. *E. coli* cells were acid and nonacid-adapted during overnight growth in controlled acidic pH (5.5) and neutral pH (7.0), respectively, in buffered Lysogenic Broth (LB). Then, they were heat inactivated at 58 °C in non-buffered LB adjusted to pH 6.2 and 7.0. Thus, four conditions were tested in total by combining the different pH values during growth/thermal inactivation: 5.5/6.2, 5.5/7.0, 7.0/6.2 and 7.0/7.0. Acid adaptation in buffered LB at pH 5.5 increased the heat resistance of *E. coli* strains in comparison with nonacid-adaptation at pH 7.0. For instance, the median D_{58}-value of strains inactivated at pH 7.0 was approximately 6 and 4 min for acid-adapted and nonacid-adapted strains, respectively. For the nonacid-adapted strains, the thermal inactivation at pH 6.2 and 7.0 was not significantly ($p = 0.06$) different, while for the acid-adapted strains, the thermal treatment at pH 6.2 showed a higher heat resistance than at pH 7.0. The correlation between the odds of growth under low pH previously determined and the heat resistance was significant ($p < 0.05$). Remarkably, a great variability in heat resistance among the strains was observed for all pH combinations, with D_{58}-values varying between 1.0 and 69.0 min. In addition, highly heat resistant strains were detected. Those strains exhibited D_{58}-values between 17.6 and 69.0 min, while *E. coli* O157:H7 (ATCC 43888) showed D_{58}-values between 1.2 and 3.1 min. In summary, results clearly showed that adaptation of *E. coli* cells to constant acidic pH results in cross-protection against thermal inactivation.

1. Introduction

Bacteria are capable to adapt to different stress environments encountered in their natural habitat through highly coordinated cell mechanisms. When this adaptation occurs during exposure to a nonlethal stress factor, bacteria can further exhibit higher resistance to the same or to different stress factors, a phenomenon known as cross-protection (Battesti et al., 2011). Cross-protection has important implications in food safety and in food processing optimizations. For instance, the use of hurdle technologies, where sub-lethal or mild stresses are applied, may induce multiple stress responses reducing the efficacy of subsequent treatments (Capozzi et al., 2009).

In literature, cross-protection between different stresses was studied for several foodborne pathogens such as *Salmonella* (Tassou et al., 2009; Xu et al., 2008), *Listeria monocytogenes* (Gabriel and Arellano, 2014; Koutsoumanis et al., 2003; Pittman et al., 2014) and *Escherichia coli* (Cheng et al., 2003; Stopforth et al., 2007; Usaga et al., 2014a; Velliou et al., 2011). Specifically, studies about cross-protection between low pH and other stress factors applied different acid adaptation methodologies, such as exposing the cells to gradual acidification as a result of glucose fermentation (Buchanan and Edelson, 1996; Sharma et al., 2005) or growing the cells in unbuffered media initially adjusted to low pH (Cheng et al., 2003; Gabriel and Nakano, 2011; Singh and

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Jiang, 2012; Usaga et al., 2014b; Velliou et al., 2011). In both methodologies the exact pH profile during adaptation is unknown. It has been observed that complex media initially adjusted to low or neutral pH showed a further pH reduction during growth due to the production of organic acids. For instance, during growth of *E. coli* O157:H7 in TSB (tryptone soya broth), the initial pH of 5.0 and 6.0 dropped to 4.5 and 5.0, respectively (Yuk and Marshall, 2004). Similarly, during *Salmonella enterica* ser. Enteritidis growth in TSB, the initial pH of 5.3 and 6.3 dropped to 4.9 and 5.2, respectively (Yang et al., 2014). Although considerable research has been devoted to acid adaptation of cells exposed to gradual acidification as a result of glucose fermentation on one hand or immediately exposed to media initially adjusted to low pH on the other hand, less attention has been paid to acid adaptation in a controlled pH environment, with Koutsoumanis and Sofos (2004) being a notable exception.

Phenotypic variability among strains of the same species has been studied for different aspects and for different bacterial species, like *E. coli* (Haberbeck et al., 2015; Oh et al., 2009; Saridakis et al., 2004; Whiting and Golden, 2002), *S. enterica* (Lianou and Koutsoumanis, 2013), *Listeria monocytogenes* (Aryani et al., 2015a, 2015b; Lianou et al., 2006) and *Lactobacillus plantarum* (Parente et al., 2010). The mechanisms causing phenotypic variability among strains of the same species can be attributed to genomic and epigenetic differences (Smits et al., 2006). How genetic differences in a population influences phenotypic variation and evolution is a major concern in modern biology (Bergström et al., 2014). Genetic variation can occur, for instance, by gene loss/genome reduction, genome rearrangement, expansion of functional capabilities through gene duplication, acquisition of functional capabilities through lateral gene transfer and gene expression differences (Carreto et al., 2011; Luo et al., 2010). Moreover, many factors have been considered to contribute to the variability in thermal resistance of microorganisms, such as strain differences, physiological state of the cell, growth and experimental conditions (Aryani et al., 2015b). In the current study, variability is defined as in Aryani et al. (2015b): strain variability is the variability between strains from the same species, biological variability is the variability between biologically independent repetitions for each strain and experimental variability is the repeatability of parallel experimental replicates.

In the present work, we investigated three hypotheses. The first hypothesis is that growth of *E. coli* strains under low pH condition (acid-adaptation) induces cross-protection against subsequent thermal inactivation at 58 °C. Forty-eight *E. coli* strains were selected among 188 strains previously characterized according to their odds of growth under low pH (Haberbeck et al., 2015). Differently from previous studies, the strains were acid-adapted by growing the cells at pH 5.5 or pH 7.0 in buffered lysogenic broth (LB) which maintained the pH constant during the adaptation phase. This approach was chosen to evaluate the effectiveness of acid adaptation knowing the exact pH conditions during growth. Besides that, a constant pH in a buffered medium simulates more closely foods such as meat products, which contain naturally a range of potentially buffering molecules (Pösö and Puolanne, 2005). The second hypothesis is that the cells would be more heat resistant in heating medium of pH 6.2 than those in pH 7.0. It has been reported that the maximum heat resistance of *E. coli*, *Salmonella* Enteritidis and *Cronobacter* ssp. is obtained in slightly acidified media (Arroyo et al., 2009; Blackburn et al., 1997; Mañas et al., 2003). Four conditions were tested combining two pH values during growth (5.5 and 7.0) and two pH values for the heating menstruum (6.2 and 7.0). In summary, combining this two first hypotheses, we assume the following order of heat resistance: 5.5/6.2 > 5.5/7.0 > 7.0/6.2 > 7.0/7.0. The third hypothesis is that that strains with relatively high odds of growth under low pH (3.8 to 4.2), defined in Haberbeck et al. (2015), would show to be more heat resistant. Finally, we also had the objective to quantify the impact of strain variability on the thermal resistance (phenotypic response) of the *E. coli* strains tested.

2. Material and methods

2.1. Bacterial strains

The 48 *E. coli* strains used in this study are listed in Table 1. They were selected from the 188 strains in the previous study (Haberbeck et al., 2015) using a stratified sampling method. Since they have been isolated from a large number of diverse sources, most of these bacteria can be considered unique strains. However, it cannot be excluded that some isolates originating from the same source and having similar odds of growth in acidic conditions (Table 1) are clonal. Stock cultures were maintained at −80 °C in LB with 25% v/v glycerol. Strains were activated by loop-streaking the stock cultures onto LB agar stock plates, which were then incubated for 24 h at 37 °C. Forty-seven strains plus the reference *E. coli* O157:H7 (ATCC 43888) were selected according to their odds of growth previously determined (Haberbeck et al., 2015). The selection was done in a stratified way as follows: firstly, 14 strains were randomly selected among the ones with higher odds of growth than the reference, secondly, 9 strains were randomly selected among the ones with lower odds of growth, and lastly 24 strains were randomly selected among the ones with higher and lower odds. By doing so, the stratification ensured to cover the different possibilities concerning lower and higher odds of growth.

2.2. Acid adaptation

Acid adaptation was achieved during pre-inoculum growth. LB was buffered with 0.1 M of 2-(N, morpholino) ethanesulfonic acid (MES) (AppliChem, Darmstadt, Germany) at pH 5.5 for the acid-adapted cells, and with 0.1 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, Bornem, Belgium) at pH 7.0 for the nonacid-adapted cells. pH was adjusted with 1 M of NaOH and verified using a digital pH meter (Hanna, HI9125). Then, buffered LB was filter-sterilized with 0.22 μm cellulose acetate sterile filters (VWR International, Belgium) and kept at 4 °C until use. Prior to thermal treatments, single colonies were inoculated in microcentrifuge tubes with 400 μl buffered LB. The pre-inoculum was incubated at 37 °C overnight under shaking at 200 rpm. After overnight growth and before proceeding with thermal treatment, pH stability was checked and confirmed for a random selection of pre-cultures. The working culture was obtained by centrifuging the pre-inoculum at 3600 g for 10 min (Centrifuge 5430, Eppendorf, Hamburg, Germany). The cell pellets were then washed once with 10 mM of potassium phosphate buffer (PPB) and finally resuspended in LB at pH 7.0 or 6.2 just before the thermal inactivation. The pH of LB for the thermal inactivation was adjusted with 1 M of NaOH or 37% HCl followed by filter sterilization. In total, four conditions were tested combining two pH values during growth (5.5 and 7.0) and two during thermal inactivation (6.2 and 7.0). Along the text, the pH conditions are referred to as the combination of pH during growth/pH during thermal inactivation, i.e. 5.5/6.2, 5.5/7.0, 7.0/6.2 and 7.0/7.0.

2.3. Thermal inactivation

Thermal inactivation was accomplished at 58 °C using a thermal cycler (Biometra®, Westburg, The Netherlands). The experiments were carried out using thin walled PCR tubes (Bioplastics, Landgraaf, The Netherlands) with 30 μl of the working culture. The initial cell concentration was on average 10^8 CFU/ml. Samples were placed in the thermal cycler and to reduce and standardize the come-up time (time to reach the target temperature), temperature was initially set at 37 °C for 30 s. Then the thermocycler block started to increase the temperature, and when the inactivation temperature of 58 °C was reached time zero samples were taken and immersed immediately in an ice-water bath. After 10 min, PCR tubes were removed and placed in the ice-water bath. Some strains were subjected to a second set of heat treatment experiments, limiting the duration at 58 °C to 5 min; and some strains were