Physiologically distinct subpopulations formed in *Escherichia coli* cultures in response to heat shock

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Bacteria can form heterogeneous populations containing phenotypic variants of genetically identical cells. The heterogeneity of populations can be considered a bet-hedging strategy allowing adaptation to unknown environmental changes – at least some individual subpopulations or cells might be able to withstand future adverse conditions. Using Percoll gradient centrifugation, we demonstrated that in an *Escherichia coli* culture exposed to heat shock at 50 °C, two physiologically distinct subpopulations were formed. A high-density subpopulation (HD$_{50}$) demonstrated continued growth immediately after its transfer to LB medium, whereas the growth of a low-density subpopulation (LD$_{50}$) was considerably postponed. The LD$_{50}$ subpopulation contained mainly viable but non-culturable bacteria and exhibited higher tolerance to sublethal concentrations of antibiotics or H$_2$O$_2$ than HD$_{50}$ cells. The levels of aggregated proteins and main molecular chaperones were comparable in both subpopulations; however, a decreased number of ribosomes and a significant increase in protein oxidation were observed in the LD$_{50}$ subpopulation as compared with the HD$_{50}$ subpopulation. Interestingly, under anaerobic heat stress, the formation of the HD$_{50}$ subpopulation was decreased and culturability of the LD$_{50}$ subpopulation was significantly increased. In both subpopulations the level of protein aggregates formed under anaerobic and aerobic heat stress was comparable. We concluded that the formation of protein aggregates was independent of oxidative damage induced by heat stress, and that oxidative stress and not protein aggregation limited growth and caused loss of LD$_{50}$ culturability. Our results indicate that heat stress induces the formation of distinct subpopulations differing in their ability to grow under standard and stress conditions.

1. Introduction

There is increasing evidence that genetically identical bacteria form heterogeneous populations containing phenotypic variants of cells which differ in active metabolic processes (Nikolic et al., 2013; Taniguchi et al., 2010). This phenotypic heterogeneity, or phenotypic noise, may result from stochastic gene expression or accumulation of point mutations (Ackermann, 2015; Elowitz et al., 2002). Stressful conditions can further increase population heterogeneity and Silander et al. (2012) revealed that genes involved in adaptation to stress show increased levels of phenotypic noise. The asymmetrical inheritance of protein aggregates in aging or heat-stressed *E. coli* may also contribute to population heterogeneity. The aggregates are formed at old poles and

after division only the old-pole cell inherits the aggregate, resulting in a decreased growth rate, whereas the new-pole cell is damage-free and able to resume growth faster (Stewart et al., 2005; Lindner et al., 2008, Winkler et al., 2010).

The heterogeneity of populations can be considered a bet-hedging strategy which is helpful during adaptation to uncertain environments. For heterogeneous populations, at least some individual subpopulations or cells can withstand future adverse conditions (Ackermann, 2015). Persister cells are an example of a subpopulation that is often formed stochastically in bacterial cultures (Balaban et al., 2004). Persisters are dormant or slow growing cells that are transiently capable of surviving exposure to lethal concentrations of antibiotics. Mechanisms underlying persistence are complex and include the stringent response alarmon (p)

ppGpp, toxin-antitoxin systems and indole signaling (Harms et al., 2017; Leszczynska et al., 2013; Maisonneuve et al., 2013; Vega et al., 2012). The formation of persisters is enhanced by external stimuli including acid, heat or oxidative stress, antibiotics or metabolic shift (Dörr et al., 2010; Hong et al., 2012; Kwan et al., 2013; Vega et al., 2012; Wu et al., 2012). Persisters usually constitute a small fraction of whole populations and in our previous studies only ~0.02% of cells submitted to 50 °C appeared to be persistent (Kuczyńska-Wiśnik et al., 2015).

In this study, we aimed to further characterize the heterogeneity of *E. coli* populations exposed to heat stress. We assumed that, apart from the low-level persister fraction, more abundant subpopulations could be isolated from *E. coli* cultures. This assumption was supported by the results presented earlier by Van Derlinden et al. (2011). They found that *E. coli* showed non-sigmoid growth curves at temperatures near the maximum growth temperature. Mathematical simulations demonstrated that this behavior could be explained by the coexistence of two subpopulations: temperature-resistant and temperature-sensitive.

To observe *E. coli* subpopulations formed under different growth conditions, density-gradient centrifugation has been widely used. Culturable and non-culturable subpopulations were isolated from starved or stationary-phase *E. coli* cultures using equilibrium density-gradient centrifugation in Radioselectan. The non-culturable high-density fraction accumulated increased levels of oxidized and aggregated proteins in comparison with the low-density subpopulation (Desnues et al., 2003; Maisonneuve et al., 2008). *E. coli* cultures are separated into numerous discrete subpopulations by Percoll-gradient centrifugation during growth transition from exponential to stationary phase. This heterogeneity of the stationary culture is caused by an increase in the buoyant density of *E. coli* cells due to accumulation of glycogen (Makinoshima et al., 2002). Percoll-gradient centrifugation has previously been used to sort *E. coli* cells with different buoyant densities depending on the level of overproduced inclusion bodies (Cheng, 1983; Pandey et al., 2013). We assumed that Percoll-gradient centrifugation allows to separate physiologically distinct *E. coli* subpopulations differing in their levels of heat-denatured and aggregated proteins. Our studies revealed that indeed, after heat shock two distinct *E. coli* subpopulations were separated in the Percoll gradient; however, their formation was not related to the level of aggregated proteins. These subpopulations differed in the content of oxidized proteins and ability to grow under standard and stress conditions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli MC4100 [F$^-$ *araD139 (argF-lac)U169 rpsL150 deoC1 relA1 thiA ptsF25 flbB5301 rbsR*], BW25113 [Δ(*araD-araB*)567 Δ(*rhaD-rhaB*)568 Δ*lacZ4787*(::rrnB-3) *hsdR514 rph-1*] and MC4100Δ*glgA*:kan derivatives (Kuczyńska-Wiśnik et al., 2015) were used in this study. The strains were grown in Lysogeny broth (LB) medium in Erlenmeyer flasks with agitation (200 rpm). The cultures submitted to heat stress were first incubated at 30 °C to an OD$_{595}$ of 0.3, and then transferred to flasks (aerobic conditions) or screw-cap tubes filled to the top (anaerobic conditions) and kept at 50 °C for 15 min.

2.2. Percoll-gradient centrifugation of E. coli cultures

To prepare the Percoll gradient, 9 ml of Percoll (Sigma) and 1 ml of 10-fold concentrated PBS were mixed and centrifuged for 1 h at 20,000 rpm in a Beckman SW41Ti rotor. Bacteria from a 100 ml culture were washed, resuspended in 0.5 ml of PBS buffer and layered on top of 10 ml of the preformed Percoll (Sigma) gradient. After 1 h centrifugation at 20,000 rpm, 24 0.4 ml aliquots were collected from the top of the gradient. OD$_{595}$ was measured and used to estimate the percentage of cells in each subfraction. Subfractions 8–11 and 20–24 from heat

stressed cultures were combined and denoted LD$_{50}$ and HD$_{50}$, respectively. A control sample contained subfractions 7–10 from an unstressed culture.

2.3. Determination of the number of VBNC, persister, live and dead cells

Total cell counts, and the level of dead and live cells were estimated using a Neubauer chamber at 1000-fold magnification and an epifluorescence microscope (Zeiss Axio Scope.A1) after staining with a LIVE/DEAD BacLight viability kit (Molecular Probes) according to the manufacturer's protocol. CFU counts were estimated by plating serial dilutions on LB agar. The number of VBNC cells was calculated by subtracting CFU counts from the number of live cells. To determine the level of persisters, subpopulations were diluted to an OD$_{595}$ of 0.03 in fresh LB medium, supplemented with 200 mg/ml ampicillin and incubated at 37 °C for 5 h. The surviving persisters were plated on LB agar for colony counts.

2.4. Determination of colony lag time and growth rate

The delay in growth (lag time) and growth rate of individual LD$_{50}$ and HD$_{50}$ colonies on LB agar plates incubated at 37 °C. Before plating bacteria were diluted to obtain 100–150 colonies on a plate. After initial 10 h of incubation, serial digital images of the plates were acquired every 2–3 h using the Azure system. The size and position of colonies in each image were analyzed with OpenCFU v3.9.0 software (Geissmann, 2013). A linear dependence of colony size as a function of time was observed. On the basis of these data growth curves were plotted for each unique colony and a linear dependence of colony size on time was observed. *Lag time* was calculated as the *x-intercept* of the linear equation. Growth rate was defined as an increase in colony area size (mm^2) per hour.

2.5. Immunodetection of molecular chaperones and oxidized proteins

SDS-PAGE and Western blotting were performed according to Sambrook et al. (1989). Polyclonal rabbit antisera against ClpB, DnaK, and IbpA/B (Kuczyńska-Wiśnik et al., 2002) and anti-rabbit peroxidase conjugate (Sigma) were used for immunodetection.

Carbonyl groups, the major products of protein oxidation, were immunodetected after reaction with 2,4-dinitrophenylhydrazine (DNPH), as described previously (Kuczyńska-Wiśnik et al., 2015). Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Protein-bound 2,4-dinitrophenylhydrazones were detected using anti-2,4-dinitrophenol (DNP) antibodies (Sigma) and ECL Western blotting detection reagents (Roche).

2.6. Light and transmission electron microscopy

LD$_{50}$ (subfractions 8–10) and HD$_{50}$ (subfractions 20–24) cells were stained with fuchsin and visualized by light microscopy at 1000× magnification The average length and width of the cells were estimated using Zen imaging software (Zeiss). The dimensions of at least 200 individual cells were measured.

For transmission electron microscopy bacteria from the LD$_{50}$ and HD$_{50}$ subpopulations were washed with PBS, fixed overnight with 2.5% glutaraldehyde, followed by postfixation with 1% osmium tetroxide for 2 h. Following ethanol dehydration (30°, 50%, 70%, 90%, 100% x2), probes were embedded in Epon resin, cut on an ultramicrotome Leica UC7 and contrasted in uranyl acetate and lead citrate. Transmission electron microscopy studies were performed using a Philips CM100 electron microscope.

2.7. Glycogen and SOD assay

To determine the amounts of glycogen, cell extracts were heated at