Chapter 20

Survival of *Escherichia coli* under Nutrient-Deprived Conditions: Effect on Cell Envelope Subproteome

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Abstract

In the aquatic ecosystems, microorganisms are exposed to seasonal and circadian cycles. Abiotic factors (e.g. low temperature, nutrient deprivation) can cause morphological and physiological changes in bacteria, thereby facilitating cell survival. While representing the interface between the cells and external environment, the cell envelope plays a major role in bacterial response to stress and characterization of the changes it undergoes can help to understand the adaptation process. In this study, analysis of the morphological and physiological changes as well as variations in protein composition of the Escherichia coli cell envelope was carried out for populations maintained for 21 days under nutrient deprivation and suboptimal temperatures (4°C and 20°C). It was found that the absence of nutrients led to a temperature-dependent reduction of cell culturability but had no effect on cell viability and integrity. The concentration of membrane proteins playing the key roles in cellular transport, maintenance of cell structure or bioenergetics processes remained mainly unchanged. In contrast, the level of several proteins such as the elongation factor EFTu 1, components of Bam complex or proteins implicated in chemotaxis was altered, thus indicating that cells were readily responding and adapting to stress.

Keywords: starvation, suboptimal temperature, cell envelope subproteome

1. Introduction

In their natural environments, including aquatic ecosystems, microorganisms are usually exposed to seasonal and circadian cycles significantly dependent on environmental

conditions. Moreover, during transfer from their natural environments to other ecosystems, bacteria can change their status from autochthonous to allochthonous one. This scenario is very typical for enteric bacteria, which are regularly transferred from their hosts to aquatic systems, a process accompanied by changes in the temperature and nutrient content of their habitats.

Escherichia coli (allochthonous, copiotroph, mesophile bacterium) is an indicator of fecal pollution and, therefore, its detection, quantification, as well as assessment of its ability to survive in aquatic environments are important subjects of the study and monitoring pursued by both public administrations and the scientific community.

Many bacteria, including *E. coli*, can cope with adverse conditions and successfully thrive in new environments by adjusting their physiology and metabolism. *E. coli* survival in a hostile aquatic environment depends on both biotic and abiotic factors [1–3]. Several abiotic factors including suboptimal temperature [4, 5], limitation of nutrients [6–8], and exposure to solar radiation [9–11] can lead to the loss of culturability. Barcina and Arana [12], Lothigius et al. [13], and others have demonstrated that, under these conditions, cells still remain physiologically active and intact. Transition from culturable to non-culturable state is known to involve considerable changes in the biochemical content of the cells [14–16].

To learn more about *E. coli* adaptation in aquatic systems, we undertook the present study to focus on adaptation changes affecting the composition of cell envelope and appearance of *E. coli* cells. While representing the interface between the cytoplasm and external environment, the cell envelope plays a major role in how bacteria sense and respond to stress [17] during its adaptation to changing environments [18, 19]. These functions of the cell envelope prompted us to characterize the morphological and physiological changes undertaken by *E. coli* populations during their permanence at suboptimal temperatures and under limitation of nutrients. Moreover, we have analyzed the variations in the subproteome of cell envelopes accompanying this survival process.

2. Materials and methods

To prepare experimental samples, *E. coli* ATCC 27325 cells were cultured aerobically in Tryptone soy broth (TSB) at 37°C with shaking (120 rpm) until they reached the stationary growth phase (24 h). The cells were harvested by centrifugation (4000 g, 4°C, 20 min) and washed three times with sterile saline solution (0.9% NaCl, w/v). All the glass flasks used for handling *E. coli* cultures were cleaned with H_2SO_4 (96%, v/v) beforehand, rinsed with deionized water, and kept at 250°C for 24 h to get rid of residual organic compounds.

Cells were inoculated in Erlenmeyer flasks containing sterile saline solution (absence of nutrients) to obtain a final density of 10^8 cells ml^{-1} and further incubated for 21 days at 4 and 20°C (suboptimal temperatures) with shaking (120 rpm) in darkness.

Subsamples were collected at the beginning of the experiments and after 3, 6, 12, and 21 days of nutrient deprivation to determine the number of total, viable, and culturable cells and to

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estimate the size of the cells (see below). Subsamples were also collected at 0 (P0), 6 (P1), 12 (P2), and 21 days (P3) to extract membrane proteins further analyzed by mass spectroscopy (see below).

The results from survival experiments are presented as the means of three independent experiments, with coefficient of variation between replicates less than 12%. The one-way analysis of variance (ANOVA) was used to determine the differences between the means. Probabilities less than (or equal to) 0.05 were considered significant.

The total number of bacteria was determined according to the procedure described by Hobbie et al. [20]. Namely, aliquots of cell suspensions from survival assays were filtered throughout 0.22 μm pore size black polycarbonate filters, stained with acridine orange (0.01%, w/v), and examined through epifluorescence microscopy. Viable bacteria, estimated as bacteria with intact cytoplasmic membranes (MEMB+), were counted with Live/Dead BacLight™ kit (Invitrogen) as described by Joux et al. [21]. The bacteria with intact (green fluorescence, MEMB+) and permeabilized (red fluorescence) cytoplasmic membranes were enumerated separately. The number of culturable bacteria was determined by the spread plate method on Tryptone soy agar (TSA) followed by their incubation for 24 h at 37°C.

The length variations of *E. coli* cells during their survival at 4 and 20°C were estimated through image analysis of epifluorescence preparations [22] by using an image analysis system, which included a high-resolution video camera (Hamamatsu 2400). Images of microscopic fields with enough bacteria were selected to be digitized and analyzed by Scion Image 1.62[a] software. For each subsample, 200 bacteria were measured. The values of mean size (x = 1.45 μm) and standard deviation (SD = 0.32) of initial population were used to establish three size categories (≤ x−SD, > x−SD – ≤ x + SD, > x + SD). Therefore, according to their length, the cells fell into one of the following size ranges: ≤ 1.12 μm, >1.12–≤1.77 μm, or >1.77 μm.

Membrane protein preparations were obtained according to the method described by Molloy et al. [23] with minor variations [24]. Subsequent analysis of these proteins was performed by the Proteomics Core Facility-SGIKER at the University of the Basque Country, using the protocol previously described by Gonzalez-Fernandez et al. [25]. Briefly, after protein precipitation by using a 2D Clean-Up Kit (GE Healthcare), the pellet was suspended in RapiGest solution (0.2%) (Waters Corporation) and heated at 85°C for 15 min. Then, the preheated suspension was reduced with DTT (5 mM), alkylated with iodoacetamide (15 mM), and digested with trypsin (2 μg per sample) overnight. MassPREP Enolase Digestion Standard (Waters Corporation) was added to the supernatants collected after centrifugation (16,000 g, 10 min) of HCl-treated samples (inactivation of RapiGest) and was used as a standard for protein absolute quantification. A nanoACQUITY UPLC System coupled to a SYNAPT HDMS (Waters Corporation) was used for data-independent acquisition analyses. Subsamples with tryptic peptides and MassPREP Enolase Digestion Standard were loaded onto a Symmetry300 C18, 180 μm × 20 mm precolumn (Waters Corporation) connected to a BEH130 C18 column (75 μm × 200 mm, 1.7 μm [Waters Corporation]). Peptides were eluted with a linear gradient of acetonitrile (120 min from 3 to 40% and 15 min from 40 to 60% [v/v]). Mass spectra were acquired using a data-independent acquisition mode (MSE) [26] as previously described by

Gonzalez-Fernandez et al. [25] and processed with ProteinLynx Global SERVER v2.4 Build RC7 (Waters Corporation). Protein identification was carried out using the database search algorithm of the program [27] and the parameters specified by Parada et al. [24]. The absolute protein quantification based on peak area intensity of peptide precursors was calculated by the program using enolase peptides as an internal standard [28].

Among proteins confirmed by the presence of at least three protein-derived peptides in the tryptic digests, those detected in two or three of the biological replicates were considered for further analysis. Quantification values of individual proteins were normalized *versus* the total protein in the samples. Only those proteins showing a 1.5-fold increase or a 0.6-fold decrease in their relative abundance (with respect to the previous sampling time) were considered differentially affected by survival conditions.

UniProt and KEGG databases were used to verify the identity and function of proteins. For the prediction of the bacterial protein subcellular localization, the PSORTb 3.0 program [29] was used. According to their main biological functions specified in UniProt database, selected proteins were further grouped to form the categories of proteins that (i) play structural roles involved in (ii) transport, (iii) bioenergetics, (iv) synthesis, degradation, and turnover of protein, (v) stress response, or (vi) have miscellaneous functions.

3. Results and discussion

Consistent with the results of previous studies [4, 11, 30, 31], the total number of *E. coli* cells remained unchanged throughout the experimentation time under nutrient deprivation. The percentages of viable and culturable cells were calculated with respect to the total bacteria for each analyzed time (**Figure 1A** and **C**). Although the viable population did not show significant variations throughout the survival period, the culturable fraction declined progressively. Moreover, the loss of culturability of the cells incubated at 20°C occurred faster (already after 6 days of incubation). This result agrees with those obtained in previous works [4, 32] in which it was established that, in the absence of natural microbiota, the survival of *E. coli* reduces at higher temperatures.

Cellular dwarfing has been described as a typical response of bacteria exposed to adverse conditions. However, our work did not reveal any significant changes in the size of starved *E. coli* cells during 21 days (**Figure 1C** and **D**). For the main fraction of starved *E. coli* cells (63.5–73.5%), the cell length was preserved within the same range (>1.1–≤1.8 μm) during the incubation time. Similar results were obtained by Muela et al. [33], who found no changes in the cell size throughout the long-term survival of *E. coli* in sterile river water.

Thus, analysis of cell physiology and morphology revealed that, although *E. coli* cells remained active and maintained their integrity and size, starvation led to a decrease in the number of culturable cells. Moreover, these changes were temperature dependent. Similar behavior has been previously described for this bacterium [4], and it was attributed to the differences in metabolic activities of cells cultured at low and normal temperatures [34, 35].

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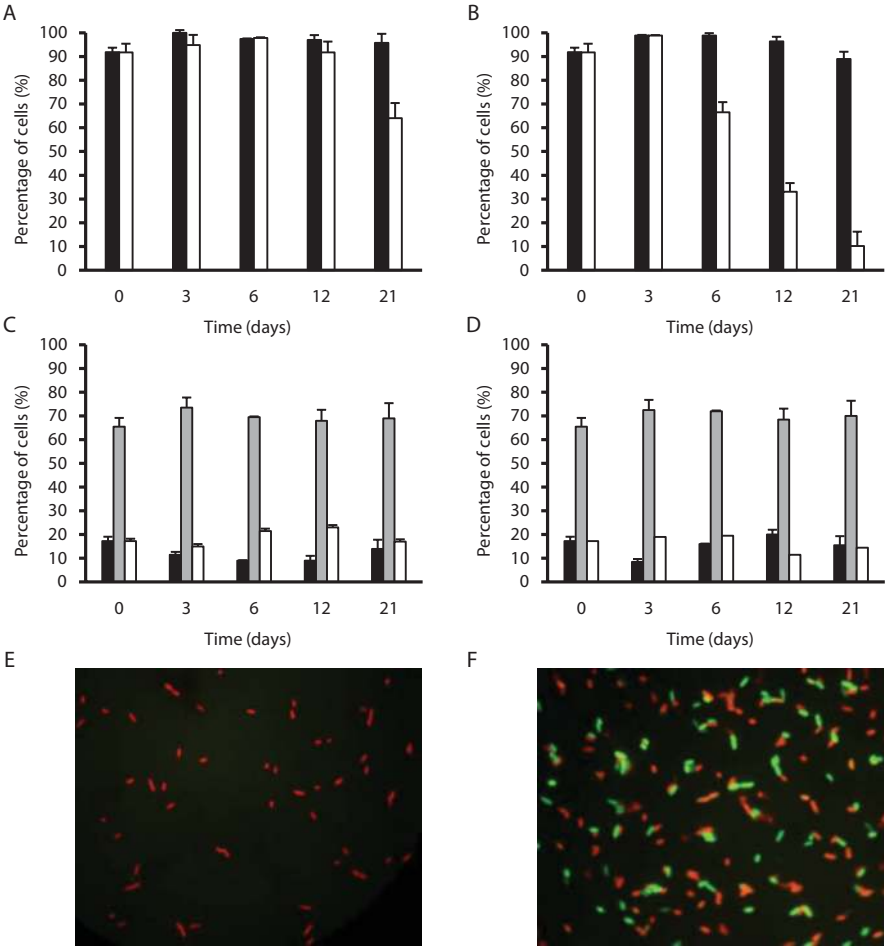

Figure 1. *Escherichia coli* ATCC 27325 survival in the absence of nutrients at 4°C (A, C) and 20°C (B, D). (A and B) Variations in the percentages of viable (■) and culturable (□) bacteria obtained with respect to the total count at each period. (C and D) Variations in cell size distribution (■ ≤ 1.12 μm; ▒ >1.12–≤1.77 μm; □ >1.77 μm). The data are mean values from three independent experiments with error bars representing the standard deviations calculated. Pannels (E and F), images of *E. coli* preparations stained with acridine orange (E) or Live/Dead BacLight™ kit (F) and examined through epifluorescence microscopy.

From survival assays carried out under starvation conditions, the samples for membrane subproteome analysis were collected at different incubation times: 0 (P0), 6 (P1), 12 (P2), and 21 days (P3). Despite the use of membrane fraction for mass spectrometry analysis, the PSORTb 3.0 program revealed that the resulting dataset potentially contained predicted cytosolic proteins (22%), including cytosolic subunits of ATP synthase or proteins that can conditionally be associated with the membrane (**Tables 1** and **2**). The fortuitous presence of cytoplasmic proteins in the membrane fractions was somewhat anticipated, as it was also observed in previous studies [24, 31].

Category	Protein accession number	Location[a]	Protein name
Cell structure	LPP_ECOLI	OM	Major outer membrane lipoprotein Lpp
	PAL_ECOLI	OM	Peptidoglycan-associated lipoprotein
	SLP_ECOLI	OM	Slp
	METQ_ECOLI	CM	D-Methionine-binding lipoprotein MetQ
	DACC_ECOLI	CM	D-Alanyl-D-alanine carboxypeptidase DacC
	SLYB_ECOLI	OM	Outer membrane protein SlyB
	YDGA_ECOLI	CM	YdgA
Transport	OMPA_ECOLI	OM	OmpA
	OMPC_ECOLI	OM	OmpC
	OMPW_ECOLI	OM	OmpW
	OMPX_ECOLI	OM	OmpX
	TOLC_ECOLI	OM	TolC
	GLPT_ECOLI	CM	Glycerol 3 phosphate transporter
	PTNAB_ECOLI	Cyt	PTS system mannose-specific EIIAB component
	PTND_ECOLI	CM	Mannose permease IID component
	COPA_ECOLI	CM	Copper-exporting P-type ATPase A
	ACRA_ECOLI	CM	Multidrug efflux pump subunit AcrA
	YHII_ECOLI	CM	Uncharacterized protein YhiI
	DCUA_ECOLI	CM	Anaerobic C4-dicarboxylate transporter DcuA
Bioenergetics	ATPA_ECOLI	Cyt	ATP synthase, subunit alpha
	ATPB_ECOLI; ATPL_ECOLI	CM	ATP synthase, subunits beta and c
	CYDA_ECOLI; CYDB_ECOLI	CM	Cytochrome bd-I ubiquinol oxidase, subunits 1 and 2
	FRDB_ECOLI; FRDA_ECOLI	CM	Fumarate reductase iron-sulfur subunit and flavoprotein subunit
	DHSA_ECOLI DHSB_ECOLI	CM	Succinate dehydrogenase flavoprotein subunit and iron-sulfur subunit
	NARG_ECOLI	CM	Respiratory nitrate reductase 1 alpha chain
Synthesis, degradation, and turnover of proteins	HFLK_ECOLI	Cyt	Modulator of FtsH protease HflK
	HFLC_ECOLI	CM	Modulator of FtsH protease HflC
Stress responses	YQJD_ECOLI	?	Uncharacterized protein YqjD
	ELAB_ECOLI	?	ElaB
Others	MIND_ECOLI	CM	Septum site-determining protein MinD

[a]OM, outer membrane; CM, cytoplasmic membrane; Cyt, cytosolic protein; ?, unknown.

Table 1. Membrane proteins that did not show significant changes in their level after 0, 6, 12, and 21 days of *E. coli* starvation in saline solution (NaCl 0.9%).

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Category	Protein accession number	Location[a]	Protein name	4°C		20°C		
				P1/P0	P3/P1	P1/P0	P2/P1	P3/P2
Cell structure	YBJP_ECOLI	?	Uncharacterized YbjP	NC[b]	NC	0.59[c]	NC	NC
	OSME_ECOLI	?	Osmotically inducible lipoprotein E	1.93	NC	NC	ND	ND
	YIDC_ECOLI	CM	Membrane protein insertase YidC	NC	ND	NC	NC	ND
	BAMA_ECOLI	OM	Outer membrane protein assembly factor BamA	ND	ND	ND	ND	ND
	BAMB_ECOLI	OM	Outer membrane protein assembly factor BamB	ND	ND	ND	ND	ND
Transport	PTW3C_ECOLI	CM	PTS system N-acetylglucosamine-specific EIICBA component	NC	ND	NC	NC	NC
	SECD_ECOLI	CM	Protein translocase subunit SecD	NC	NC	NC	ND	ND
	PTM3C_ECOLI	CM	PTS system mannitol specific EIICBA component	NC	ND	NC	ND	ND
Bioenergetics	NUOCD_ ECOLI	Cyt	NADH-quinone oxidoreductase subunits C/D	NC	ND	NC	NC	NC
	DHNA_ECOLI	CM	NADH dehydrogenase	ND	ND	ND	ND	ND
Synthesis, degradation and turnover of proteins	FTSH_ECOLI	CM	ATP-dependent zinc metalloprotease FtsH	NC	NC	NC	0.57	NC
Stress response	BFR_ECOLI	Cyt	Bacterioferritin	NC	NC	ND	ND	ND

Category	Protein accession number	Location[a]	Protein name	4°C		20°C		
				P1/P0	P3/P1	P1/P0	P2/P1	P3/P2
Others	EFTU1_ECOLI	Cyt	Elongation factor Tu 1	2.81	NC	4.22	NC	NC
	HEMX_ECOLI	CM	Putative uroporphyrinogen-III C methyltransferase	NC	NC	0.59	NC	NC
	HEMY_ECOLI	CM	Protein HemY	NC	ND	NC	NC	NC
	QMCA_ECOLI	?	Protein QmcA	NC	NC	NC	ND	ND
	PPID_ECOLI	CM	Peptidyl-prolyl *cis*-trans isomerase D	NC	NC	NC	ND	ND
	FLIC_ECOLI	Ex	Flagellin	NC	ND	NC	NC	ND
	MCP1_ECOLI	CM	Methyl accepting chemotaxis protein I	ND	ND	ND	ND	ND

[a]OM, outer membrane; CM, cytoplasmic membrane; Cyt, cytosolic; ?, unknown; Ex, extracellular.

[b]NC, no significant changes with respect to the previous sample; ND, not detected.

[c]Values higher than 1.5 indicate significant increases, and values lower than 0.6 indicate significant decreases of protein level with respect to the previous time.

Table 2. Membrane proteins that exhibited significant changes in their level at 6 (P1), 12 (P2), and 21 days (P3) of *E. coli* starvation in sterile saline solution (NaCl 0.9%, w/v).

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A large group of proteins (**Table 1**) did not show any significant upregulation (>1.5-fold) or downregulation (<0.6-fold) during the survival experiments. This group included proteins related to the maintenance of cell structure (some lipoproteins, YdgA, and other) and/or the transport (porins such as OmpA, OmpW, OmpC, and TolC) (**Table 1**). Noteworthy, some of these proteins (namely, Lpp lipoprotein and OmpA and OmpC porins) belong to the group of the most abundant polypeptides detected in all samples. The above data suggest a role for these proteins in the maintenance of cell integrity observed here and in previous studies [12, 15, 16, 36, 37] upon *E. coli* exposure to adverse conditions. While some of them (e.g., lipoproteins) may be critical for maintaining the lipid bilayer, others (e.g., OmpA and OmpW) are likely involved in sustaining the integrity of the outer membrane [38–40]. No changes in protein level were also observed for different proteases implicated in synthesis, degradation, and turnover of membrane proteins (HflK and HflC) (**Table 1**). It seems that their presence is critical for preservation of cell stability as these proteases might degrade damaged or unnecessary proteins that could potentially accumulate in the lipid bilayer, thus restricting membrane permeability [41, 42], which is one of the fundamental functions of biological membranes [41]. This idea is supported by the results of staining with the Live/Dead BacLight™ kit used to differentiate *live* and *dead* cells (**Figure 1A** and **B**), demonstrating that the membranes of the starved cells remain intact and preserve their selective permeability.

We also observed that the level of numerous proteins implicated in bioenergetics (namely, different subunits of ATP synthase, cytochromes, and reductases) and transport (porins, mannose permease, components of PTS systems, or glycerol 3 phosphate transporter) was nearly the same in the control sample (P0) and samples (P3) mainly containing non-culturable bacteria (**Figure 1** and **Table 1**). Despite the constant presence of these proteins in cell envelope, several studies suggested that starving cells likely preserve a minimal level of metabolic activities. For instance, Ozkanca and Flint [43] indicated that respiration rates greatly decreased to almost undetectable levels in *E. coli* cells exposed to starvation during their incubation in sterile lake water. Likewise, Barcina et al. [44] detected a decrease of glucose uptake for populations maintained in freshwater. Thus, the constant presence of the energy- and sugar metabolism-related proteins seems to indicate that starving cells still stay alarmed and prepared to quickly respond to favorable environmental conditions. Indeed, analysis of glucose uptake by the starving cells revealed a quick response and, as a result, an increase in the respiration rate [44]. Consistently, several authors have demonstrated the function of the electron transport chains in non-culturable bacteria by showing their ability to reduce intracellularly tetrazolium salts [31, 45, 46]. Moreover, in a previous work, Arana et al. [30] found that some *E. coli* cells could release nutrients (mainly monomeric carbohydrates and amino acids) into the surrounding medium under stress conditions. The released nutrients are taken up by other cells, thus could aid in the survival of remaining culturable cells and, therefore, ensure the persistence of the species in adverse environments.

Other constantly present proteins include YqjD and its paralogous protein ElaB, known to be abundant in the stationary growth phase. These proteins seem to be involved in inhibition of ribosomal activity and in localization of ribosomes on the inner membrane during the stationary phase of growth. In cells exposed to some stress conditions (e.g., starvation), both ribosomal biogenesis and protein synthesis are known to be suppressed. Thus, the negative

regulation of these processes by YqjD and ElaB could be important for bacterial adaptation and survival in harsh environments [47].

Table 2 shows the membrane proteins (accounted for 17–19% of the total analyzed polypeptides) that became less or more abundant upon starvation of *E. coli* in sterile saline solution. Some of these proteins underwent variations dependent on nutrient status and/or incubation temperature. For example, the level of two proteins (namely, BamA and BamB) belonging to the outer membrane complex Bam (additionally containing BamB, BamC, BamD, and BamE [48, 49]) as well as the membrane protein insertase YidC sharply declined and became undetectable in the starved cells (**Table 2**). Since the β-barrel assembly machinery (BAM) is essential for maintaining the bacterial cell envelope and is involved in OMP recognition, folding, and assembly [48, 50, 51], its depletion with BamA, one of the key components of the *E. coli* Bam complex, after 3 days of incubation under starvation conditions could indicate the reduction in the production and/or active assembly of proteins in the outer membrane. Volokhina et al. [48] suggested that loss of activity of this protein promotes accumulation of proteins in the outer membrane that cannot be inserted therein. This accumulation could be lethal for the bacterium since aggregates would be formed in the periplasmic space. Moreover, this could lead to the incorporation of these OMPs into inner bacterial membranes, which would dissipate the proton-motive force and kill bacteria [52]. However, in this study, we have not detected dead cells (**Figure 1**). This fact could indicate that the Bam complex might become redundant in the nondividing *E. coli* cells due to reduction of production and maturation of OMPs in bacterial cells exposed to starvation.

Similar to BamA and BamB, the membrane protein insertase YidC also was not detectable after 21 days of starvation. This protein has been proposed to mediate the transfer of transmembrane segments of hydrophilic polypeptide chains from the Sec-translocon into the lipid bilayer and can assist folding of inner membrane proteins [53] including ATP synthase subunit c [17]. This finding together with the data obtained for BamA and BamB (see above) suggests that limitation of nutrients leads to the overall reduction of cell envelope biogenesis.

Other proteins that became undetectable in starved cells were the methyl-accepting chemotaxis protein I and the flagellin FLIC_ECOLI. Chen and Chen [54] demonstrated that under starvation, *Vibrio vulnificus* populations exhibited reduced motility. Lemke et al. [55] and Chandrangsu et al. [56] concluded that DksA (protein required for the regulation of certain promoters) and the alarmone ppGpp inhibit expression of the flagellar cascade during cells' entry into the stationary phase or during their starvation. This mechanism could prevent unnecessary waste of energy on synthesis of macromolecular complexes and generation of proton-motive force used to rotate the flagella apparatus [57] and, therefore, would free more energy to sustain the survival process.

Unlike chemotaxis protein I and the flagellin FLIC_ECOLI, the elongation factor Tu 1 became one of the most abundant proteins in populations maintained 6 days at 20°C. This elongation factor is known as a cytoplasmic chaperone implicated in protein synthesis, growth regulation, and stress responses [58, 59]. The high level of this protein in starving cells is consistent with the data presented by Muela et al. [31]. They observed an increase in the level of EFTu in *E. coli* populations under starvation conditions and its subsequent localization on the membrane.

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As shown in **Figure 1**, *E. coli* response to starvation was temperature dependent. We identified several proteins whose level was differently affected by temperature. Namely, the protein translocase subunit SecD and bacterioferritin became undetectable in starved cells maintained at 20°C, whereas PTS system N-acetylglucosamine-specific EIICBA component was gradually lost in populations maintained at 4°C (**Table 2**). Unlike above examples, the temperature-dependent regulation of osmotically inducible lipoprotein E (OSME_ECOLI) and ATP-dependent zinc metalloprotease FtsH did not reveal a clear regulation pattern.

Interestingly, the levels of three proteins were below detection at the starting point (P0), but their level was increased afterward. This group of proteins includes the structural protein MreB (MREB_ECOLI), putative porin NmpC, and the cytosolic universal stress protein F (USPF_ECOLI). MreB is a homolog of eukaryotic actin, which has been found to be associated with the membrane in several bacteria [60–62]. Shih et al. [63] stated that the MreB system is required for establishment of the rod shape of cells. MreB proteins form actin-like cables lying beneath the inner cell membrane. The cables are required to guide longitudinal cell wall synthesis. Chiu et al. [64] demonstrated that, in non-culturable *Vibrio parahaemolyticus* populations appeared upon starvation, MreB protein was located near the cytoplasmic membrane. Moreover, these authors reported a reduction in cellular size associated with the increase in the expression of the *mreB* gene. However, Ben Abdallah et al. [65] and Parada et al. [24] described the decline in the expression of the *mreB* gene and MreB protein content, respectively, in *Vibrio* species undergoing morphological changes in response to stress. Kruse et al. [66] demonstrated that a decrease of MreB concentration leads to merodiploid spherical and inflated *E. coli* cells prone to cell lysis. Moreover, Defeu Soufo et al. [67] demonstrated a relationship between MreB and EFTu for *E. coli* and *Bacillus subtilis* cells, and they suggested that EFTu could affect the cellular morphology through interaction with MreB. EFTu improves the ability of MreB to form filaments functioning as a basal structure. Our work suggests that both proteins could interact, as both of them are present during the cell response to stress.

Thus, the present study showed that, although incubation in the absence of nutrients reduced cell culturability in a temperature-dependent manner, the cells still remained active and preserved their integrity and size. In addition, proteome analysis of the cell's envelope revealed that the concentration of membrane proteins playing the key roles in cellular transport, maintenance of cell structure, as well as bioenergetic processes remained almost unchanged, indicating their crucial roles in *E. coli* survival under nutrient-limiting conditions. Moreover, some of the proteins critical for preservation of cell stability and membrane permeability (such as the modulators of FtsH protease, HflK, and HflC) appeared to be steadily present in the populations of mainly non-culturable cells. We also found the continuous increase in the level of elongation factor EFTU1 along the survival process, thus suggesting its essential role in the adaptation process. Interestingly, the level of some proteins (e.g., bacterioferritin) was differently affected by temperature (see above). Finally, the observed depletion of the key components of the Bam complex, insertase YidC, and/or proteins implicated in chemotaxis suggested their redundancy for preserving cell integrity and therefore allowed to save energy during *E. coli* adaptation and survival.

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References

[1] Barcina I, Lebaron P, Vives-Rego J. Survival of allochthonous bacteria in aquatic systems: A biological approach. FEMS Microbiology Ecology. 1997. 23:1-9. DOI: 10.1016/S0168-6496(97)00016-0.

[2] Noble RT, Lee IM, Schiff KC. Inactivation of indicator microorganisms from various sources of faecal contamination in seawater and freshwater. Journal of Applied Microbiology. 2004. 96:464-472. DOI: 10.1111/j.1365-2672.2004.02155.x.

[3] John DE, Rose JB. Review of factors affecting microbial survival in groundwater. Environmental Science and Technology. 2005. 39:7345-7356. DOI: 10.1021/es047995w.

[4] Arana I, Muela A, Orruño M, Seco C, Garaizabal I, Barcina I. Effect of temperature and starvation upon survival strategies of *Pseudomonas fluorescens* CHA0: Comparison with *Escherichia coli*. FEMS Microbiology Ecology. 2010. 74:500-509. DOI: 10.1111/j.1574-6941.2010.00979.x.

[5] Blaustein RA, Pachepsky Y, Hill RL, Shelton DR, Whelan G. *Escherichia coli* survival in waters: Temperature dependence. Water Research. 2013. 47:569-578. DOI:10.1016/j.watres.2012.10.027.

[6] Ramos JL, Gallegos MT, Marqués S, Ramos-González MI, Espinosa-Urgel M, Segura A. Responses of Gram-negative bacteria to certain environmental stressors. Current Opinion in Microbiology. 2001. 4:166-171. DOI: 10.1016/S1369-5274(00)00183-1.

[7] Ihssen J, Grasselli E, Bassin C, François P, Piffaretti JC, Köster W, Schrenzel J, Egli T. Comparative genomic hybridization and physiological characterization of environmental isolates indicate that significant (eco-)physiological properties are highly conserved in the species *Escherichia coli*. Microbiology. 2007. 153:2052-2066. DOI: 10.1099/mic.0.2006/002006-0.

[8] van Elsas JD, Semenov AV, Costa R, Trevors JT. Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. ISME Journal. 2011. 5:173-183. DOI: 10.1038/ismej.2010.80.

[9] Muela A, García-Bringas JM, Arana I, Barcina I. The effect of simulated solar radiation on *Escherichia coli*. The relative roles of UV-B, UV-A, and photosynthetically active radiation. Microbial Ecology. 2000. 39:65-71. DOI:10.1007/s002489900181.

[10] Whitman RL, Nevers MB, Korinek GC, Byappanahalli MN. Solar and temporal effects on *Escherichia coli* concentration at a Lake Michigan swimming beach. Applied and Environmental Microbiology. 2004. 70:4276-4285. DOI: 10.1128/AEM.70.7.4276-4285.2004.

[11] Arana I, Orruño M, Pérez-Pascual D, Seco C, Muela A, Barcina I. Inability of *Escherichia col*i to resuscitate from the viable but nonculturable state. FEMS Microbiology Ecology. 2007. 62(1):1-11. DOI:10.1111/j.1574-6941.2007.00362.x.

[12] Barcina I, Arana I. The viable but nonculturable phenotype, a crossroad in the live cycle of non differentiating bacteria?. Reviews in Environmental Science and BioTechnology. 2009. 8:245-255. DOI:10.1007/s11157-009-9159-x.

[13] Lothigius Å, Sjöling Å, Svennerholm AM, Bölin I. Survival and gene expression of enterotoxigenic *Escherichia coli* during long-term incubation in sea water and freshwater. Journal of Applied Microbiology. 2010. 108:1441-1449. DOI:10.1111/j.1365-2672.2009.04548.x.

[14] McDougald DM, Rice SA, Kjelleberg S. New perspectives on the viable but nonculturable response. Biologia Bratislava. 1999. 54:617-623.

[15] Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiology Reviews. 2010. 34:415-425. DOI:10.1111/j.1574-6976.2009.00200.x.

[16] Pinto D, Santos MA, Chambel L. Thirty years of viable but nonculturable state research: unsolved molecular mechanisms. Critical Reviews in Microbiology. 2015. 41:61-76. DOI :10.3109/1040841X.2013.794127. Epub 2013 Jul 12.

[17] Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. Cold Spring Harbor Perspectives in Biology. 2010. 2:a000414. DOI:10.1101/cshperspect.a000414.

[18] Darcan C. Expression of OmpC and OmpF porin proteins and survival of *Escherichia coli* under photooxidative stress in Black Sea water. Aquatic Biology. 2012. 17:97-105. DOI:10.3354/ab00458.

[19] Shimizu K. Regulation systems of bacteria such as *Escherichia coli* in response to nutrient limitation and environmental stresses. Metabolites. 2013. 4:1-35. DOI: 10.3390/metabo4010001.

[20] Hobbie JE, Daley RJ, Jasper S. Use of nucleopore filters for counting bacteria by epifluorescence microscopy. Applied and Environmental Microbiology. 1977. 33:1225-1228.

[21] Joux F, Lebaron P, Troussellier M. Succession of cellular states in *Salmonella typhimurium* population during starvation in artificial seawater microcosms. FEMS Microbiology Ecology. 1997. 22:65-76. DOI:10.1111/j.1574-6941.1997.tb00357.x.

[22] Massana R, Gasol JM, Bjørnsen PK, Blackburn N, Hagström A, Hietanen S, Hygum BH, Kuparinen J, Pedrós-Alió C. Measurement of bacterial size via image analysis of epifluorescence preparations: Description of an inexpensive system and solutions to some of the most common problems. Scientia Marina. 1997. 61:397-407.

[23] Molloy MP, Herbert BR, Slade MB, Rabilloud T, Nouwens AS, Williams KL, Gooley AA. Proteomic analysis of the *Escherichia coli* outer membrane. European Journal of Biochemistry. 2000. 267:2871-2881. DOI:10.1046/j.1432-1327.2000.01296.x.

[24] Parada C, Orruño M, Kaberdin V, Bravo Z, Barcina I, Arana I. Changes in the *Vibrio harveyi* cell envelope subproteome during permanence in cold seawater. Microbial Ecology. 2016. 72:549-558. DOI:10.1007/s00248-016-0802-0.

[25] Gonzalez-Fernandez R, Aloria K, Arizmendi JM, Jorrin-Novo JV. Application of label-free shotgun nUPLC-MS(E) and 2-DE approaches in the study of *Botrytis cinerea* mycelium. Journal of Proteome Research. 2013. 12:3042-3056. DOI: 10.1021/pr3010937.

[26] Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ, McKenna T, Nold MJ, Richardson K, Young P, Geromanos SJ. Quantitative proteomic analysis by accurate mass retention time pairs. Analytical Chemistry. 2005. 77:2187-2200. DOI:10.1021/ac048455k.

[27] Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ. Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. Proteomics. 2009. 9:1696-1719. DOI:10.1002/pmic.200800564.

[28] Silva JC, Gorenstein MV, Li GZ, Vissers JP, Geromanos SJ. Absolute quantification of proteins by LCMSE: A virtue of parallel MS acquisition. Molecular and Cellular Proteomics. 2006. 5:144-156. DOI:10.1074/mcp.M500230-MCP200.

[29] Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, Dao P, Sahinalp SC, Ester M, Foster LJ, Brinkman FSL. PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics. 2010. 26:1608-1615. DOI:10.1093/bioinformatics/btq249.

[30] Arana I, Seco C, Epelde K, Muela A, Fernández-Astorga A, Barcina I. Relationships between *Escherichia coli* cells and the surrounding medium during survival processes. Antonie Van Leeuwenhoek. 2004. 86:189-199. DOI:10.1023/B:ANTO.0000036146.28808.93.

[31] Muela A, Seco C, Orruño M, Camafeita E, Arana I, López JA, Barcina I. Changes in *Escherichia coli* outer membrane subproteome under environmental conditions inducing the viable but nonculturable state. FEMS Microbiology Ecology. 2008. 64:28-36. DOI: 10.1111/j.1574-6941.2008.00453.x.

[32] Flint KP. The long-term survival of *Escherichia coli* in river water. Journal of Applied Bacteriology. 1987. 63:261-270.

[33] Muela A, Pocino M, Arana I, Justo JI, Iriberri J, Barcina I. Effect of growth phase and parental cell survival in river water on plasmid transfer between *Escherichia coli* strains. Applied and Environmental Microbiology. 1994. 60:4273-4278.

[34] Morita RY. The starvation-survival state of microorganisms in nature and its relationship to the bioavailable energy. Experientia. 1990. 46:813-817.

[35] Semenov AV, van Bruggen AHC, van Overbeek L, Termorshuizen AJ, Semenov AM. Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cow manure. FEMS Microbiology Ecology. 2007. 60:419-428. DOI:10.1111/j.1574-6941.2007.00306.x.

[36] Roszak DB, Colwell RR. Survival strategies of bacteria in the natural environment. Microbiological Reviews. 1987. 51:365-379.

[37] Oliver JD. The viable but nonculturable state in bacteria. Journal of Microbiology. 2005. 43:93-100.

[38] Sonntag I, Schwarz H, Hirota Y, Henning U. Cell envelope and shape of *Escherichia coli*: Multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. Journal of Bacteriology. 1978. 136:280-285.

[39] Koebnik R, Locher KP, Van Gelder P. Structure and function of bacterial outer membrane proteins: Barrels in a nutshell. Molecular Microbiology. 2000. 37:239-253. DOI:10.1046/j.1365-2958.2000.01983.x.

[40] Asakura H, Kawamoto K, Haishima Y, Igimi S, Yamamoto S, Makino S. Differential expression of the outer membrane protein W (OmpW) stress response in enterohemorrhagic *Escherichia coli* O157:H7 corresponds to the viable but non-culturable state. Research in Microbiology. 2008. 159:709-717. DOI:10.1016/j.resmic.2008.08.005.

[41] Ito K, Akiyama Y. Cellular functions, mechanism of action, and regulation of FtsH protease. Annual Review of Microbiology. 2005. 59:211-231. DOI:10.1146/annurev.micro.59.030804.121316.

[42] Narberhaus F, Obrist M, Führer F, Langklotz S. Degradation of cytoplasmic substrates by FtsH, a membrane-anchored protease with many talents. Research in Microbiology. 2009. 160:652-659. DOI:10.1016/j.resmic.2009.08.011.

[43] Ozkanca R, Flint KP. Relationship between respiratory enzymes and survival of *Escherichia coli* under starvation stress in lake water. Journal of Applied Microbiology. 1997. 82:301-309.

[44] Barcina I, González JM, Iriberri J, Egea L. Survival strategy of *Escherichia coli* and *Enterococcus faecalis* in illuminated fresh and marine systems. Journal of Applied Bacteriology. 1990. 68:189-198.

[45] Besnard V, Federighi M, Declerq E, Jugiau F, Cappelier JM. Environmental and physico-chemical factors induce VBNC state in *Listeria monocytogenes*. Veterinary Research. 2002. 33:359-370. DOI:10.1051/vetres:2002022.

[46] Falcioni T, Papa S, Campana R, Manti A, Battistelli M, Baffone W. State transitions of *Vibrio parahaemolyticus* VBNC cells evaluated by flow cytometry. Cytometry Part B: Clinical Cytometry. 2008. 74:272-281. DOI:10.1002/cyto.b.20427.

[47] Yoshida H, Maki Y, Furuike S, Sakai A, Ueta M, Wada A. YqjD is an inner membrane protein associated with stationary-phase ribosomes in *Escherichia coli*. Journal of Bacteriology. 2012. 194:4178-4183. DOI: 10.1128/JB.00396-12.

[48] Volokhina EB, Grijpstra J, Beckers F, Lindh E, Robert V, Tommassen J, Bos MP. Species-specificity of the BamA component of the bacterial outer membrane protein-assembly machinery. PLoS One. 2013. 8:e85799. DOI:10.1371/journal.pone.0085799.

[49] Wu T, Malinverni J, Ruiz N, Kim S, Silhavy TJ, Kahne D. Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. Cell. 2005. 121:235-245. DOI:10.1016/j.cell.2005.02.015.

[50] Hagan CL, Kahne D. The reconstituted *Escherichia coli* Bam complex catalyzes multiple rounds of β-barrel assembly. Biochemistry. 2011. 50:7444-7446. DOI:10.1021/bi2010784.

[51] Albrecht R, Schütz M, Oberhettinger P, Faulstich M, Bermejo I, Rudel T, Diederichs K, Zeth K. Structure of BamA, an essential factor in outer membrane protein biogenesis. Acta Crystallographica Section D. Structural Biology Crystallography. 2014. 70:1779-1789. DOI:10.1107/S1399004714007482.

[52] Gessmann D, Chung YH, Danoff EJ, Plummer AM, Sandlin CW, Zaccai NR, Fleming KG. Outer membrane β-barrel protein folding is physically controlled by periplasmic lipid head groups and BamA. Proceedings of the National Academy of Sciences of the United States of America. 2014. 111:5878-5883. DOI:10.1073/pnas.1322473111.

[53] Luirink J, Yu Z, Wagner S, de Gier W. Biogenesis of inner membrane proteins in *Escherichia coli*. Biochimica et Biophysica Acta. 2012. 1817:965-976. DOI:10.1016/j.bbabio.2011.12.006.

[54] Chen H, Chen CY. Starvation induces phenotypic diversification and convergent evolution in *Vibrio vulnificus*. PLoS One. 2014. 9:e88658. DOI:10.1371/journal.pone.0088658.

[55] Lemke JJ, Durfee T, Gourse RL. DksA and ppGpp directly regulate transcription of the *Escherichia coli* flagellar cascade. Molecular Microbiology. 2009. 74:1368-1379. DOI:10.1111/j.1365-2958.2009.06939.x.

[56] Chandrangsu P, Lemke JJ, Gourse RL. The dksA promoter is negatively feedback regulated by DksA and ppGpp. Molecular Microbiology. 2011. 80:1337-1348. DOI:10.1111/j.1365-2958.2011.07649.x.

[57] Häse CC, Barquera B. Role of sodium bioenergetics in *Vibrio cholerae*. Biochimica et Biophysica Acta (BBA)—Bioenergetics. 2001. 1505:169-178. DOI:10.1016/S0005-2728(00)00286-3.

[58] Yu F, Inouye S, Inouye M. Lipoprotein-28, a cytoplasmic membrane lipoprotein from *Escherichia coli*. Cloning, DNA sequence, and expression of its gene. Journal of Biological Chemistry. 1986. 261:2284-2288.

[59] Caldas TD, El Yaagoubi A, Kohiyama M, Richarme G. Purification of elongation factors EF-Tu and EF-G from *Escherichia coli* by covalent chromatography on thiol-sepharose. Protein Expression and Purification. 1998. 14:65-70. DOI:10.1006/prep.1998.0922.

[60] Wachi M, Doi M, Okada Y, Matsuhashi M. New *mre* genes *mreC* and *mreD*, responsible for formation of the rod shape of *Escherichia coli* cells. Journal of Bacteriology. 1989. 171:6511-6516.

[61] Figge RM, Divakaruni AV, Gober JW. MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in *Caulobacter crescentus*. Molecular Microbiology. 2004. 51:1321-1332. DOI:10.1111/j.1365-2958.2003.03936.x.

[62] Mazza P, Noens EE, Schirner K, Grantcharova N, Mommaas AM, Koerten HK, Muth G, Flärdh K, van Wezel GP, Wohlleben W. MreB of *Streptomyces coelicolor* is not essential for vegetative growth but is required for the integrity of aerial hyphae and spores. Molecular Microbiology. 2006:60:838-852. DOI:10.1111/j.1365-2958.2006.05134.x.

[63] Shih YL, Kawagishi I, Rothfield L. The MreB and Min cytoskeletal-like systems play independent roles in prokaryotic polar differentiation. Molecular Microbiology. 2005. 58:917-928. DOI:10.1111/j.1365-2958.2005.04841.x.

[64] Chiu SW, Chen SY, Wong HC. Dynamic localization of MreB in *Vibrio parahaemolyticus* and in the ectopic host bacterium *Escherichia coli*. Applied and Environmental Microbiology. 2008. 74:6739-6745. DOI:10.1128/AEM.01021-08.

[65] Ben Abdallah F, Ellafi A, Lagha R, Bakhrouf A, Namane A, Rousselle JC, Lenormand P, Kallel H. Identification of outer membrane proteins of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* altered in response to γ-irradiation or long-term starvation. Research in Microbiology. 2010. 161:869-875. DOI:10.1016/j.resmic.2010.10.009.

[66] Kruse T, Bork-Jensen J, Gerdes K. The morphogenetic MreBCD proteins of *Escherichia coli* form an essential membrane-bound complex. Molecular Microbiology. 2005. 55:78-89. DOI:10.1111/j.1365-2958.2004.04367.x.

[67] Defeu Soufo HJ, Reimold C, Breddermann H, Mannherz HG, Graumann PL. Translation elongation factor EF-Tu modulates filament formation of actin-like MreB protein *in vitro*. Journal of Molecular Biology. 2015. 427:1715-1727. DOI:10.1016/j.jmb.2015.01.025.