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ORIGINAL PAPER

Proteomic changes of viable but nonculturable (VBNC) *Escherichia coli* O157:H7 induced by low moisture in an artificial soil

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Abstract

We studied the viable but nonculturable (VBNC) state of the human pathogen *Escherichia coli* O157:H7 during desiccation using genomic, proteomic, and direct methods in a model soil under controlled conditions. During desiccation, the bacterial cells reduced their culturability, protein synthesis, and DNA replication, and changed their morphology. Four days after rewetting of dry soil, the culturability of *E. coli* recovered, and protein synthesis was restored. A total of 2,324 proteins were differently expressed in the VBNC state compared to the culturable state and identified. Morphological changes during the VBNC state paralleled the down-regulation of cytoskeletal proteins and enzymes of the tricarboxylic acid cycle. On the contrary, proteins involved in nutrient transport, membrane, chemotactic, flagellar, virulence, and adhesion were upregulated. Overall, our results indicated that low soil moisture can induce the VBNC state in *E. coli* O157:H7 but this state can revert to culturability and full metabolic activity upon soil rewetting. These findings can be important aspects in assessing the environmental risks posed by microbial pathogens in soil.

Keywords E. coli O157:H7 · Soil proteomics · VBNC state · Drying · Carbon availability

Introduction

E. coli O157:H7 is a dangerous pathogen, even with a low infective dose (as few as 10 cells) (Tilden Jr. et al. 1996), and can persist in soil from several days to more than 5 months (Islam et al. 2004). Its survival depends on temperature (Semenov et al. 2007), oxygen (Fremaux et al. 2007), pH (Darcan et al. 2009), dissolved organic C (Gitanjali et al. 2018), composition of microbial communities (Semenov et al. 2007), and management practices (Semenov et al. 2009). It is plausible to hypothesize that *E. coli* O157:H7 may reach man transferred by fruits and vegetables, and this may cause serious risks to human health.

Soil drying-rewetting can affect microbial survival by decreasing the diffusion of resources to microorganisms and causing osmotic stress (Schimel et al. 2007; Sun et al. 2018). Resource availability, such as C substrate availability, is likely the main factor affecting *E. coli* survival in soil (van Elsas et al. 2010). Under nutrient-limiting conditions, some bacterial cells may become dormant; for example, Gram-positive bacteria can form spores as main survival strategy (Semenov et al. 2007). In hot summers, pathogens, such as *Salmonella* sp. and fecal coliforms, can become undetectable by culturable techniques due to the decreased soil moisture but these pathogens regrow if soil moisture is increased (Gibbs 1997). When facing unfavorable environments, such as limited nutrients, water availability, or other stress factors, microbes can enter a viable but nonculturable (VBNC) state to survive. The VBNC state was first characterized by in vitro studies of pathogenic bacteria, such as *E. coli* and *Vibrio cholerae* (*V. cholera*) (Xu et al. 1982). *E. coli* can enter the VBNC state due to starvation, suboptimal temperature, chlorination, osmotic stress, high-pressure CO_2, oxidative stress, visible radiation, sunlight, and pH variations (Darcan et al. 2009). The mechanisms responsible for resuscitation from the VBNC state of pathogens, such as *E. coli* in soil, are poorly known. Both RNA polymerase sigma S (RpoS) and (p)ppGpp modulated

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by protein RelA play crucial roles in affecting the formation of VBNC cells (Zhao et al. 2017). Magnusson et al. (2005) reported that RpoS expression could promote VBNC-state formation in *E. coli* O157:H7 under conditions of osmotic and oxidative stress.

Nowadays, techniques based on DNA analysis, mRNA synthesis, de novo sequencing, and microarrays have been used to describe the VBNC state (Coutard et al. 2005; Randa et al. 2004; Rosche et al. 2005), but it is difficult to isolate the short-lived and unstable mRNA (Kim et al. 2014) and only proteomics holds the potential to detect changes in microbial activity because proteins are the final results of gene expression (Nannipieri et al. 2020). In addition, soil proteomics can be used to understand the underlying mechanisms of bacterial transition from the culturable state to VBNC state and vice versa in soil (Giagnoni et al. 2018); however, low protein extraction yields and identification interferences by soil components, such as montmorillonite and humic acids, are technical challenges (Giagnoni et al. 2012, 2013). Soil proteolytic activity and geochemical denaturation also pose challenges in soil proteomics (Renella et al. 2014). In addition, the microbial proteins in soil may influence the identification and quantification of the target protein. Soil is hard to sterilize and the native microbe will regrow even after sterilization via gamma irradiation (Xing et al. 2020), and the proteins of dead cells can persist in soil. Therefore, artificial soil is preferred to the sterilized soil in studies about changes in the proteome due to the VBNC state of bacteria. Indeed, artificial soil was used for this purpose by Giagnoni et al. (2018).

This study was conducted to answer the following questions. (1) Can *E. coli* O157:H7 enter the VBNC state under dry soil condition? (2) Can *E. coli* O157:H7 in the VBNC state be resuscitated by wetting dry soil? (3) What are the different morphological and protein expression features of the VBNC state in soil? The starting hypothesis was that the VBNC state and the resuscitation in soil could change bacterial properties including its pathogenicity. To verify this hypothesis, we inoculated *E. coli* O157:H7 in soils of definite composition until the appearance of the VBNC state under dry conditions. Then we reverted its physiological state to culturability by wetting the dry soil. We studied protein expression and used transmission electron microscopy (TEM), laser scanning confocal microscopy (LSCM) Syto9/PI (Aurass et al. 2011) and TMT-labeled to monitor the VBNC-state *E. coli* O157:H7 in an artificial soil.

Materials and methods

Bacterial inoculation into artificial soil and its culturability

Artificial soil was prepared by mixing quartz sand (Sigma Aldrich); kaolinite (Clay Minerals Society, USA), with a cation exchange capacity (CEC) of 2 cmol kg^{-1} and 10 m^2 g^{-1} of surface area; montmorillonite (Clay Minerals Society, USA), with a CEC of 120 cmol kg^{-1} and 97.4 m^2 g^{-1} of surface area; goethite (Sigma Aldrich); and humic acids (Sigma Aldrich) (Giagnoni et al. 2011). The weight ratio of these components was 78:18:2:1:1.

The artificial soil was sterilized 3 times in an autoclave (20 min at 121 °C, 1 bar pressure) with an intermittent incubation at 20 °C for 2 days between each sterilization step. The artificial soil was then dried in a heater at 50 °C prior to bacterial inoculation. We prepared three independent replicates of each treatment, and the total amount of soil in each microcosm was 10 g. The microcosms consisted of sterile triangular bottles with aseptic sealing film; they were placed into a 37 °C incubator, and the soil pH was 7.0 during the incubation period.

The soil was inoculated with 3 mL containing 10^8 CFU of *Escherichia coli* O157:H7 EDL933 (ATCC 43895), giving 10^7 cells per gram of soil, and subjected to two treatments. In the first treatment, the soil moisture content (w/w) of the microcosms was not adjusted during incubation giving the following values: 30.0% at 0 day (T0); 17.4% at 1 day (T1); 12.3% at 3 days (T3); 7% at 7 days (T7); and 1.6% at 11 days (T11). After, soil moisture was increased to 30% and kept constant until the VBNC cells resuscitated at 15 days (T15). In the second treatment, the soil moisture was kept to 30% and sterile deionized H_2O was added if needed; the incubation lasted 11 days.

Counting of total, viable, and culturable cells

The total, viable, and culturable cells were counted after 1 day (T1), 3 days (T3), 7 days (T7), 11 days (T11), and 15 day (T15) of incubation by the heterotrophic plate count (HPC) method (Zhang et al. 2018a).

The viable cell numbers and total cell numbers were enumerated by PMA-quantitative polymerase chain reaction (PMA-qPCR) and quantitative polymerase chain reaction (qPCR), respectively. Soil DNA was extracted using a Power Soil DNA isolation kit (Mo Bio) with minor modifications; the set of PCRs always included negative controls. After DNA extraction from the soil, we applied PMA-qPCR to detect viable *E. coli* O157:H7 cells (Burkert et al. 2019). The extracted DNA concentrations were determined by using a NanoDrop spectrophotometer (NanoDrop technologies, Wilmington, USA); then samples were stored at − 80 °C before determining the abundance of *E. coli* O157:H7 by qPCR on a 7500 real-time PCR system (Applied Biosystem, MA, US) according to Burkert et al. (2019). The *Z3276* gene was amplified in 10 μL reaction volume containing 5 μL of 2 × Probe Premix (Takara, Dalian, China), 0.2 μM of each primer, 0.35 of μM probe, 0.1 of μL ROX, and 1 μL of DNA template.

The qPCR conditions were as follows: activation of TaqMan probe at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing at 60 °C for 1 min. Standard curves of the *Z3276* gene were generated with serial 10-fold dilutions of plasmids (synthesized by Sangon, Shanghai, China). As qPCR target gene *Z3276* is a single copy, the calculation of the cell equivalents was based on the known genome sizes of the *Z3276* (Jager et al. 2018). The limit of quantification (LOQ) for all qPCRs ranged from 10 to 100 gene copies per reaction and was implemented as appropriate for each specific run, as described in the supplementary materials.

Morphology assays

The morphology of *E. coli* O157:H7 cells was analyzed by laser scanning confocal microscopy (LSCM, LSM780, Zeiss), after staining with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR), and by transmission electron microscopy (TEM, H-7650, Hitachi). A BacLight solution containing 3.34 mM SYTO 9 (green fluorescence) and 20 mM PI (red fluorescence) in dimethyl sulfoxide (DMSO) was used. SYTO 9 stained all cells green, whereas dead cells were stained red by PI. Briefly, equal volumes of SYTO 9 and PI were mixed thoroughly and 3 μL of mixture was then added to 1 mL of bacterial suspension according to the manufacturer's instructions. After incubation under darkness at room temperature for 15 min, 5 μL of each suspension was trapped between a slide and a square coverslip, before LSCM observation.

Bacterial protein extraction

Soil samples (T1 and T11) were ground by liquid nitrogen, treated with a lysis buffer (1% Triton X-100, 10 mM dithiothreitol, 1% Protease Inhibitor Cocktail, 50 μM PR-619, 3 μM TSA, 50 mM NAM, and 2 mM EDTA), and then sonicated three times on ice using a high-intensity ultrasonic processor (Scientz). Next, tris-saturated phenol (pH 8.0) of equal volume was added and vortexed for 5 min. The mixture was centrifuged at 4 °C and 5,000*g* for 10 min, and the upper phenol phase was transferred to a new centrifuge tube. Four volumes of ammonium sulfate–saturated methanol were added to precipitate the proteins at − 20 °C for at least 6 h. After centrifugation at 4 °C for 10 min, the supernatant was discarded. The remaining precipitate was washed with ice-cold methanol one time, and then with ice-cold acetone three times. The protein precipitate was dissolved in 8 M urea and the protein concentrations were determined with a BCA kit (Beyotime) according to the manufacturer's instructions.

Trypsin digestion

The protein solution was treated with dithiothreitol (to give a concentration of 5 mM), incubated at 56 °C for 30 min, alkylated by adding 11 mM iodoacetamide, and incubated for 15 min at room temperature in the dark. Finally, the urea concentration of the sample was diluted to less than 2 M with distilled water. Trypsin was added at a mass ratio of 1:50 (pancreatin:protein) and the obtained solution was incubated overnight at 37 °C. Trypsin was then added at a mass ratio of 1:100 (pancreatin:protein) and proteins were digested for 4 h (Zhang et al. 2018b).

TMT labeling

The trypsin-digested peptide was desalted by Strata X (Phenomenex) and vacuum-dried. The precipitate was solubilized by 0.5 M TEAB and then peptides were labeled according to the labeling kit (Phenomenex) instructions. Briefly, the labeled reagent was thawed, dissolved in acetonitrile, mixed with peptides, and incubated for 2 h at room temperature. The labeled peptides were mixed, desalted by stage-tip, and dried under a vacuum before the mass spectrometry analysis (1 μ of peptide) (Zhang et al. 2018b).

LC-MS/MS analysis

Tryptic peptides were solubilized by a solution containing 0.1% formic acid and 2% acetonitrile and separated by the EASY-nLC 1000 ultra-high-performance liquid system with the same solution used to solubilize tryptic peptides. Then tryptic peptides were injected into an NSI ion source for ionization and analyzed using Orbitrap Fusion TM mass spectrometry. The ion source voltage was set to 2.0 kV, and the peptide parent ion and its secondary fragments were analyzed by high-resolution Orbitrap (Zhang et al. 2018b).

Database search

The MS/MS data were processed using the Maxquant search engine (v.1.5.2.8) and matched in the proteome database using the anti-library to rule out false positive rates (FDR) caused by random matches. The minimum peptide length was set to 7 amino acid residues, the maximum number of missing sites was set to 2, the mass tolerance of the primary parent ions was set as 20 mg/L in the first search and 5 mg/L in the main search, and the mass tolerance for the fragment ions was set to 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and oxidation on Met was specified as variable modifications. The FDR was adjusted to < 1% and the minimum score for peptides was > 40 (Zhang et al. 2018b).

The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. Proteins with fold change ratios of 1.2 and *P* values of 0.05 were considered to be significantly and differentially expressed. Functional annotations of the proteins were conducted using the Blast 3, 4, 5 GO program against the non-redundant protein database (NR; NCBI). The KEGG database (http://www.genome.jp/kegg/) was used to classify and group the identified proteins. The proteomics and bioinformatics analysis was helped by Jingjie PTM BioLab (Hangzhou) Co. Ltd.

Results

Total, viable, and culturable cell counts and morphological characteristics of culturable and VBNC cells

The number of culturable bacterial cells decreased in both treatments with the incubation time and after 11 days cells were undetectable (< 10 CFU per gram of dry soil) in the dry soil; upon rewetting cells, number increased and accounted for 344 CFU per gram of dry soil after 15 days (Fig. 1). It took 96 h from being undetectable to be counted as 344 CFU per gram of dry soil. If this was due to the regrowth of surviving culturable cells, the generation time of *E. coli* would be 18.8 h: However, by considering that the generation time was about 2.11 h in a preliminary independent experiment, the growth would have given of 2.6×10^4 CFU per gram of dry soil in only 24 h. Therefore, we suggest that the high generation time (18.8 h) observed in our study is attributable to the resuscitation of VBNC cells, rather than the regrowth of undetected culturable cells. The total and viable cell counts determined by the qPCR and PMA-qPCR methods, respectively, declined in the drying soil being 1.50×10^6 and 1.52×10^5 after 11 days (Fig. 1).

The *E. coli* showed rod-like shapes and dense cytoplasm at 30% soil water content after 11 days (Fig. 1c, d, and e), while the VBNC cells in the air-dried soil were spherical, with thick cell walls, empty cytosol, and intact membranes. And part of the structure of the cell organelles was still visible after 11 days (Fig. 1f, g, and h). The *E. coli* recovered their culturability after the water content was restored, but were still spherical with some short rod shapes (Fig. 1i, j, and k).

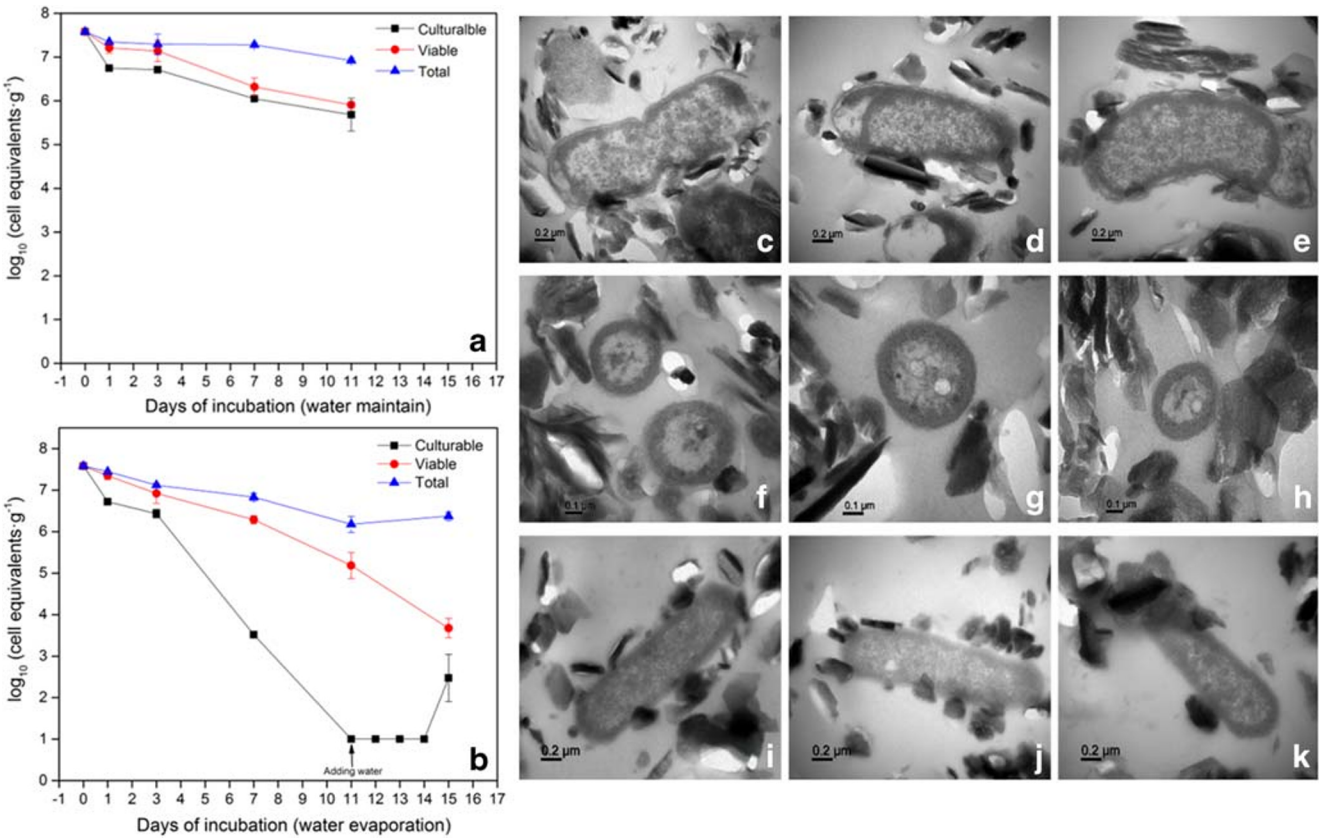

Fig. 1 Total, viable, and culturable *E. coli* cell counts during the incubation of the moist soil (**a**). Total, viable, and culturable *E. coli* cell counts during the incubation of drying soil (**b**). Morphology of *E. coli* in the wet soil after 11 days (**c**, **d**, **e**). Morphology of *E. coli* present as VBNC state in the drying soil after 11 days (**f**, **g**, **h**). Morphology of *E. coli* cells after 15 days, that is after rewetting the dry soil (**i**, **j**, **k**)

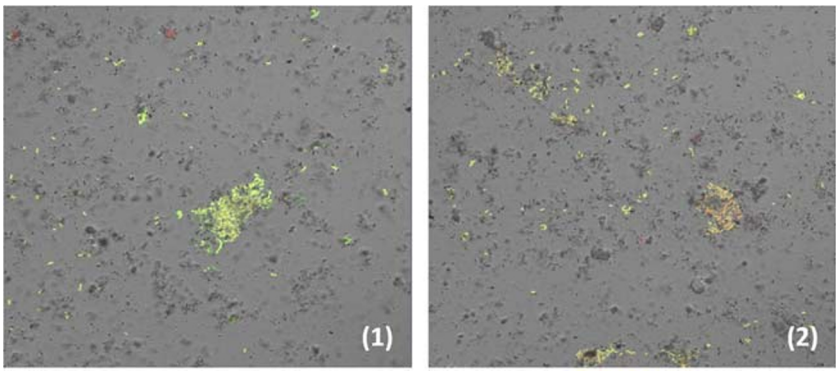

Fig. 2 LSCM microphotographs. (1) *E. coli* incubated in wet soil (control); (2) *E. coli* cells in the air-dried; both microphotographs were taken in soils incubated for 11 days

The *E. coli* cells of soil with constant moisture were mainly the VBNC cells (with green fluorescence), while those of the air-dried soil were mainly dead (with red fluorescence) or close to death (with yellow fluorescence) when both soils were analyzed after 11 days (Fig. 2).

Proteomic analysis

Differentially expressed proteins by comparing the culturable and the VBNC state

The VBNC state of *E. coli* showed that 1,219 proteins were differentially expressed which makes the data reliable. For the differentially expressed proteins, 802 (66%) were in the cytoplasm, 200 (16%) in the periplasm, 127 (10%) in the cell inner membrane, 54 (5%) in the cell outer membrane, and 36 (3%) extracellular proteins (Fig. 3); 657 proteins were upregulated and 561 downregulated.

GO annotation and GO enrichment of the significant DEPs

All of the identified proteins were subjected to the GO annotation to assess their biological functions (Fig. 4); 1,219 differently expressed proteins in the VBNC state were involved in biological process and molecular functions or were cellular components. At the 3rd level of the GO annotation, it showed that proteins involved in metabolic processes, cell activities, and enzyme activities were the most important in biological process, cellular component, and molecular function, respectively. At the 4th level, proteins involved in macromolecular metabolic processes, intracellular location, and anion binding were the most important in biological process, cellular component, and molecular function, respectively. At the 5th level, proteins related to the biosynthetic processes of organic N compounds, integral components of membrane, and nucleotide binding were the most significant ones by considering the above categories. Interestingly, proteins involved in the functions related to transport, signaling receptors, and flagellum were upregulated, whereas those involved in the DNA repair,

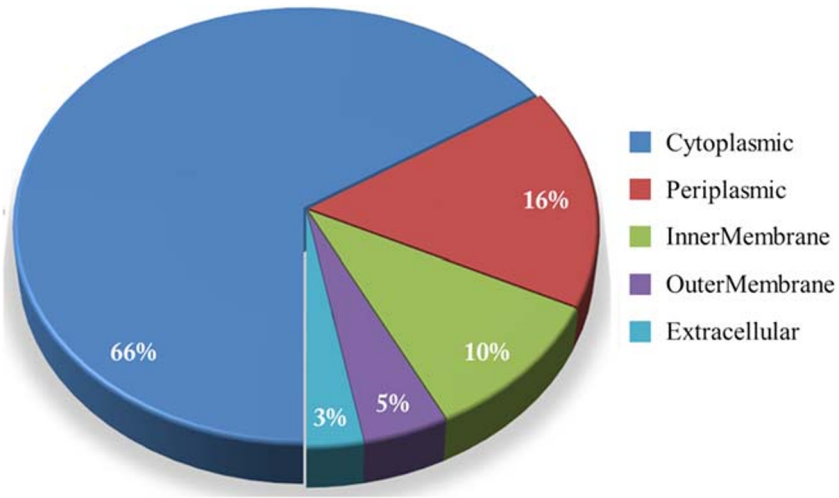

Fig. 3 Subcellular locations of differentially expressed proteins (DEPs) of the VBNC cells compared with the culturable cells

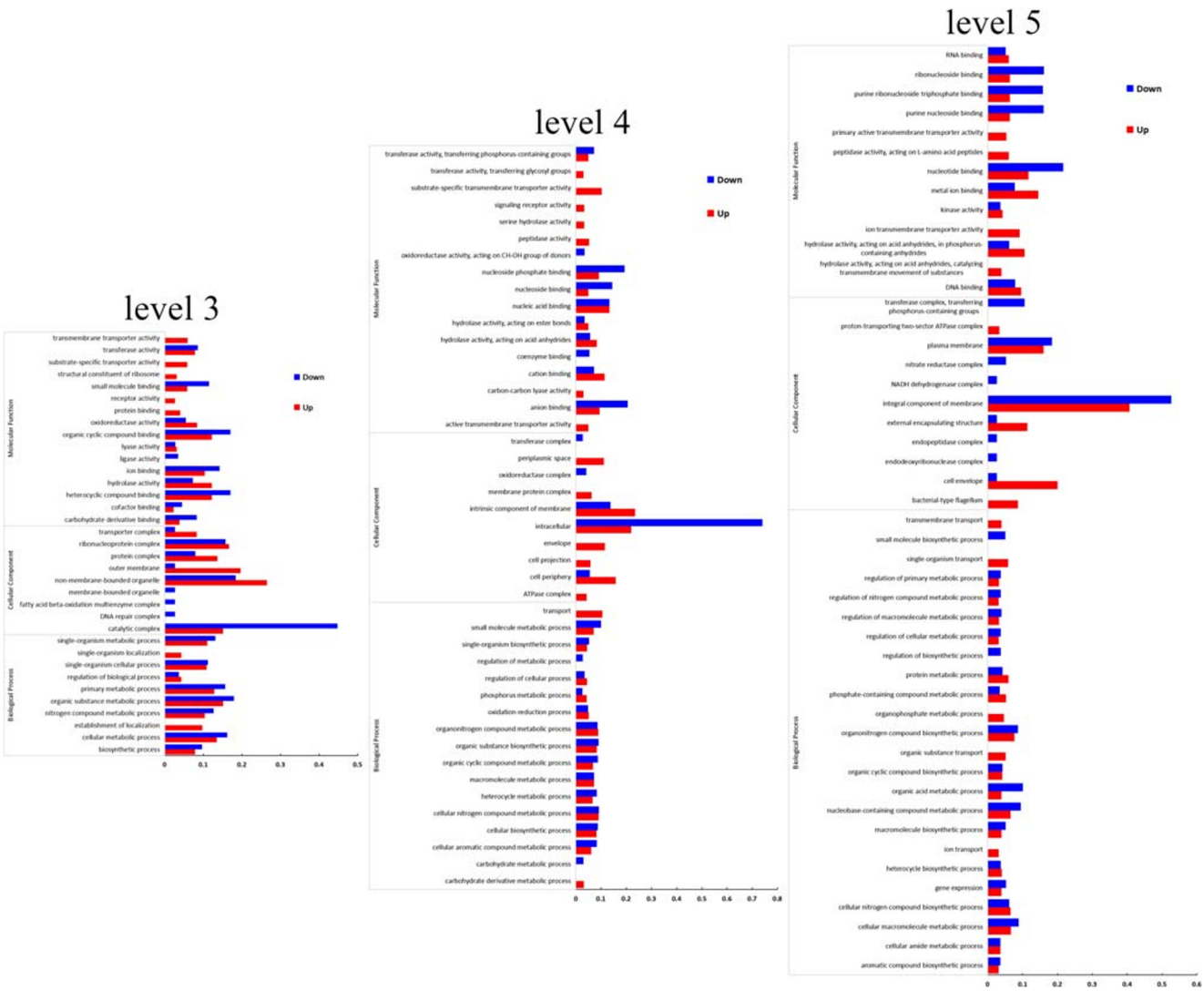

Fig. 4 GO annotation of the significant differently expressed proteins of the VBNC cells compared with the culturable cells at the 3rd, 4th, and 5th level

carbohydrate metabolism, and regulation of metabolism were downregulated.

To analyze the biological properties and functional alterations of the VBNC state compared to the culturable state, we performed the GO enrichment analysis (Fig. 5). Among the biological processes, the upregulated proteins were mainly those related to cilium or flagellum-dependent cell motility, protein transport, and iron chelate transport while the main downregulated proteins were those related to oxoacid and carboxylic acid metabolisms. As it concerns the cellular location, some upregulated proteins were enriched in periplasmic space, outer membrane, and cell envelope, whereas some downregulated proteins were enriched in the cytoplasm. According to the molecular function analysis, we found that proteins related to transmembrane receptor and signaling activity and transporter activity were primarily enriched as upregulated proteins, while most of the enriched and downregulated proteins were involved in ligase activity, anion binding, and nucleotide binding.

KEGG pathway analysis of the significant DEPs

One thousand one hundred five differentially expressed proteins (DEPs) of the VBNC state were enriched according to the KEGG pathways analysis (Fig. 6). Among the upregulated proteins, those related to the ABC transporter, bacterial chemotaxis, flagellar assembly, protein export, and pathogenic *E. coli* infection pathways were enriched, whereas the enriched and the downregulated proteins were related to the aminoacyl-tRNA biosynthesis, glycine biosynthesis, biofilm formation, glycolysis, citrate cycle (TCA cycle), and DNA replication (Fig. 7 and Fig. S1-S25).

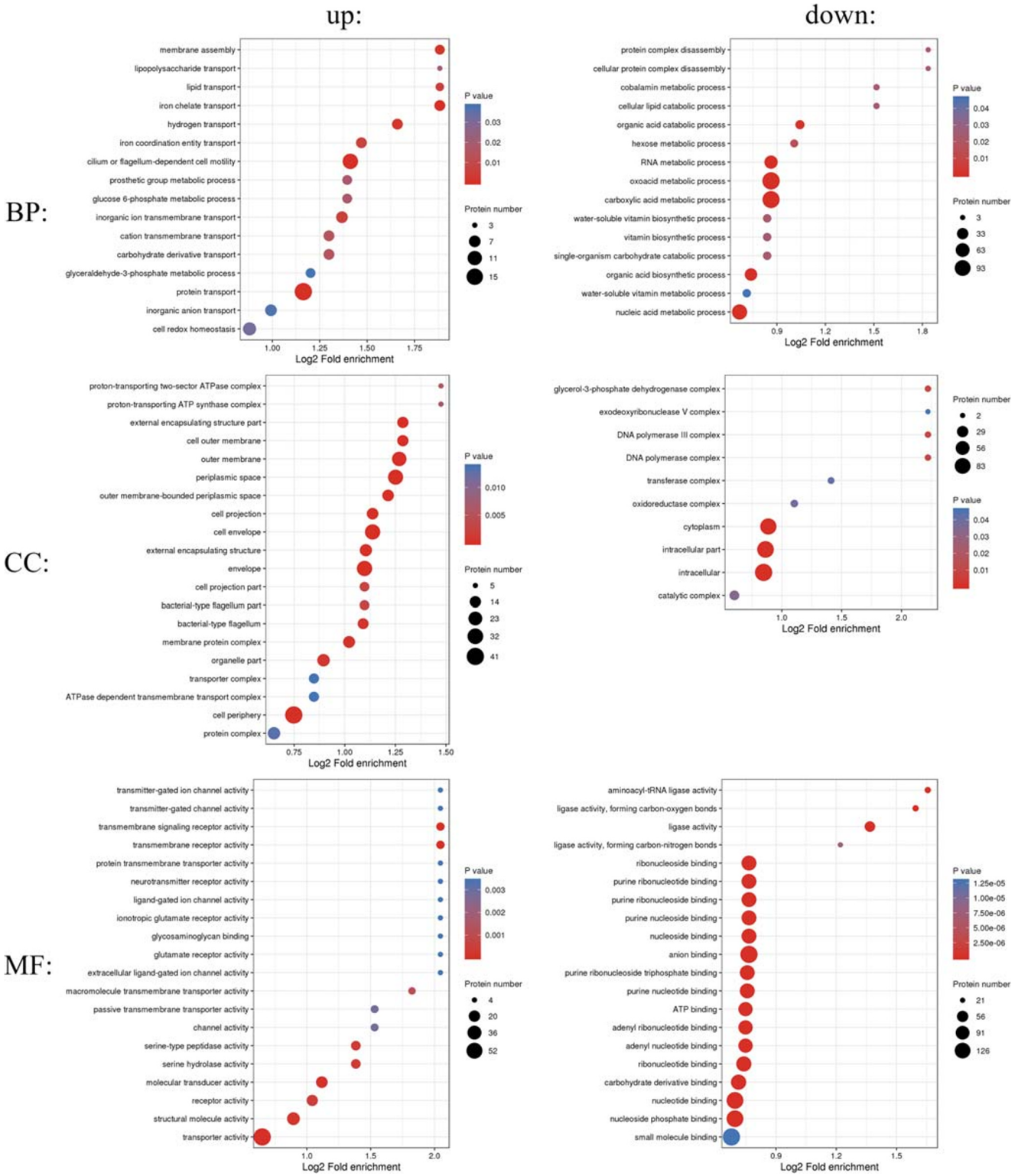

Fig. 5 GO enrichment of the differently expressed proteins of the VBNC cells compared with the culturable cells (BP: biological process, CC: cellular component, MF: molecular function)

Network analysis of protein-protein interaction

The network analysis of protein-protein interaction (PPI) identified the major nodes and important linkers among the differentially expressed proteins of the VBNC state. This analysis showed that the main interactions occurred between 150 proteins; those related to 30S and 50S ribosomes were significantly upregulated, while GMP

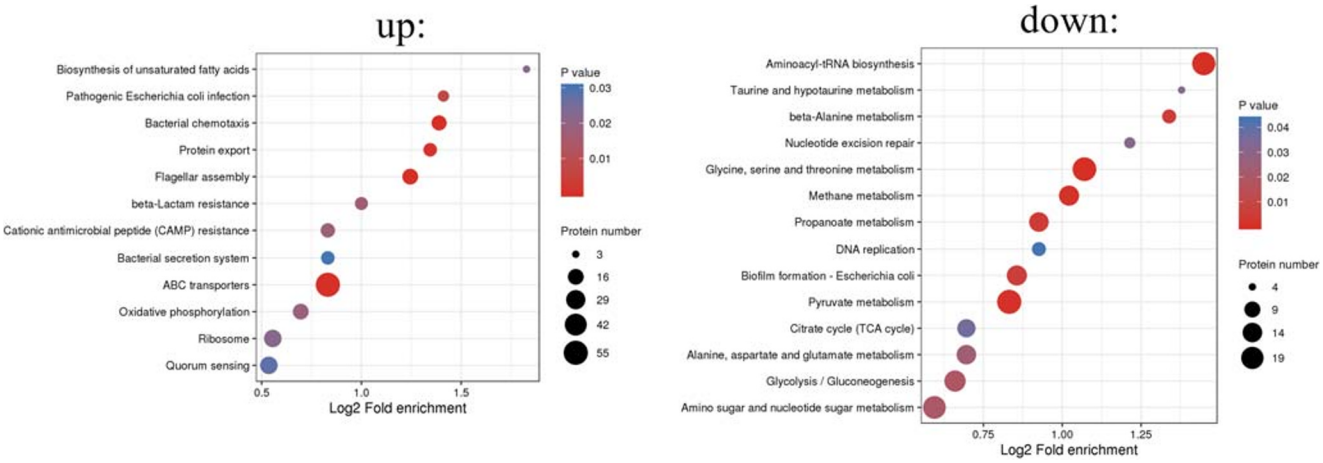

Fig. 6 Enrichment of the differently expressed proteins of the VBNC cells compared with the culturable cells as analyzed by the KEGG pathway

synthase, DNA-directed RNA polymerase, and DNA polymerase were downregulated (Fig. 8).

Discussion

Culturability and morphology of *E. coli* O157:H7

Cells of *C. metallidurans* strain CH34 entered the VBNC state by decreasing soil moisture (Giagnoni et al. 2018), probably due to the shortage of available C sources. *E. coli* O157:H7 needs a high soil moisture content to survive (Nyberg et al. 2014); indeed, cells entered the VBNC state as the artificial soil dried and *E. coli* O157:H7 regained the culturable state as soil was rewetted. Likely, sharp changes of osmotic pressure and the decrease in nutrient availability led to a decline in the number of culturable cells of *E. coli* O157:H7. We confirmed what was reported for morphological changes of the chlorine-induced VBNC state of *E. coli* (Chen et al. 2009) because cells of *E. coli* O157:H7 in the drying soil assumed globular shapes

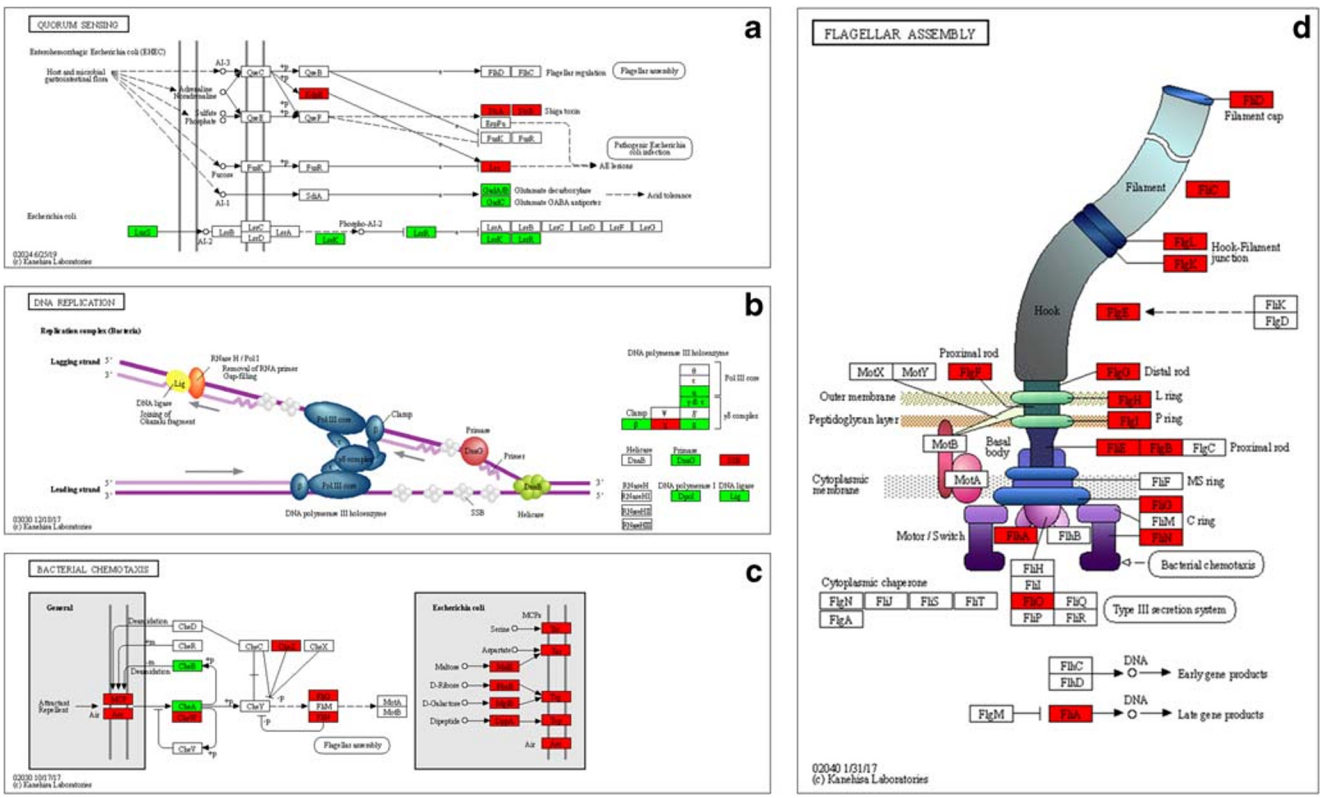

Fig. 7 Regulation of differently expressed proteins involved in quorum sensing (**a**), DNA replication (**b**), bacterial chemotaxis (**c**), and flagellar assembly complement and coagulation cascades (**d**) as showed by the KEGG pathway analysis. The proteins in red were upregulated whereas those in green were downregulated

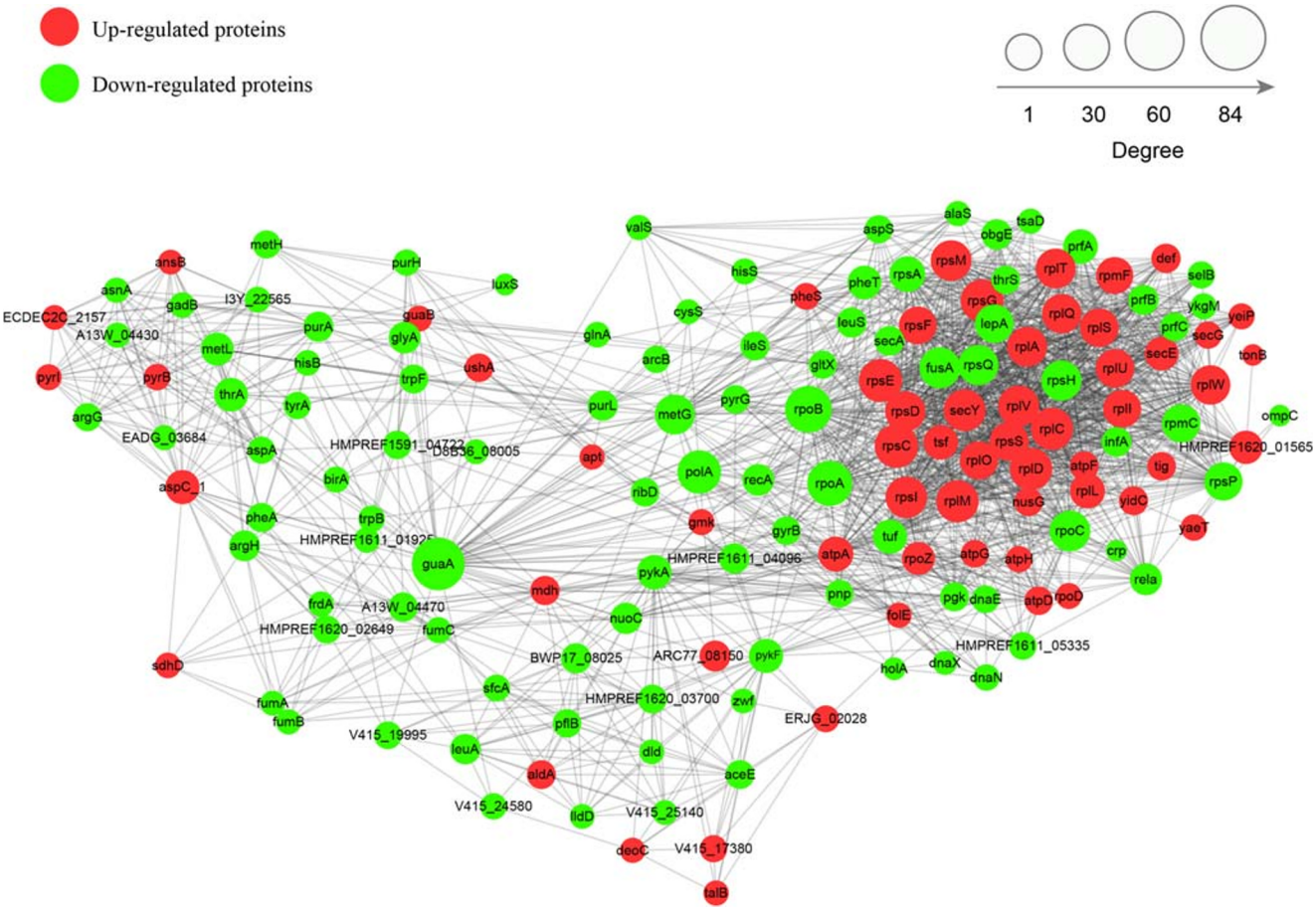

Fig. 8 Network analysis of protein-protein interaction

from long rod shapes, and observation by TEM revealed that the cytoplasm was reduced. Zhao et al. (2016) showed that *E. coli* O157:H7 could enter the VBNC state under high CO_2 pressure and the cell shapes changed from rods to short or to curved rods with a decrease in nuclear and cytoplasmic density. In addition, these cells showed loose nuclear material, a gap between the intima and the adventitia, and the decreased ribosome number. Other researches also reported that poor nutrition (Chen et al. 2009), low temperature (Chen et al. 2009), or extreme pH (Giotis et al. 2007) decreased cell size with transitions from bacilli to cocci in the VBNC state (Colwell 2000; Krebs and Taylor 2011). These changes enabled cells to have the largest surface area for nutrient uptake while maintaining the least amount of cell mass (Krebs and Taylor 2011). When conditions changed to favorable ones, the cells returned to their normal shapes (Chiu et al. 2008).

Our study mainly focused on protein expression. The fact that a total of 2,324 proteins were identified indicates that artificial soil was a good model for studying the proteomic features of *E. coli* in the soil environment. Proteins related to the cell morphology, such as the MreB protein, a cytoskeletal protein present in all non-spheroidal bacteria and involved in cell polarity, chromosome separation, sporulation, and cell-shape control (Chiu et al. 2008), were downregulated in the VBNC cells in this research. The starvation state of *V. parahaemolyticus* decreased the MreB expression and this changed the morphology of cells from rod shapes to small spheres (Chiu et al. 2008). In our study, the peptidoglycan polymerase MrdB protein was upregulated 1.45-fold in the VBNC *E. coli* compared to the culturable cells. This protein is involved in the cell wall elongation and the peptidoglycan biosynthesis pathway which is important for the cell wall synthesis. Besides, the DacB protein involved in the cell morphological changes (Hung et al. 2013) was also upregulated, and this may be a potential reason for the formation of small spheres in VBNC cells in our study. Likely, the cell walls of *E. coli* under the VBNC state thickened to resist the hostile environment.

Membrane proteins

Distinguishing between viable (including culturable and VBNC) and dead cells by measuring the integrity of the cell membranes is a widely accepted approach, because only dead cells lose integrity of the cell membranes (Vives-Rego et al.

2000). Membranes of VBNC cells were stained by SYTO9 providing that they were intact.

The outer membrane proteins of gram-negative bacteria, like *E. coli* O157:H7, play important roles in many cellular and physiological processes (Qian et al. 2008) and for adapting cells to variations in environmental conditions (Xu et al. 2005), and their expression changes when bacteria are in the VBNC state (Zhang et al. 2019a; Zhao et al. 2016). We observed that several membrane proteins, mainly involved in transport functions, were differentially expressed in the VBNC cells compared to culturable cells. This was the behavior of three representative outer membrane proteins, CusC, OmpW, and OmpR. CusC was involved in the excretion of monovalent copper ions or monovalent silver ions from the cytoplasm to outside (Kim et al. 2011a). The efflux of copper ions may help microorganisms to withstand damage caused by any reactive oxygen produced, for example by phagocytic cells (Munson et al. 2000). Therefore, upregulation of CusC may be beneficial for the survival of the VBNC cells under oxidative stress. OmpF is one of the most abundant proteins in the outer membrane of *E. coli*, exists as a stable trimer, forms an electronegative channel on the outer membrane, and is responsible for the diffusion of positively charged molecules with a molecular weight lower than 600 Da to pass through cell membrane. OmpF is also directly involved in the absorption of large toxic proteins such as colicin (Housden et al. 2010; Nikaido 2003), and it is mainly expressed under nutrient deficiencies and hypotonic pressures at room temperatures, thereby contributing to the efficient absorption of nutrients by *E. coli* in nutrient-deficient media (Yoshida et al. 2006). Likely, the upregulated expression of OmpF (2.04-fold) might have maintained the integrity of the extracellular membrane and allowed the cells to acquire nutrients in large quantities for a rapid recovery when conditions were appropriate (Zhao et al. 2016). The *E. coli* can increase OmpW protein expression under H_2O_2 oxidative stress to survive; it is important to underline that the *ompw* mutant strain was more resuscitable under VBNC state than the wild strain (Asakura et al. 2008).

Proteins involved in the DNA replication and recombination

Under the VBNC state, DnaA, DnaE, DnaG, DnaN, and DnaX proteins, which are involved in DNA polymerase activity (Cohen-Fix and Livneh 1994), were all downregulated. The DnaA protein is the starting protein for chromosome replication in *E. coli* and plays an important role in the initiation and regulation of chromosome replication. The *dnaE* gene mutations cause defects in DNA replication and elongation, decreasing the rate of DNA synthesis and inhibiting cell division (Strauss et al. 2004). The DnaG protein is an RNA polymerase and catalyzes the synthesis of short RNA molecules acting as primers for DNA polymerase during DNA replication. The DNA polymerase III tau subunit encoded by the *dnaX* gene is part of a beta sliding clamp loading complex that hydrolyzes ATP to load beta clamp onto the primed DNA. The downregulation of the above proteins reduced the rate of DNA synthesis and inhibited cell division, and this seems to explain why the VBNC cells could not divide.

The SeqA that is encoded by the *seqA* gene affects DNA replication in the VBNC cells (Zhao et al. 2016). Likely, its upregulation inhibited the initiation of DNA replication and delayed the separation of nuclei and cell division, leading to the formation of VBNC state. In addition, the presence of loose nuclei in VBNC cells might also depend on the upregulated expression of SeqA.

Proteins involved in carbohydrate transport and metabolism

The proteins involved in the tricarboxylic acid cycle (TCA cycle), glyoxylate cycle, glycolysis, and disaccharide metabolism were downregulated in the VBNC state, indicating that the carbohydrate metabolism was inhibited.

The carbohydrate phosphotransferase system is the major carbohydrate uptake system that transports and phosphorylates a variety of carbohydrates by consuming phosphoenolpyruvate in bacteria (Jamal et al. 2013). The expressed proteins of the VBNC state, which were related to the phosphotransferase system such as enzyme I (E1) of phosphotransferase system (encoded by PtsI, PtsP, and PtsA), were downregulated, thus decreasing carbohydrate metabolism. The phosphotransferase system is involved not only in the absorption of carbohydrates but also in the control of bacterial organic C dynamic. A decrease in the activity of the phosphotransferase system under the VBNC state increased the amount of PEP; besides, it has been reported that PEP content was increased in persister and starved-state *E. coli* (Radzikowski et al. 2016). In addition, the growth rate of *E. coli* was reduced after mutation of the *ptsI* gene (Nilsson et al. 2003). Therefore, the downregulation of EI expression may be one of the causes for the inability of VBNC cells to be culturable.

In *E. coli*, glucose-6-phosphate is mainly involved in the glycolytic pathway and pentose phosphate pathway, both of which are regulated by glucose-6-phosphate dehydrogenase (G6PDH) encoded by the *zwf* gene (Sandoval et al. 2011). The overexpression of G6PDH increased the C flux through the pentose phosphate pathway (Kim et al. 2011b; Nicolas et al. 2007). In our experiment, the G6PDH was downregulated under the VBNC state, because the glycolysis pathway was downregulated and the overall metabolism was slowed down under unfavorable conditions.

The network analysis of the protein interaction in this study showed that the pyruvate kinase, encoded by *pyka* and *pykf* and catalyzing the rate-limiting reaction in the glycolysis (Xie et al. 2016), was a high-integration-degree kinase suggesting

that pyruvate and the glycolysis pathway was probably important in the formation of the VBNC state. In addition, pyruvate kinase M2 (PKM2) plays an important role in maintaining redox homeostasis in cells and this role has not received enough attention so far (Liang et al. 2016). Monahan and Harry (2016) reported that the pyruvate kinase enzyme (PKY), which produces pyruvate in the final reaction of glycolysis, is involved in the Z-ring formation of *B. subtilis* cells. The addition of exogenous pyruvate restored normal division in the absence of the pyruvate kinase activity, thus suggesting that pyruvate was a key metabolite in the coordination of bacterial growth. The pyruvate accumulation was involved in the elimination of ROS, whose over-accumulation caused the VBNC state (Zhang et al. 2019b). In our study, the two pyruvate kinases and the eight proteins involved in pyruvate synthesis were all downregulated, suggesting that this overall downregulation might have also caused the VBNC-state formation.

Proteins of the transport systems

ABC transporters responsible for the transport of extracellular substances into cells represent the main nutrient uptake by *E. coli*. In addition, they can export toxic molecules out of cells (Davidson et al. 2008). In our study, the ABC transporters were all upregulated in the VBNC state. The TonB protein was upregulated by 2.38-fold, and the TonB system–like proteins were significantly upregulated and enriched in the VBNC state. The TonB system is generally involved in the transport of many important substances, such as iron, heme, vitamin B12, carbohydrates, and various transition metal elements in Gram-negative bacteria (Schauer et al. 2008). The main 5 MCPs proteins of *E. coli*, namely, Tsr, Tar, Trg, Tap, and Aer, were all upregulated as well as most of the chemotaxis proteins under the VBNC state in our study.

In dry soil environments, the migration of organic and inorganic substrates and extracellular enzymes is restricted, thereby affecting microbial access to nutrients. As the soil water potential and water film thickness decreased, microbial activity and passive mobility also decreased. Despite that the intracellular metabolism slowed down in the VBNC state, the expression of TonB and the ABC transporters was upregulated, likely because the cells could be immediately active to uptake nutrients when they resuscitate from the VBNC state.

Proteins involved in the flagellar movement

The flagellum is a key feature affecting the morphology, chemotaxis, and survival of bacteria (Chilcott and Hughes 2000). Several reports have shown that flagella directly mediates bacterial adhesion (Haiko and Westerlund 2013).

In our research, 19 proteins involved in the flagellar synthesis, rotary motor, hook (universal joint), and fibril (spiral propeller) were all upregulated in the VBNC state. Bacteria steer the direction of rotation of the flagellar motor to avoid adverse environments, and the change in the direction of rotation of the flagellar motor depends on the chemotaxis signal (Fukuoka et al. 2007; Kojima and Blair 2004). Among the stator proteins located on the cell membrane and responsible for the flagellar movement, the FliN, FliG, and FliM proteins together formed a "rotating complex" involved in flagella assembly, rotation, and clockwise-counterclockwise conversion (Morimoto et al. 2010). In this study, FliN and FliG were upregulated, which was likely to increase the sensitivity of the VBNC cells to chemotactic signals. It also suggests that those cells, once resuscitating from the VBNC state, were ready to start searching for nutrients especially under nutrient limitations. Indeed, the genes related to "flagella motility" were all downregulated when *E. coli* responded to five different perturbations (Jozefczuk et al. 2010). Zhong et al. (2018) also reported that the proteins associated with the flagellum were significantly downregulated in the VBNC state of *Vibrio parahaemolyticus* when the state was induced by low temperature and oligotrophic conditions. The different results were probably due to the fact that those studies have been conducted in pure culture with uniform nutrient distribution and without concentration. On the contrary, soils are solid and nutrients are not evenly distributed. Such concentration differences may lead to chemotaxis of microorganisms toward nutrients using flagella. A similar study showed that *E. coli* had the ability to chemotactically move toward glucose even when it entered dormancy state (Yamasaki et al. 2020).

Moreover, the flagellar cascade inhibitor protein DksA was downregulated in our study. Lemke et al. (2009) reported that DksA/ppGpp of *E. coli* inhibited the expression of the flagellar cascade during the stationary phase and following starvation. Likely, DksA/ppGpp coordinated flagella and ribosome assembly to save energy, thus confirming what was observed for the flagellar protein data in this study.

Proteins involved in pathogenicity

E. coli O157:H7 is known as one of the most dangerous foodborne pathogenic bacteria due to its ability to cause diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (Harris 1990). All of these diseases arise from the A/E damage on the surface of intestinal epithelial cells via the production of Shiga toxin, hemolysin, and adhesion (Donnenberg et al. 1993; Elliott et al. 1998).

The proteins related to pathogen properties of *E. coli* O157:H7 examined in our study can be broadly classified into three categories. The first category participates in the synthesis and function of the type III secretion system (T3SS). We observed a 1.6-fold downregulation of type III structural proteins such as sepQ. The *tir* gene encoding the type III secreted protein was upregulated in the VBNC state. This multifunctional protein

is required for efficient pedestal formation in host epithelial cells during infection (Battle et al. 2014). Tir, Ler (type III secretion system LEE master regulator), and Espd (LEE-encoded effector which is involved in pili synthesis) were all upregulated in the VBNC state in our study.

The second category concerned pili chaperone proteins expressed by the *espD* gene and the *fimC* gene (Low et al. 2006); these proteins were upregulated. They accelerate the folding and stabilization of the pili subunit; in addition, they are involved in the transport of pili subunits through the periplasmic space to the pili assembly station of the outer membrane (Larsson et al. 2005). The upregulation of those two proteins suggested the enhanced cell adhesion ability in the VBNC state, which likely depended on the uneven distribution of humic acids in the artificial soil; *E. coli* cells might have adhered to the surface of humic acids' particles because humic acids are a nutrient source among the surface-reactive particles of the artificial soil; this adhesion may be one of the causes of *E. coli* survival in the dry soil thus maintaining the risk of potential pathogenicity.

The third category is pathogenicity-related virulence factors. In the present study, we have analyzed maltoporin encoded by the *lamB* gene (Werts et al. 1992), which was downregulated 1.29-fold. This category also includes hydroxymethyl oxoglutarate aldolase encoded by the *eda* gene (Chattopadhyay et al. 1991), which was upregulated 1.69-fold, and the outer membrane protein OmpA and virulence protein, encoded by the *stx1A*, *stx2A*, and *stxB* genes (Kim et al. 2010), which were upregulated.

Yaron and Matthews (2002) observed that virulence genes *stx1* and *stx2* were expressed in the VBNC state of *E. coli* O157:H7. Lothigius et al. (2010) found that although enterotoxins were not produced when *E. coli* entered the VBNC state, the expression of virulence genes *eltB* and *estA* encoding the LT and STh enterotoxins was maintained. In addition, VBNC cells still present potential danger for causing fatal diseases because of their rapid resuscitation and activation of the virulent proteins under suitable conditions (Du et al. 2007). Patrone et al. (2013) observed that *C. jejuni* in the VBNC state still maintained the ability to adhere to intestinal cells. These studies demonstrated that the virulence genes in VBNC cells could still be expressed.

Proteins regulating VBNC state

RpoS protein is a key regulator in the formation of the VBNC state (Zhao et al. 2017). The network analysis of the protein interaction showed that the DNA-directed RNA polymerase subunit alpha, encoded by *rpoA*, *rpoB*, and *rpoC* genes, had the highest degree of interaction and it was downregulated, suggesting the decreased transcription which is important in the formation of VBNC state. The SpoT and RelA proteins catalyze the formation of ppGpp, which played a key role in the formation of *E. coli* VBNC state (Ayrapetyan et al. 2015). Both proteins were downregulated in our research. In addition, the proteins regulated by the *relA* gene interacted with 33 proteins in the PPI network analysis, indicating that the expression of the *relA* gene may be important in the VBNC-state formation. The polyphosphate kinase is involved in various cell functions, including cell survival, stress responses, host colonization, and virulence (Brown and Kornberg 2008; Gangaiah et al. 2010). Gangaiah et al. (2009) reported that polyphosphate kinase 1 (PPK1) could promote the entry of *Campylobacter jejuni* into the VBNC state by increasing the accumulation of Poly-P. In our research, polyphosphate kinase, expressed by the *ppk* gene, was downregulated. The PPK is involved in various intracellular reactions, whose role in the formation of the VBNC state is still unknown (Pinto et al. 2015).

Pu et al. (2019) reported that the ATP content of *E. coli* was gradually reduced under nutrient-limiting conditions. In addition, many proteins involved in cellular functions formed aggresomes, when the cells entered the VBNC state. The ATP content of cells was replenished upon restoring optimal nutrient conditions and with the resuscitation from the VBNC state. The DnaK-ClpB bichaperone system can facilitate the disaggregation. In our study, the proteins encoded by the *clpB* and *dnaK* genes were downregulated in the VBNC state suggesting that some aggresomes may have formed during the VBNC state. Three possible mechanisms regarding the formation of VBNC *E. coli* in this study are as follows:

1. Dry conditions lead not only to a decrease in water content but also to the decrease in nutrient availability in the soil. When cells were stressed to produce a stringent response (SR), RelA and SpoT as alarm signal (p)ppGpp (alarmone nucleotides) synthase proteins were downregulated. This process further leads to the downregulation of downstream RpoS, which causes most of the *E. coli* to die whereas the survivors entered the VBNC state.
2. Water and nutrient stress result in the ROS accumulation in cells and antioxidant enzymes were upregulated to remove ROS. When ROS accumulation reached a certain level, some *E. coli* will enter the VBNC state to survive in adverse environment.
3. Both water and nutrient stress decrease the ATP content of *E. coli* cells. Moreover, DnaK and ClpB proteins were downregulated and protein aggregates were condensed, thus causing them to enter the VBNC state.

Conclusions

This study demonstrated that *E. coli* O157:H7 could enter the VBNC state after soil drying and could be resuscitated by

rewetting. Furthermore, when compared with cultivable cells, cells in VBNC state were spherical, with thicker cell walls and emptier cytosol. Besides, the proteomic profiles of the VBNC cells showed that the proteins involved in the DNA replication and recombination, carbohydrate transportation, and metabolism were downregulated, which implies the low metabolic activity and unculturable state of VBNC *E. coli* O157:H7. However, the proteins related to the flagellar movement, pathogenicity, and nutrient uptake were all upregulated in the VBNC cells, indicating that VBNC *E. coli* could still cause fatal diseases. In addition, because of their rapid resuscitation when suitable conditions were again established in soil, the VBNC *E. coli* O157:H7 can still pose potential environmental risk.

Despite that this study was conducted in an artificial soil, it has the potential to understand the mechanisms underlying the VBNC state of *E. coli* in soil where the soil proteomic approach is still a technical challenge due to several factors including the huge amounts of expressed proteins, which cannot all be extracted from soil and detected. The search of expressed proteins in soil might be limited to those playing an important role in soil quality as those involved in the expression of pathogenicity. According to Schloter et al. (2018), molecular indicators of soil quality are required; these novel indicators should represent soil status in an accurate manner and indicate the range of values being acceptable for defining soil quality. Future research should select proteins representing microbial pathogenicity to be included in novel framework for soil quality assessment.

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