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Bacterial culturability and the viable but non-culturable (VBNC) state studied by a proteomic approach using an artificial soil

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ABSTRACT

Gram-negative bacteria in soil rapidly adapt to various stresses, including nutrient limitation and desiccation, by adopting the viable but non-culturable (VBNC) state as a survival strategy. Due to the physico-chemical and microbiological complexity of soils, little is understood on the effects of nutrient availability and moisture level on the transition from the VBNC state to culturability in soil. We evaluated the effects of gluconate or water on the transition of the soil borne bacterium *C. metallidurans* strain CH34 from the VBNC state to culturability by experiments of inoculation into artificial soils and bacterial metaproteomic analysis. Incubation without water or nutrients reduced the bacterial culturability to zero in 12 d, and addition of both water or gluconate restored the bacterial culturability to high levels within 24 h. The proteomic analysis showed that under water and nutrient limitation, proteins related to the cell shape and protein synthesis were rapidly down-regulated and stress-related proteins were quickly up-regulated during the transition from culturability to VBNC state. Reversion from the VBNC state to a culturable state with water or gluconate led to highly different bacterial proteomic profiles of *C. metallidurans*. Gluconate availability restored main protein biosynthesis and energy metabolic pathways, whereas water addition led to up-regulation of only six proteins, one of which degrade sigma factors involved in expression of genes controlling bacterial resistance under nutrient limitation. Proteins regulated during the transition between culturable and VBNC states could also be involved in the phenotypic VBNC for other soil bacteria, and can highlight some of the microbial genetic mechanisms allowing the entering and exiting from the VBNC state. Implications of the VBNC in microbial diversity and soil functionality are discussed.

1. Introduction

Soil hosts one of the most complex microbial communities performing key ecological functions, such as organic matter decomposition, nutrient mineralization, plant growth promoting activity, and the rate of soil functions depends on the active biochemical pathways in soil microorganisms. In most soils, microorganisms are subjected to daily and seasonal variations of temperature, moisture level, vegetation cover and availability of nutrients. A central question in soil microbiology is how microbial cellular activities are influenced by the fluctuations in key soil properties and nutrient availability. Except for microbial communities inhabiting niches with available C such as the rhizosphere, soil microorganisms are under metabolically resting conditions due to C limitation (Anderson and Domsch, 1985); moreover, it was estimated that more than 80% of microbial cells and about 60% of soil microbial species are inactive (Lennon and Jones, 2011). This implies that the highly diverse soil microbial communities have generally limited expression of their metabolic potential, but their quiescence can be suddenly reverted by the availability of C sources (De Nobili et al., 2001). While Gram-positive bacteria form spores as main survival strategy to nutrient limiting conditions, Gram-negative bacteria have evolved a metabolic strategy, termed viable but non-culturable (VBNC) state, to survive under adverse conditions such as limiting nutrient and water availability or stressing factors (Atlas and Bartha, 1981). It is well established that less than 1% of soil microorganisms are cultivable on growth media (Rappe and Giovannoni, 2003). During the VBNC state, non-spore-forming bacteria assume a metabolic quiescent state, undergo to morphological modifications, but retain the cell integrity and their replicative potential (Colwell, 2009). The VBNC state has been first characterized for pathogenic bacteria such as *E. coli* and *V. cholera* by *in vitro* studies (Xu et al., 1982; Oliver, 2005, 2010). Laboratory experiments, based on the inoculation of bacteria into estuarine or see waters with varying temperature and nutrient concentrations, have allowed the study of the VBNC state in several environmental bacterial strains (Epstein, 2009). While dormancy in all forms contributes to the maintenance of microbial diversity in the environment by the 'storage effect' and increase resilience and recovery of microbial

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communities under variable environmental conditions (Stevenson, 1977; Chesson and Warner, 1981; Jones and Lennon, 2010), there is still poor information on the relative effects of moisture and C availability on the VBNC state in soil-borne bacteria. This is mainly due to the high complexity of both the soil matrix and natural microbial communities.

The VBNC state in bacteria can be studied by microscopic observations after fluorescent stain (Daley and Hobbie, 1975; Zimmerman et al., 1978; Kogure et al., 1979), by immunological methods using fluorescent probes also coupled to flow cytometry (Grimes and Colwell, 1986; Wallner et al., 1999). However, microscopic techniques do not provide information on the changes in cell metabolic activity and their use in soil studies can not discriminate labelled bacteria from fluorescent soil particles. Genomic techniques based on the analysis of DNA, mRNA synthesis, *de novo* sequencing or microarray techniques, also based on the use of isotopic or fluorescent probes, have been used for describing the VBNC state (Randa et al., 2004; Coutard et al., 2005; Rosche et al., 2005). The main limitations of genetic-based detection of the VBNC state in soil microorganisms is the difficult isolation of the short-lived and unstable mRNA from soil (Kim et al., 2014), along with the still limited annotation of environmental strains in the proteomic databases (Renella et al., 2014). Moreover, Oliver (1993) showed that changes in the quality of DNA can limit the PCR technique for studying the transition of bacteria in the VBNC state. Proteomics holds the potential to detect changes in the activity of soil microorganisms in relation to C availability (Williams et al., 2010), and it is a suitable approach to understand the underlying mechanisms that induce the transition from the culturability to the VBNC and *viceversa* of bacteria in soil. Proteomic studies on the VBNC state of bacteria extracted from various environmental matrices have been performed (Graves and Haystead, 2002; Heim et al., 2002; Muela et al., 2008; Vidovic et al., 2012) and up- and down-regulation of several proteins occur during the transition to the VBNC state (Nyström, 2003; Muela et al., 2008; Trevors, 2011; Vidovic et al., 2012). However, the proteomic studies were mainly performed *in vitro*, whereas there is still few studies of changes of the proteomic profile of bacteria under VBNC state in soil.

For characterization of bacterial VBNC, it is important to examine the resuscitation, i.e. the recovery of culturability of bacteria under VBNC state. Bacterial resuscitation should ideally restore the metabolic pathways down-regulated in VBNC cells (Baffone et al., 2006), upon removal of stressing factors (Wong and Houry, 2004; Zhong et al., 2009). The best characterized resuscitation mechanism is based on quorum sensing and involves the production of resuscitation-promoting factor (rpf) proteins, and the metagenomic analysis has allowed the discovery rpf homologues in microorganisms from various ecosystems, including in soil (Lennon and Jones, 2011).

The starting hypothesis of this research was that the VBNC state of soil bacteria depends on nutrient and water availability. We tested this hypothesis by monitoring the transition of the soil-borne bacterium *Cupriavidus metallidurans* CH34 from the culturable to the VBNC state and the recovery of its culturability within microcosms inoculated with this bacterial species. The proteome of *C. metallidurans* CH34 is fully annotated and this allowed to overcome the problems related to the limited proteomic databases of soil-borne bacteria (Renella et al., 2014). We inoculated bacterium in soils of definied composition until the emergence of the VBNC state, and reverted its physiological state to culturability by supplying a C source or water. This study can serve as a model for understanding the metabolic changes during the transition from the culturability to the VBNC state of bacteria in soil. The findings of this study may also improve the culturability of soil bacteria by identifying the *loci* involved in the bacterial reversion from the VBNC with a proteomic approach.

2. Materials and methods

2.1. Bacterial inoculation into artificial soils and culturability

Cupriavidus metallidurans strain CH34 was isolated on Petri dishes of solid broth Luria-Bertani (LB). The bacterial colonies were grown in a mineral salt medium containing 0.2% of gluconate and trace concentration of several elements (i.e. Na, K, Mg, Ca, PO_4, SO_4, Fe; Mergeay et al., 1985) on a rotary shaker at 30 °C until the mid exponential phase giving an optical density (O.D.) of 0.6 at 660 nm wavelength, corresponding to a cell density of 10^8 cells mL^{-1}. The bacterial cells were centrifuged at 5.000 × *g* for 15 min at 4 °C to reach 10 OD corresponding to 10^9 cells mL^{-1}. The cell pellets were suspended in 1 mL of the mineral salt medium and inoculated into soil microcosms.

The artificial soil was prepared according to Giagnoni et al. (2011), and contained quartz sand (Sigma Aldrich), kaolinite (Clay Minerals Society, USA) with cation exchange capacity (CEC) of 2 cmol kg^{-1} and 10 m^2 g^{-1} of surface area, montmorillonite (Clay Minerals Society, USA) with CEC of 120 cmol kg^{-1} and 97.4 m^2 g^{-1} of surface area, goethite (Sigma Aldrich) and humic acids (Sigma Aldrich) in the following weight ratio: 78:18:2:1:1. The artificial soil was sterilized in autoclave (20 min at 121 °C, 1 bar pressure) and dried in heater at 50 °C prior to bacterial inoculation, and the total amount of soil in each microcosm was 4 g. The *C. metallidurans* CH34 was incubated into the artificial soil at 30 °C for 12 days, without any C source or H_2O additions for the whole incubation period. The moisture content (w/w) of the microcosms during the incubation were: T0 22.1%, T1 20.4%, T3 12.8%, T5 5.9% and T12 2.4%. After 12 d, three microcosms were amended with 1 mL of growth minimal medium (C-source) containing 0.5 mg gluconate C g^{-1} soil (T12 + C) and three microcosms were treated with 1 mL of sterile deionized H_2O (T12 + H_2O). These amended microcosms were analysed for bacterial culturability and protein profiles after further 24 h of incubation at 30 °C.

Three independent replicates for each sampling time and amendment were prepared, and the whole microcosm was used for both CFU counts and proteome analysis.

2.2. Bacterial culturability and protein extraction

Both CFU and proteome analyses were analysed on three independent replicates using the entire microcosms. Bacterial culturability was determined by counting the colony forming units (CFU), according to Lorch et al. (1995), after 30 min (T0), 1 d (T1), 3 d (T3), 5 d (T5), 12 d (T12) of incubation in soil microcosms and after C-source (T12 + C) and water addition (T12 + H_2O) in soil microcosm. The bacterial proteome was extracted and analysed in pure culture, at T0, T1, T12 and after C-source and water addition (T12 + C and T12 + H_2O).

Proteins from microcosms inoculated with *C. metallidurans* CH34 were extracted with 4 mL of lysis buffer containing 20 mM phosphate buffer saline (PBS) (Sigma Aldrich), 1% SDS (Sigma Aldrich), DNAse and RNAse (Roche Diagnostic) and a microbial protease inhibitor cocktail (Sigma Aldrich), under sonication on ice at 400 W (BioClass UP 400 s) for 2 min, as described by Giagnoni et al. (2011). Soil extracts were immediately centrifuged at 8.000 × *g* for 10 min using by VivaSpin molecular sieve with molecular weight cut off at 3 kDa (Sartorius, Japanese Minebea Co.) to eliminate polyphenolic compounds. Proteins were purified by precipitation with 0.1 mL of deoxycolate and 0.1 mL 72% trichloroacetic acid (Bensadoun and Weinstein, 1976), followed by centrifugation at 5.000 × *g* for 10 min. Proteins were then precipitated overnight in 16 mL of acetone at −20 °C.

2.3. Protein analysis

The protein pellet was resuspended in ultrapure sterile water and the protein concentration was determined using the Bradford assay (Bradford, 1976). One-hundred μg of proteins were analysed for each sampling time and soil amendment. We added 5 μL of the 200 mM Tris (2-carboxyethyl) phosphine (TCEP) and we incubated samples at 55 °C for 1 h. We added 5 μL of the 375 mM iodoacetamide to the samples and we incubated the samples for 30 min protected from light at room temperature. Then 2.5 μL of trypsin (equivalent to 2.5 μg) were mixed

with the 100 μg of protein and digestion was conducted overnight at 37 °C. Immediately before use, the Tandem Mass Tags (TMT) Label Reagents were equilibrated at room temperature. The reduced and alkylated protein digests were transferred to the TMT Reagent vial and incubated for reaction for 1 h at room temperature. Then, 8 μL of 5% hydroxylamine were added to the samples and incubated for 15 min to quench the reaction. Finally, the samples were combined at equal amounts in new microcentrifuge tubes and stored at −80 °C. The TMT-labelled peptide concentration was measured using the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay.

The TMT labeling of proteins was performed according to the manufacturer protocol (https://www.piercenet.com/instructions/2162073.pdf); each of the three biological replicates from each sampling time and amendment were labelled with the 126, 127, 128, 129, 130 and 131 TMT tags, before being combined and lyophilized. For the high pH reverse-phase fractionation of the TMT-labelled samples, the following liquid chromatography (LC) conditions were used. Desalted peptides were suspended in 0.1 mL 20 mM ammonium formiate (pH 10.0) plus 4% (v/v) acetonitrile. Fifty mg of the combined peptides were loaded onto an Acquity bridged ethyl hybrid C18 UPLC column (Waters; 2.1 mm i.d. X 150 mm, 1.7 μm particle size), and eluted with a linear gradient of 5–60% acetonitrile plus 20 mM ammonium formiate (pH 10.0) over 60 min, at a flow-rate of 0.25 mL min^{-1}. Elutes were monitored with a diode array detector (Acquity UPLC, Waters) scanning between wavelengths of 200 and 400 nm. Samples were collected in 1 min increments and dried by vacuum centrifugation. Only the fractions containing peptides eluted (from 10 to 43 min) were analysed, whereas fractions of the initial and end periods of the gradient, which did not contain peptides were discarded. In total, 34 fractions were collected for each LC run and the fractions were pooled together in the following way: fraction 1 with fraction 18, fraction 2 with fraction 19, fraction 3 with fraction 20 etc. This ensured that the 17 pooled peptides fractions had a good range of hydrophobicity for each LC-MS/MS run. The LC fractionation was performed for each of the three biological replicates.

For the LC-MS/MS analysis, dried fractions from the high pH reverse-phase separations were dried and suspended in 30 μl of 0.1% formic acid and placed into a glass vial. One μL of each fraction was injected by the HPLC autosampler and peptides separated by reverse-phase chromatography using a Waters reverse-phase nano column (BEH C18, 75 μm i.d. X 250 mm, 1.7 μm particle size) at flow rate of 300 nL min^{-1}. Peptides were initially eluted onto a pre-column (Waters UPLC Trap Symmetry C18, 180 μm internal diameter x 20 mm, 5 μm particle size) from the nanoAcquity sample manager with 0.1% formic acid for 3 min at a flow rate of 10 μL min^{-1}. After this period, the column valve was switched to allow the elution of peptides from the pre-column onto the analytical column. Solvent A was water plus 0.1% formic acid and solvent B was acetonitrile plus 0.1% formic acid. The linear gradient employed was 5–30% B in 100 min, followed by a washing and re-equilibration step (120 min total run). The eluted LC was sprayed into the mass spectrometer by a nanospray source. All the mass-to-charge (m/z) values of eluting ions were measured in the Orbitrap Velos mass analyzer, set at a resolution of 30.000. The fragment ions were passed into the Orbitrap (7500 resolution), via the C-trap for mass analysis, and this resulted in the generation of MS/MS spectra. Ions with charges of $^{2+}$ and above were selected for fragmentation. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by higher-energy collisional dissociation (HCD, Normalised collision energy:45) in the HCD cell.

All MS/MS data were converted to mgf files and submitted to the Mascot search algorithm (Matrix Science, London UK). We used the Uniprot_C_metallidurans_CH34 database with a fixed modification of carbamidomethyl (C) and variable modification of oxidation (M) and TMT 6-plex (K/N-term), with a peptide tolerance of 25 ppm. Peptides were identified at similarity greater than 95% probability, considering false discovery rates (FDRs) based on a random decoy database. The peak areas of each of the TMT reporter ions was then calculated by the Proteome Discover software (version 1.3. ThermoFisher). For protein identification at least 2 peptides were identified.

2.4. Statistical analysis and analysis of protein functions

The identified proteins were analysed by two-way ANOVA for assessing their expression as affected by treatments, using Past3 software (Hammer, 2009). Only proteins present in all the three replicates of a given treatment were statistically analysed. When the differences between treatments was significant ($P < 0.05$), the protein fold change (FC) was calculated and FC > 1.5 was assumed as index of protein up- or down-regulation. The pathway analysis was conducted by UniprotKB, KEGG pathway (http://www.genome.jp/kegg/pathway.ht μl), BioCyc.org and the STRING database (http://string-db.org/), all selecting *Cupriavidus metallidurans* CH34 as target organism.

3. Results

3.1. Bacterial culturability analysis

At T0, i.e. 30 min after its inoculation into artificial soils, the *C. metallidurans* CH34 viability was $2.08.10^8$ CFU per g of dry soil, then it declined during the incubation to undetectable CFU after 12 d (Fig. 1). Amendment of microcosm with C-source or H_2O restored the bacterial culturability at $5.2.10^6$ and $2.6.10^6$ CFU per g of dry soil, respectively (Fig. 1).

3.2. Proteomic analysis of bacteria undergoing to VBNC state

The LC-MS/MS analysis allowed the identification and relative quantitation of 304 proteins of *C. metallidurans* CH34: 220 proteins (72.4%) in cytoplasm, 23 proteins (7.6%) in plasma membrane, 4 proteins (1.3%) in periplasmic space, 3 (1%) in cell inner membrane, 1 protein (0.3%) in extracellular space, 1 protein (0.3%) in cell outer membrane, 1 protein (0.3%) in cell surface, whereas the cell compartment of 51 proteins could not be defined. Most of the identified proteins were located in the cytoplasm and in the cell membrane, and less in the extracellular space. The proteome profile in pure culture and at T0 (30 min after inoculation) was similar, but at T0, 19 proteins were up-regulated and 6 proteins were down-regulated (Table S1). Concerning the 19 up regulated proteins, 9 proteins were involved in information storage and processing (A, J symbols in Tables S1) and 8 proteins related to metabolism and 2 proteins related to cellular processes and signalling. The down-regulation involved 4 proteins related to information storage and processing (J symbol) and 2 proteins related to fundamental metabolism (H symbol) (Table S1).

At T1, 105 proteins mainly involved in the basic bacterial

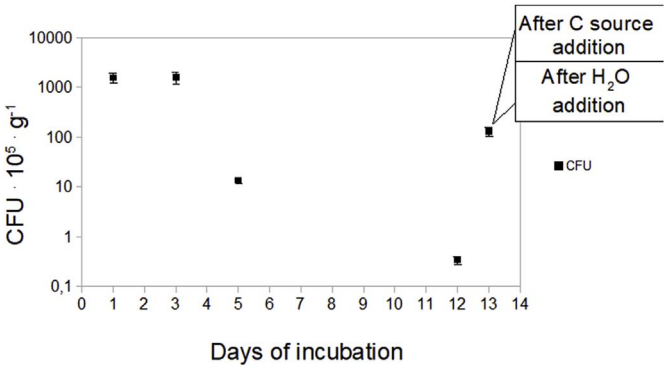

Fig. 1. Bacterial culturability of *C. metallidurans* CH34 during the incubation and after the amendment with C source (gluconate) or H_2O in the artificial soil.

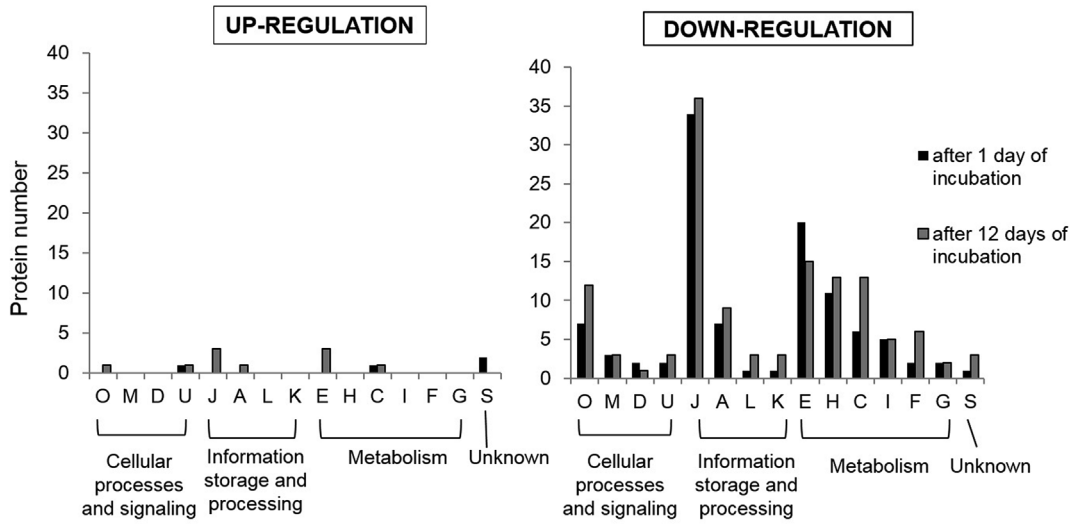

Fig. 2. Category classification of the total number of up- and down-regulation of protein synthesis during the transition of *C. metallidurans* CH34 to the VBNC state. The letters in the graphs correspond to the cluster of orthologous groups (COGs).

metabolism were down-regulated. Of these, 48 proteins were involved in metabolism (C, E, F, G and H symbols - Table S2 and Fig. 2), 43 proteins were involved in information storage and processing (A, J, K, L symbols – Table S2 and Fig. 2) and 14 proteins were involved in cellular processes and signalling (D, M, O, U symbols, see Table S2 and Fig. 2). Of the 105 proteins down-regulated at T1, 77 proteins maintained a lower level of expression until T12 (Fig. 2). The down-regulation involved a large number of proteins related to information storage and processing (A, J, K, L symbols Table S2, Fig. 2) and fundamental metabolism (C, E, F symbols in Table S2, Fig. 2). Moreover some proteins related to cellular processes as cell division (D symbol in Table S2, Fig. 2) and protein turnover (O symbol in Table S2, Fig. 2), were down-regulated at T1 and T12. Other proteins, such as 50S ribosomal protein L21, 50S ribosomal protein L24, imidazoleglycerol-phosphate dehydratase and NADPH-dependent 7-cyano-7-deazaguanine reductase, 10 kDa chaperonin, outer-membrane lipoprotein carrier protein and 50S ribosomal protein L24 were down-regulated at T1 but showed higher expression levels at T12 (Table S2). In particular, at T12 the amounts of imidazoleglycerol-phosphate dehydratase NADPH-dependent 7-cyano-7-deazaguanine reductase and 50S ribosomal protein L21 were higher than at T1 and were similar at T0.

At T1, the following 4 were significantly up-regulated when compared with T0: ATP synthase subunit delta, phosphate import ATP-binding protein PstB, acyl carrier protein and nucleoid-associated protein Rmet_2128 (Table S2).

At T12, 117 proteins were down-regulated when compared with T0 (Table S3 and Fig. 2).

Most of these proteins (80 proteins, see Table S2) were already down-regulated at T1, whereas the other 37 proteins were down-regulated at T12. The amount of proteins up or down regulated after 1 day and 12 days of incubation artificial soil and the cluster of orthologous groups (COGs) are shown in Fig. 2. Most of the down-regulated proteins were involved in the bacterial basic metabolism and information storage and processing (Fig. 2). Nucleoid-associated protein Rmet_2128 was the only one protein up-regulated in T12 (Table S3). Globally, during the transition from culturable to the VBNC state, we observed a strong decrease in the expression of proteins involved in basal bacterial metabolism, cellular processes, signalling and information storage, the pathways of protein synthesis, energetic processes and regulation of cell shape, whereas some proteins involved in energetic processes production and red-ox reactions were up-regulated (Fig. 2).

3.3. Proteomic analysis of bacteria reverting from VBNC to culturable state

In microcosms amended with minimal growth medium (C-source) containing gluconate (T12 + C), the expression of 40 proteins did not increase with the C-source addition and the quantification of these proteins were similar to T1 and T12 and lower in comparison to their expression at T0 (Table S3). A peculiar behaviour was observed for the protein expression of nucleoid-associated protein Rmet_2128: the protein was only up-regulated after 1 day of incubation and after C-source addition the protein expression was comparable to that at T0 and T12.

The C-source addition induced the up-regulation of 38 proteins in the bacteria under VBNC state (Table S3). Among them, 8 proteins (imidazole glycerol phosphate synthase subunit HisF, 50S ribosomal protein L15, 50S ribosomal protein L28, 30S ribosomal protein S6, ribosomal RNA small subunit methyltransferase A, ribosome-binding factor A, outer-membrane lipoprotein carrier protein and 50S ribosomal protein L24) were up-regulated in comparison to their expression levels at T1 and T12, whereas 29 proteins were up-regulated to an expression level comparable to that of T0. The phosphate import ATP-binding protein PstB had a particular quantification, because the protein was significantly up-regulated in T1 and after C-source addition when compared with the expression at T0 (Table S3).

Addition of water (T12 + H_2O) had no effect on the expression of 119 proteins, which remained down-regulated at similar level as in the VBNC state, whereas 6 proteins (60 kDa chaperonin, Methionine import ATP-binding protein MetN, Phosphoribosylformylglycinamidine cycloligase, 50S ribosomal protein L9, Uroporphyrinogen decarboxylase, Holliday junction ATP-dependent DNA helicase RuvA) were significantly up-regulated in comparison to expression at T12 (Table S4). Among them, 2 proteins (Phosphoribosylformylglycinamidine cyclo-ligase and Uroporphyrinogen decarboxylase) showed the same behaviour in both the T12 + C and T12 + H_2O treatments (cfr Tables S3 and S4), whereas the other 4 proteins were up-regulated only after water treatment.

Globally, during the reversion from the VBNC to the culturable state, we observed that the C-source availability up-regulated the expression of several proteins related to the fundamental bacterial metabolism whereas the addition of water only up-regulated six proteins (Fig. 3).

The amount of proteins up or down regulated after C-source or water and the COGs are shown in Fig. 3. Several proteins related to

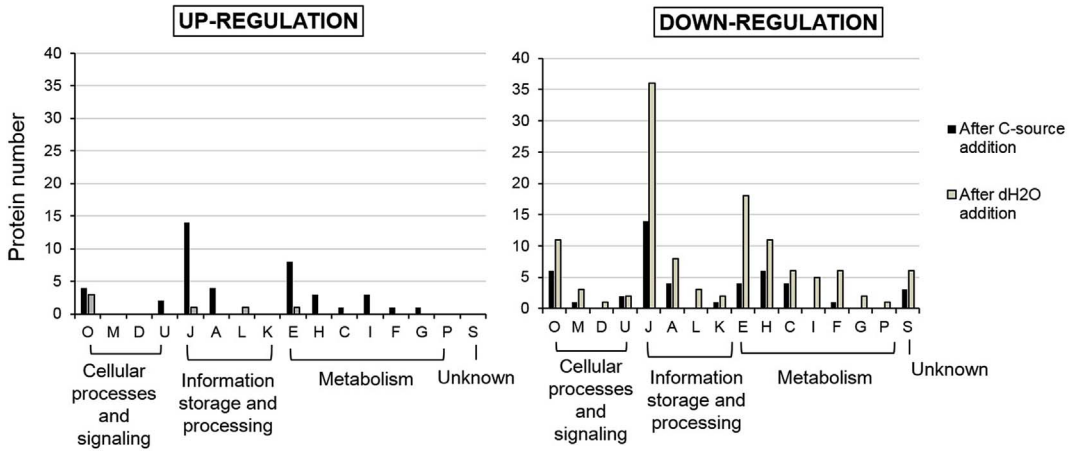

Fig. 3. Category classification of the total number of up- and down-regulation of protein synthesis after the artificial soil amendment with C-source (gluconate) and water (dH2O). The letters in the graphs correspond to the cluster of orthologous groups (COGs) category classification.

information storage, metabolism and signalling remained down-regulated even after C-source addition to artificial soil, in comparison to their expression levels at T0.

4. Discussion

The number of predicted proteins of *C. metallidurans* CH34 on the base of its genome annotated in the NCBI genomic database is 6316 (https://www.ncbi.nlm.nih.gov/genome/?term=Cupriavidus%20metallidurans%5Borganism%5D&ccm=DetailsSearch, accessed on July 27, 207). Because we overall identified 304 proteins, our results confirm that a large fraction of the hypothetical proteins can not be easily extracted from soil and identified because not expressed under the adopted experimental conditions, structurally unstable (e.g. metallo-proteins), or proteins losses during the various protein purification steps such as the 1002 membrane transporters (von Rozycki and Nies, 2009) or proteins modified by the contact with humic substances (Arenella et al., 2014). The identified proteins of *C. metallidurans* CH34 in the various sampling times and treatments in this study were all indicative of the bacterial physiological state because involved in the fundamental bacterial metabolism and fundamental cell processes (Fig. s 2, 3). Moreover, a good match of the bacterial proteome in liquid pure culture and bacterial proteome immediately after contact with the artificial soil (T0) also obtained in the previous work by Giagnoni et al. (2011) under the same experimental conditions, indicating that some of the hypothetical proteins were not expressed under the adopted experimental conditions.

The proteomic analysis showed that the rapid onset of the VBNC state in *C. metallidurans* was due to the rapid C-source depletion caused by both microbial use and chemisorption, and confirmed the previous results that soil-borne Gram-negative bacteria exposed to nutrient starvation in soil can adopt the VBNC state as survival strategy (e.g. Oliver, 2010). As compared to previous studies, the proteomic analysis of *C. metallidurans* CH34 demonstrated that the bacterial transition to the VBNC state was induced rapidly after its contact with the soil solid phases. The contact with artificial soil immediately changed the protein expression profile of *C. metallidurans* CH34, inducing the down-regulation of proteins related to information storage and cellular processing, and the overexpression of several proteins related to metabolism and cellular processes. As a soil bacterium, the bacterial metabolic response of *C. metallidurans* CH34 was very fast and permitted its adaptation to survive in the artificial soil microcosms. This behaviour confirmed the observation by Giagnoni et al. (2011) that the proteome profile obtained by 2 DE, after contact with artificial soil was different in comparison to the pure culture proteome profile. Among the down-regulated proteins, the 10 kDa chaperonin and the outer-membrane lipoprotein carrier protein are related to microbial stress adaptation (Yousef and Juneja, 2002). The VBNC stage is induced in many non-spore-forming bacterial species under various environmental stresses (Oliver et al., 1991; Makino et al., 2000; Oliver, 2005, 2010; Aurass et al., 2011). For example, Kong et al. (2014) reported the induction of the VBNC stage in *R. solanacearum* strains upon low temperature in a soil microcosm. The rapid increase in the expression of proteins involved in the RNA metabolism and heat shock response indicating a stress response (Bügl et al., 2000) could be considered a further indication of the induction of VBNC state in *C. metallidurans* CH34. Although the microcosms desiccation could be a factor inducing the bacterial VBNC state, rapid metabolic changes were observed in the beginning of the incubation when the moisture level was not significantly changed. Moreover, because we did not observe changes in proteins responding to osmotic stress in C. metallidurans (e.g. trascriptional regulators envZ-OmpR), we suggest that the transition to the VBNC state was due to C shortage.

Upon increasing of contact time, several proteins involved in the regulation of cell shape such as the bifunctional protein GlmU, the UDP-N-acetylglucosamine 1-carboxyvinyltransferase, the UDP-N-acetylmuramate-L-alanine ligase, and D-alanine-D-alanine ligase were down-regulated, confirming previous observations (Muela et al., 2008; Oliver, 2010). The alanine racemase was down-regulated after 1 day of incubation and its expression was restored after 12 d of incubation. The behaviour of this protein indicates the VBNC state because the alanine racemase catalyzes the D-alanine synthesis, involved in the formation of aberrant-shaped cells during the VBNC state of *Vibrio parahaemolyticus* (Hung et al., 2013).

The up-regulation of ATP synthase subunit delta, phosphate import ATP-binding protein PstB, acyl carrier protein and nucleoid-associated protein Rmet_2128, after 1 day of incubation, could indicate a relationship between these proteins and the bacterial transition from culturability to the VBNC state. The down-regulation of proteins related to information storage and cellular oxidation-processes in *C. metallidurans* CH34 during the transition to the VBNC state was paralleled the response of non spore forming bacteria to environmental stresses and that in *R. solanacearum* strains in soil microcosms exposed to low temperature (Oliver et al., 1991; Makino et al., 2000; Oliver, 2005, 2010; Lai et al., 2009; Aurass et al., 2011). The up-regulation of the outer-membrane proteins was observed in *E. coli* cells under VBNC state by Asakura et al. (2007). After 12 days of incubation several proteins were permanently down-regulated in *C. metallidurans* CH34. Among these proteins, lower expression levels of ribosome-associated proteins and oxidation-responsive factors were also observed in *E.coli* (EHEC) O157 cells under VBNC state (Asakura et al., 2007), and further indicated that bacterial cells entering into the VBNC state, undergo to

profound cellular changes caused by depletion of energy pools. These changes can also cause cell leakage, altered expression of genes and DNA replication (Trevors et al., 2012). At T12, *C. metallidurans* CH34 showed 7 up-regulated proteins in comparison to protein expression at T1; these proteins (50S ribosomal protein L21, 50S ribosomal protein L24, imidazoleglycerol-phosphate dehydratase, NADPH-dependent 7-cyano-7 deazaguanine reductase, 10 kDa chaperonin, outer-membrane lipoprotein carrier protein and elongation factor G2) were related to translation, metabolism processes and transport. This behaviour confirms previous results on the role of chaperonins, heat shock and elongation factors in the transition of bacteria from culturable to the VBNC state induced by oxidative, thermal and osmotic stresses (Nyström, 2003; Muela et al., 2008; Vidovic et al., 2012). Chaperonines are ubiquitous proteins and facilitate the proper folding of newly synthesized proteins, and their function is essential to prevent protein misbehavior, due to loss of conformations (Wong and Houry, 2004). The permanent down-regulation at T12 of the putative phosphoenolpyruvate synthase (a regulatory protein involved in glycolysis), ATP synthase (involved in oxidative phosphorylation), and the elongation factor EF-Tu (involved in protein synthesis), confirmed the decrease in metabolic activity observed in VBNC cells by Heim et al. (2002). The down-regulation of the acetyl-coenzyme A carboxylase carboxyl transferase involved in pyruvate carboxylate was also observed in *Pelagibacter ubique* during the VBNC state (Sowell et al., 2008). Globally, the changes in the proteomic profile of *C. metallidurans* CH34 during its transition from the culturable to the VBNC state (T0, T1), and after the consolidation into the VBNC state (T12), paralleled those of bacterial proteomic responses to various environmental stresses (Lai et al., 2009).

We succeeded in reverting the state of *C. metallidurans* CH34 strain from VBNC to culturability at comparable levels into the artificial soil microcosm by suppling either gluconate or H_2O (Fig. 1). However, only gluconate induced a significant activation of the fundamental cell metabolism. After the gluconate amendment, 48% of up-regulated proteins were involved in information storage and processing, 46% were involved in metabolism and 6% involved in cellular processes and signalling. The ATP-dependent Clp protease, up-regulated after gluconate addition, regulates the quality of intracellular proteins by protein unfolding and polypeptide translocation (Baker and Sauer, 2012), and also degrading sigma factors such as RpoS (Zhou and Gottesman, 1998), which are essential for survival of bacteria in the stationary phase (Subsin et al., 2003). The ATP-dependent Clp protease is also responsible for bacterial virulence (Li et al., 2010; Alleron et al., 2013), and degrade the transcriptional regulator (CtrA), which controls several cell cycle events such as DNA replication, DNA methylation, and cell division (Domian et al., 1997). These results paralleled those of Bastida et al. (2016) whose reported that in a comparative metaproteomic study of soils with different dissolved organic C (DOC) contents, proteins involved in replication and nucleic acids integrity and metabolism were significantly higher in soils with higher DOC content, particularly those of Proteobacteria (such as *C. metallidurans*).

After H_2O addition to soil microcosms only six proteins were up-regulated, and were involved in transport (chaperonin and methionine transport), translation (50S ribosomal protein L9) and protein folding (uroporphyrinogen decarboxylase and phosphoribosylformylglycinamidine cyclo-ligase). The uroporphyrinogen decarboxylase and phosphoribosylformylglycinamidine cyclo-ligase were also up-regulated after gluconate amendment. Therefore, these two proteins may be important for the recovery of culturable state of *C.metallidurans* CH34.

The adopted proteomic approach allowed us to distinguish between the re-growth of culturable cells and the reversion of the VBNC stage to culturable cells, as suggested by Mukamolova et al. (2003). In structured polyphasic environments such as soil, bacterial cells may exist in different physiological states depending on the water and C availability within aggregates, and it could not be excluded that VBNC could co-exist with sparse cultivable cells of *C. metallidurans*, as reported for mixed microbial cultures by Bogosian et al. (1998). The growth of *C. metallidurans* CH34 from undetectable to 10^5 CFU (Fig. 1) in 24 h occurred in 76.3 and 67.5 min after addition of C-source and H_2O, respectively, periods of time higher than that of 26.5 min occurring in liquid culture and measured in a preliminary independent experiment. Another result that confirmed the transition from the VBNC to culturable state rather than a re-growth of the *C. metallidurans* strain, was that soil amendment with C-source or H_2O did not induce up-regulation of proteins related to cell-growth, DNA replication and cell division, but up-regulated proteins related to RNA and protein synthesis, and cell uptake. Finally, it is highly unlikely that H_2O could induce a re-growth rate comparable to that induced by C-source.

Effectiveness of gluconate as sole C-source in reverting the VBNC was comparable to the resuscitation of *Bacillus* spores by alanine (Setlow, 2003) or meso-diaminopimelic acid (m-DAP) (Shah et al., 2008) in about 24 h, a time period similar to our experiments. Metabolic activation of the resuscitated *C. metallidurans* CH34 by gluconate was also confirmed by the regulation of kinase enzymes, as protein and metabolite phosphorylation generally initiate cellular metabolic transformations. Although our study focused on the transition of a single soil-borne bacterial species, our results support the conclusions by Bastida et al. (2015) indicating that an intense turnover of the soil biomass not necessarily associated to microbial growth can occur in nutrient limited soils. Moreover, Bastida et al. (2016) reported that the dissolved organic C (DOC) can influence the activity of soil microbial communities in arid soils. The results of our model study suggest that some key microbial functions may indicate the role of two key soil factors, such as labile C and water amendment, in influencing the intracellular metabolism and the associated lifestyles of soil bacterial community inhabiting restored soils. Therefore, our study may contribute to better understanding how these two factors can define the ecological functional niches (*sensu* Fierer et al., 2009).

In conclusion, our model proteomic study showed that bacterial VBNC state in soil is been initiated by reduced availability of C in soil, and that C limitation elicits gene expression and protein synthesis that lead to the VBNC state. The main metabolic response induced by C limitation concerned the intracellular protein translation, protein rearrangement and energy production processes, typical microbial responses to intracellular C depletion. The proteomic approach allowed to demonstrate that the availability of gluconate as sole C source or H_2O are sufficient to restore the bacterial culturability, indicating an important role of σ-factors, regulators of transcription, in the reversion from the VBNC to the culturable state. However, the response to H_2O could be interpreted as a 'permissive' soil condition, with gluconate availability leading to a significant restoration of the fundamental bacterial metabolic activity.

Results from this study can advance the understanding on the functioning of selected bacterial populations in soil and explain important phenomena observed in soil ecology such as the spatial distributions of microorganisms in soils from different environments. In fact, the VBNC state may reduce the negative selection and regulate the microbial dominance in soil hotspots such as the rhizosphere, where plants could induce selective bacterial resuscitation through the release of selected organic compounds thus influencing the species richness and evenness (Fierer et al., 2010). However, although *C. metallidurans* is considered a representative model of Gram-negative soil-borne bacteria and belongs to Proteobacteria, whose proteins are broadly retrieved in soil metaproteomic studies (Bastida et al., 2015, 2016), caution is needed in extrapolating our results to complex microbial communities as those of soils where more ecological factors (e.g. quality of energy substrates, temperature) and more complex microbial ecological interactions (e.g. mutualism, symbiosis, quorum sensing, plant-induced microbial selection) than those of our study may play important roles in regulating microbial activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.12.004.

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