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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