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Different efficiencies of the same mechanisms result in distinct Cd tolerance within *Rhizobium*

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ABSTRACT

Soil contamination with metals is a widespread problem posing risks to humans and ecosystems. Metal contaminated soils often hold poor microbial density and biodiversity. Among soil bacteria, rhizobia have a great agronomic and environmental significance and are major contributors to a sustainable maintenance of soil fertility. This group of microorganisms are severely affected by metals, such as cadmium (Cd), but information about metal resistance mechanisms in rhizobia is still limited. A concerted approach of the different mechanisms conferring Cd tolerance to rhizobia was conducted using two *Rhizobium* strains with contrasting tolerances to Cd. Results show that both strains resort to the same mechanisms (extracellular immobilization, periplasmic allocation, cytoplasmic sequestration and biotransformation of toxic products) to overcome stress, but differences in the efficiencies of some mechanisms were noticed. The ability of *Rhizobium* to increase glutathione in the presence of Cd emerges as a central factor in the tolerance to Cd and is as a feature to be looked for when screening or transforming microorganisms to integrate plant-microbe consortia. These could promote plant growth at contaminated sites, being more efficient for the cleanup of metals from contaminated sites and the restoration of soil quality.

1. Introduction

After the onset of the industrial era, soil contamination increased continuously, resulting in a widespread problem (Lemire et al., 2013). Among soil contaminants metals are the most frequent (Science Communication Unit, 2013). Metal contamination of soils might pose risks to humans and ecosystems through direct contact with contaminated soil and the food chain (Wuana and Okieimen, 2011). Metal contaminated soils often present poor plant growth and are low vegetated areas, or in more severe cases, barren soils (Hao et al., 2015). Phosphate fertilizers are a major route of cadmium (Cd) entry into soils and contribute to soil contamination in addition to industry, sludge amendment, and mining activities (Volesky and Holan, 1995; Garbisu and Alkorta, 2001). In soil solution, Cd concentrations are usually found between 0.3 μg and 6 mg L^{-1} (0.0027–53 μM) (Helmke, 1999; Kabata-Pendias, 2011), but this element can reach concentrations higher than 300 mg L^{-1} (2669 μM) at highly contaminated sites (Itoh and Yumura, 1979). Cd is classified as the seventh more toxic substance on the 2015 Priority List of Hazardous Substances by the Agency for Toxic Substances and Disease Registry (2015). Therefore even at low concentrations the potential to affect soil microbial communities should be high (Shentu et al., 2008). The reactivity of Cd with thiol groups and its ability to displace essential biological metals results in protein denaturation and mismetallation ablating protein function, inhibiting electron transport chains and inducing oxidative stress (Nies, 1999; Prévéral et al., 2009; Cuypers et al., 2010; Pacheco et al., 2008). Cd also induces membrane damage through peroxidation of membrane lipids (Lemire et al., 2013; Nies, 1999; Cardoso et al., 2017). Lipid peroxidation products, such as aldehydes, are highly toxic to cells through oxidation of proteins and formation of DNA adducts, thus being mutagenic to bacteria (Lemire et al., 2013). Moreover, Cd interacts with calcium and zinc metabolism (Nies, 1999). All these effects culminate in reduced growth, long log phases, lower cell densities, and ultimately in bacterial death (Les and Walker, 1984; Sinha and Mukherjee, 2009).

Due to these multiple effects only in rare cases a single mechanism has been found to effectively protect bacterial cells from Cd toxicity (Nies, 1999). In fact, a multitude of mechanisms conferring Cd tolerance were described in bacteria, and different bacteria differ in the strategies used to tolerate this element (Prévéral et al., 2009). Down-regulation of influx transporters or induction of efflux pumps are crucial for metal resistance, and different bacterial species have distinct complements of these systems (Nies, 1999, 2003; Ma et al., 2009; Outten

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and O'Halloran, 2001; Silver and Phung, 1996; Gadd, 2010; Teitzel and Parsek, 2003). Precipitation of metal ions (Harrison et al., 2007; Chaturvedi et al., 2012; Johnston et al., 2013) and interactions involving proteins or cell-associated polysaccharides (Langley and Beveridge, 1999; Pereira et al., 2006a; Purchase et al., 1997) in the extracellular environment also contribute to bacteria tolerance. The described mechanisms rely on the avoidance of high intracellular metal concentration to increase tolerance, circumventing Cd ions to interfere with cellular metabolism. However, in bacteria of the same species, higher tolerance to Cd was reported in strains accumulating higher concentrations of Cd intracellularly (Pereira et al., 2006a; Figueira et al., 2005), which is possible if tolerant bacteria reduce Cd toxicity inside cells. In gram-negative bacteria metals can be allocated in the periplasmic space (Zannoni et al., 2007),yet the concentration of metals in this compartment and its contribution in the tolerance of bacteria to metals remains to be elucidated. Chelation of metal ions to proteins or to glutathione also decreases metal toxicity in bacteria (Silver and Phung, 1996; Carrondo, 2003; Lima et al., 2006), but it remains to be clarified if the protection conferred by this mechanism changes with the level of stress imposed. Glutathione (GSH) was shown to be important for stress protection in bacteria (Chesney et al., 1996; Ferguson and Booth, 1998; Riccillo et al., 2000), acting as a radical scavenger producing oxidized glutathione (GSSG) (Sies, 1999) or complexing Cd ions through the formation of bisglutathionate-Cd complexes (Lima et al., 2006). The activity of gluthathione S-transferases (GSTs), a family of detoxifying enzymes catalyzing the conjugation of GSH with xenobiotics, was shown to be involved in the formation of bisglutathionate-Cd complexes in *Rhizobium* (Corticeiro et al., 2013) and in the detoxification of lipid peroxidation products highly toxic to cells (Cardoso et al., 2017). The efficiency of this metal chelation mechanism is dependent upon a steady supply of GSH from the enzyme glutamate cysteine ligase (GCL). After all these defense mechanisms, there are Cd ions that remain free in the cytoplasm and that will interact with cell metabolism, inactivating enzymes, bursting oxidative stress and causing cell damage.

The identification of the determinants that confer tolerance to soil bacteria provides information to understand how bacteria can survive in contaminated environments. Survival is important given the services that soil bacteria provide to ecosystems, such as mineralization of organic compounds, solubilization of nutrients and synthesis of plant growth promoting substances. Rhizobia are ubiquitous soil bacteria that besides these ecosystems services are able to fix N_2 endosymbiotically with legumes, and thus have an increased role on plant growth (Marschner, 1995). Since areas contaminated with metals often exhibit low nitrogen levels (Zribi et al., 2012), legumes establishing effective symbiosis with rhizobia, and thus not depending on soil nitrogen, can be pioneer plants and have shown great potential to revegetate degraded soils (Hao et al., 2015). Persistence of N_2-fixing microorganisms in contaminated soils may minimize the effects of contamination and increase the resilience of communities from contaminated sites, thus decreasing the impact on density and biodiversity of these communities.

Since information about metal resistance mechanisms in rhizobia is still limited (Hao et al., 2015) the aim of the present study is to identify the determinants that confer tolerance to *Rhizobium*. For this a concerted approach of the different mechanisms acting on rhizobia Cd tolerance was pursued. In a previous study distinct tolerances to Cd were identified among *Rhizobium* strains (Figueira et al., 2005). Two *Rhizobium* strains with contrasting Cd tolerances were used to highlight the relative importance of each mechanism (extracellular immobilization, periplasmic allocation, and cytoplasmic sequestration) at different levels of Cd toxicity and to evaluate the variability of Cd tolerance within *Rhizobium*.

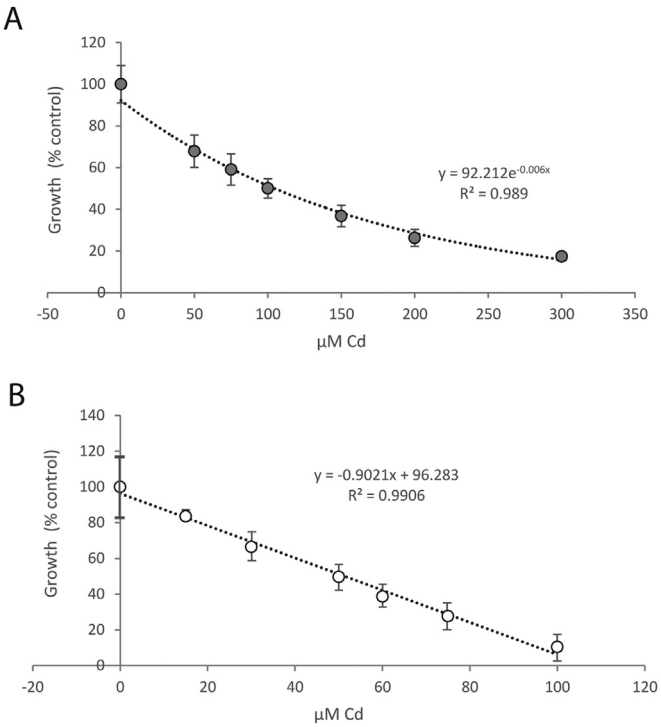

Fig. 1. Growth of two *Rhizobium* strains during 72 h at different ranges of Cd concentration. A) Growth of the tolerant strain at 0, 50, 75, 100, 150, 200 and 300 µM Cd. B) Growth of the sensitive strain at 0, 15, 30, 50, 60, 75 and 100 µM Cd. Values are means (± standard deviation) of 3 independent experiments with 3 replicates each. Equations were used for the determination of Cd concentrations inhibiting 50% (low stress) and 70% (high stress) the growth of each Rhizobium strain.

2. Material and methods

2.1. Bacterial strains and growth conditions

Rhizobium sp. strains E20-8 and NII-1, isolated from root nodules of *Pisum sativum* L. grown in a non-contaminated field in Southern Portugal, were previously classified as tolerant and sensitive to Cd, respectively (Figueira et al., 2005). These strains were cultured in yeast extract mannitol (YEM) (control condition) and in YEM supplemented with $CdCl_2$ (15, 30, 50, 60, 75 and 100 µM for the sensitive and 50, 75, 100, 150, 200 and 300 µM for the tolerant strain) to choose two levels of Cd stress. Inoculated tubes were incubated at 26 °C in an orbital shaker (200 rpm), during 72 h. Growth was determined by measuring optical density at 620 nm. The relationship between optical density and cell concentration was obtained by direct cell counting in a Neubauer chamber. Cell concentration was expressed in million cells per milliliter (M cells ml^{-1}).

Considering the different Cd tolerances displayed by the two strains (Fig. 1), parameters (besides growth) were determined at two stress levels: low stress (inducing 50% growth inhibition), achieved by growing the tolerant strain at 100 µM Cd and the sensitive strain at 50 µM; and high stress (inducing 70% growth inhibition), achieved by growing the tolerant strain at 200 µM and the sensitive strain at 75 µM.

2.2. Analysis of Cd complexes in the periplasm and cytoplasm

2.2.1. Purification of periplasmic and cytoplasmic fractions

Rhizobium cells were harvested by centrifugation, for 10 min at 10,000 *g* at room temperature. Cell pellets were washed in 1 ml deionized H_2O and centrifuged at 10,000 *g* at room temperature. The pellets were again resuspended in 1 ml deionized H_2O, and the procedure was repeated three more times, to assure that Cd from the culture medium was not present in cell pellets. The isolation and identification of the