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# Transcriptional analysis and molecular dynamics simulations reveal the mechanism of toxic metals removal and efflux pumps in *Lysinibacillus sphaericus* OT4b.31

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ABSTRACT

*Lysinibacillus sphaericus* strain OT4b.31 is a bacterium widely applied in bioremediation processes of hydrocarbon and metal polluted environments. In this study, we identified the molecular mechanism underlying the $Pb^{2+}$ and $Cr^{6+}$ resistance. Metal uptake and temporal transcription patterns of metal resistance operons were evaluated using reverse-transcribed quantitative PCR amplification. The function of the resistance determinants was studied applying docking and *in silico* mutagenesis methods. The results revealed that the adaptation of *Lysinibacillus sphaericus* OT4b.31 to elevated levels of lead and chromium involves the *pbr* and *chr* operons which comprise a transcriptional regulatory component (*pbrR* and *chrB*) and efflux ATPases (*pbrA* and *chrA*) to expel ions from the cytoplasm. Expression of metal resistance genes was constitutive and specifically inducible to the exposure of $Pb^{2+}$ and $Cr^{6+}$. The simultaneous presence of cations didn't affect the bioaccumulation of metals, evidencing the multimetal resistance of *L. sphaericus*. Docking analysis revealed the key metal-protein interactions and the conformational changes after metal or ATP binding. Results showed that residues with aromatic rings or imidazole in the catalytic domain are crucial for metal binding and achievement of the function. To our knowledge, this is the first report of a specific mechanism for lead and chromium resistance in *Lysinibacillus* genus. From the findings of this study, it is possible to suggest the bacterium as a suitable candidate for rapid toxic metals bioremediation processes.

## 1. Introduction

Waste from anthropogenic activities such as mining, petroleum and metallurgical industry have increased environmental deterioration due to the accumulation of inorganic pollutants that can't be degraded such as toxic metals (Hullmann et al., 2012; Mohan and Dubey, 2013). Also, they have become a major issue in public health as their high solubility promotes rapid transport across biological membranes and once internalized them, they produce a variety of toxic effects in cells (Hullmann et al., 2012). Some transition metals are used for cellular processes and as enzymatic cofactors. However, in excess they are also toxic causing damage to the membrane, DNA structure, generating free radical species or replacing other metals from their native binding sites in metalloenzymes (Alexandrino et al., 2011; Liang et al., 2016; Xiong et al., 2011). Metals such as lead, cadmium, and mercury don't have known biological function and are toxic at any concentration (Jarosławiecka and Piotrowska-Seget, 2015; Mohan and Dubey, 2013). Thus, regulatory systems are necessary to control intracellular levels of metals. The most common toxic ions present in crude oil are chromium, lead, arsenic, copper, cadmium, cobalt and zinc (Duyck et al., 2002; Stigter et al., 2000). Chromium and lead are found in the highest concentrations and are predominantly associated with the hydrocarbon matrix (Duyck et al., 2002; Sarma et al., 2016). Lead has increased more than 1000-fold over the past three centuries as a result of activities in metallurgic and petroleum industry (Mohan and Dubey, 2013). Lead is mutagenic and teratogenic, causing effects in the human body such as neurodegenerative diseases, renal failure, reproductive damage and different types of cancer (Mohan and Dubey, 2013; Naik and Dubey, 2017). Similar to lead, chromium is considered as a priority environmental pollutant because also cause mutagenicity and carcinogenicity. Although chromium exists in different oxidation states, $Cr^{6+}$ compounds are extremely toxic due to their high solubility in water, rapid permeability through biological membranes and interactions with proteins and nucleic acids (Thatoi et al., 2014). Conventional

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technologies for lead and chromium remediation includes physical and chemical precipitation, activated carbon adsorption, ion exchange, reverse osmosis and graphene oxide adsorption (He et al., 2011; Luo et al., 2014; Mohan and Dubey, 2013). However, these methods demand high energy input, are found to be very expensive and are not efficient on a large scale and low metal concentration (Thatoi et al., 2014). Therefore, are required novel, economical, safe and sustainable technologies for detoxification of hazardous metals.

Microorganisms have developed mechanisms of tolerance and resistance to maintain an optimal intracellular concentration of metals (Jarosławiecka and Piotrowska-Seget, 2015; Viti et al., 2014). Tolerance is the ability of a microorganism to survive metal toxicity using intrinsic properties, metabolism-independent ways such as adsorption by membranes, cell wall or surface layers (S-Layer) (Thatoi et al., 2014; Velásquez and Dussan, 2009). On the other hand, resistance is the ability of a microorganism to survive the toxic effects of metal exposure using genetic and energy-dependent mechanisms produced in direct response to a particular metal. Among the broad diversity of resistance mechanisms in prokaryotes, the extrusion of cations by efflux pumps are the main energy-dependent homeostasis system used to control intracellular metal concentrations (Chong et al., 2016; Nies, 2003). In gram-negative bacteria, metal resistance through efflux pumps has been well studied (Nies, 2003). P-type ATPases or members of the Cation Diffusion Facilitator (CDF) protein family drive by proton motive force the transport of heavy metal from the cytoplasm to periplasm, or across the outer membrane from the periplasm to outside of the cell (Nies, 2003; Vaccaro et al., 2016). In contrast to gram-negative bacteria, gram-positive bacteria are less studied, and there is a gap of knowledge in the mechanism of heavy metal resistance. Several efflux systems have been described in bacteria, but the most common for chromium and lead detoxification are encoded by *chr* and *pbr* operons respectively (Monchy et al., 2007). These operons harbor the *pbrA* and *chrA* genes that encode membrane transporters that catalyze the efflux of ions from the cytoplasm. The most-studied are the *Cupriavidus metallidurans* CH34 PbrA and ChrA efflux pumps (Monchy et al., 2007). ChrA belongs to the chromate ion transporter (CHR) superfamily and PbrA to the P-type ATPase (P-ATPase) superfamily (Díaz-Magaña et al., 2009; Martínez-Valencia et al., 2012). These hydrophobic membrane proteins work as chemiosmotic pumps that extrude lead and chromate from the cytoplasm using the proton motive force (Mergeay and Lelie, 2009; Thatoi et al., 2014). The mere presence of *chrA* or *pbrA* genes cannot explain the vast difference in resistance levels between bacteria, suggesting that different mechanisms could be working in parallel (Henne et al., 2009). The expression of metal homeostasis systems is controlled transcriptionally. ChrB and PbrR act as metal-sensitive regulators of the transcription of the structural genes of the *chr* and *pbr* operons respectively (Monchy et al., 2007). ChrB act as repressor binding the operator in apo-form and release the operator when bound to chromium (Branco et al., 2008b; Branco and Morais, 2013). In contrast, PbrR act as inducer that remodels the promoter region to facilitate transcription initiation when is in the presence of lead (Gaballa et al., 2003). These regulators are known for preferential high-affinity binding of certain metal ions. Nevertheless, they can still be cross-activated by chemically similar metal ions (Hložková et al., 2013). Studies of the expression of efflux pump genes have been carried out under metal pressure, but only concentration-dependent responses were considered (Branco et al., 2008b; Hložková et al., 2013; Zulfiqar and Shakoori, 2012). These studies did not monitor how the expression changed over time or in the simultaneous presence of different cations. It is essential to understand these bacterial resistance mechanisms open the possibility of developing novel, economical and environmentally friendly alternatives for detoxification of metal-polluted environments.

Transmembrane ATPases perform a crucial task maintaining intracellular concentrations of ions by controlling the flux of ions and molecules across the membrane. This implies a high specificity in the metal they are binding and a structure very well conserved to avoid extrusion of essential metals (Sitsel et al., 2015). Considering the high degree of sequence and structure conservation among efflux ATPases, a uniform mechanism should be anticipated. The number of known metal ATPase proteins exceeds 180.000 entries (UniProtKB, November 2017) and more than 2000 crystallography structures (PDB, November 2017). However, despite the substantial diversity, efflux pump ATPases share common topology and are likely to exploit the same general reaction cycle (Sitsel et al., 2015). The common and conserved topology opens the possibility of use bioinformatics approaches to predict structures by direct homology. Also, cocrystallization of transmembrane protein-ligand complexes are not always possible, and although three-dimensional structures accurately define structural binding epitopes (i.e., residues in direct contact with a ligand), they do not address the energetics of a binding interaction (Weiss et al., 2000). To fill these gaps and gain comprehensive understanding of ATPase-metal and/or regulator-metal interaction, computational studies have gained significant attention. Computational methods such as docking can identify hot spots for protein-ligand interaction, preferred orientation of a ligand molecule in a protein and predict structural changes after the interaction with the ligand (Bromberg and Rost, 2008). Ideal docking approach should be able to suggest potential binders, as molecules with highly negative binding energy, and discard all nonbinders. To gain further insight into the behavior of protein-ligand interaction, multiple *in silico* approaches to predict how mutations affect protein function have been developed over the past twenty years based on various evolutionary and physicochemical hypotheses (Pires et al., 2016). Although many different mutagenesis strategies have been proposed, site-directed mutagenesis by alanine-scanning has been successful in systematically mapping functional binding sites (Weiss et al., 2000; Bromberg and Rost, 2008). Alanine eliminates the side chain beyond the β-carbon, does not alter the primary chain conformation and does not inflict extreme steric or electrostatic effects. Substitution with alanine allows quick and detailed mapping of functional binding residues without protein purification or biophysical analysis. Instead, the binding free energy contributions of individual side chains can be determined from statistical and energy binding analysis (Weiss et al., 2000). In this study, we applied computation approaches to study the functional traits of toxic metal ATPases and its regulators. The bioinformatics approach described here, helped us to explain in detail the metal-protein interactions.

Mechanisms of Cr and Pb resistance have been extensively studied in model organisms such as *Cupriavidus metallidurans* CH34 (Monchy et al., 2007), *Klebsiella* sp. (Zulfiqar and Shakoori, 2012), *Pseudomonas aeruginosa* (Alvarez et al., 1999) and gram-positive bacterium *Bacillus subtilis* (Díaz-Magaña et al., 2009). However, molecular determinants of Cr and Pb resistance have not been identified yet in gram-positive bacteria of *Lysinibacillus* genus. *Lysinibacillus* is a remarkably diverse genus that comprises several species and strains of relevant interest for biotechnological purposes (Lozano and Dussán, 2013; Velásquez and Dussan, 2009). In this study, the focus is on *Lysinibacillus sphaericus,* a soil bacterium applied in bioremediation processes of hydrocarbon-polluted environments, usually under high concentrations of toxic metals (Manchola and Dussán, 2014). Previous studies exposed the ability of *Lysinibacillus* to tolerate metals such as Cr, Pb, As, Cu, Ni, Co, Hg, Cd, Ag and Ni (He et al., 2011; Prithviraj et al., 2014; Rahman et al., 2015; Velásquez and Dussan, 2009). Also, the genomic information of sequenced *Lysinibacillus sphaericus* strains revealed several protein encoding sequences possibly involved in the resistance against metals (Hernández-santana et al., 2016; Peña-montenegro et al., 2015; Peña-Montenegro and Dussán, 2013; Rey et al., 2016). Therefore, the objective of this study was to explore the efflux pump-mediated lead and chromium resistance in *Lysinibacillus sphaericus* OT4b.31. Our results provide insights of the molecular mechanism of tolerance and resistance of *Lysinibacillus sphaericus* OT4b.31 in the presence of chromium and/or lead and its temporal variation. To our knowledge, this is the first report which characterized the molecular determinants of lead