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Pb^{2+} biosorption by *Serratia marcescens* CCMA 1010 and its relation with *zntR* gene expression and ZntA efflux pump regulation

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ABSTRACT

Global concerns about the preservation and restoration of aquatic environments are rising and pollution related to heavy metals is one of the main worries. Indeed, this issue has been a challenge for the metallurgical industry and other activities associated with metal contamination. Thus, over time, several physical and chemical methods have been developed and applied to remove metals from water. However, these methods can be associated with high costs, and bioremediation using plants, fungi, and bacteria is considered a viable alternative. This paper reports experiments on lead removal from an aqueous medium using active and inactive *Serratia marcescens* CCMA 1010 and a study on the effect of lead in this bacterial strain regarding the expression of the *zntR* gene, which produces the *zntR* protein known to have a role as a regulator of the ZntA efflux pump of metals (Pb^{2+}, Cd^{2+}, Zn^{2+}). The amount of removed Pb^{2+} by active biomass remained below ~25 mg/L for the initial concentrations tested up to 120 mg/L but increased to removals of ~70 and ~167 mg/L for the tested concentrations of 220 and 300 mg/L, respectively. On the other hand, the removal of Pb^{2+} by inactive biomass increased in direct relation to the initial tested concentration, with removed percentages around 25 %. Interestingly, in cultures with 15 and 60 mg/L Pb^{2+} the expression of *zntR* was 27 to 74 and 87 to 177 times lower (respectively) than in cultures without Pb^{2+}, while in cultures with 120 mg/L Pb^{2+}, the *zntR* expression was just 1.3 to 9.3 times lower than in the absence of Pb^{2+}. The results confirm the potential of *S. marcescens* CCMA 1010 for Pb^{2+} biosorption, the presence of Pb^{2+} resistance mechanisms in this strain, and contribute to a better understanding of the ZntA transmembrane protein regulation.

Introduction

Controlling environmental pollution related to heavy metals has been a challenge for industries. There has been an increase in concern for the preservation and maintenance of the environment, and in this context, there is also a greater interest in viable low-cost alternatives for treating waste with metals to prevent the accumulation of these pollutants in the environment (Gavrilescu et al., 2015; Briffa et al., 2020). In this sense, using microorganisms for bioremediation is a viable alternative to physical and chemical methods, which can have high costs (Fu and Wang, 2011; Sengupta et al., 2017; Wang et al., 2023). However, there is a complex interaction between media compounds and microorganisms, which can directly affect the efficiency of these processes, therefore, studies are needed to address the different conditions found in contaminated environments, the resistance profile of microorganisms, and the biological mechanisms allowing metals bioremediation (Fu and Wang, 2011; Naik and Dubey, 2013; Abo-Alkasem et al., 2023). The use of microbial biomass to remove heavy metals is an alternative that has been discussed for a long time, and it is known that applying these processes in real treatment systems requires optimization studies due to the variations found in the contaminated waste (e.g. Migahed et al. 2017). It is possible to use active or inactive biomass according to the specificities of the environment and of each microorganism, which can release products that induce the precipitation of metals, have absorption capacity by mechanisms of bioaccumulation of metals inside the cells or in the periplasmic region, or have adsorption capacity on the surface of the cell wall (Huang et al., 2013; Ahemad and Kibret, 2013; Abo-Alkasem et al., 2023). In cases where active microorganisms can remove metals from the environment that surrounds them, several genes may be essential for this aptitude, whether related to

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the mechanisms contributing to lower the concentration of metals outside the cells, or genes conferring resistance to exposure of microorganisms to metals (e.g. Giovanella et al. 2017; Tiquia-Arashiro et al. 2018; Yadav et al. 2023). Moreover, studies on the expression of heavy metal resistance genes, in addition to providing greater knowledge regarding the physiology of microorganisms in contaminated environments, are also useful in the development of biosensors for the detection of such pollutants (Kim et al., 2018; Gutiérrez et al., 2015; Kannappan and Ramisetty 2022).

Lead is a highly polluting element, even at low concentrations, found in paints, gasoline, the water distribution system, and food contaminated by waste from industrial or agro-industrial activities (Chakraborty et al., 2017). Usually, the removal of this metal from aqueous wastes is based on methods of chemical precipitation, flocculation and coagulation, flotation, ion exchange using resins, adsorption using activated carbon, and membrane filtration; nevertheless, several studies have demonstrated the potential of lead removal using plants, fungi or bacteria (Bahadir et al., 2007; Fu and Wang, 2011; Tabaraki et al., 2014; Carolin et al., 2017; Wagh et al., 2023).

Bacterial lead resistance, which is of major importance for the bacterial lead removal potential, may be determined by different mechanisms, such as efflux from the cytoplasm, sequestration by metallothionein, sequestration in exopolysaccharide, cell surface adsorption, biosorption in the cell wall and periplasmic space, precipitation catalyzed by Phosphatase enzyme, and even precipitation by reaction with sulfide released by sulfate-reducing bacteria (Naik and Dubey 2013). Yet, it can be considered that real resistance is fundamentally based on the efflux of lead ions to prevent its harmful effects in the cell. In bacteria, there are three main families of efflux transporters: (1) P-type ATPases (adenosine triphosphatases) transporting metal ions from the cytoplasm to the periplasm using ATP as an energy source, (2) Capsule Biogenesis Assembly (CBA) transporters working as chemiosmotic antiporters transporting cations from cytoplasm and periplasm to outside the cell mostly in gram-negative bacteria, and (3) Cation Diffusion Facilitator (CDF) transporters acting as chemiosmotic ion-proton exchangers transporting cations from cell cytoplasm to periplasm in exchange for a proton entering into the cytoplasm, which provide low resistance to bacteria despite having a crucial role at low concentrations of heavy metals in the cell cytoplasm (Sevak et al., 2021).

The resistance of bacteria to Pb^{2+} ions, according to proteomics and transcriptomics studies combined with functional tests by mutagenesis, is related to the *pbrUTRABCD* plasmid operon, which has the peculiarity of combining uptake, efflux, and accumulation functions, or to the *pbrR2 cadA pbrC2* operon, and to the *zntA* operon, both chromosomal and with an efflux function only. Interestingly, the ATPase PbrA membrane protein of Pb^{2+} transport encoded by the *pbrA* gene of the pbrUTRABCD operon is phylogenetically close to the ATPase CadA protein and its homolog ZntA encoded by *zntA* gene (Taghavi et al., 2009; Helmann, et al., 2007). These CadA and ZntA proteins were initially associated with membrane transport of Cd^{2+} and Zn^{2+} but it has been shown they also play a role in Pb^{2+} efflux (Rensing et al. 1998a,b; Binet and Poole, 2000; Hou and Mitra 2003). Furthermore, it has been reported that the *zntA* gene is regulated by the MerR-like ZntR regulatory protein encoded by the *zntR* gene, which is related to the control of Zn^{2+} in bacterial cells but seems to have a role in the control of other metals as well (Rensing et al., 1997; Brocklehurst et al., 1999; Outten et al., 1999; Singh et al., 1999; Helmann et al., 2007; Permina et al., 2006; Schulz et al. 2021). Indeed, Binet and Poole (2000) confirmed that the transcriptional regulation of the *zntA* gene, when induced by Cd^{2+}, Zn^{2+}, or Pb^{2+}, is mediated by the regulatory protein ZntR.

On the other hand, the regulatory protein ZntR has also been indicated as a regulator of the expression of genes of the VI (T6SS) type secretion system in *Yersinia pseudotuberculosis* (Wang and Shen 2017). The T6SS system is probably involved in different functions in the bacterial life cycle and is regulated by several regulators (Bingle et al., 2008; Boyer et al., 2009). In *Y. pseudotuberculosis* was found that T6SS4 is related to importing Zn^{2+} ions from the environment into bacterial cells, which alleviates the accumulation of hydroxyl radicals induced by multiple stress factors (Wang et al., 2015). Interestingly, it has been considered that microorganisms with this type of gene system can be useful in treating waste contaminated with metals due to the protection against oxidative stress caused by these pollutants (Wang et al., 2017).

The isolation and identification of microorganisms with useful capabilities for bioremediation processes is always interesting, and microorganisms isolated from contaminated areas have an important potential for this purpose (Afzal et al., 2017). For instance, *S. marcescens* CCMA 1010 strain was isolated from a coffee-processing wastewater treatment system with high concentrations of Cd^{2+} (130 mg/L) (Pires et al., 2017) and is able to grow in Pb^{2+} concentrations of up to 4.0 mM (dos Reis Ferreira et al. 2023), which suggests it may have resistance genes conferring the ability to live in extreme conditions of different metals (Iguchi et al., 2014; Khan et al., 2015; 2017). In fact, several bacterial strains of *Serratia marcescens* have already been used in bioremediation research and have shown potential for the removal of various metals, such as Pb, Zn, Cd, Cr, and Ni (Cristani et al., 2012; Nwagwu et al., 2017). On the other hand, the *zntA* and *zntR* genes (which play a role in controlling the concentrations of Cd^{2+}, Zn^{2+}, or Pb^{2+} in cells, as described above) have already been identified in a sequenced genome of a *Serratia* sp. (Iguchi et al., 2014) and the *zntR* gene of *S. marcescens* CCMA 1010 was already fully sequenced (dos Reis Ferreira et al. 2023).

The work here presented reports: (1) studies on Pb^{2+}removal from an aqueous medium using active and inactive biomass of *S. marcescens* CCMA 1010, (2) studies on the expression of the *zntR* gene in this strain when exposed to different concentrations of Pb^{2+}, and (3) a discussion of results aiming to better understand the mechanisms involved in the observed Pb^{2+} resistance and removal capacity as well as the role of *zntR* in their regulation.

Materials and methods

Microorganism reactivation

The bacterial strain *S. marcescens* CCMA 1010 resistant to Pb^{2+}, belonging to the agricultural microbiology culture collection of the Federal University of Lavras (CCMA/UFLA), was reactivated in nutrient broth (NB) culture medium (5 % peptone and 3 % yeast extract) in stirring at 150 rpm for 24 h at 28°C until reaching a concentration of 10^9 CFU/ml.

$Pb^{2\pm}$ removal from the aqueous medium with S. marcescens CCMA 1010 biomass

Experiments were carried out with active and inactive biomass to study the type of mechanism that gives *S. marcescens* CCMA 1010 the ability to remove Pb^{2+} from aqueous media.

Removal of Pb^{2+} with active biomass

1 ml aliquots of *S. marcescens* CCMA 1010 reactivated culture were inoculated into flasks with 100 ml of LB culture medium (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl) modified with supplements of lead nitrate (N_2O_6Pb) to make different concentrations of Pb^{2+} (15, 40, 60, 120, 220 and 300 mg/L) in the test cultures, and without N_2O_6Pb in the control culture. The assays were performed in triplicate, and samples were taken at the beginning (time 0) and at 24h, 48h, and 168h after inoculation and incubation at 28°C with orbital shaking at 150 rpm. The samples were centrifuged at ~7000 g for 10 min at room temperature, and the liquid phases were collected and diluted in nitric acid (5 %) for analysis of the lead concentration by flame atomic absorption spectrometry in a novAA 350 equipment (Analytik Jena), and calculation of removed lead (initial-final lead concentration) and lead removal rate (100·removed/initial lead concentration) of this metal from the culture

medium.

Removal of Pb^{2+} with inactive biomass

In this experiment, 1 ml aliquots of reactivated *S. marcescens* CCMA 1010 cultures were also used, but now with cells inactivated in the autoclave at 120°C for 30 min immediately after reactivation. The inactivated microbial biomass was added to flasks with 100 mL of LB medium containing N_2O_6Pb supplements at initial concentrations of 15, 40, 60, 120, 220, and 300 mg/L of Pb^{2+}. These assays were also performed in triplicate, but the Pb^{2+} concentration was evaluated only at the beginning and after 24 h of incubation at 28°C with orbital shaking at 150 rpm. The collection of samples and the analysis of Pb concentrations to calculate this metal's removal rate were carried out as described above.

Analysis of zntR gene expression in S. marcescens CCMA 1010 in the presence of $Pb^{2\pm}$

The *zntR* gene expression was studied by Real-Time Quantitative PCR (qPCR) using complementary DNA (cDNA) synthesized immediately after total RNA extraction and following a Relative Quantitation of Gene Expression approach. The 16S rRNA gene was used as an endogenous control gene for normalization since it is one of the most tested and validated genes in qPCR studies (Rocha et al. 2015).

The study was carried out in LB medium cultures with supplements of 15, 60, and 120 mg/L of Pb^{2+} and cultures without this metal (as calibrator – reference sample) prepared as described above for active biomass. 4 biological replicates were used (4 cultures for each Pb^{2+} concentration), and 3 technical replicates were performed (3 independent qPCR reactions for each biological replicate).

RNA extraction and cDNA synthesis

After incubating the *S. marcescens* CCMA 1010 cultures for a period of 12 h, total RNA was extracted using the NZY Total RNA Isolation Kit (NZYTech, Portugal) according to the manufacturer's instructions. Immediately after, the efficiency of the extraction was confirmed by electrophoresis in 1 % (w/v) agarose gel in 1× TAE buffer (AMRESCO, USA) with nucleic acids stained by addition of 50 μL/L of GreenSafe Premium (NZYTech, Portugal) to the gel, and then cDNA synthesis was performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) following the manufacturer's instructions. Finally, the cDNA concentration was estimated using the Qubit® 2.0 Fluorometer with the Qubit ssDNA Assay Kit (Thermofisher Scientific, UK), and all samples were diluted in MiliQ sterile water to make 0.25 ng/μL.

qPCR primers design

The primers for qPCR reactions to analyze the expression of the *zntR* and 16s rRNA genes of *S. marcescens* CCMA 1010 were designed using DNA sequences from this strain (published in NCBI GenBank with accessions MH844628 and ON454114, respectively for *zntR* and 16S rRNA), which in turn were obtained with primers designed in the genomic sequence of *Serratia marcescens* subsp. *marcescens* Db11 available at the NCBI genomes database (GenBank assembly accession: GCA_000513215.1) (Iguchi et al., 2014).

When designing the qPCR primers, the following precautions were taken to try to achieve good PCR efficiencies: (1) products larger than 200 bp were avoided, (2) the online application "The mfold Web Server" was used to identify and choose regions with less folding in the possible arrangements of the amplified product, and (3) the NCBI "Primer-BLAST" tool was used with the genome of *S. marcescens* subsp. *marcescens* Db11 as a reference to identify and avoid the possibility of amplifying multiple PCR products. The primers designed and used for qPCR amplification of the *zntR* gene were ZntR-f: CTCTTGGCAGGTATGATGCTC and ZntR-r: AGGGCATGATGGATCACAATG. The primers for qPCR amplification of the 16s rRNA gene were 16s-f: GTTTGATCATGGCTCAGATTGAAC and 16s-r: CATCAGGCAGTTTCCCAGA.

qPCR conditions

The NZY qPCR Green Master Mix (2x) (NZYTech, Portugal) was used for qPCR. The reaction consisted of 5 μL of the Master Mix, 0.4 μL forward primer, 0.4 μL reverse primer (10 μM solutions), 4.0 μL of the cDNA (after adjusting the samples to appropriate cDNA concentrations), and 0.2 μL of miliQ water. The qPCR amplification took place in a CFX Connect real-time PCR thermocycler (Bio-Rad, USA) with the following program: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min.

zntR gene expression

First, qPCR amplification tests of the target gene (*zntR*) and of the endogenous control gene (16S rRNA) were performed with different amounts of cDNA from a mix of all samples to confirm the specificity of the primers used and to evaluate the amounts of cDNA to amplify each gene to obtain Cycle Thresholds (CT) values in a similar range.

Afterward, the study of *zntR* gene expression in the presence of Pb^{2+} was carried out by Relative Quantitation of Gene Expression. For that: (1) qPCR amplifications of the target gene (*zntR*) and the endogenous control gene (16S rRNA) were carried out using the previously estimated amounts of cDNA from the cultures not exposed to Pb^{2+} (calibrator or reference sample) and from the cultures exposed to different Pb^{2+} concentrations (tests); and (2) qPCR amplifications were carried out with different amounts of cDNA (1 to 0.000001 ng) prepared by successive 1:10 dilutions of a mix of all samples, to construct standard curves for both genes.

The qPCR amplification efficiencies for both genes based on regression line plots of CT value *vs*. log of input cDNA were used to determine if the Comparative CT Method (ΔΔ CT Method) or the Standard Curve Method was used (Schmittgen and Livak 2008). Then, the calculations for Relative Quantitation of Gene Expression were as described in the Applied Biosystems User Bulletin No. 2 (P/N 4303859): https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_040980.pdf.

Results

$Pb^{2\pm}$ removal from the aqueous medium with S. marcescens CCMA 1010 biomass

Removal of Pb^{2+} with active biomass

In the first 24 and 48 h of incubation, the Pb^{2+} concentration dropped by an order of magnitude between 4 and 21 mg/L in all the *S. marcescens* CCMA 1010 cultures inoculated with active cells, regardless of the tested initial concentration of this ion (15, 40, 60, 120, 220 and 300 mg/L). After 168 h of incubation, the Pb^{2+} removed by active *S. marcescens* CCMA 1010 remained below 25 mg/L for the tested concentrations up to 120 mg/L, but it was much higher for the tested concentrations of 220 and 300 mg/L: 70 and 167 mg/L Pb^{2+} removed, respectively (Fig. 1A).

Although these results allow a first inference of removal capacity, additional studies with a more comprehensive set of experimental variables (different Pb^{2+} concentrations, temperatures, pH, and amounts of biomass) will be necessary to determine the best conditions and which is the model that defines the Pb^{2+} biosorption by *S. marcescens* CCMA 1010. However, that was not the objective of our work, but rather to look at the relationship between Pb^{2+} removal by this strain with its *zntR* gene expression and the ZntA efflux pump regulation system.

Even so, the results were also represented as Pb^{2+} removal percentages to help the interpretation of identified trends. In the first 24 and 48 h of incubation, the Pb^{2+} removal percentages varied between ~35 and ~4 % with a general trend of decreasing percentages from the

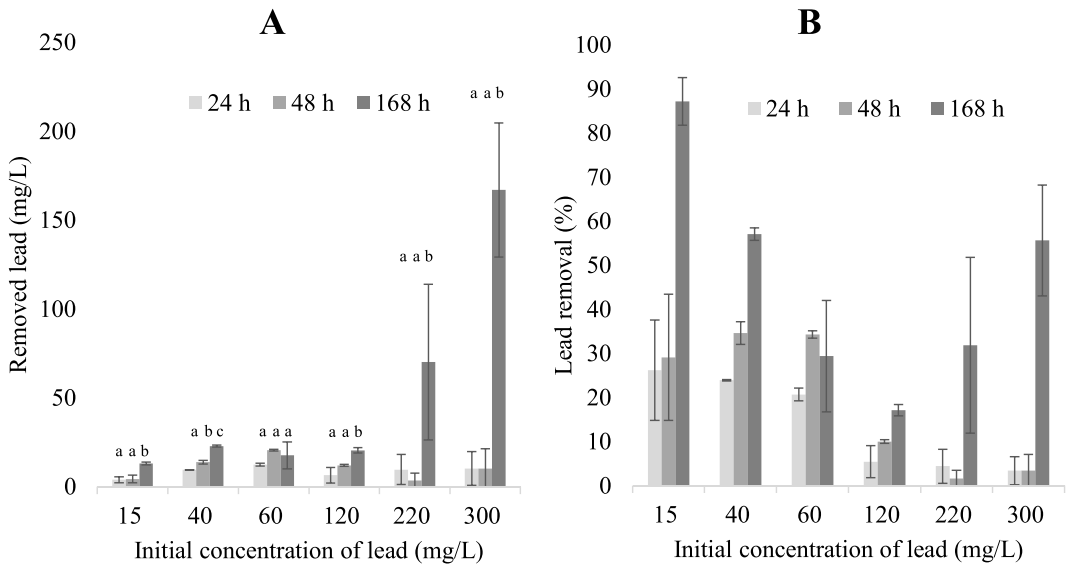

Fig. 1. Lead (Pb^{2+}) removals in LB culture medium 14, 48 and 168 h after inoculation with active *S. marcescens* CCMA 1010 cells. A) concentration removed - bars with the same letters do not significantly differ at 0.05 level (ANOVA and Tukey Kramer's tests). B) removal percentages.

lowest to the highest tested concentrations of this ion. After 168 h incubation, the removed Pb^{2+} concentrations are reflected in a removal percentage decay from ~87 to ~17 % as the tested concentration increased from 15 to 120 mg/L, followed by a removal percentage rise to ~32 and ~56 % for the tested concentrations of 220 and 300 mg/L Pb^{2+}, respectively (Fig. 1B).

The observed differences in Pb^{2+} removals with active cells of *S. marcescens* CCMA 1010 between the initial incubation times (24 and 48 h) and the final incubation time (168 h) reflect the cumulative effects of exposure to the different tested concentrations of this ion on the increasingly larger number of cells in the cultures over time. Therefore, the results observed at the final incubation time allow these effects to be detected more easily, thus helping the final integrated discussion of all results reported in this article.

Removal of Pb^{2+} with inactive biomass

The amount of removed Pb^{2+} by inactive *S. marcescens* CCMA 1010 in the liquid culture medium increased as the initial tested concentration raised (Fig. 2A). Although, as stated above, the objective is not to characterize the removal model, it seems that the amount of removed concentration is directly related to the initial concentration of Pb^{2+}, as the removal percentage average remained in the range of 19–27 % for all initial concentrations tested (Fig. 2B). Indeed, the linear correlation between the removed Pd^{2+} and the tested concentrations has an R^2 of 0.9037 and is represented by $y = 0.234x + 0.4567$ (where x is the initial and y the removed concentration). The maximum removed Pb^{2+} was, achieved when the maximum concentration of 300 mg/L Pb^{2+} was tested.

Analysis of zntR gene expression in S. marcescens CCMA 1010 in the presence of $Pb^{2\pm}$

The initial qPCR amplification tests revealed unique fragments for both genes, confirming the primers' specificity. Moreover, these tests

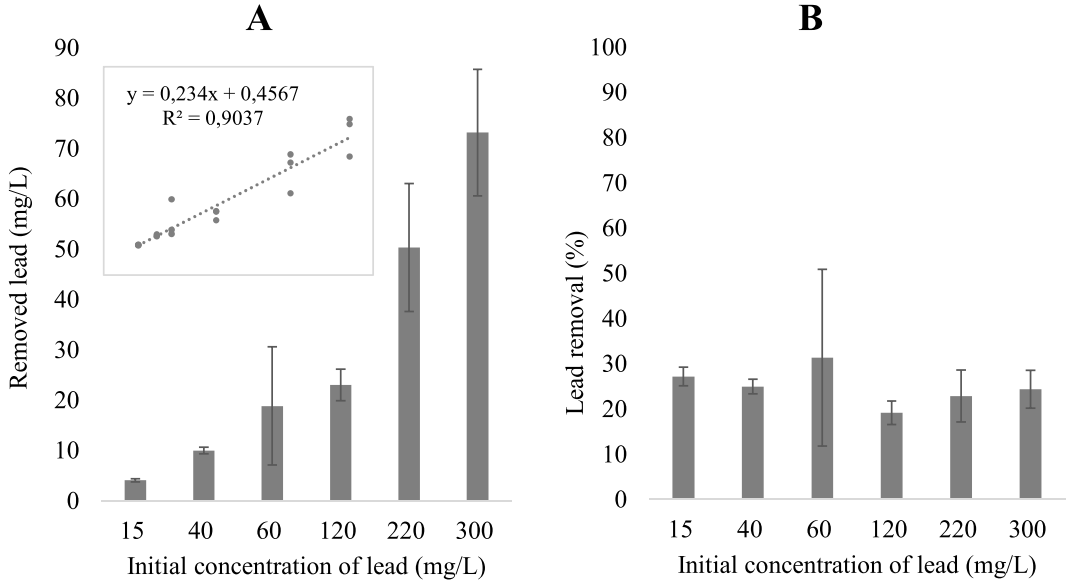

Fig. 2. Lead (Pb^{2+}) removals in LB culture medium 24 h after adding inactive *S. marcescens* CCMA 1010 cells. A) concentration removed - a linear correlation between the removed Pd^{2+} and the tested concentrations is represented using a dispersion graph. B) removal percentages.

showed that the *zntR* amplification would need to start with 0.1 ng of cDNA while the 16S rRNA amplification with 0.001 ng of cDNA to obtain cycle threshold (CT) values close to ~20 for both genes.

In what concerns the qPCR amplifications for the expression study, the slopes of the real-time PCR standard curves represented as regression line plots of CT value vs. log of input cDNA were different for both genes (-3.04 for the *zntR* and -5.11 for the 16S rRNA), which indicates different PCR efficiencies and excludes the possibility of applying the Comparative CT Method (ΔΔ CT Method) (Schmittgen and Livak 2008). Indeed, in the validation test, the regression line of log input RNA amount (ng) vs. ΔCT revealed a slope > 0.1 confirming the different qPCR efficiencies for the target and reference genes. Therefore, the *zntR* gene expression was studied following the Relative Quantitation of Gene Expression approach by the Standard Curve Method (Suplementary Material).

The results demonstrated that the *zntR* gene expression in *S. marcescens* CCMA 1010 decreases in the presence of lead up to certain concentrations, but when the lead concentration reaches higher values, the gene expression returns to values close to the expression in the absence of this metal. In the cultures with 15 mg/L of Pb^{2+}, the expression of the *zntR* gene decreased to values 27 to 74 times lower than the expression in the culture without Pb^{2+}, and in the culture with 60 mg/L of Pb^{2+}, the expression of *zntR* decreased to values 87 to 177 times lower. However, when the Pb^{2+} concentration was 120 mg/L, the *zntR* gene expression was only 1.3 to 9.3 times lower than in the absence of Pb^{2+} (Table 1).

Discussion

Pb^{2+} removal by *S. marcescens CCMA 1010* in LB culture medium occurred with active and inactivated biomass, indicating that biosorption processes (passive removal by absorption and/or adsorption) must have played a fundamental role. In this case, it is known that the higher the metal concentrations in the medium, the faster the binding sites in bacteria tend to become saturated, with limited removal capacity after that (Wen et al., 2018; Aryal and Kyriakides, 2015). In the tests with inactive biomass, the removal of Pb^{2+} increased proportionally to the increase in the tested concentration of this ion, suggesting that the removal depended mainly on the probability of encounters between the ions and the bacteria, thus corroborating the hypothesis of removal by biosorption without achieving saturation. With the active biomass, however, the removed amounts of Pb^{2+} were always below 25 mg/L for the different tested concentrations up to 120 mg/L but increased (to ~70 and 167 mg/L) for the higher tested concentrations of 220 and 300 mg/L. This indicates that in the presence of Pb^{2+} up to a certain concentration biological mechanisms are operating to limit the amount of these ions retained by the bacterial cells but those mechanisms stop in higher concentrations.

ZntR is a MerR-like protein that regulates the transcription of the *zntA* gene, which produces a protein (ZntA) that transports Pb^{2+}, Cd^{2+}, Zn^{2+}, and Co^{2+} out of the cell. At the time of ZntR discovery, it was revealed that when binding to these ions, it becomes a transcription factor of *zntA*, and with exposure of cells to those metal ions, the production of ZntA increases (Rensing et al. 1997; Brocklehurst et al. 1999; Singh et al. 1999; Binet and Poole 2000; Pennella and Giedroc 2005; Helmann et al. 2007). Initially, information about the regulation of the *zntR* gene itself was scarce, though the presence of a partial copy of the *zntA* gene promoter in the *zntR* coding region (Brocklehurst et al. 1999) raised the hypothesis of negative autoregulation. Indeed, electrophoretic mobility shift assays (EMSAs) and *in vitro* transcription assays using *Staphylococcus aureus* revealed that ZntR binds to the znt promoter and represses *zntR* transcription together with the *zntA* gene in a concentration-dependent way (Singh et al. 1999). Furthermore, the similarities of the *zntA* promoter (*PzntA*) sequence with the promoters' sequences of the mercury resistance operons *mer* T21 and *mer* Tn501 (Rensing et al. 1997; Brocklehurst et al. 1999; Brown et al. 2003) also suggested a process of negative autoregulation for the MerR-like proteins. Indeed, it has been suggested that MerR proteins (which originated the name of the family of MerR-like regulators), in response to the recognition of the Hg^{2+} ion, bind to the promoters of the *mer* operons to activate the transcription of several genes involved in the mechanism of detoxification of this ion, while the same binding sites also negatively regulate the transcription of *merR* gene promoters which are in overlapping positions and in the genome's divergent direction (Helmann et al. 2007). Afterward, the hypothesis of negative self-regulation of the *zntR* gene was reinforced in a study with mutant lines of *Brucella abortus* (Sheehan et al. 2015). In addition, that work also showed that the deletion of the *zntR* gene resulted in increased expression of *zntA*, thus confirming that ZntR also negatively controls the expression of *zntA* but proving (in our opinion) that ZntA production does not depend on ZntR (with or without metal ions effectors) as a transcription factor of *zntA*. Nevertheless, more recently, a study with the metallophilic *Cupriavidus metallidurans* CH34 strain showed using a *zntA-lacZ* reporter gene fusion technique that the expression of the ZntA efflux pump is upregulated when 200 nM to 100 mM zinc is present in the medium and proved trough EMSAs that ZntR bounds to the *zntA* promoter region, at both ratios of 0.43 and 0.14 Zn atoms per ZntR polypeptide (Schulz et al. 2021).

Finally, we consider that according to all these findings, it can be deduced that when the cell "needs" to increase the production of ZntA efflux protein to raise the export of Pb^{2+} (or another metal ion), it must decrease the production of ZntR. Therefore, it may be that ZntR inhibits the transcription of both the *zntR* and *zntA* genes (as suggested by other authors). However, it is possible that ZntR bound to Pb^{2+} may inhibit the transcription of *zntR* but not of *zntA*.

This hypothesis is supported by our results obtained in this work with *S. marcescescens* CCMA 1010 (a wild bacterial strain) in which the *zntR* gene is present: the presence of Pb^{2+} ions caused a reduction of *zntR* expression. Moreover, this work revealed that the mechanisms regulated by the ZntR protein play an important role in the resistance of this bacterium to certain concentrations of these ions, but for higher

Table 1
Relative expression of *zntR* in *S. marcescens* CCMA as calculated using the relative standard curve method.

Cultures (mg/L Pb)	Cycle threshold (Ct)		Log. of cDNA ng input		cDNA ng imput		*zntR* normalized to 16S	*zntR* relative to 0 Pb	Fold-difference range of *zntR* relative to 0 Pb	
	zntR	16S	*zntR*	16S	*zntR*	16S				
0	23.0 ± 0.7	17 ± 1	-0.3 ± 0.2	-2.7 ± 0.2	0.5 ± 0.3	0.002 ± 0.001	241 ± 168	1.0 ± 0.7		
15	28.7 ± ± 0.3	18.5 ± 0.9	-2.2 ± 0.1	-3.0 ± 0.2	0.006 ± 0.002	0.0010 ± 0.0004	6 ± 3	0.03 ± 0.01	-27	-74
60	28.5 ± 0.3	15.7 ± 0.6	-2.1 ± 0.1	-2.5 ± 0.1	0.008 ± 0.002	0.0036 ± 0.0009	2.1 ± 0.7	0.009 ± 0.003	-87	-177
120	24 ± ± 1	17 ± 1	-0.7 ± 0.3	-2.6 ± 0.2	0.28 ± 0.15	0.003 ± 0.001	105 ± 79	0.4 ± 0.3	-1.3	-9.3

concentrations, these mechanisms are no longer active. At the concentrations of 15 and 60 mg/L of Pb^{2+}, the expression of *zntR* was 50.5 ±23.5 and 132±45, respectively, times below the "normal" expression in the culture without Pb^{2+} supplement, while at the concentration of 120 mg/L Pb^{2+}, the expression of this gene was only 5.3±4 times lower. Interestingly, as mentioned above, it was also at initial concentrations above 120 mg/L Pb^{2+} that the active biomass began to remove a greater amount of this ion from the aqueous medium, which corroborates a greater biosorption of this metal, possibly due to a lower efflux caused by the lower *zntR* expression and ZntR production, thus increased *zntA* expression and ZntA production. This is in agreement with a recent work using engineered *Escherichia coli* strains ΔzntA with suppressed ZntA efflux system and pYYDT-zntA with strengthened efflux system, in which it was observed 20 % more Cd^{2+} accumulated in ΔzntA and 17 % less in pYYDT-zntA, compared to the wild type strain (Zhu et al. 2021).

In a previous study, we showed by minimal inhibitory concentration analysis that *S. marcescens* CCMA 1010 could grow in a solid medium supplemented with 4.0 mM of Pb^{2+} but not with supplements of 4.5 mM and higher (dos Reis Ferreira et al. 2023). Interestingly, the *C. metallidurans* CH34 strain referred to above can tolerate Zn^{2+} in solid medium up to the same concentration of 4.5 mM (Mergeay et al. 1985, von Rozycki et al. 2008), which is yet another indication of the existence of similar and/or crossed resistance mechanisms for Zn^{2+} and its heavy-metal mimetics such as Cd^{2+} and Pb^{2+}.

Regarding the resistance of *S. marcescescens* CCMA 1010 to Pb^{2+} at higher concentrations as 120 mg/L, when the expression of *zntR* is close to "normal", a global stress response like that described by Choudhary and Sar (2016) may have a role. These authors, in a study on the expression of metals resistance genes in *Pseudomonas aeruginosa*, observed an increase in the expression of *copA* and *czcA* genes for sequestration and efflux of metallic ions when exposed to low concentrations of metals for a short duration. In contrast, the expression of the *sodA* gene of global stress response, which encodes the enzyme superoxide dismutase, only increased at higher metal concentrations or longer exposure time. Interestingly, Yoshida et al. (2023) showed that ZntR and other metal-responsive transcription factors (TFs) following the addition of effectors (Zn^{2+} was used for ZntR) bind to the promoter regions of *rsd* and *rmf* genes, which enconde the Rsd and RMF protein regulators of transcriptional and translational activities under stress. Thus, it may be that ZntR when bound to Pb^{2+} also causes some global stress response. Indeed, in our previous study referred to above, we observed a decay in the growth and in the biological activity of *S. marcescens* CCMA 1010 when exposed to 0.88 g/L (4 mM) of Pb^{2+} (dos Reis Ferreira et al. 2023), which indicates a stress response.

Conclusion

This work confirms the promising potential of *S. marcescens* CCMA 1010 biomass in Pb^{2+} treatment systems, as it shows that this strain is efficient in the biosorption of these ions and presents functional resistance mechanisms that allow its proliferation in environments with Pb^{2+} contamination.

Furthermore, this study contributes to a better understanding of the regulatory mechanism of ZntR protein production, which regulates the expression of the transmembrane protein ZntA associated with the efflux of Pb^{2+} and other metal ions (Cd^{2+} and Zn^{2+}).

CRediT authorship contribution statement

Jorge Dias Carlier: Supervision, Methodology, Investigation, Formal analysis, Project administration, Writing – original draft. **Gustavo Magno dos Reis Ferreira:** Investigation, Writing – review & editing. **Rosane Freitas Schwan:** Resources, Supervision, Writing – review & editing. **Cristina Ferreira da Silva:** Resources, Supervision. **Maria Clara Costa:** Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2023.100479.

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