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Burkholderia sp. cadmium tolerance mechanism and its influence in phytoremediation

Manuella Nóbrega Dourado Ribeiro

Thesis presented to obtain the degree of Doctor in Science. Program: International Plant Cell and Molecular Biology

Piracicaba
2013

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I dedicate this thesis to
my parents, Durval and Valéria,
and my husband Rafel
for the incondicional love and suport
that guide my life.

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“The best of all things is to learn.
Money can be lost or stolen,
health and strength may fail
but what you have committed
to your mind is yours forever."

Louis L’Amour

CONTENTS

RESUMO

Mecanismos de tolerância ao cádmio em *Burkholderia* sp. e sua aplicação na fitorremediação

O cádmio (Cd) tem contaminado solos pelo uso de fertilizantes, calcário, agrotóxicos e resíduos industriais e/ou domésticos. Podendo ser lixiviado ao lençol freático ou absorvido pelas plantas,resultando na redução do crescimento e da produtividade. Esse metal afeta todos os organismos vivos e causa diferentes danos às células. A tolerância a esse metal se deve principalmente ao balanço do estado redox da célula para manter a integridade celular e metabolismo.Assim, foram isoladas bactérias de solo contaminado e não contaminado com Cd, selecionando isolados tolerantes a altas concentrações de diferentes metais (Cd, Ni e Zn), em seguida, foi observado a resposta do sistema antioxidante da bactéria na presença do Cd, a fim de auxiliar no desenvolvimento de metodologias para biorremediar solos contaminados. Foi quantificado MDA e peróxido de hidrogênio e a atividade de diferentes enzimas do sistema antioxidante (SOD, CAT, GR e GST) de duas estirpes do gênero *Burkholderia* tolerantes a todos os metais testados, uma isolada do solo contaminado com altas concentrações de Cd (estirpe SCMS54) e a outra do solo sem Cd (estirpe SNMS32) em dois tempos de exposição (5 e 12 h). Na estirpe SCMS54, as medidas de estresse (peroxidação lipídica e peróxido de hidrogênio) e a atividade das enzimas antioxidantes (SOD, CAT, GR e GST) da maioria dos tratamento com cádmio aumentaram, esta estirpe também expressa mais isoformas de SOD, CAT e GR, além de acumular 50% mais Cd. Esses resultados mostram que a estirpe SCMS54 (isolada do solo contaminado com Cd) apresenta uma maior diversidade metabólica e plasticidade. Foram analisadas também a resposta dessas duas estirpes ao Ni, observando uma resposta semelhante ao Cd, exceto na expressão da enzima GST, que no estirpe SCMS54 foi induzida na presença do Ni, indicando que essa enzima pode ser essencial na tolerância ao Ni. Portanto, a estirpe isoladado solo contaminado com Cd (SCMS54) foi selecionada para prosseguir os estudos e avaliar os benefícios da interação entre microrganismos tolerantes-plantas de tomate na fitorremediação. Essa técnica é usada remover para metais pesados do solo com um menor impacto e baixos custos. Os microrganismos do solo podem solubilizar e mobilizar metais do solo, atuando como biorremediador. Além da alta tolerância ao Cd, a estirpe SCMS54 produz ácido indol acético (AIA), solubiliza fosfato inorgânico e produz sideroforo, mostrando seu potencial na interação benéfica planta-microorganismo. Quando interagindo com as plantas de tomate expostas ao Cd, essa bactéria diminui a concentração de peróxido da planta e a clorose ocasionado pelo Cd,e reduz a absorção de Cd pela raiz resultando em um aumento da tolerância da planta ao metal pesado altamente tóxico. Assim, a inoculação de plantas de tomate com *Burkholderia* sp. SCMS54 promove crescimento da planta na presença de Cd, desencadeando um mecanismo que diminui a concentração de Cd nas raízes devido a essa interação benéfica bactéria-raiz da planta.

Palavras-chave: Metal pesado; Estresse oxidativo; Bactéria Promotora do Crescimento de Planta (PGPB)

ABSTRACT

Burkholderia sp. cadmium tolerance mechanism and its influence in phytoremediation

Soils have been contaminated with cadmium (Cd) by the use of fertilizers, calcareous, pesticides and industrial and/or domestic effluents. It can be leached to groundwater, as well as be taken up by plants potentially leading to reduce growth and yield. It causes different damages to the cell, generating oxidative stress which is responsible for its toxicity, affecting all living organism. A balance in the redox state of the cell to maintain cellular integrity and metabolism is essential for organism tolerance. Thus, the antioxidant response of bacteria exposed to Cd was studied to understand the tolerance mechanism, and be able to develop a methodology to bioremediate contaminated soils. MDA and hydrogen peroxide contents and different enzymes activity of antioxidant system (SOD, CAT, GR and GST) of two strains from *Burkholderia* genus, one from a soil contaminated with Cd in high concentrations (strain SCMS54) and the other from soil without Cd (strain SNMS32) in two exposure time (5 and 12 h), were analyzed. Stress measurement (MDA and hydrogen peroxide content) and antioxidant enzyme activities (SOD, CAT, GR and GST) increased in almost all treatments in the presence of Cd. These results also indicate that strain SCMS54 (isolated from Cd contaminated soil) presents a higher metabolic diversity and plasticity due the expression of more isoforms of the enzymes SOD, CAT and GR. The strain also accumulates 50% more Cd. We also analyzed the response to Ni of these two strain, observing a similar response to Cd, except for GST enzyme expression, which in strain SCMS54 this enzyme was induced in the presence of Ni, indicating that this enzyme can be essential on Ni tolerance. After that, the strain isolated from Cd contaminated soil (SCMS54) was selected to proceed the studies to evaluate the benefits of tolerant microorganism-tomato plant interaction. The use of plants to remove heavy metals from contaminated soilhas less impact and a lower cost. Soil microorganisms can be able to solubilize or mobilize soil metals acting also as bioremediator. Besides the high tolerance to Cd, the strain SCMS54 can produce indole-acetic acid (IAA), solubilize inorganic phosphate and produce siderophore, revealing its potential in plant-microorganism mutual and beneficial interaction. When interacting with tomato plants exposed to Cd, this bacterium led to decrease in plant peroxide concentration and chlorosis levels, promoted relative plant growth and reduced the root absorption of Cd resulting in an increase in plant tolerance to this highly toxic heavy metal. Indicating that inoculation of tomato plants with *Burkholderia* sp. SCMS54 promotes better growth when cultivated in the presence of Cd by a mechanism that appears to decrease Cd concentration in roots as a result of a bacterial-plant root beneficial interaction.

Keywords: Heavy metal; Oxidative stress; Plant Growth Promoting Bacteria (PGPB)

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1 INTRODUCTION

Metals and metalloids with atomic specific mass greater than 5 g cm^{-3} are classified as heavy metals. Most of these metals are present in the environment as trace elements, but in high concentrations they can be toxic to all living organisms (BHARGAVA et al., 2012). The presence of heavy metals have increased in the environment due to agriculture intensification (using chemical fertilizers and pesticides) and industry residues (MATSON et al., 1997).

Since heavy metals present high toxicity to plants, animals and humans, it is essential to remediate contaminated soil to enable the conduction of agronomical crops. Having this in view, there are different available methods: (i) phytoremediation, using hyperaccumulatorplants that absorb metal from soil, allowing, subsequently, the growth of non-tolerant plants (CHANEY et al., 1997); (ii) biosurfactants produced by fungi and bacteria,whichincludes peptides, glycolipids, glycopeptides, fatty acids and phospholipids, that do not bind to soil particles, but complex metals with high affinity, not allowing plants to absorb these complexes (HAZEN; TABAK, 2005); (iii) biolixiviation that consists in washing the soil, removing soil solution metals and part of these metals adsorbed on clay soil (GADD, 2004; SEIDEL et al., 2004); and (iv) organic matter, such as sludge biosolids or vermiculite that bind the metallic cation, turning into a complex compound in the soil, remaining not available to plants (KUKIER et al., 2010).

Phytoremediation is the technique with less impact and at a lower cost, than other processes such as soil excavation or soil washing. It is carried out *in situ* reducing exposure to pollutants to human health, being an environmental friendly technology. The requirements for an ideal plant for phytoremediation are fast growth, tolerance to the contaminants and high biomass production. In order to enhance phytoremediation, it is important to know the biological processes involved, such as plant-microbe interaction, plant translocation and uptake, and tolerance mechanisms (PILON-SMITS, 2005).

Microorganisms cooperate with the efficiency of phytoremediation techniques (PILON-SMITS, 2005) and they are also responsible for biosurfactants production. In this way, it is important to understand Cd tolerance in microorganisms. According to Haferburg and Kothe (2007), there are four main tolerance mechanisms to heavy metals in bacteria (Figure 1): (i) biosorption, the metal binds to bacterial cell wall, becoming not available; (ii) intracellular sequestration, the metal is chelated to a compound inside of the cell; (iii) efflux transporter, the metal is expelled from of the cell by the membrane pump (ZHANG et al., 2008); and (iv) extracellular chelation, a chelating compound is pumped out of a bacterial

cell, complexing the metal, turning it unavailable to living organisms.An example is the siderophore molecule (DIMKPA et al., 2009; SINHA; MUKHERJEE, 2008).

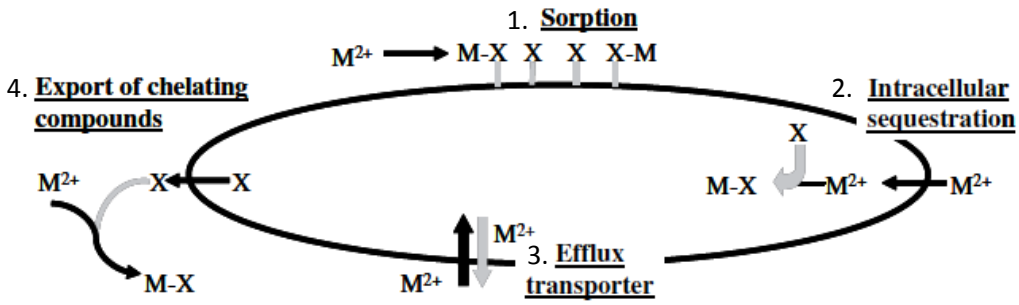

Figure 1.1 - Overview of bacteria resistance mechanisms (HAFERBURG; KOTHE, 2007)

Heavy metal toxicity is also attributed to the metal capacity of inducing reactive oxygen species (ROS) (GRATÃO et al., 2005). Therefore, besides these four tolerance mechanisms listed above, an efficient antioxidant system is essential for Cd tolerance, since Cd induces the generation of ROS, damaging lipids, proteins and DNA (THORSEN et al., 2009).

Genomic and Proteomic studies have shown that in the presence of Cd there is an increase in antioxidant gene expression (e.g. *sod* and *gst*) in *Caulobacter crescentus* (HU et al., 2005) and of proteins related to oxidative stress in *Pseudomonas putida* (MILLER et al., 2009). Moreover, Harrison et al. (2009), using different *E. coli* mutants, showed that genes related to antioxidative stress (*sodAB, gor and gsh*) are involved in the tolerance of different metals, reaffirming that Cd tolerance is due an efficient antioxidant response.

Most studies of antioxidant enzymes in bacteria are based in genomic analysis. There are few studies that measure bacteria enzyme activity (EERD et al., 2003). It is necessary to perform biochemical studies to determine the role of these enzymes in metal tolerance. The present study began due to soil contamination in a coffee crop area at University of São Paulo (Esalq), Piracicaba, SP, Brazil. The soil was intentionally contaminated with Cd, nickel (Ni) and zinc (Zn) in previous experiments, and the high concentrations of Cd in these areas lead to the death of coffee plants.

Figure 1.2 - Coffee crop at University of São Paulo(Esalq): (A) non contaminated and (B) contaminated soil with Cd

Therefore, the aim of this research was to study Cd tolerance mechanisms in microorganisms from the contaminated area in order to obtain more information about the soil microrganisms and potential use in soil phytoremediation. Tolerant bacteria from non contaminated and Cd contaminated areas were isolated and identified. We selected bacteria tolerant to various metals, that additionally bioaccumulate Cd. The selected bacteria were used as a model to describe antioxidant response in the presence of Cd, in order to understand the bacterial defense mechanisms under such stress. To verify the potential use in bioremediation of contaminated soils, one tolerant strain was also inoculated on Cd-sensitive tomato plants in the presence of Cd to evaluate bacteria-plant-Cd interaction.

It is important to emphasize that this thesis is one of the first thesis of the International Cellular and Molecular Plant Biology graduate program, which has collaboration with The Ohio State University, Columbus, Ohio, USA and The State University of New Jersey (Rutgers), New Brunswick, New Jersey, USA. Therefore, part of the work was performed at Dr. Robert Tabita's Laboratory (co-adviser) during an internship in Ohio. The research Project developed abroad was not closely related to the thesis theme; however it was essential to work at a distinguished laboratory, whose main research line is bacterial ribulose biphosphate carboxylase/oxygenase (RubisCO). The student was able to learn different bacterial cultivation methods and a number of molecular technics, besides the rich cultural experience that was of great value, not only with North Americans, but also with citizens from all over the world.

The internship project aimed to find new environmental RubisCO (Form I, II or IV), in order to understand their differences and improve efficiency, that would have high carboxylation rates (V_{CO2}), high oxygen tolerance, that happens with high CO_2 specificity

(high $S_{C/O}$) and a high affinity for CO_2 (low Km). Even not directly related, previous research from Azevedo's research group (PIOTTO, 2012) showed that the presence of Cd in tomato plants represses significantly the RubisCO protein expression. The used technics could be applied to find an environmental RubisCO enzyme that would maintain its expression in the presence of Cd, enabling the increase of production (by improving photosynthesis) of a tolerant plant.

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2 ANTIOXIDANT ENZYMES AFFECTS *Burkholderia* sp. CADMIUM TOLERANCE

Abstract

Cadmium (Cd) is a heavy metal that has contaminated soils in high concentration due to the increased use of chemical fertilizers and pesticides. This metal affects microorganisms, plants and humans, causing different damages to the cell, generating oxidative stress. A balance in the redox state of the cell to maintain cellular integrity and metabolism is essential for organism tolerance. Thus, antioxidant response of bacteria exposed to Cd was studied to understand the tolerance mechanisms, enabling the development of a bioremediation methodology for contaminated soils. We quantified MDA (malondialdehyde), hydrogen peroxide content and different enzyme activities of the antioxidant system (SOD, CAT, GR and GST) of two strains: one from a soil contaminated with Cd in high concentrations (strain SCMS54) and the other from soil without Cd (strain SNMS32) in two exposure times (5 and 12 h). Results indicate that strain SCMS54 (isolated from the Cd contaminated soil) presents a higher metabolic diversity and plasticity due the activity of more enzyme isoforms in SOD, CAT and GR.This strain also accumulates 50% more Cd. SOD was the only enzyme that had its activity increased in both strains in treatments in the presence of Cd. Moreover, SOD activity in a *E. coliΔsodB* (lacking Fe-SOD) assay (using mainly Mn-SOD), presented a higher hydrogen peroxide content with Cd. Therefore, the Fe-SOD isoform (constitutively expressed in *Burkholderia*sp.) presents an essential role in Cd tolerance.

Keywords: Superoxide dismutase; Catalase; Glutathione reductase; Heavy metal

2.1 Introduction

Cadmium (Cd) is a transition metal, atomic number 48, atomic weight 112.4 and specific mass 8.642 g.cm^{-3}. In soil it can be found as: ion (Cd^{2+}), complexed ($CdCl^{+}$, $CdOH^{+}$, $CdHCO_3^{+}$, $CdCl_3^{-}$, $CdCl_4^{2-}$, $Cd(OH)_3^{-}$ and $Cd(OH)_4^{2-}$) or chelated by organic compounds (KABATA-PENDIAS, PENDIAS, 2001).

Cd is used in fungicide manufacture, rubber treatment, pigments production as well as in batteries (JARUP, 2003; MOORE; RAMAMOORTHY, 1984). Furthermore, this metal can be added to the soil through phosphate fertilizers, pesticides, industrial and domestic effluents, which can lixiviate to the groundwater, can be absorbed by plants and ingested by humans (POMBO, 1995).

In soils, Cd affects microbe population in number and activity (BROOKES, 1995; KHAN et al., 2010) and plant growth, mainly in high concentrations (GRATÃO et al., 2005a; MOBIN; KHAN, 2007; WAHID; GHANI, 2008; GRATÃO et al., 2008; 2012; TEZOTTO et al., 2012). In plants, this metal affects water transportation, mitochondria oxidative phosphorylation, photosynthesis and chlorophyll content (VITÓRIA et al., 2006), as well as

inhibits cellular division, promotes chromosome aberration and induces pyknosis on root cells (DAS et al., 1997; PIZZAIA, 2013). All these alterations depend on metal concentration, plant species, organ, tissue and time length of exposure (BENAVIDES et al., 2005).

This toxicity occurs according to metal chemical and physical properties, that confer three main molecular mechanisms of metal toxicity: (i) production of reactive oxygen species (ROS) by auto-oxidation, (ii) blockade of functional groups essentials to a molecule and (iii) substitution of essential metallic ions in the molecule (SCHÜTZENDÜBEL; POLLE; ANDREA, 2002).

Heavy metals (HM), including Cd, generate oxidative stress in the cells and its toxicity is partially attributed to the metal capacity of inducing reactive oxygen species (ROS), damaging lipids, proteins and DNA (GRATÃO et al., 2005b; THORSEN et al., 2009). Tolerance occurs when there is a balance in the redox state of the cell maintaining cellular integrity and metabolism (GREEN; PAGET, 2004).

The first line of defense is superoxide dismutase (SOD, EC 1.15.1.1.) that is found in aerobic and facultative anaerobic organisms, acting on the detoxification of superoxide radical (O_2^{-}) in a dismutation reaction, generating hydrogen peroxide (H_2O_2) and O_2 (Figure 2.1) (ALSCHER et al., 1997; MCCORD; FRIDOVICH, 1969). SODs are classified in four groups, according to their cofactor: manganese (Mn) SOD-A, iron (Fe) SOD-B, copper/zinc (Cu/Zn) SOD-C and nickel (Ni) SOD (KEITH; VALVANO, 2007). Next, catalase (CAT, EC.1.11.1.6.) has a crucial role removing hydrogen peroxide (Figure 2.1) generated as product of aerobic cellular respiration (GRATÃO et al., 2005a).

Glutathione (GSH) is described as the compound responsible for the main cellular detoxification, including Cd in*Escherichia coli* (MASIP et al., 2006) and in *Candida tropicalis* 2222 (REHMAN; ANJUM, 2011). Besides binding to toxic molecules, the reduced glutathione (GSH) is also responsible for scavenging of ROS (mainly $O_2^{-\cdot}$ and H_2O_2) (SCRUTTON et al., 1987), these reactive compounds oxidize glutathione, inactivating molecules. Glutathione reductase is the enzyme that reduces oxidized glutathione (GSSG) using NADPH (Figure 2.1) (GRATÃO et al., 2005b).

GSH is also a thiol donor to the glutathione S-transferase (GST; EC 2.5.1.18) enzyme, which catalyzes the conjugation of glutathione electrophilic group to different toxic hydrophobic compounds (Figure 2.1) (ALLOCATI et al., 2008). The GST enzyme is responsible for the detoxification process of xenobiotic and toxins (GHELFI et al., 2011). Studies that investigatethe GST function and its metabolic diversity present a great potential

to future biotechnological application in environmental decontamination (VUILLEUMIER; PAGNI, 2002).

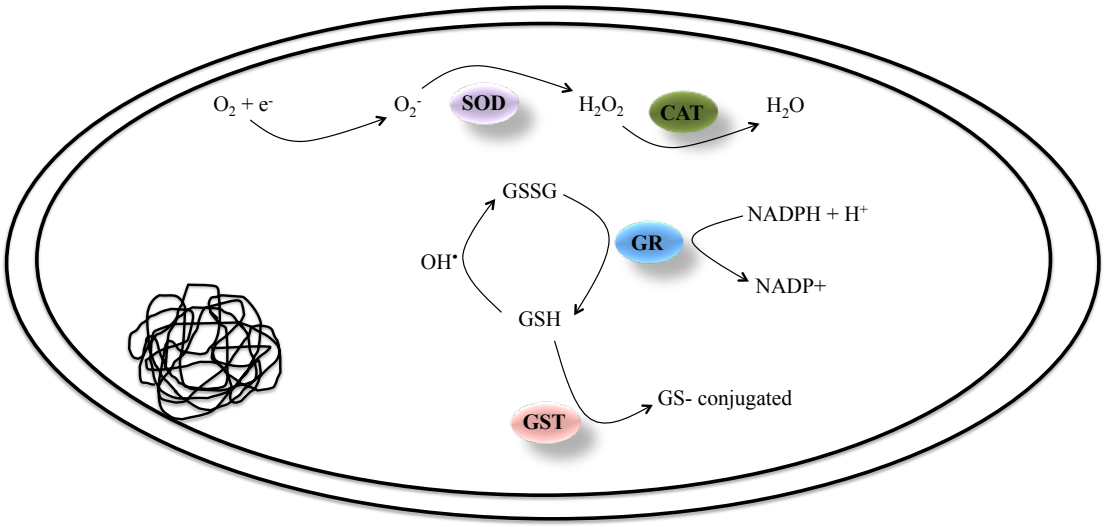

Figure 2.1- Illustrative scheme of the studied enzymes of antioxidant system in a cell. Superoxide dismutase (SOD): dismutase O_2-• radical generating H_2O_2; catalase (CAT): breaks H_2O_2 into H_2O; glutathione reductase (GR): reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH and glutathione s-transferase (GST): catalyzes the conjugation of reduced glutathione to different hydrophobic compounds

Genomic and proteomic studies show that the presence of Cd increases expression of genes (HU et al., 2005)and proteins (MILLER et al., 2009) related to oxidative stress system. Harrison et al. (2009) confirmed using different *E. coli* lacking important genes for redox homeostasis (*sod*AB*, gor and gsh*) that these tested genes are involved in tolerance to different metals. Most studies of antioxidant enzymes in bacteria are based on genomic or proteomic analysis, few studies measured the enzymatic activity in the bacteria. It is important to quantify the activity of the enzyme to verify if the transcript gene and protein are functional or not (EERD et al., 2003).

In this study, bacteria tolerant to Cd were isolated from Cd-contaminated and non Cd-contaminated soil, seeking for multi-tolerant strains able to interact positively with the plant in the phytoremediation process.The aim was to understand the bacterial tolerance to Cd-induced oxidative stress, characterizing the antioxidant response of these bacteria exposed to the heavy metal Cd, identifying which enzymes are essential in Cd tolerance.

2.2 Material and Methods

2.2.1 Soil sampling

Soil samples were collected at 10 cm depth in the projection of the coffee tree, near the roots.The soil is a clay latosolic eutroferric alfisol of an artificially Cd-contaminated coffee experimental field at the University of São Paulo, Piracicaba, Brazil (23°51´16"S/46°08´19"W). Around each coffee tree (2 m^2) 45 and 90 $g.kg^{-1}$of Cd were applied to the soil as a sulphate salt[1].Three replicates of soil samples were dried and weighed, and Cd was sequentially extracted by H_20, $Mg(NO_3)_2$, DTPA, NaOH and nitric acid (MANN; RITCHIE, 1993). The Cd contents were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).

2.2.2 DNA extraction of environmental sample

Soil DNA was obtained from 0.5 g of soil. Soil DNA was isolated according to the kit MoBio UltraCleanTM soil DNA kit (MoBio Laboratories, USA). The DNA extraction and integrity were checked in 1% agarose gel (*w/v*).

2.2.3 PCR-DGGE (Denaturing Gradient Gel Electrophoresis)

PCR-DGGE profile was used in order to analyze bacteria diversity. The amplification of 16S rRNA from total DNA was performed with a semi-nested PCR. For the first PCR, we used 20 ng of soil DNA and primers 2F and R1387, submitted to 25 cycles of 94ºC for 30 s, 62.5ºC for 1 min and 72ºC for 1 min. The second PCR was performed using 20 ng of the first PCR. In a total volume of 50 µL, 400 nM of universal primers U968_GC and R1387 were used in similar conditions of the first PCR, except for the annealing temperature at 55ºC with 35 cycles. PCR products were analyzed by agarose gel electrophoresis (*w/v*) in TBE buffer 0.5X.

PCR-DGGE were performed as previously reported by Muyzer, Wall and Uitterlinden(1993). The PCR products wereplaced on apolyacrylamide gel 6% (*w/v*) in TAE buffer 0.5 X. The polyacrylamide gel presented a denaturant gradient of 45 to 65 % (in which 100% of denaturation contains 7 M of urea and 40% of formamide). The gel ran for 16 hours at 26 100 V and 60°C, and then it was stained with silver and photographed under white light.

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[1]See Tezotto et al. (2012) for full details about the experimental conditions adopted for coffee cultivation and soil contamination with Cd.

2.2.4 Bacteria isolation

For bacterial cell isolation, 1 g from two soil samples of Cd contaminated soil (90 $mg.dm^{-3}$of Cd) and non Cd contaminated soil (control) (TEZOTTO et al., 2012) it was added to sterile phosphate-buffered saline (PBS, containing ($g.L^{-1}$) Na_2HPO_4, 1.44; KH_2PO_4, 0.24; KCl, 0.20; NaCl, 8.0; pH 7.4) 1:100 (w:v) and maintained at 28°C under 150 rpm agitation for 1 hour. Appropriate dilutions were subsequently plated onto Minimal Media (DWORKIN; FOSTER, 1958) supplemented with 128 µM of $CdCl_2$. Plates were incubated at 28°C for 10 days and the number of colony forming units (CFU) were determined at the end of this period. Following colony purification; single colonies were suspended in Nutrient Broth containing 20% glycerol solution and stored at -80°C.

2.2.5 Selection and identification of bacteria lines tolerant to multiple heavy metals

All 33 Cd tolerant bacteria isolated from the coffee grown soil were assessed for growth on Nutrient Broth medium supplemented separately with three different HM: Cd (1.28 and 5.00 mM $CdCl_2$), Zn (12.50 and 15.00 mM $ZnCl_2$) and Ni (4.36 and 6.00 mM $NiCl_2$). All isolates were exposed to each tested HM and the experiments performed in triplicate. The plates were incubated for three days at 28°C. The isolatesgrowth was assessed in a comparative form to the control treatment (no metal) and classified as tolerant or not.

After HM selection, two isolates were selected, one from the control soil and another from Cd-contaminated soil. DNA of these isolates was extracted as reported by Araújo et al. (2002) and a partial sequence of the 16S rRNA gene was amplified with primers R1387 (5' – CGGTGTGTACAAGGCCCGGGAACG - 3') (HEUER et al., 1997) and P027F (5' – GAGAGTTTGATCCTGGCTCAG - 3') (LANE et al., 1985). PCR were performed in 50 µL reaction mixture containing 1 X enzyme buffer, 3.75 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer and 0.1 $U.\mu L^{-1}$ of Taq DNA Polymerase (Invitrogen, Brazil). An initial denaturation was carried out at 94°C for 4 min, followed by 35 thermal cycles of 30 s at 94°C, 1 min at 62.5°C and 1 min at 72°C, with a final extension performed at 72°C for 7 min. PCR amplicons were checked by electrophoresis on agarose gel (1.5% *w/v* agarose) and visualized by an UV apparatus for ethidium bromide stained gels. For bacterial identification the 16S rRNA gene PCR products were purified with polyethylene glycol (PEG) (20% PEG 8000; 2.5 mM NaCl) and sequenced with R1387 primer in the 377 DNA sequencer (ABI, USA). The sequences were evaluated in BLASTn (National Center for Biotechnology Information website) against the database of the GenBank.

2.2.6 Sequence analysis

Firstly, all the electropherograms were trimmed for high quality bases (80% of bases with quality > 20) at Phred software and the trimmedsequences were used for comparison in the Ribosomal Data Project (16S rRNA gene). After the phylogenetic affiliation of the sequences, the best hits of well-characterized strains were retrieved from the databases and used for alignment and phylogeny analysis. The evolutionary history was inferred using the Neighbor-Joining method (SAITOU; NEI, 1987), using MEGA software version 4.0 (TAMURA et al., 2007).

2.2.7 Nucleotide sequence accession numbers

A total of 29 DNA sequences of the partial 16S rRNA genes were deposited in the GenBank database under accession numbers KF002971 to KF002999.

2.2.8 Bacterial strains

Burkholderia sp. strains SNMS32 and SCMS54 were isolated from the coffee crop soil. SNMS32 was isolated from Cd non contaminated soil and SCMS54 from a Cd contaminated soil (with 1.28 mM of Cd).

Escherichia coli BW25133 (also described as K-12 strain) and simple mutation strains were used from "KEIO Knockout Collection" (BABA et al., 2006) gently given by Dr. Michael Ibba (Ohio State University). The mutant strain *E. coli* Δ*sod*A and Δ*sod*B had its genes replaced by kanamycin resistance gene by *rep swat* method (DATSENKO; WANNER, 2000).

2.2.9 Growth determination

Bacterial growth was monitored by measuring the colonyforming unit (CFU) per mL, as reported by Sangali and Brandelli (2000). *Burkholderia* sp. strains were grown in 50 mL of nutrient medium in three treatments: 0.3 mM; 1.28 mM and without Cd (control), both related to concentrations found in contaminated soil with coffee crop. The bacteria were incubated on a rotary shaker at 150 rpm, at 30ºC, for 36 h. *Escherichia coli* strains were grown in 50 mL of LB, in the absence and presence of Cd (0.3 mM), and incubated on a rotary shaker at 150 rpm at 37ºC for 12 h. The bacterial suspension was diluted and samples (100 μL) were loaded in triplicate onto nutrient agar plates. After 24 hours of incubation at 37ºC, the CFU number was recorded.

2.2.10 Cd biosorption

The biosorption assay was carried out as reported by Kanazawa and Mori (1996) with modifications. Bacteria were grown in 100 mL of nutrient broth until reaching optical density 1 at 600 ηm. Then, the cells were separated by centrifugation at 8,000 g for 15 min, and the bacterial pellet was washed twice with phosphate buffered saline [PBS, containing ($g.L^{-1}$) Na_2HPO_4, 1.44; KH_2PO_4, 0.24; KCl, 0.20; NaCl, 8.00; pH 7.4]. The collected biomass was incubated for 5 h (end of adaptation period) and 12 h (stabilization period) at 28°C in Minimal Media Dworkin and Foster (1958) pH = 5.3 with 0.3 mM and 1.28 mM Cd. The suspension was then centrifuged at 8,000 g and filtered through a 0.22-µm Millipore membrane to separate the biomass from the filtrate. The biomass was dried, weighed, and heavy metals were extracted by nitric-percloric acid solution (5:1) digestion (24 h) (AZCÓN et al., 2010; VIVAS et al., 2005). The Cd contents were determined by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES).

2.2.11 Stress measurements (lipid peroxidation and hydrogen peroxide content)

Lipid peroxidation was determined by estimating the content of the thiobarbituric acid reactive substance (TBARS) as reported by Heath andPacker (1968). The malondialdehyde (MDA) was quantified by measuring the absorbance at 535 and 600 ηm with a Perkin Elmer Lambda 40 spectophotometer, and the concentration calculated using an extinction coefficient of 155 $mM.cm^{-1}$.

The content of H_2O_2 was determined according to Alexieva et al. (2001). Bacterial cells were homogenized in 0.1% (*w/v*) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,100 g, 15 min, 4°C, and 200 µL of the supernatant was added to200 µL of 100 mM potassium phosphate buffer (pH 7.0) and 800 µL of 1 M KI. The absorbance was read at 390 ηm. H_2O_2 content for allsamples was determined using H_2O_2 as a standard.

2.2.12 Protein extraction and quantification

Liquid cultures were centrifuged at 12,000 g for 15 min at 4°C and the pellets macerated with liquid nitrogen. The extracts were homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol (DTT) and 5% (w/w) polyvinylpolypyrrolidone (PVP) (GRATÃO et al., 2008; MARTINS et al., 2011). The homogenates were centrifuged at 12,000 g for 30 min and the supernatants were stored in separate aliquots at -80°C for further biochemical analysis. The protein concentration in all the samples was determined by the method of Bradford (1976).

SDS-denaturing and non-denaturing polyacrylamide gel electrophoresis (PAGE) were carried out as reported by Laemmli (1970) modified by Gratão et al. (2008). Equal amounts of protein were loaded on to each lane.

2.2.13 SOD total activity determination (SOD, EC 1.15.1.1)

SOD activity was performed according to Giannopolitis and Ries (1977), adding 50 µL of protein extract to a mix of 5 mL with sodium phosphate buffer 50 $mmol.L^{-1}$, pH 7.8, containing methionine 13 $mmol.L^{-1}$, NBT 75 $\mu mol.L^{-1}$, EDTA 0.1 $mmol.L^{-1}$, riboflavin 2 $\mu mol.L^{-1}$. The reaction was performed at 25^{o}C in a chamber (covered in aluminum foil) with a fluorescent light of 15 W, after 5 min of light exposure, light was turned off and blue formazane compound, produced by NBT photoreduction was measured at 560 ηm. The negative control was a sample with all reagents (but without protein extract) maintained in dark for the same period of time and was subtracted from all samples that received light.

2.2.14 SOD activity staining

SOD activity staining was carried out as reported by Beauchamp and Fridovich (1971) modified by Medici et al. (2004). After non-denaturing-PAGE, the gel was rinsed in distilled-deionized water and incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium, and 0.3% N,N,N´,N´-tetramethylethyllenediamine. One unit of bovine liver SOD (Sigma, St. Louis, USA) was used as a positive control of activity. After 30 min, the gels were rinsed with distilled–deionized water and then illuminated in water until the development of achromatic bands of SOD activity on a purple-stained gel. In order to classify SOD isoenzymes from the different bacterial strains, samples were subjected to non-denaturing PAGE and the SOD bands classified as reported by Guelfi et al. (2003). SOD isoforms were distinguished by their sensitivity to inhibition by 2 mM potassium cyanide (KCN) and 5 mM hydrogen peroxide (H_2O_2).

2.2.15 CAT total activity determination (CAT, EC 1.11.1.6)

CAT activity was assayed as reported previously by Kraus, Mckersie and Fletcher (1995) modified by Gratão et al. (2008) at 25^{o}C in a reaction mixture containing 1 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 2.5 µL H_2O_2 (30% solution). The reaction was initiated by the addition of 25 µL of protein extract and the activity determined

by following the decomposition of H_2O_2 as a change in absorbance at 240 ηm in a spectrophotometer.

2.2.16 CAT activity staining

CAT activity following non-denaturing PAGE, was determined as reported by Woodbury, Spencer and Stahman (1971) modified by Ferreira et al. (2002). Gels were incubated in 0.003% H_2O_2 for 10 min and developed in a 1% (*w/v*) $FeCl_3$ and 1% (*w/v*) $K_3Fe(CN_6)$ solution for 10 min. One unit of bovine liver CAT (Sigma, St. Louis, USA) was used as a positive control of activity. The relative intensity of the stained bands was determined using an ImageScanner (Amershan Bioscience) and the Shortcut program.

2.2.17 GR total activity determination (GR, EC 1.6.4.2)

GR activity was assayed as reported previously by Smith, Vierheller and Thorne (1988) at 30^{o}C in a mixture consisting of 1.7 mL 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 1 mM oxidized glutathione (GSSG) and 0.1 mM NADPH. The reaction was started by the addition of 50 µL of protein extract. The rate of reduction of oxidized glutathione was followed in a spectrophotometer by monitoring the change in absorbance at 412 ηm for 1 min.

2.2.18 GR activity staining

GR activity following non-denaturing PAGE, was determined as reported by Rao, Paliyath and Ormrod (1996) with modification by Medici et al. (2004). The gels were rinsed in distilled-deionized water and incubated in the dark for 30 min at room temperature in a reaction mixture containing 250 mM Tris (pH 7.5), 0.5 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*- tetrazolium bromide (MTT), 0.7 mM 2,6-dichloro-*N*-(4-hydroxylphenyl)-1,4-benzoquinoneimine sodium salt (DPIP), 3.4 mM GSSG and 0.5 mM NADPH. One unit of bovine liver GR (Sigma, St. Louis, USA) was used as a positive control of activity.

2.2.19 GST total activity determination (GST; EC 2.5.1.18)

GST activity was assayed spectrophotometrically at 30ºC in a mixture containing 900 µL 100 mM potassium phosphate buffer (pH 6.5), 25 µL 40 mM 1-chloro-2,4-dinitrobenzene (CDNB), 50 µL 1 mM GSH and 25 µL enzyme extract. The reaction mixture was followed by monitoring the increase absorbance at 340 ηm over 5 min (ZABLOTOWICZ et al., 1995). GST activity is expressed as $\mu mol.min^{-1}.mg^{-1}$ protein.

2.2.20 Statistical analysis

Enzyme activity determinations were performed on three replicates for each treatment, and the significance of the observed differences was verified using a one-way analysis of variance (ANOVA) followed by Duncan's test. Differences with a p-value inferior to 0.05 were considered significant. All statistical analysis were carried out using the software R program, version 2.15.1.

2.3 Results

The maintenance of soil physical-chemical, biological and microbiological integrity is essential to productive crops (VAL-MORAES et al., 2009). In order to minimize contamination of soil and hydric resources, this study aimed to understand the genetic and physiology of tolerant soil microorganisms, to develop and optimize bioremediation techniques.

2.3.1 Soil Cd content

Cd was found in both soils; in control soil (without added Cd) 1.46 $mg.kg^{-1}$ of Cd was detected, while in soil with applied Cd, 54.87 $mg.kg^{-1}$ of Cd were detected, which represents around 60% of the initially applied Cd (90 $mg.kg^{-1}$). In this contaminated soil, around 30% of the Cd is exchangeable, 30% is bound to organic matter and 30% bound to oxides/clays (Table 2.1).

Table 2.1- Soil Cd quantification in control and contaminated soil

Added to Soil	Cd quantification ($mg.kg^{-1}$ soil)					
$mg.kg^{-1}$ Cd	Soluble in water	Exchangeable	Bound to organic matter	Bound to oxides/clays	Residual	Total
0	0.09	0.08	0.49	0.07	0.73	1.46
45	0.18	4.77	8.33	11.53	3.10	27.91
90	0.77	17.13	17.72	16.42	2.83	54.87

2.3.2 Microbial diversity in Cd contaminated and non contaminated soils

The non-cultivable microbial diversity was analyzed by the DGGE profile (Figure 2.2 A). There was a change in the bacterial community in the presence of 45 and 90 $kg.ha^{-1}$ of Cd, proving that in the same area, the presence of Cd changes microbial community, acting as a

selective agent (Figure 2.2 B). Multivariate analysis showed that the bacterial community of the control soil (0 Cd) and soils with 45 and 90 kg.ha^{-1} of Cd are different from each other, indicating that the doses also influences bacterial selection. The high stress caused by this heavy metal could induce a decrease in bacterial biomass and microbial diversity, and the DGGE profile showed that there is not anunbalance in contaminated soil, but there is a selection of tolerant microorganisms.

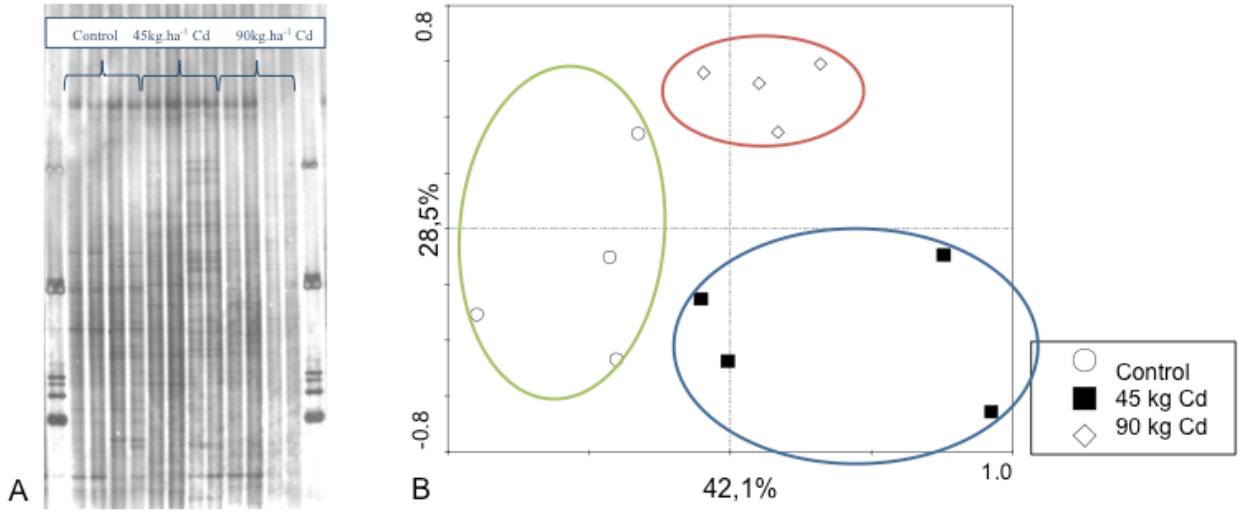

Figure 2.2- (A) DGGE fingerprint in non-contaminated (control) and contaminatedsoil with Cd (45 and 90 kg.ha^{-1}of Cd). (B) Multivariate analyzes of contaminated and non-contaminated soil

A total of 27 bacteria were isolated and purified from both soils: control and contaminated with the highest concentration of Cd (90 kg.ha^{-1}).After that, all 27 isolates had part of 16S rRNA gene sequenced (around 400 bp) and a phylogenetic tree was built (Figure 2.3).

Eight different genera were found in both soils. Three genera belong to Actinobacteria phylum (*Arthrobacter, Leifsonia* and *Mycobacterium*) and five to Proteobacteria phylum (*Methylobacterium*, *Bosea*, *Burkholderia*, *Pantoea* and *Pseudomonas*). Most genera were isolated from both contaminated and non Cd contaminated soils. However, *Bosea* were isolated exclusively from control soil, despite of this isolate being tolerant to Cd since it was isolated in Minimal Media with Cd. *Artrobacter* and *Mycobacterium* were isolated only in Cd contaminated soil. However, our soil samples were not large enough to affirm which genera are exclusive to Cd contaminated or non Cd contaminated soils. This soil bacterial isolation was performed in order to obtain tolerant isolates, not to study microbial cultivable diversity.

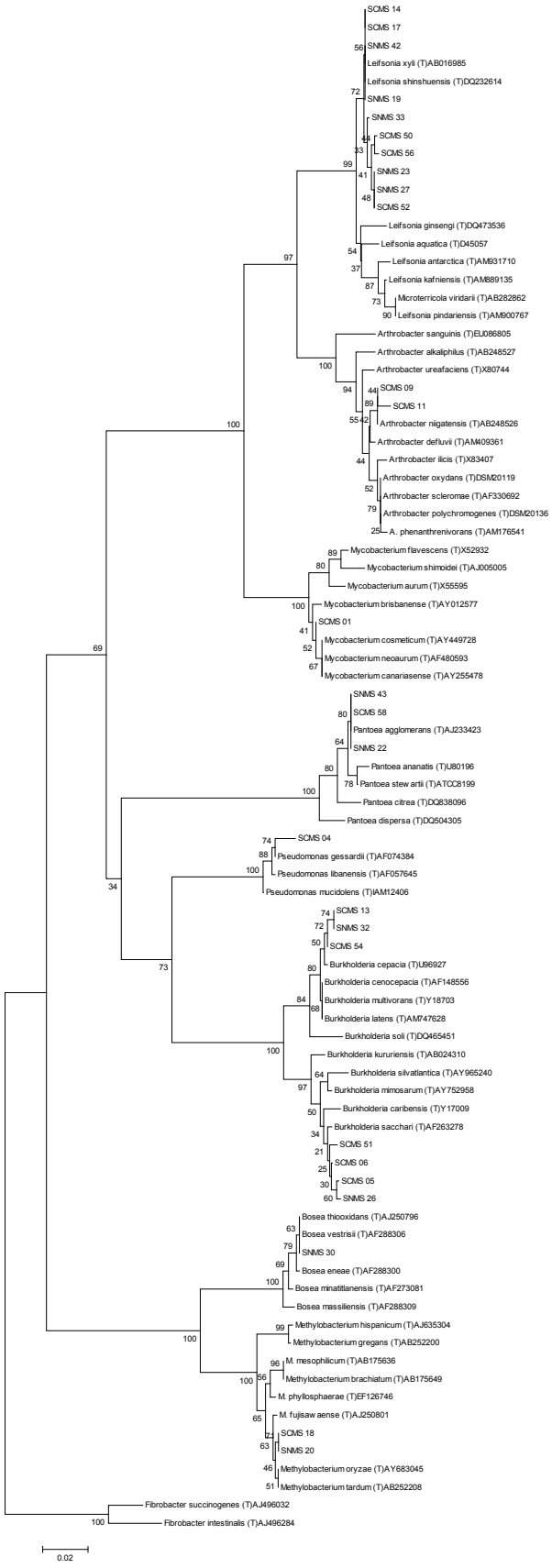

Figure 2.3- Phylogenetic analyses of 16S rRNA gene with sequences available in RDP Query database, using *Neighbor-joining*. Bootstrap values (1,000 replications). There were a total of 400 nucleotide positions in the final dataset, and *Fibrobacter* served as outgroup

2.3.3 Selection of microorganisms tolerant to multiple metals

All isolates were tested for Cd, Ni and Zn tolerance, in two different concentrations of each metal (Figure 2.4). With this tolerance tested we observed that Ni was the most toxic metal, followed by Cd, and then Zn. Ni inhibited growth in 75% of isolates. In soils, the contamination of different metals occurs simultaneously, for this reason we selected isolates tolerant to all tested metals in the highest tested concentration.

Thereby, two isolates multi-tolerant to all tested metals were isolated: SCMS54 (isolated from Cd contaminated soil) and SNMS32 (isolated from control soil), both classified, by 16S rRNA gene sequence and phylogenetic tree as *Burkholderia* sp. (Figure 2.3).

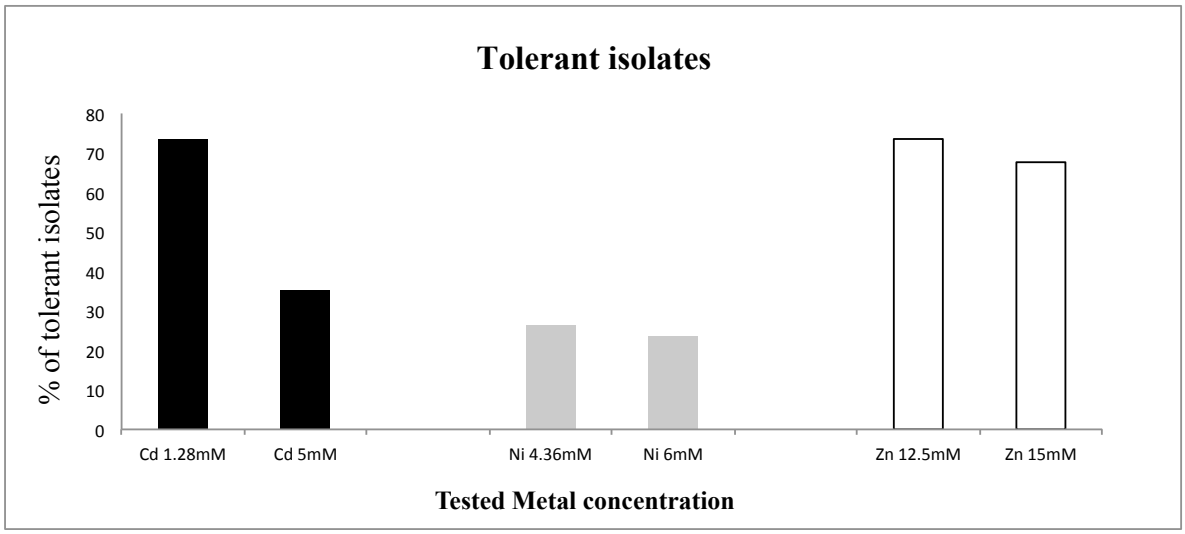

Figure 2.4- Percentage of isolates tolerant to three tested metals (Cd, Ni and Zn) in two different concentrations

2.3.4 Characterization of bacterial growth in the presence of Cd and Cd biosorption

Each strain isolated from contaminated (SCMS54) and non-contaminated (SNMS32) soils grew in the presence of 0.3 mM and 1.28 mM of Cd (the same Cd concentrations found in the coffee crop soil), in nutrient broth, were monitored for 32 hours (Figure 2.5 A and B).Each bacterium was isolated under different conditions. However, both strains presented a similar growth curve, (even in the presence of Cd) decreasing their growth at 0.3 mM of Cd, and almost not growing at 1.28 mM Cd. The main difference is that it seems that SCMS54

(from contaminated soil) with 0.3 mM of Cd is still growing after 32 h, while strain SNMS32 (in treatment and time) is already in stationary phase.

For Cd biosorption, the increase of Cd concentration results in an increase of Cd biosorption in both strains. Besides that, the strainSCMS54 (from Cd contaminated soil) showed 50% higher Cd bioaccumulation in both times and concentration, when compared to SNMS32 strain (Figure 2.5 C and D).

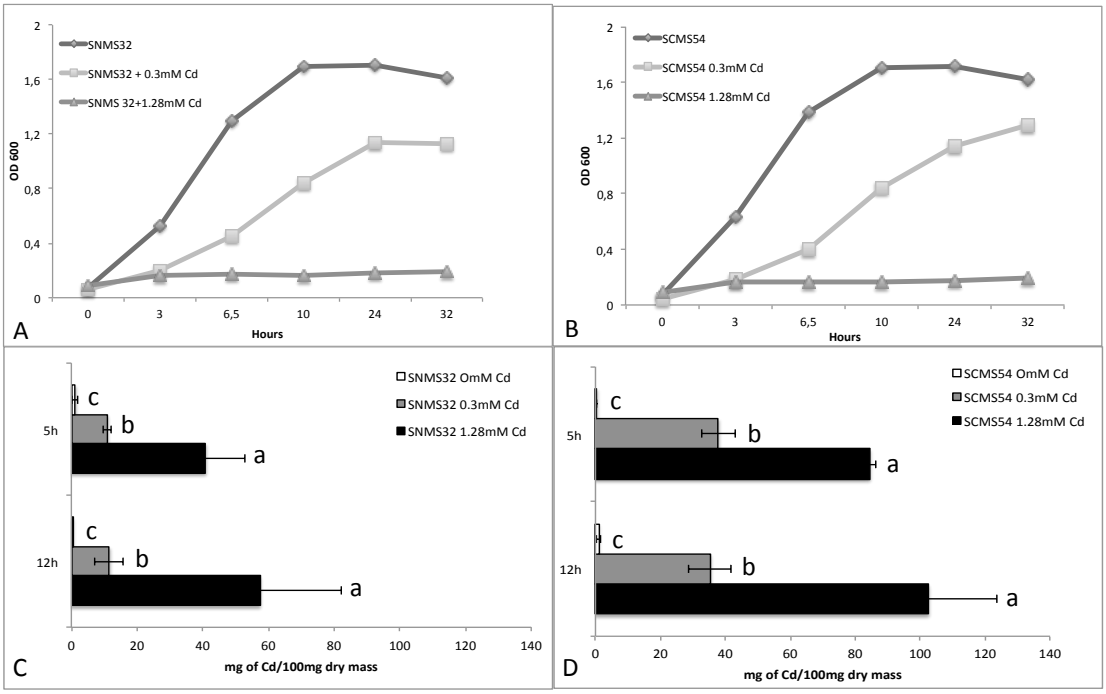

Figure 2.5- (A and B) Bacteria growth curve,(C and D) Bacterial biosorption of Cd with the same Cd concentrations of Esalq/Usp coffee crop soils: 0; 0.3 mM and 1.28 mM of Cd. (A and C) *Burkholderia* sp. strain SNMS32; (B and D) *Burkholderia* sp. strain SCMS54

In the SDS-denaturant gel we did not observe significant difference inthe protein profile for treatments with and without Cd and between 5 h and 12 h of Cd exposure. However, strains SCMS54 and SNMS32 present a different protein profile (Figure 2.6).

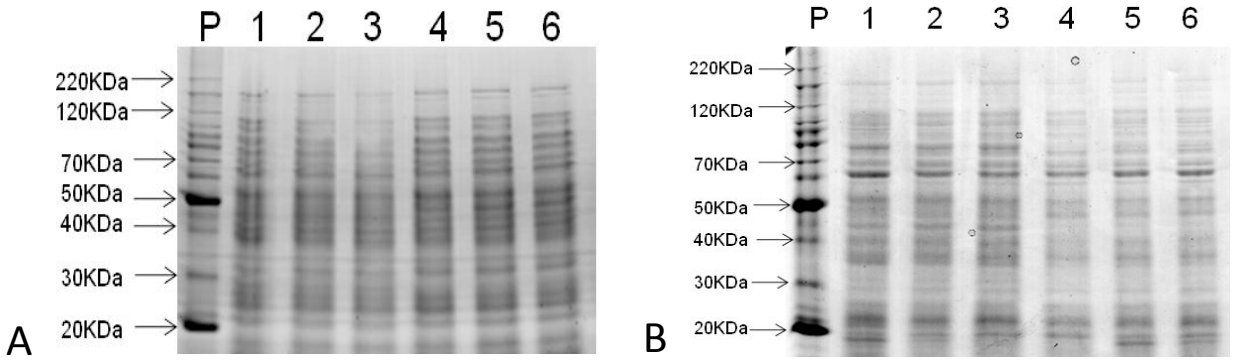

Figure 2.6- Protein profile in SDS-denaturing gel. (A) *Burkholderia* sp. strain SNMS32 and (B) *Burkholderia* sp. strain SCMS54. Lanes: 1. Strain + 0 mM of Cd (control); 2. Strain + 0.3 mM of Cd; 3. Strain + 1.28 mM of Cd (after 5 h Cd was added); 4. Strain+ 0 mM of Cd (control); 5. Strain + 0.3 mM of Cd; 6. Strain + 1.28 mM of Cd (after 12 h Cd was added)

2.3.5 Stress measurement (lipid peroxidation and hydrogen peroxide content)

To evaluate the direct effect of Cd toxicity in the antioxidant system, lipid peroxidation through MDA concentration (nmol.g^{-1} dry mass) and hydrogen peroxide content (μmol.g^{-1}) were measured. MDA content did not change in the presence of Cd in SCMS54 strain (Table 2.2), but increased in SNMS32 after 5 h in the presence of Cd. Hydrogen peroxide content was increased in the presence of Cd in 5 h and 12 h only in strain SNMS32, different from strain SCMS54 that decreased peroxide content with Cd (Table 2.2).

2.3.6 Effect of Cd on antioxidant enzymes activity of *Burkholderia*sp. strains

The first enzyme activated in the antioxidant system issuperoxide dismutase, which was up regulated in the presence of 0.3 mM Cd for SCMS54 after 5 h and 12 h of Cd exposure; while for the strain SNMS32, its activity increased only 12 h after Cd exposure (Table 2.2).

In non-denaturing gel, the two strains SNMS32 and SCMS54 showed different SOD isoenzyme profiles (Figure 2.7). Both strains presented a Fe-SOD, but only the strain SCMS54 exhibited a differential isoenzyme (Mn-SOD). When analyzed the treatments in the absence and presence of Cd, Fe-SOD isoform was present in both strains and all tested treatments, independent of the exposure time, showing that it is a constitutively expressed isoform. However, the Mn-SOD from SCMS54 strain exhibited differences in the band intensity, mainly between the 5 and 12 hours of Cd exposure.

For catalase enzyme total activity, SCMS54 strain showed increase in the activity after 5 hours in the Cd presence. However, after 12 hours, the same strain did not present any

statistical differences in CAT activity between tested treatments (Table 2.2). However, SNMS32 strain did not exhibit any difference between the control and the 0.3 mM Cd treatment for both times, but a decrease in CAT activity was observed when this strain was submitted to higher Cd concentration in both times (Table 2.2).

Agreeing with SOD activity in non-denaturing gel, two isoforms (II and III) (Figure 2.7) were found present in both strains in all treatments. In the strain SCMS54 an extra isoform (I) was found for all treatments with 5 h of Cd exposure (in both Cd treatments and control). Strain from the Cd contaminated soil (SCMS54) had a significant higher CAT activity at 5 h after Cd exposure, and after 12 h this activity decreased.

Glutathione reductase total activity was also increased only in strain SCMS54 after 12 h of Cd exposure, but did not present statistical difference (Table 2.2). Similar to the previously described enzymes, GR activity in non-denaturing gel showed the expression of six isoforms.Five were found in all treatments for both strains (I, II, III, V and VI): one isoform is exclusive of strain SCMS54 (IV) (Figure 2.6). Additionally, in strain SCMS54 isoform I is only present in 5 h after Cd exposure. Moreover, isoenzyme III is the most expressed isoenzyme, being constitutively expressed in both strains and in all treatments.

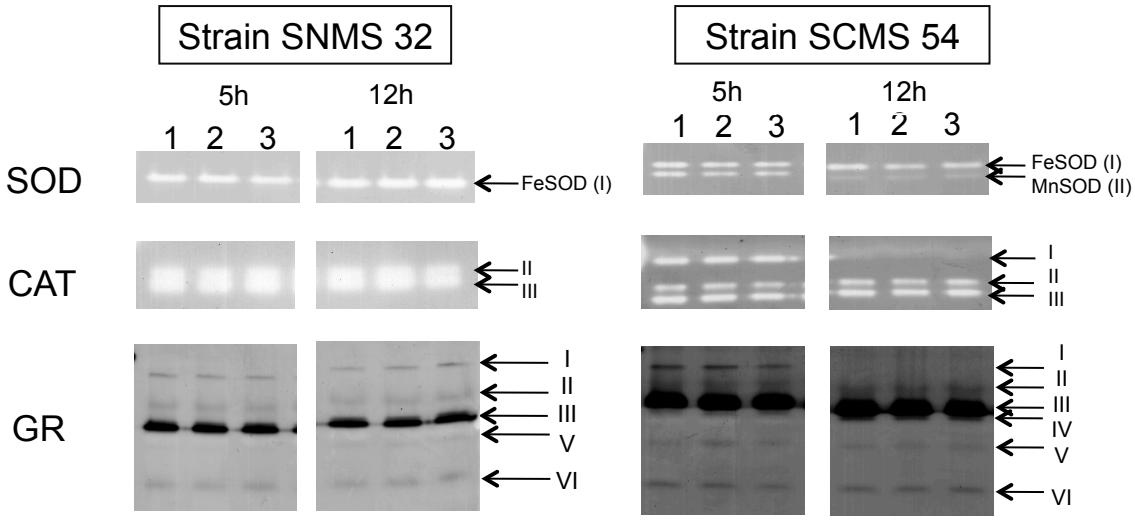

Figure 2.7- Enzymes activity (SOD, CAT and GR) in non-denaturing gel Lanes: 1. Strain+ 0 mM of Cd (control); 2. Strain + 0.3 mM of Cd; 3. Strain + 1.28 mM of Cd (after 5 h or 12 h Cd was added)

In the presence of Cd, GST activity in the strain isolated from non Cd contaminated soil (SNMS32) did not present statistical differences between the treatments, whereas for the Cd contaminated soil strain (SCMS54), GST activity decreased in treatments after 12h in the presence of Cd (Table 2.2).

Table 2.2- MDA, peroxide and antioxidant enzymes activity

Strain	Exposition time	Cd concentration	MDA mmol/g dry mass	Peroxid (µmol/g)	SOD units of SOD/mg	CAT	GR µmol/min mg	GST
		0	6.17±0.05 b	34.57± 1.91 b	7.63±0.27 a	21.23±0.37 a	0.175±0,037 a	0.0032 ±0.0006 a
	5h	0.3mM	4.65±0.02 b	40.49 ± 5.09 ab	6.88±0.60 ab	27.27±4.32 a	0.170±0.027 a	0.0041 ±0.0009 a
		1.28mM	8.56±0.07 a	67.42± 34.04 a	5.54±0.04 b	9.71±0.60 b	0.214±0.011 a	0.0044 ±0.0004 a
SNMS32								
		0	8.70±0.04 a	26.12 ± 7.83 b	4.36±0.17 c	20.05±0.87 a	0.144±0.035 a	0.0035 ±0.0004 a
	12h	0.3mM	5.74±0.09 b	48,41 ± 8.85 ab	8.37±0.18 b	24.22±1.20 a	0.174±0.039 a	0.0043 ±0.0005 a
		1.28mM	5.97±0.01 b	65.27 ± 10.12 a	10.75±1.09 a	17.76±8.19 b	0.203±0.073 a	0.0030 ±0.0007 a
		0	6.20±0.06 A	50.68± 0.43 A	2.97±0.544 b	56.11±7.21 B	0.125±0.032 A	0.0038 ±0.0007 A
	5h	0.3mM	7.71±0.29 A	41.03 ± 9.12 A	7.37±0.74 a	93.70±4.64 A	0.126±0.031 A	0.0031 ±0.0002 A
		1.28mM	9.51±0.20 A	12.62± 2.49 B	3.80±1.11 ab	104.21±1.71 A	0.118±0.015 A	0.0032 ±0.0003 A
SCMS54								
		0	11.21±0.15 A	13.22 ± 0.09 A	1.34±0.06 B	7.52±0.91 A	0.120±0.037 A	0.0054 ±0.0002 A
	12h	0.3mM	11.63±0.16 A	5.05 ± 1.79 B	4.08±0.58 A	9.29±0.34 A	0.132±0.021 A	0.0051 ±0.0005 A
		1.28mM	9.51±0.07 A	10.24 ± 4.02 A	3.27±0.84 AB	9.95±1.11 A	0.194±0.031 A	0.0009 ±0.0001 B

2.3.7 Effect of Cd on antioxidant enzymes activity of *Escherichia coli* mutants

Growth curve showed that Cd at the highest tested concentration (1.28 mM) inhibit significantly the growth of all tested *E. coli* (wild type, Δ*sod*A and Δ*sod*B) (Figure 2.8) similar to both *Burkholderia*sp. strain, while 0.3 mM of Cd delayed bacteria growth of all strains when compared to the control (without Cd), the wild type was less affected by Cd when compared to *ΔsodA* and *ΔsodB* (Figure 2.8), showing that SOD isoforms are important on bacterial Cd tolerance.

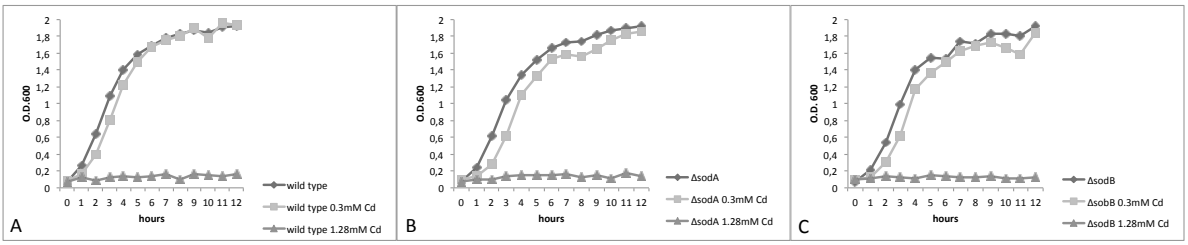

Figure 2.8- *E. coli* BW25133 growth curve of (A) wild type, (B) Δ*sod*A (lacking Mn-SOD) and (C) Δ*sod*B (lacking Fe-SOD) in the presence of 0; 0.3 mM and 1.28 mM of Cd

The mutations of *sodA* and *sodB* genes influenced the expression of other genes changing also the protein profile (in denaturing SDS-PAGE) (Figure 2.9 A). In *E. coli* wild type, Δ*sodA* and Δ*sodB* (in non-denaturing gel), the presence of 0.3 mM of Cd (when compared to the control) did not influence SOD isoform expression (Figure 2.9 B), however in both mutants Δ*sodA* (lacking Mn-SOD) and Δ*sodB* (lacking Fe-SOD) the expression *sodC* (Cu/Zn-SOD) decreased (Figure 2.9 B).

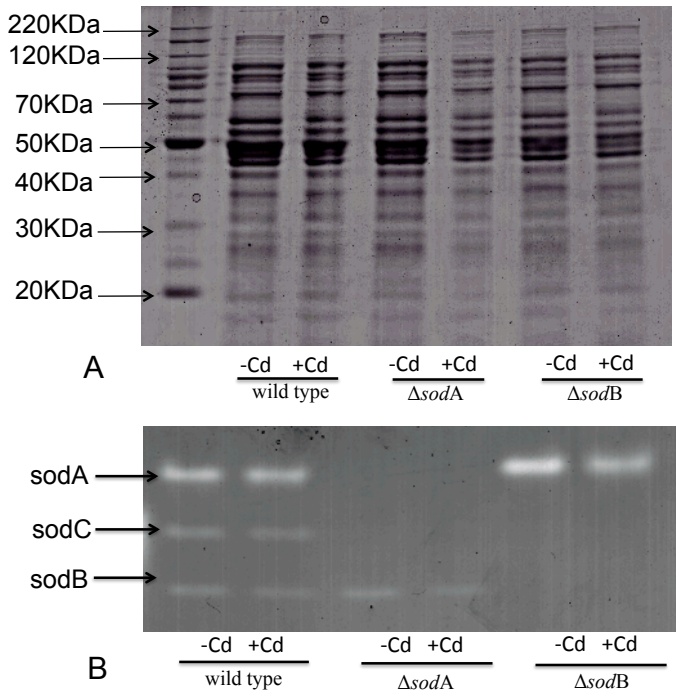

Figure 2.9- (A) Protein profile in denaturing SDS-PAGE of wild type, Δ*sod*A (lacking Mn-SOD) and Δ*sod*B (lacking Fe-SOD) in the presence of 0 and 0.3 mM of Cd. (B) Superoxide dismutase isoforms (*sod*A, *sod*B and *sod*C) in wild type, Δ*sod*A and Δ*sod*B in the presence of 0 and 0.3 mM of Cd in non-denaturing polyacrylamide gel electrophoresis (PAGE)

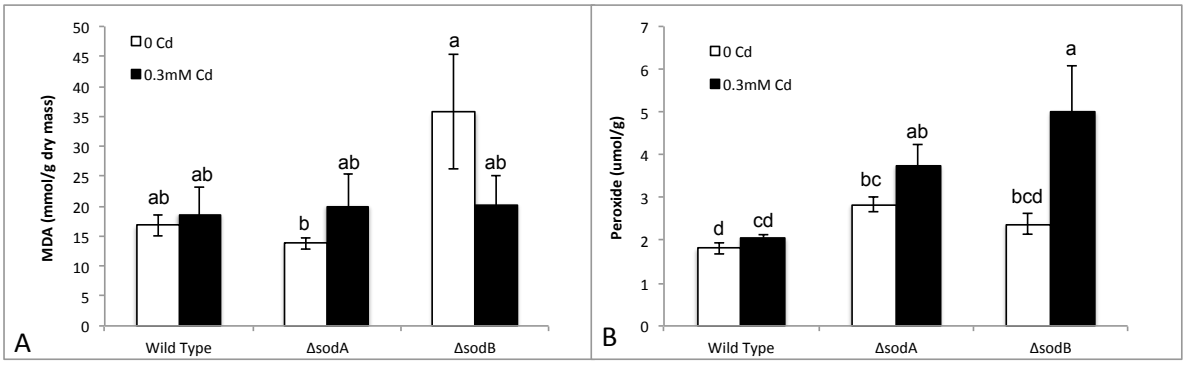

Figure 2.10- Stress measurement of wild type, Δ*sod*A and Δ*sod*B in 0 and 0.3 mM of Cd. (A) MDA quantification (B) Peroxide quantification

MDA quantification did not vary statistically in the presence of Cd in any strain (Figure 2.10 A). Peroxide quantification revealed that the presence of Cd (0.3 mM) increased peroxide content in all strains (wild type, Δ*sodA* and Δ*sodB*), indicating that despite of the increase in peroxide content there is an efficient antioxidant system that prevents lipid peroxidation in this bacterial cell. Moreover, Δ*sodB* was the most affected and the only one that presented a statistically significant difference of peroxide production (Figure 2.10 B).

This indicates that the lack of *sodB* isoenzyme causes an unbalance in bacterium metabolism, becoming less efficient in ROS scavenging, and consequently decreasing bacteria tolerance.

2.4 Discussion

Cultivable bacteria represent 1% of the total soil microorganisms. Since microbial cultivability is determined by nutritional requirements, which is different for each species (TAYLOR et al., 2002), we checked if soil with and without Cd presented different microbial communities, applying the DGGE technique. Results showed that Cd and different Cd concentrations interfere in the soil bacteria community, probably by selecting Cd tolerant bacteria.

Yet, Cd tolerant bacteria were isolated from both soils since there is a different bacterial community in Cd contaminated and non Cd contaminated soils (shown in DGGE analysis) and there were previous reports that bacteria from soils with high concentrations of heavy metals are more tolerant than bacteria from non-heavy metal-contaminated soils (ABOU-SHANAB et al., 2007). In fact, most of the reported genera were isolated from both soils, but due to the low number of obtained isolates it was not possible to infer about the microbial abundance or which genera are exclusive to each soil (contaminated or non-contaminated).

The purpose of Cd tolerant bacteria isolation was to select bacteria tolerant to different soil heavy metal contaminants (Cd, Ni and Zn), which could be used in the study of Cd tolerance mechanisms and present a great potential in helping the development of techniques for bioremediation of heavy metal contaminated areas. Therefore, we selected the only two isolates tolerant to the highest concentration of each metal (Cd, Ni and Zn). One from the Cd contaminated soil (SCMS54) and the other from the non Cd contaminated soil (SNMS32), both classified as *Burkholderia* sp. (according to 16S rRNA sequencing).

Studies show the beneficial effects of the *Burkholderia* genus in agriculture; it is an antagonist of phytopathogens and a plant growth promoter; it establishes symbiotic relation in the rhizosphere; it contributes to nitrogen fixation (MINERDI et al., 2001; PAYNE et al., 2006), phosphorus solubilization and xenobiotic catabolism (COENYE et al., 2001; GORIS et al., 2004; PAYNE et al., 2006). This genus is commonly found in soils and rhizospheres (VALVERDE et al., 2006), including species tolerant to heavy metal, tolerating until 2.5 mM of Cd (ABOU-SHANAB et al., 2007). Coenye et al. (2001) also reported that *B. cepacia* presents a huge metabolic diversity, enabling it to use herbicides and hydrocarbons as an energy source, showing its potential for bioremediation of contaminated soils.

*Burkholderia*sp. strains from Cd contaminated and non Cd contaminated soils are both tolerant to Cd and able to grow in high concentrations of Cd, the main difference is the Cd biosorption. The strain from contaminated soil (SCMS54) is able to adsorb higher concentrations of Cd than strain SNMS32. Indicating that the mechanism of tolerance is due to the Cd adsorption (probably bind to the cell wall) (BIANUCCI et al., 2012), not due to a Cd extrusion mechanism (WEI et al., 2009).

The tolerance to heavy metals, such as Cd, is deeply associated to the scavenging of Cd-induced ROS generation by the metal, therefore an efficient antioxidant enzyme system is essential for bacteria tolerance (APEL; HIRT, 2004; HARRISON et al., 2009). This study reports the stress (shown by the peroxide hydrogen quantification) only in the strain SNMS32 (isolated from control soil), indicating that Cd causes stress only in non-adapted strain.

Thus, the first enzyme of defense against ROS is SOD (that dismutase superoxide to peroxide), which showed an increase in its activity in the presence of Cd in both strains of *Burkholderia*sp. (SNMS32 and SCMS54), corroborating with other studies in which Cd also induces *sod* gene expression in *Caulobacter* (HU et al., 2005) and the SOD enzyme activity in *Aspergillus niger* (TODOROVA et al., 2008) and *Aspergillus nidulans* (GUELFI et al., 2003).

Periplasmatic isoform Cu/Zn SOD, associated to extracellular O_2^- scavenging (BENOV; FRIDOVICHG, 1994), was not found. In *E. coli,* Fe-SOD is synthesized constitutively (agreeing with the present work) and it is responsible for the protection of lipids and cytoplasmic enzymes while Mn-SOD is expressed in response to stress induced by the environment (GESLIN et al., 2001). However, it is not defined if different SOD isoforms have influence on Cd tolerance; it varies according to the organism. For instance, *Bacillus subtilis* presents only Mn-SOD and the absence of this gene (*B. subtilis*⁻*sod*A) does not affect Cd tolerance (INAOKA et al., 1998), while *E. coli* presents two SOD isoforms (Mn-SOD and Fe-SOD), the double mutant (⁻*sod*A⁻*sod*B) looses the ability to grow in the presence of Cd, influencing directly Cd tolerance (GESLIN et al., 2001). The present assays with *E. coli sodA* and *sodB* mutants confirmed that both SOD isoforms are important in Cd tolerance in *E. coli*. Mutants that lack any *sod* gene (Fe or Mn) in the presence of Cd increased significantly peroxide content when compared to wild type, but the production of hydrogen peroxide is higher in *E. coli* Δ*sodB* (lacking Fe-SOD). This was not reported for *Burkholderia*sp. In this way the modulated expression of Mn-SOD, in the present work, can be responsible for the higher Cd accumulation in strain SCMS54 (bacterium isolated from contaminated soil).

The next studied enzyme, CAT, which is responsible for converting hydrogen peroxide into water, showed increased activity in the presence of Cd only in strain SCMS54

from contaminated soil. Catalase genes also increase in *Sinorhizobium meliloti* in the presence of Cd (ROSSBACH et al., 2008) and in gel activity in *Xanthomonas campestris* (BANJERDKIJ et al., 2005) and *Aspergillus nidulans* (GUELFI et al., 2003). Despite the increase in CAT activity in the presence of Cd there are no reports showing that catalase is essential for Cd tolerance, since the lack of *kat*A gene in *Xanthomonas campestris* mutants did not affect Cd tolerance (BANJERDKIJ et al., 2005). Moreover, single mutants in different antioxidant system genes in *E. coli* and *Salmonella typhimurium* showed that Cd sensibility is more related to SOD than CAT enzyme activity (LAROSSA et al., 1995).

The non-denaturing CAT electrophoresis analysis complements the spectrophotometer data. Strain SCMS54 (from contaminated soil) presented a CAT isoform (I) that responds to growth stage (5 h), isoform I is not present in strain SNMS32. Therefore, the expression of isoform I can represent the significant increase observed in spectrophotometer CAT activity. Different CAT isoforms present the same role, but are expressed under different conditions in the intracellular medium. For exemple, *E. coli* has two catalases, HPI and HPII, codified by *katG* and *katE*, respectively. The expression of HPI is under OxyR control (stress response system) and HPII expression, which is not induced by H_2O_2, but is regulated by the KatF system, which varies according to bacteria growth phase (FARR; KOGOMA, 1991). Thus, since there is no difference between treatments in the presence or absence of Cd, but due to the different exposure time, probably all differential expressed isoforms are classified as *kat*E, being regulated by the growth stage.

Cd toxicity is due to its affinity to thiol groups, sulphides and compounds rich in sulfur (S). Cd^{2+} has a higher affinity to sulfur than to nitrogen and oxygen. In this way, GSH prevents that Cd^{2+}binds to other cytosolic proteins causing its inactivation (HELBIG et al., 2008). The importance of GSH on Cd tolerance was reported in *E. coli* and *Saccharomyces cerevisiae*, reporting that the absence of GSH (due to a mutation on a gene responsible for GSH synthesis, *ghs*A) reduces Cd tolerance (GHARIEB; GADD, 2004; HELBIG et al., 2008). Besides that, it was also reported that GSH is able to chelate 75% of intracellular Cd in *Rhizobium leguminosarum* Cd tolerant strains (FIGUEIRA et al., 2005; LIMA et al., 2006).

Thus, active glutathione (GHS) is in its reduced stateand when used to donate electrons in redox reactions it is oxidized to GSSG, therefore to regenerate GSH from GSSG glutathione reductase (GR) using NAD(P)H as a reducing agentis needed. In this way, similar to previous studies in which Cd induced increase in GR activity in tolerant *Bradyrhizobium sp.* (BIANUCCI et al., 2012) and *E. coli* (PACHECO et al., 2008), in the presence of Cd *Burkholderia* sp. SCMS54 increased GR activity mainly after 12 h of Cd exposure.

Acidithiobacillus ferrooxidans presents the same result in the presence of Cu (XIA et al., 2011) andchalcopyrite ($CuFeS_2$) (RODRIGUES et al., 2010). Cd can bind to two GSH molecules (LEVERRIER et al., 2007), so probably after only 5 h of Cd exposure, Cd was still bound to GSH, whereas after 12 h all Cd was pumped out of the cell or is bound to another component, e.g. lipid membrane, releasing oxidated glutathione (which donated electrons in redox reaction), requiring GSSG reduction by GR enzyme.

Another enzyme related to GSH is glutathione S-transferase (GST) that catalyzes the reduced glutathione conjugation to an electrophilic group of different hydrophobic toxic molecules, thus performing an important role in cellular detoxification (ALLOCATI et al., 2008; HAYES et al., 2005), mainly of aromatic compounds. Curiously, in SCMS54 (from contaminated soil) the GST enzyme presents the opposite behavior when compared to the other enzymestudies. The presence of Cd decreased GST activity in the strain from Cd contaminated soil (SCMS54) butdid not affect the strain from non Cd contaminated soil (SNMS32). Similar to the present result, the inhibition of the GST enzyme activity was also observed for *Rhizobium* strains in the presence of Cd (BIANUCCI et al., 2012). On the other hand, another study reported that the presence of metals Cd and Cu increased *gst* expression in *Caulobacter* (HU et al., 2005) and *Acidithiobacillus ferrooxidans* (XIA et al., 2011),respectively. However, these studies were performed with the *gst* gene expression; the RNA transcript can have its protein transduction blocked, and even if there is the protein transduction, the enzyme can be inactive. Besides that, Cervantes et al. (2006) reported that a moderate decrease in GST activity contributes to Cd accumulation by increasing the level of GSH in the algae *Euglena gracilis*, resulting in more GSH available for the redox reaction decreasing the damage caused by the metal. This agrees with the present result in which SCMS54 accumulated more Cd and decreased GST activity, suggesting that only in tolerant adapted strains Cd could interact with the GST enzyme, negatively inhibiting its activity. However, there are no reports as far as we are aware that GST catalyzes the ligation of GSH to metals. Therefore, there is a great diversity of GST, with a number of undescribed functions (ALLOCATI et al., 2008), confirmed by the identification of new GST enzymes in the last years (MA et al., 2009; SKOPELITOU et al., 2012) showing that more research is required to elucidate the different functions of this enzyme.

Summarizing, we compared the enzyme activity of both strains after 5 and 12 hours of Cd exposure, and observed that at 5 h (Figure 2.11A) strain SCMS54 (from contaminated soil) increased SOD and CAT enzymes activity, showing its advantage when compared to SNMS32 (from non Cd-contaminated soil). SNMS32 exhibited increased SOD activity only

at 12 h. Glutathione related enzymes (GR and GST) did not respond earlier, but after 12 h of Cd exposure GR activity increased in strain SCMS54.

In this study, we compared two strains from the same genus, but isolated from different environments. We also analyzed electrophoresis gel of all enzymes and observed that strain SCMS54 (isolated from Cd contaminated soil) seems to have a higher metabolic diversity than SNMS32 (from soil without Cd). SCMS54 expresses more SOD isoforms (isoform II), CAT (isoform I) and GR (isoform IV) than SNMS32, suggesting a higher plasticity of this strain. Besides that, the Cd biosorption of the strain SCMS54 from Cd contaminated soil is 50% higher than that shown by strain SNMS32.

This is the first report that compares antioxidant response of two strains, from the same genus and phylogenetically closely related (present a similar 16S rRNA sequence), but isolated from different environments. The environment with Cd selected the bacteria that appears to contain adapted genes that respond to the Cd-stress condition, allowing the selection of bacteria with diverse metabolism, such as *Burkholderia* sp. SCMS54.

However, SOD was the only enzyme that increased its activity in all Cd treatments in both strains and times (except for SNMS32 at 5 h) (Figure 2.11), constitutively expressing Fe-SOD in both strains (SNMS32 and SCMS54) in all treatments. The main physiological differences between SNMS32 and SCMS54 is that SCMS54 accumulated more Cd within the cell, did not increase peroxide content, presented a fast antioxidant response and expressed an extra isoform for SOD, CAT and GR enzymes. These extra isoforms can possibly be related to the higher tolerance of this strain as well as its capability to accumulate more Cd. Both *E. coli ΔsodB* (lacking Fe-SOD) and *Burkholderia* sp. SOD activities showed that Fe-SOD is the key isoenzyme for Cd tolerance, because it is constitutively expressed (in both *Burkholderia* sp. strain), whereas the lack of Fe-SOD (in *E. coli*) resulted in the highest hydrogen peroxide content observed. Therefore, these results indicate that both genus *Burkholderia* sp. and *E. coli* have a similar defense system to deal with Cd-induced ROS generation.

In conclusion, the highly diverse metabolism of *Burkholderia* sp. SCMS54 and its tolerance to Cd suggests that this strain can be potentially used in bioremediation programs immobilizing soil heavy metals or enhancing phytoextraction of heavy metals from soil, mainly after inducing Fe-SOD enzyme expression to increase Cd tolerance even more.

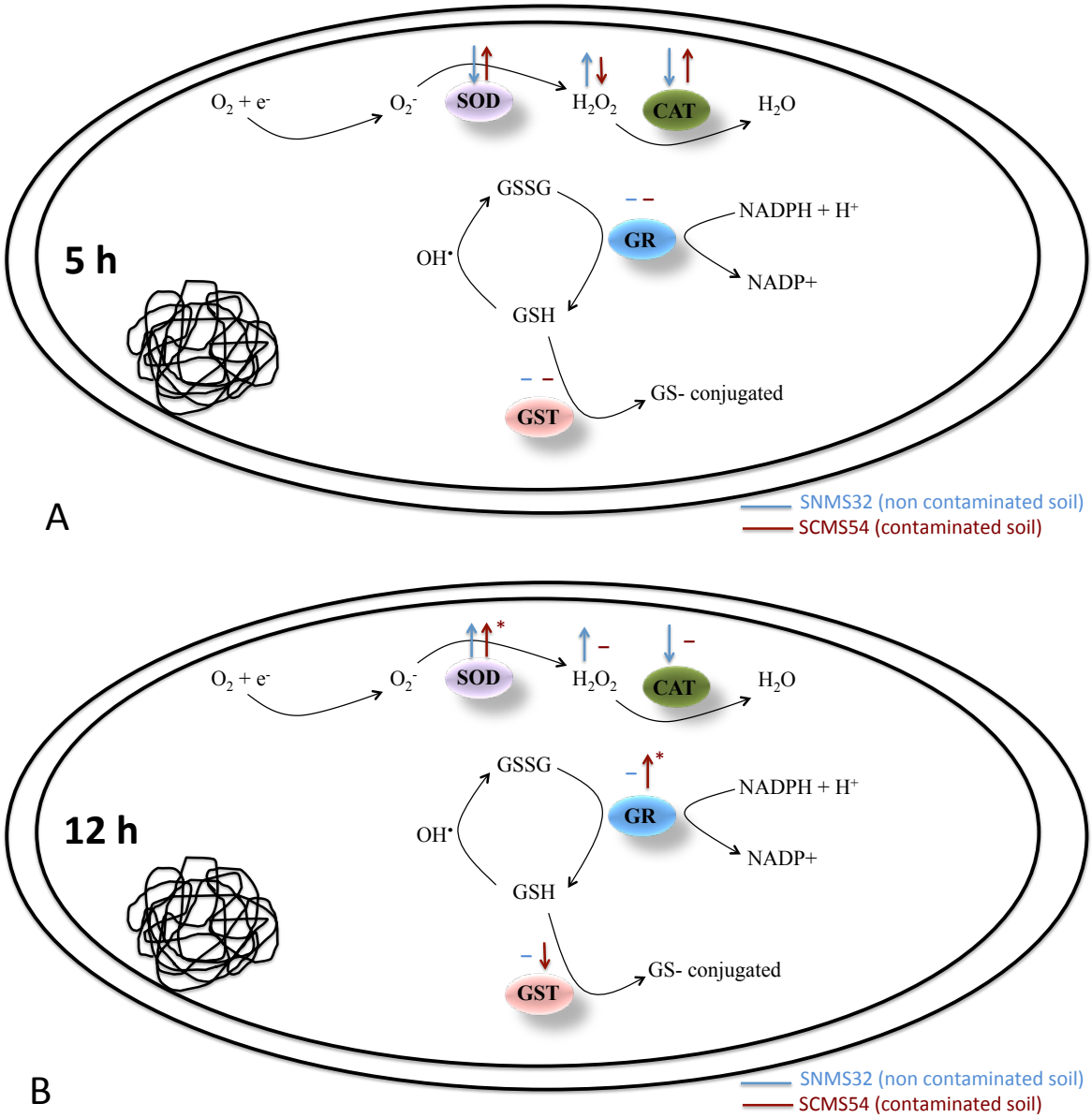

Figure 2.11- Illustrative scheme of the all studied enzymes (SOD, CAT, GR and GST) of antioxidant system and its activity in both strains from contaminated and non-contaminated soil. (A) 5 hours after Cd exposure(B) 12 hours after Cd exposure. Blue arrow represents the activity of strain from non-contaminated soil (SNMS32). Red arrow represents the activity of strain from contaminated soil (SCMS54). -: no statistical difference, arrow: increase or decrease in enzyme activity in the presence of 0.3 mM and/or 1.28 mM of Cd. * increase activity, but it is not statistically different

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3 GST ACTIVITY AFFECTS *Burkholderia* sp. TOLERANCE TO NICKEL

Abstract

The increase in agricultural productivity and the consequent increase use of herbicides, pesticides and fungicides lead to soil contamination. Nickel (Ni) was previously reported as a problem in soil and water contamination due to its high mobility. Metals, including Ni, in high concentrations are toxic to cells, generating oxidative stress in cells due to the induction of reactive oxygen species (ROS) damaging lipids, proteins and DNA. Therefore this study aimed to characterize the Ni antioxidant response of two tolerant *Burkholderia* sp. strains (one isolated from contaminated soil, SCMS54, and the other from non-contaminated soil, SNMS32), measuring SOD, CAT, GR and GST activities, together with Ni accumulation and bacterial growth in the presence of Ni. Results show that both strains present differences in Ni accumulation and in the antioxidant enzymes response. Only the strain from contaminated soil (SCMS54) presents an increase in SOD and GST activities, analyzing isoform expressions, strain SCMS54 (similar do Cd response) expresses an extra isoform in all tested enzymes (SOD, CAT and GR), moreover this strain presents a higher Ni accumulation. Some antioxidant responses of these tolerant *Burkholderia* sp. strains are similar in Ni and Cd metals (SOD and GR) suggesting that bacteria has a general metal response, however, some enzymes (such as GST) are differently modulated in the presence of Cd and Ni.

Keywords: Tolerance mechanisms; Antioxidant enzymes

3.1 Introduction

Toxic metals have been added into the environment by fertilizers, pesticides, burning of fossils fuels, as well as by mining of metalliferous ores. Metals cannot be degraded, differently from organic pollutants that can be turned into carbon dioxide, they can only be oxidized, reduced or complexed to other compounds to became less toxic (LLOYD, 2002). Therefore, metals remain a problem since they persist in the environment affecting plants, microorganisms and humans (NAGAJYOTI; LEE; SREEKANTH, 2010).

Previous study pointed high concentrations of nickel (Ni) as a problem in soil (TEZOTTO et al., 2012)and water due to its high mobility which can increase the contamination area of contamination. Despite of Ni being an essential metal in different biological processes (RAY et al., 2013) and its participation in a number of Ni containing enzymes such as hydrogenase, urease, carbon monoxide dehydrogenase and superoxide dismutase (CHENG et al., 2009), Ni is toxic in high concentrations to different organisms (GRATÃO et al., 2005), and its toxicity is usually due to the binding of Ni to sulfhydryl groups or enzymes or the replacement of another essential metal ion in a biological process (CHENG et al., 2009).

Toxic metals, including Ni, generate oxidative stress in cells and its toxicity is partially due to the induction of the reactive oxygen species (ROS) damaging lipids, proteins and DNA (GOMES-JUNIOR et al., 2006; GRATÃO et al., 2005). The antioxidative system is responsible for cellular defense, scavenging ROS produced by cellular metabolism (photosynthesis, respiration) and by abiotic stress induced conditions (heavy metals, high temperatures, drought) (CABISCOL; TAMARIT; ROS, 2010; CIA et al., 2012), presenting always some basal expression to maintain cellular balance.

When the antioxidant system is concerned, the first line of defense is superoxide dismutase (SOD, EC 1.15.1.1) which is responsible for superoxide ($O_2^{\cdot-}$) dismutation, generating hydrogen peroxide (H_2O_2) and oxygen (O_2) (GRATÃO et al., 2005, 2012; KEITH; VALVANO, 2007; LUSHCHAK, 2011). Subsequently, catalase (CAT, EC.1.11.1.6.) detoxify hydrogen peroxide into water (GRATÃO et al., 2005; MEDICI et al., 2004; NADLER; GOLDBERG; HOCHMAN, 1986). The reduced glutathione (GSH) molecule is also responsible for ROS scavenging and cellular detoxification of toxic metal (MASIP; VEERAVALLI; GEORGIOU, 2006; REHMAN; ANJUM, 2011). Glutathione reductase is responsible for glutathione balance in the cell, by reducing oxidized glutathione (GSSG) using NADPH (MASIP; VEERAVALLI; GEORGIOU, 2006). GSH is also a thiol donor to the glutathione S-transferase enzyme (GST; EC 2.5.1.18), that catalyzes the conjugation of glutathione with several electrophilic substrate (DIXON; LAPTHORN; EDWARDS, 2002; GHELFI et al., 2011), being associated to metabolizing xenobiotic compounds, secondary products, including flavonoids (GHELFI et al., 2011). The described diversity of GST indicates that there is still a great potential not yet studied for future application on bioremediation of contaminated environment (VUILLEUMIER; PAGNI, 2002).

Simple organisms as prokaryotes can be used as model to study metal tolerance. Harrison et al. (2009), using *E. coli* mutants, proved that genes involved in oxidative stress (*sodAB, gor, gsh*) are related to its tolerance to different metals. Moreover, transcriptomic and proteomic studies show that oxidative stress genes or proteins are up-regulated in the presence of the different metals (Cd, uranium, chromium) in *Caulobacter crescentus* (HU et al., 2005) and also in the presence ofNi in *Pseudomonas putida* (CHENG et al., 2009). This confirms that metal tolerance depends on an efficient antioxidant response to maintain ROS balance in the cells. Most of antioxidant enzyme studies are based in genomic or proteomic analysis. There are few studies in bacteria that measure enzyme activity, which is the final result of gene regulation (EERD et al., 2003).

Moreover, different plant associated microorganisms have been reported to increase Ni tolerance in plants (IDRIS; TRIFONOVA, 2004). They can protect against Ni toxicity, such as *Kluyvera ascorbata* bacterium in canola seedlings (BURD; DIXON; GLICK, 1998). Bacterium inoculation can promote phytoextraction like with *Psychrobacter* bacterium in *Ricinus communis* and *Helianthus annuus* plants (MA et al., 2011) or even reduce phytoextraction and increase plant growth in tomato plants inoculated with *Methylobacterium* strain (MADHAIYAN; POONGUZHALI; SA, 2007).

In this context, this research selected two bacteria tolerant to high concentrations of different metals (Cd, Zn and Ni), both classified as *Burkholderia* sp. (Chapter 2), but coming from different environments, one isolated from a contaminated soil and the other from a non-contaminated soil. This study aimed to understand the antioxidant response of these two tolerant bacteria exposed to Ni, in two different Ni concentrations and exposure time, seeking to understand the role of each tested enzyme in the Ni tolerance, enabling to unveil the bacteria tolerance mechanism. This could be used in future works to develop a method to help the bioremediation of contaminated soils.

3.2 Material and Methods

3.2.1 Bacterial strains

Burkholderia sp. strains SNMS32 and SCMS54 were isolated (Chapter 2) from a coffee crop soil from Piracicaba, SP, Brazil (TEZOTTO et al., 2012). SNMS32 was isolated from a non-contaminated soil and SCMS54 from a contaminated soil.

3.2.2 Growth determination

Bacterial growth was monitored by measuring the colony-forming unit (CFU) mL^{-1}, as described by Sangali and Brandelli (2000). *Burkholderia* sp. strains were grown in 50 mL of nutrient medium (with 1 mM; 4 mM and without Ni) were incubated in the dark on a rotary shaker at 150 rpm at 30ºC for 36 h. The bacterial suspension was diluted and samples (100 µL) were loaded in triplicate onto nutrient agar plates, which were incubated at 30ºC for 36 h for subsequent counting.

3.2.3 Ni biosorption

The biosorption assay was carried out as described by Kanazawa and Mori (1996) with modifications. Bacteria were grown in 100 mL of nutrient broth until reaching optical density 1 at 600 nm. Then, cells were separated by centrifugation at 8,000 g for 15 min, and

the bacterial pellet was washed twice with phosphate buffered saline solution [PBS, containing ($g.L^{-1}$) Na_2HPO_4, 1.44; KH_2PO_4, 0.24; KCl, 0.20; NaCl, 8.00; pH 7.4]. The collected biomass was incubated for 5 h (adaptation period) and 12 h (stabilization period) at 28ºC in Minimal Media (DWORKIN; FOSTER, 1958) with 1 mM and 4 mM Ni. The suspension was then centrifuged at 8,000 g and filtered through a 0.22 μm Millipore membrane to separate the biomass from the filtrate. The biomass was dried, weighed, and heavy metals were extracted by nitric acid digestion (24 h) (AZCÓN et al., 2010). The Ni contents were determined by ICP-OES.

3.2.4 Protein extraction and quantification

Liquid cultures were centrifuged at 12,000 g for 15 min at 4ºC and the pellets macerated with liquid nitrogen. The extracts were homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol (DTT) and 5% (w/w) polyvinylpolypyrrolidone (PVP) (GRATÃO et al., 2008; MARTINS et al., 2011). The homogenates were centrifuged at 12,000 g for 30 min and the supernatants were stored in separate aliquots at -80ºC for further biochemical analysis. The protein concentration in all the samples was determined by the Bradford (1976)method.

3.2.5 Enzymes activity

All enzyme (SOD, CAT, GR and GST) activities were performed as described in *Material and Methods* of Chapter 2.

3.2.6 Statistical analysis

Enzyme activity determinations were performed on three replicates for each treatment, and the significance of the observed differences was verified using a one-way analysis of variance (ANOVA) followed by Duncan's test. Differences with a p value of <0.05 were considered significant. All statistical analysis were carried out using the software R program, version 2.15.1.

3.3 Results

This study aimed to understand microorganism physiology to help the development of bioremediation technics. In this way, a biochemical and physiological characterization in response to Ni of two strains tolerant to high concentration of Ni, Cd and Zn was performed. The analysis of growth curves for both strains revealed similar growth in the presence or absence of Ni (Figure 3.1 A and B). They are not able to grow with 4 mM of Ni, as shown by

the growth curve, but after plating the liquid culture with 4mM of Ni, growth of colonies in petri plate was observed, showing that cells were still viable. In the presence of 1mM of Ni, there was a reduction in growth of both strains when compared to the control, with a long lag phase to adapt to the stress condition (Figure 3.1 A and B).

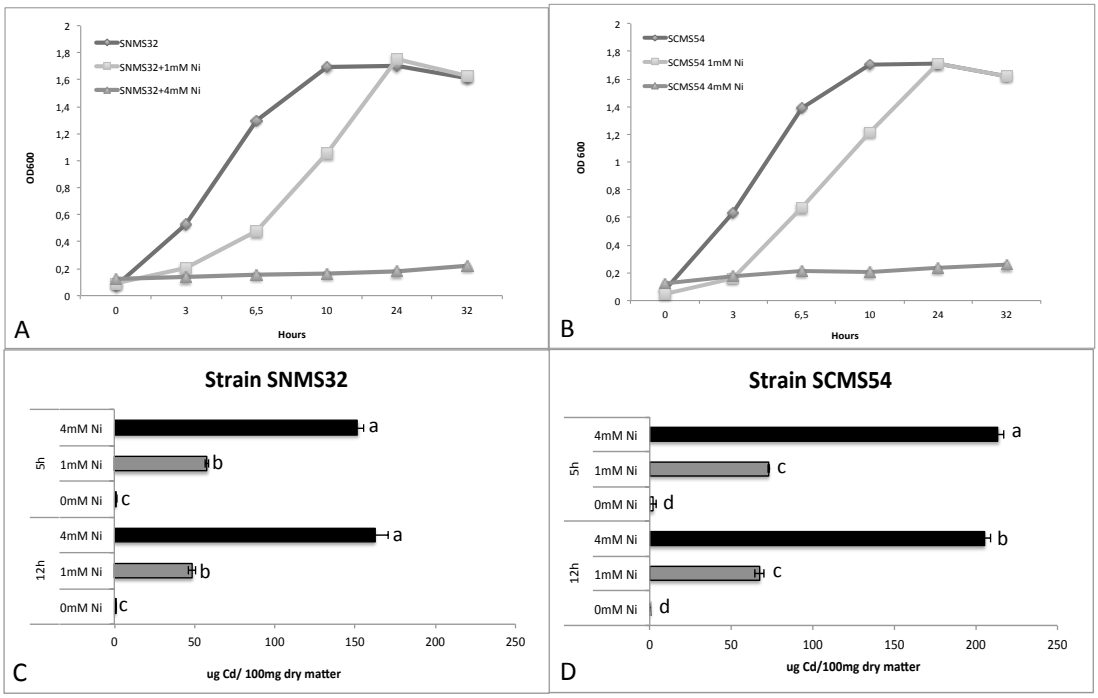

Figure 3.1- (A and B) Growth curve in the presence of Ni (C and D) Biosorption of Ni in different concentrations (0, 1 mM and 4 mM Ni); (A and C) strain SNMS32; (B and D) strain SCMS54

The measurement of Ni content in bacteria pelletsshowed a high concentration of Ni in both strains (SNMS32 and SCMS54) (Figure 3.1 C and D). Ni biosorption was increased in the treatment with higher amount of Ni (4 mM). Therefore, SCMS54 strain (isolated from contaminated soil) accumulated more Ni at both times (5 and 12 h) when compared to the strain from non-contaminated soil (SNMS32) (Figure 3.1 C and D).

The analysis of protein pattern in SDS-PAGE gel (Figure 3.2) shows that treatment with Ni and without Ni (control) did not vary in both strains (SCMS32 and SNMS54). However, when protein pattern of SCMS32 and SNMS54 strains were compared, although both belong to the same genus, a difference could be noted in their protein pattern, even comparing control treatments (without Ni) of both strains, suggesting that each strain presents a different metabolism.

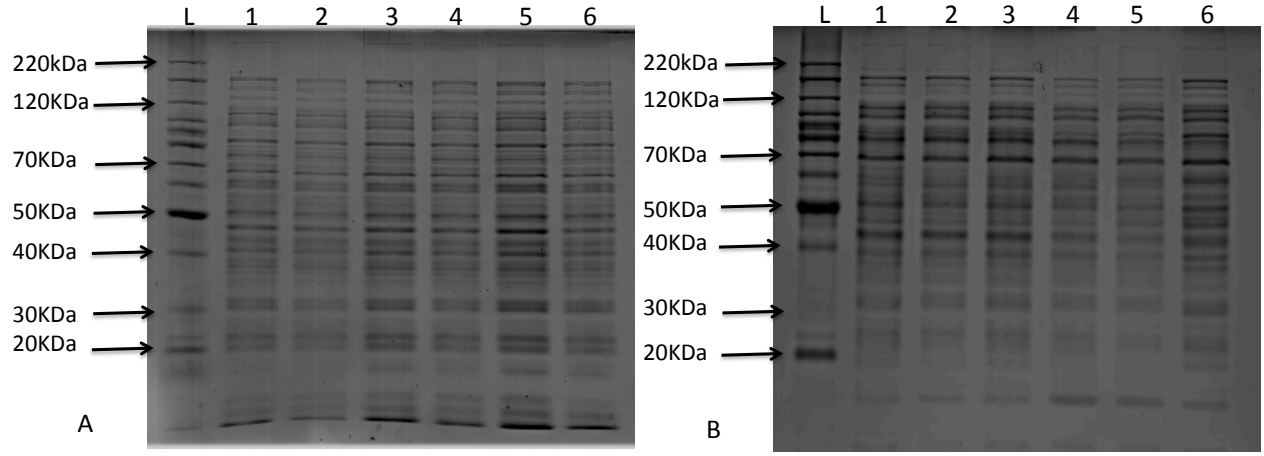

Figure 3.2- (A) Strain SNMS32; (B) Strain SCMS54. L; Invitrogen protein ladder, 1. Strain; 2. Strain + 1 mM Ni; 3. Strain + 4 mM Ni (exposure time 5 h); 4. Strain; 5. Strain + 1 mM Ni; 6. Strain + 4 mM de Ni (exposure time 12 h)

These differences in their metabolism are also present in the results of the tested enzyme activities. Each strain presents a different SOD activity response in the spectrophotometer (Figure 3.3A and B). Only in strain SNMS32 (isolated from control soil) SOD activity was inhibited after 5 hours of Cd exposure (in both tested concentrations), while in strain SCMS54 SOD activity increased at the same exposure time (5 h).

In strain SNMS32, only one SOD isoform was detected in all treatments (with and without Ni) and all exposure time (5 and 12 h) (Figure 3.4) characterized as Fe-SOD. Strain SCMS54 had the same Fe-SOD in all treatments and time, but expressed also a Mn-SOD isoform, varying with time, expressing more after 5 hours of Ni exposure and reducing its expression after 12 hours of Ni exposure.

Each strain has a different CAT activity response to Ni. Strain SNMS32 (from non-contaminated soil) presented a lower CAT activity than strain SCMS54 (from contaminated soil) mainly in the exposure time of 5 hours (Figure 3.3 C and D). Another difference is that CAT activity increased in strain SNMS32 only after 12 h of Ni exposure.

The analysis of CAT activity in non-denaturing gels (Figure 3.4), showed that strain SNMS32 expressed two isoforms (isoform II and III) in all exposure times (5 and 12 h) and in all treatments (with and without Ni), while strain SCMS54 besides the constitutive expression of this two isoforms in all times and treatments, showed an extra isoform (isoform I) in treatments with and without Ni only after 5 h of Ni exposure (absent in 12 h of Ni exposure), also similar to SOD isoform expressions.

Different from SOD and CAT, in total GR activity (spectrophotometer assay) of both strains were similar in response to Ni, and did not statistically vary (Figure 3.3E and F). However, in GR PAGE analysis similar results as previous verified for SOD and CAT were observed. Strain SNMS32 presented five isoforms in all tested treatments and times (bands I, II, III, IV and VII) (Figure 3.4), with isoform VIII being exclusive to this strain. Strain SCMS54 expressed three extras isoforms (V, VI and VIII) besides the isoform VI, which is only present in the control and 5 h of Ni exposure, but the other isoforms (bands I, II, III, IV, V, VII and VIII) were expressed in all treatments and times (Figure 3.4).

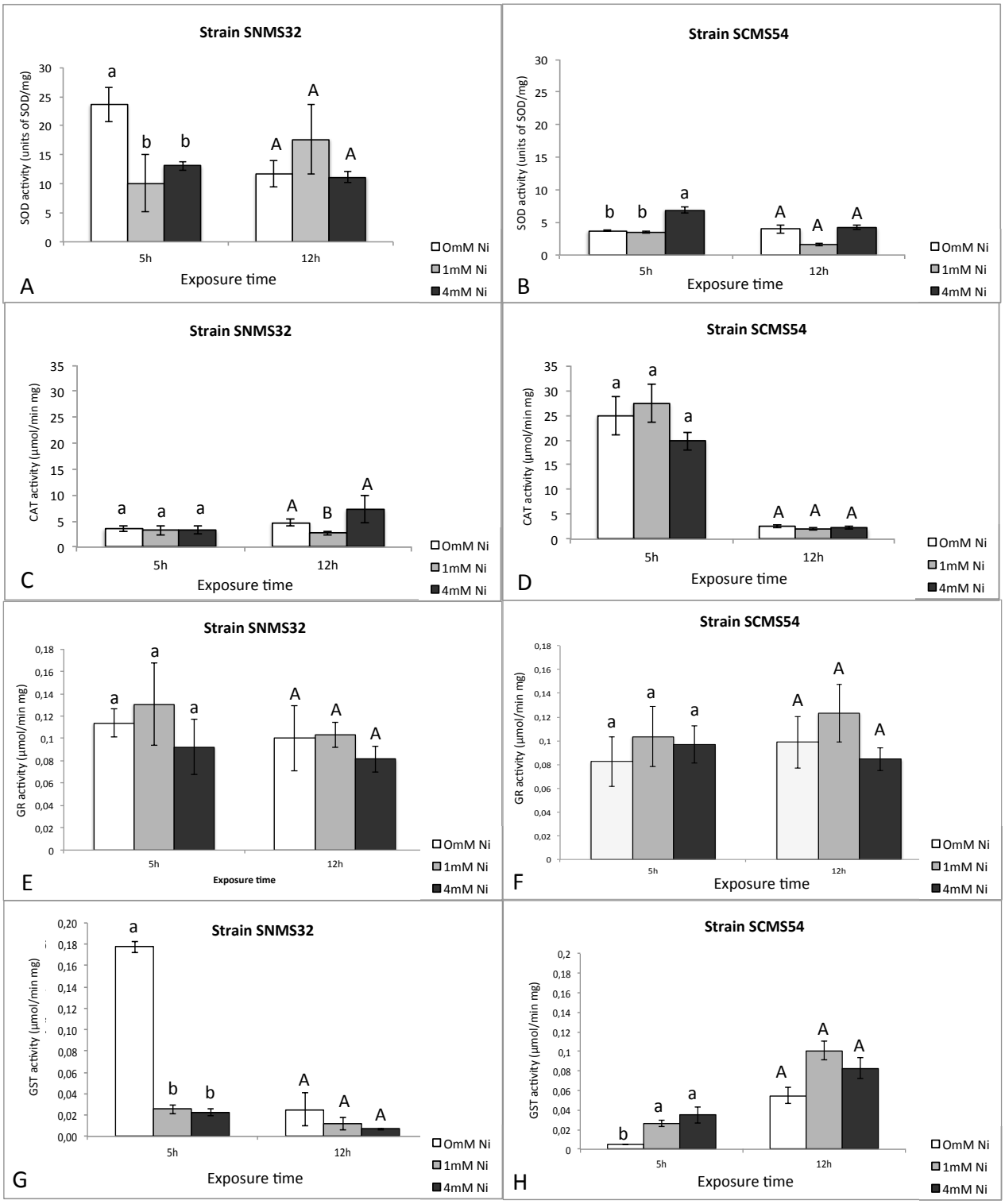

Figure 3.3- Spectrophotometer Enzyme activities of SOD (A and B), CAT (C and D), GR (E and F) and GST (G and H) of strains SNMS32 (left side -A, C, E, G) and SCMS54 (right side - B, D, F, H)

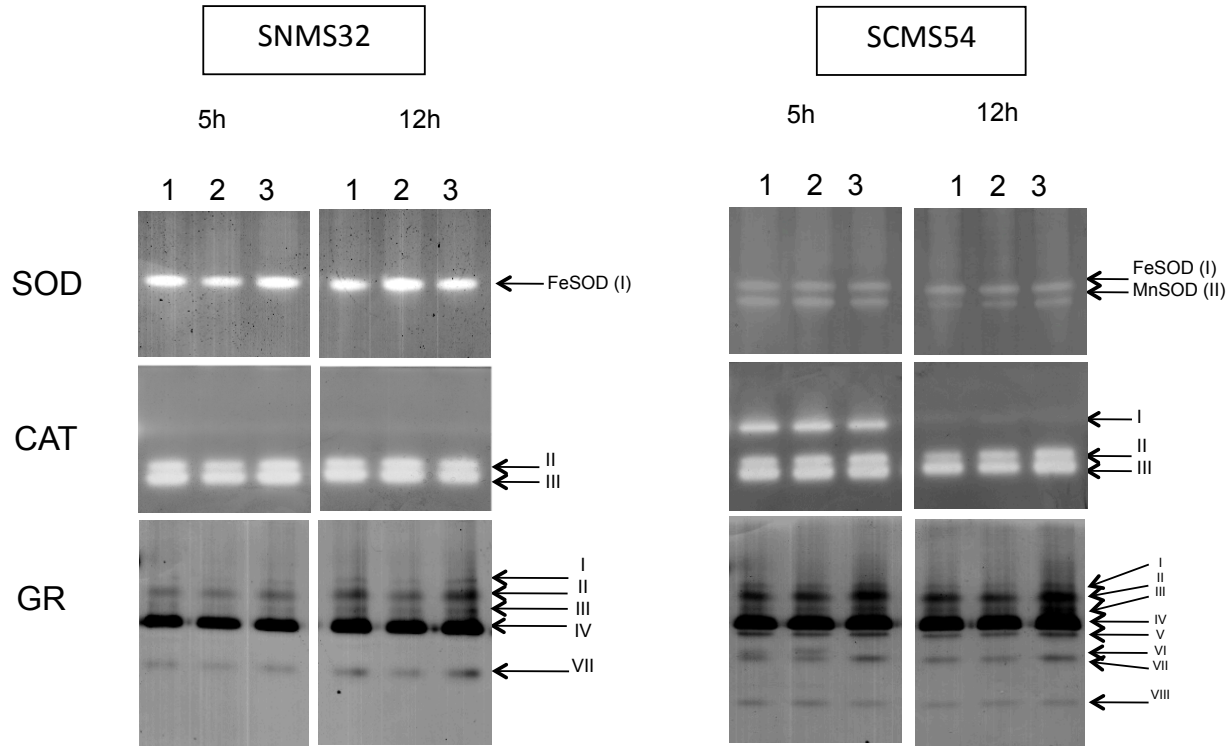

Figure 3.4- Electrophoresis gel activity of SOD, CAT and GR in non-denaturantelectrophoresis. Lanes 1. Strain + 0 mM Ni; 2. Strain + 1 mM of Ni; 3. strain + 4 mM of Ni (after 5 h or 12 h Cd was added)

Interestingly, Ni has induced GST activity only in strain SCMS54 (from contaminated soil) in both exposure times (Figure 3.3H), but the opposite occurred in strain SNMS32 (from non-contaminated soil) where Ni inhibited GST activity in both times (Figure 3.3G).

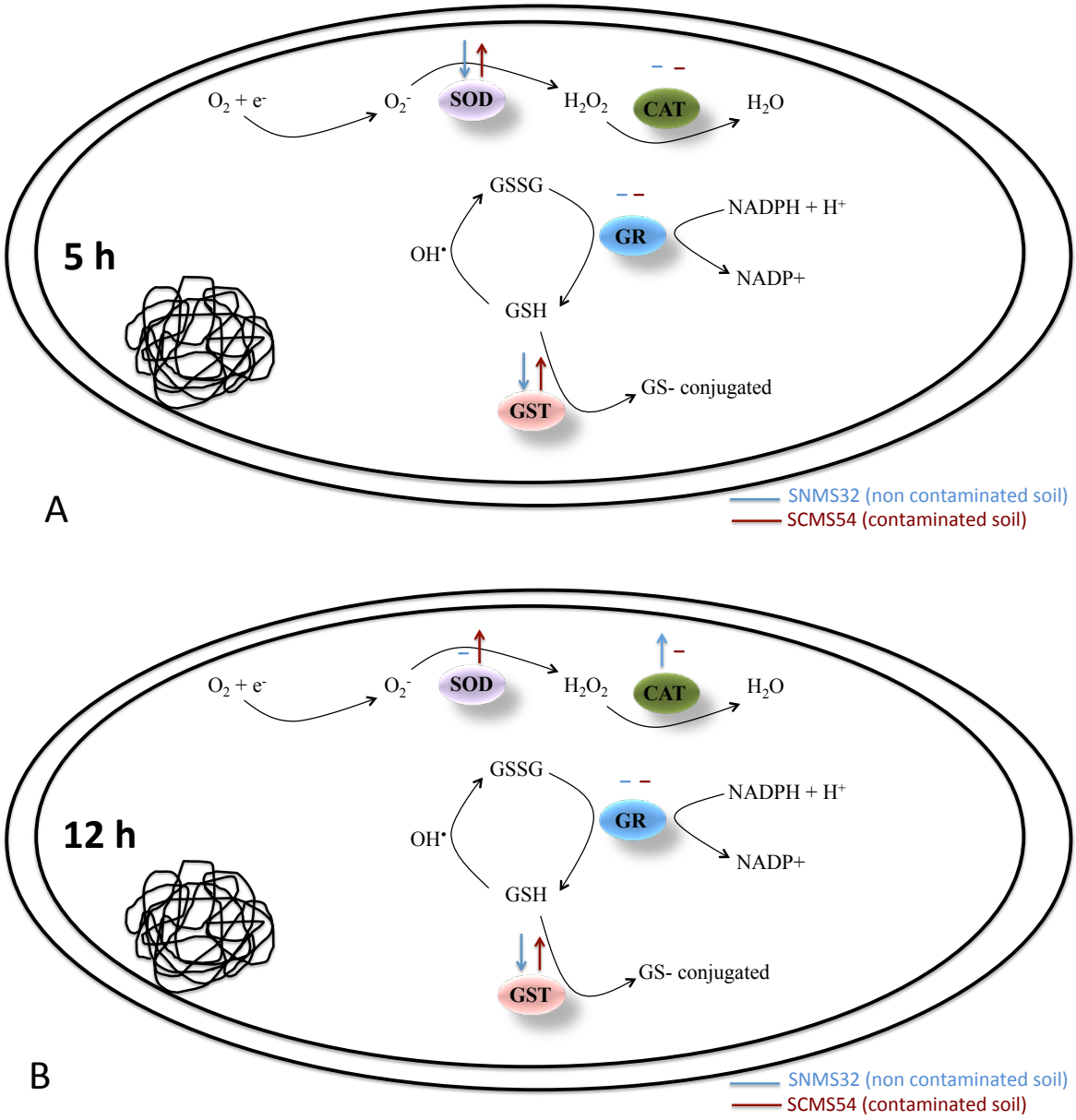

Figure 3.5- Illustrative scheme of the all studied enzymes (SOD, CAT, GR and GST) of antioxidant system and its activity in both strains from contaminated and non-contaminated soil. (A) 5 hours after **Ni** exposure (B) 12 hours after **Ni** exposure. Blue arrow represents the activity of strain from non-contaminated soil (SNMS32). Red arrow represents the activity of strain from contaminated soil (SCMS54). -: no statistical difference, arrow: indicates the increase or decrease in enzyme activity in the presence of 1 mM and/or 4 mM of Ni

3.4 Discussion

All living cells (without any stress condition) produce ROS naturally. The role of the antioxidant enzymes is to balance de level of ROS in the cell. Metals (such as Cd and Ni) disbalance ROS production by increasing it, damaging lipids and DNA. Therefore, the

purpose of this study was to investigate the potential role of antioxidant enzymes on Ni tolerance in two bacteria tolerant to multi metals.

The selected *Burkholderia* sp. strains besides being able to tolerate and grow in high concentration of Ni, are also able to accumulate 150 to 213 μg of Ni in 100 mg of bacteria dry matter. This biosorption of Ni by the bacteria can help plant growth in contaminated soils, due to its momentary unavailability of Ni. This fact together with other physiological characteristics of the strains such as solubilizing phosphate (Chapter 4; DOURADO et al., 2013; GORIS et al., 2004; LUVIZOTTO et al., 2010; PAYNE et al., 2006), competing with phytopathogen and fixing nitrogen in the rhizosphere (MINERDI et al., 2001; PAYNE et al., 2006) and also producing siderophore that can chelate Cd and Ni (Chapter 4; DIMKPA et al., 2008) may help to improve plant growth in the presence of heavy metals.

Scavengingfree radicals are an important mechanism in bacteria tolerance (HARRISON et al., 2009), with several enzymes playing a role in antioxidative defense (SETH et al., 2012). The primary ROS formed after electron transfer are the superoxide radicals, which are converted to H_2O_2 by SOD. SOD increased in the presence of Ni only in *Burkholderia* sp. strain isolated from the contaminated soil (SCMS54). Similar result was found in *Pseudomonas* (CHENG et al., 2009; RAY et al., 2013).

Both strains (SNMS32 and SCMS54) expressed cytosolic Fe-SOD in all times and treatments while Mn-SOD was exclusively expressed in the strain from contaminated soil (SCMS54). Fe-SOD is described as constitutively expressed (agreeing with this study) and Mn-SOD is expressed in response to the environmental stress (GESLIN et al., 2001). Cu/Zn-SOD was not found despite of being reported in this bacterial species (KEITH; VALVANO, 2007). The same isoform pattern occurred in response to Cd (Chapter 2), indicating that this response is not exclusive to Cd or Ni.

In the sequence, hydrogen peroxide is scavenged by CAT preventing OH^- formation (GRATÃO et al., 2005; SCHÜTZENDÜBEL; POLLE, 2002). CAT activity did not vary significantly in the presence of Ni in this work, except for an increase in strain SNMS32 after 12 h of Ni exposure. In a similar manner, CAT activity in SCMS54, based on CAT isoform distribution did not vary with or without Ni, but the only difference was the expression of an extra isoform (I) only in strain SCMS54 (from contaminated soil) after 5 h of Cd exposure. Farr and Kogoma (1991) reported in *E. coli* two catalase: one that responds to stress (*kat*G) and the other that responds to growth (*kat*E). Isoform (I) from SCMS54 should be the result of *kat*E transduction (responding to growth), since the expression of the extra isoform (I) is present only during bacterial adaptation to the new condition. Such behavior by isoform I may

explain the higher CAT activity observed by the spectrophotometer assay for total CAT activity observed at 5h. The same CAT activity profile occurred in response to Cd, probably CAT and SOD respond to metal stress the same way.

Glutathione (GSH) is also important on metal scavenging due to the presence of a thiol (-SH) group with a high affinity to metals, playing an important role in metal homeostasis, antioxidant response and signal transduction under metal stress (JOZEFCZAK et al., 2012). Metals increase GSH oxidation, however 95% of the glutathione pool is reduced to maintain GSSG:GSH balance in the cell, so glutathione reductase (GR) is responsible for the reduction of glutathione disulfide (GSSG) into its reduced form GSH using NADPH (FOYER; THEODOULOU; DELROT, 2001). In the present study GR did not vary during Ni exposure in both strains, and similar results were obtained adding Cd (after 5 h of Cd exposure) into this same *Burkholderia*sp. strain (chapter 2). The opposite occurred after 12 h of Cd exposure (chapter 2) and in coffee cell suspension cultures in response to Cd (GOMES-JUNIOR et al., 2006b), Ni (GOMES-JUNIOR et al., 2006a) and selenium (GOMES-JUNIOR et al., 2007), increasing GR activity.

Glutathione is the most important component of the redox balance in cell gran negative bacteria (SMIRNOVA; OKTYABRSKY, 2005). However, many functions of the thiol-redox system, involving the GR enzyme, can be overlapped and duplicated. For instance, *E. coli*strains containing both a *gor* (glutathione reductase) mutation or a *trxB* mutation (thioredoxin reductase deficient) were able to maintain a reduced glutathione pool (TUGGLE; FUCHS, 1985). Therefore, the thioredoxin reductase, a dimeric flavoenzyme, catalyzes the NADPH-dependent reduction of thioredoxin, contributes for the balance between thioredoxin and glutathione levels (RITZ; BECKWITH, 2001). In this way, glutathione reductase activity did not vary statistically in the different treatments, which may be because glutathione reduction can be performed by thioredoxin reductase.

Despite the lack of variation in total GR activity (in spectrophotometer), the analysis of GR isoforms by non-denaturing PAGE revealed that there were five constitutive isoforms (I, II, III, IV and VII) expressed in both strains in all times and treatments. Strain SCMS54 expresses an extra isoform (VI) only in treatment with 1 mM of Ni at 5 h. This shows that GR, different from SOD and CAT, is more diverse and is differentially expressed according to time (in strain SNMS32) or to treatment (in strain SCMS54), so more studies of this enzyme are needed in order to understand how it is regulated.

Glutathione transferases (GST) have a key role in cellular detoxification catalyzing the conjugation of reduced glutathione (GSH) to a number of electrophilic toxic substrates

(ALLOCATI et al., 2008; HAYES; FLANAGAN; JOWSEY, 2005). Interestingly, this study shows a significant increase in GST activity in the strain isolated from contaminated soil (SCMS54) in the presence of Ni at all times, but the opposite occurs with the strain isolated from non-contaminated soil (SNMS32). There are studies that report GST binding to Ni: two in human (CHAGA et al., 1994; YILMAZ et al., 1995) and one in the parasite *Schistosoma japonicum* (HAN et al., 2010), indicating that in *Burkholderia* sp. strain SCMS54 GST enzyme can bind to Ni, immobilizing it in the cell and enabling more accumulation of Ni in the cell.

There is a great diversity of GST, with a number of not yet described functions (ALLOCATI et al., 2008). Recently Ma et al. (2009) and Skopelitou et al. (2012) reported two different new GST enzymes classes, showing there is still the need for more research to elucidate the GST function, such as metal tolerance.

As described above, whencomparing both *Burkholderia*sp. selected strains (SNMS32 and SCMS54), in relation to protein pattern and enzymatic response, it becomes clear that each one presents different metabolism. In the strain isolated from contaminated soil (strain SCMS54) most of the analyzed enzymes (SOD, CAT and GST) of the oxidative stress system, increased in the presence of Ni in at least one tested time, different from strain SNMS32 (from non-contaminated soil) for which the presence of Ni decreased SOD and GST activities. Moreover, strain SCMS54 expresses one or more extra isoform in all enzymes: SOD (isoform I), CAT (isoform I) and GR (isoforms V, VI and VIII) suggesting that this strain is of more metabolic diversity, with a high plasticity and consequently can adapt to different conditions.

There are similarities between antioxidant enzyme responses to Ni and Cd. The presence of Cd and Ni induces the expression of similar isoforms during each exposure time (Figure 3.6), presenting very similar qualitative gel activity in all enzymes (SOD, CAT and GR). However, the Cd and Ni total enzyme activity (spectrophotometer assay) response differs in some enzymes (Figure 3.7). SOD and GR activity present a similar pattern in response to both Ni and Cd, suggesting that the expression of these enzymes is a global response to metal stress, while CAT and GST respond differently to each metal. In strain SNMS32 (non-contaminated soil), CAT activity decreases in the presence of Cd and increases with Ni. In strain SCMS54 (isolated from contaminated soil) GST is highly expressed in the presence of Ni, while in the presence of Cd it is repressed, indicating that CAT and GST are more responsive to Ni, playing important roles on Ni tolerance. As for Cd tolerance SOD (super expressed in SCMS54 in the presence of Cd) is probably the antioxidant key enzyme.

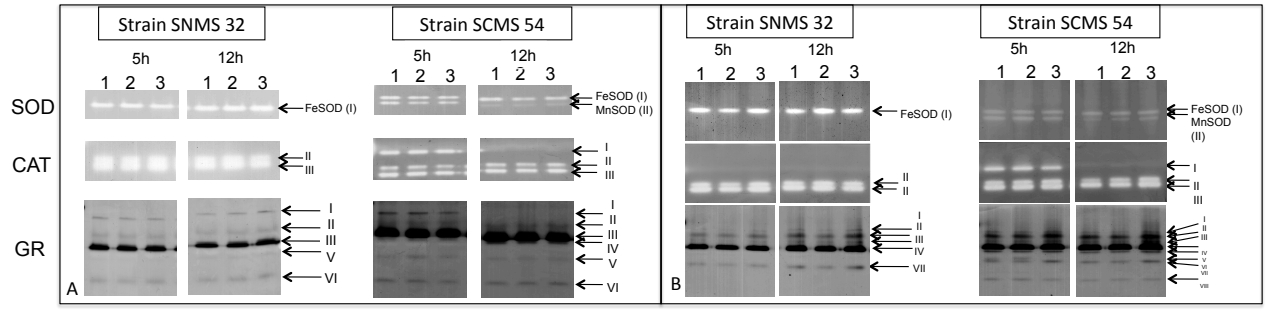

Figure 3.6- Electrophoresis gel activity of SOD, CAT and GR in non-denaturantelectrophoresis. (a) response to Cd; (b) response to Ni (after 5 h or 12 h Cd was added)

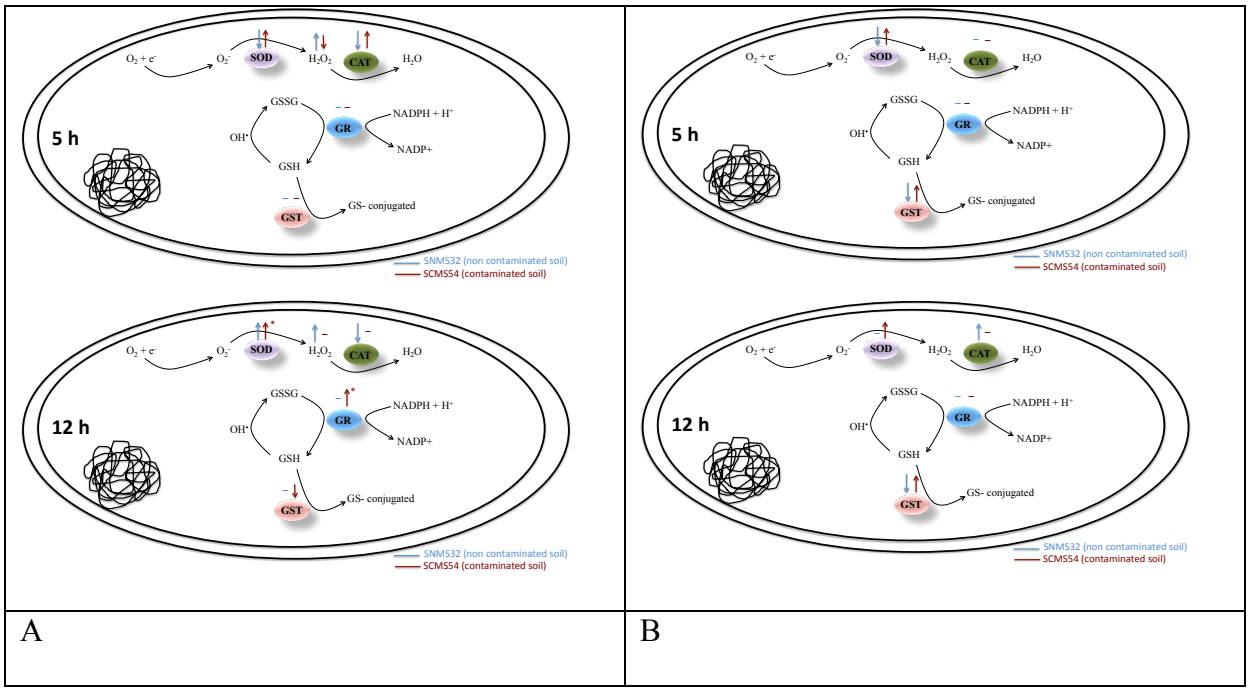

Figure 3.7- Illustrative scheme of the all studied enzymes (SOD, CAT, GR and GST) of antioxidant system and its activity in both strains (A) 5 and 12 hours after **Cd** exposure (Chapter 2) (B) 5 and 12 hours after **Ni** exposure. Blue arrow represents the activity of strain from non-contaminated soil (SNMS32). Red arrow represents the activity of strain from contaminated soil (SCMS54)

Therefore, GST plays an important role in Ni tolerance. There are eight genes that are annotated as *gst* or putative *gst* genes in *Burkholderia* sp. If we could identify which one codifies GST enzymes, we would be able to transform plants superexpressing *gst* gene aiming to increase plant tolerance.

Concluding, strain SCMS54 is indicated to proceed the phytoremediation study since it presented a greater capacity to adapt to metals due to its antioxidant response, mainly the

induction of the GST described as acting in cellular detoxification, resulting in a greater Ni bioaccumulation.

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4 *Burkholderia* sp. SCMS54 REDUCES CADMIUM TOXICITY AND PROMOTES TOMATO GROWTH

Abstract

Cadmium (Cd) has been added to the soil with the use of fertilizers, calcareous, pesticides and industrial and/or domestic effluents. It can be leached to groundwater, as well as be taken up by plants potentially leading to reduced growth and yield. Phytoremediation is a technique that uses plants to remove heavy metals from contaminated environment, as soil and water, with less impact and at a lower cost. In the soil, plant roots interact with heavy metals-tolerant microorganisms which may promote plant growth. The soil microorganisms may also be able to solubilize or mobilize soil metals acting as bioremediator. This interaction between plant-metal-microorganism-soil once understood can be used to improve phytoremediation techniques. Two multi tolerant bacteria of the *Burkholderia* genus were isolated from contaminated and uncontaminated soil of a coffee plantation. Besides the high tolerance to Cd, the strain SCMS54 produced indole-acetic acid (IAA), solubilized inorganic phosphate and produced siderophore, revealing its potential in plant-microorganism mutual and beneficial interaction. When interacting with tomato plants exposed to Cd, the bacterium led to decrease in plant peroxide concentration and chlorosis levels, promoted relative plant growth and reduced the root absorption of Cd resulting in an increase in plant tolerance to this highly toxic heavy metal. The results indicated that inoculation of tomato plants with *Burkholderia* sp. SCMS54 promotes better growth when cultivated in the presence of Cd by a mechanism that appears to decrease Cd concentration in roots as a result of a bacterial-plant root beneficial interaction.

Keywords: Heavy metal; Oxidative stress; Plant Growth Promoting Bacteria (PGPB); Phytoremediation; Tolerance mechanism

4.1 Introduction

Heavy metal (HM) pollution is a major environmental problem (ARRUDA; AZEVEDO, 2009) that reduces crop production and food quality. Unlike organic contaminants, metals are not degradable and thus remain in the environment for long periods of time; when present at high concentrations, metals can negatively affect plant metabolism (GRATÃO et al., 2005a). Cd is a transition metal whose biological function in plants is unknown. Cd can be added to the soil through the use of fertilisers, lime, pesticides and industrial and domestic effluents, thereby becoming available to plants cultivated in contaminated soil (GAMALERO et al., 2009). Cd strongly affects plants in several different ways, disturbing processes such as growth, photosynthesis and the uptake and distribution of nutrients, potentially leading to plant cell death (BENAVIDES et al., 2005; AZEVEDO, 2012; GALLEGO et al., 2012). Plants exposed to Cd (or other metals) and abiotic stresses in

general also display a wide range of antioxidant stress responses (VITORIA et al., 2003; GARCIA et al., 2006; GRATÃO et al., 2008a; GHELFI et al., 2011; CIA et al., 2012) that can help explain how oxidative metabolism is affected by such metals and how the plant responds in defence (GHELFI et al., 2011; GALLEGO et al., 2012).

A number of strategies can be employed to reduce the negative impact of HMs on plants when their bioavailable concentration in the soil is sufficiently high to cause damage. Bioremediation can be used to remove or reduce the levels of contaminants in the soil (PILON-SMITS, 2005). One important and well-described bioremediation strategy for reducing soil contaminants is phytoremediation, which uses plants that have the capability to take up HMs present in the soil, thereby allowing other, non-tolerant crops to be cultivated on the same soil after it has been remediated (YANG et al., 2005; SOUZA et al., 2013). Plant species that accumulate HMs have been effectively used to ameliorate HM contamination in soils, and a list of plant species that naturally hyper-accumulate Cd is available (GRATÃO et al., 2005b). Tomato plants in particular havebeen used as a model to study Cd phytoextraction and potential defence mechanisms in response to HMs (MADHAIYAN et al., 2007; GRATÃO et al., 2008a; MONTEIRO et al., 2011).

The interaction between microorganisms and plants can be beneficial to both under normal or stressful conditions (ANDREWS et al., 2011; 2012; KARAMANOLI et al., 2011; MARTINS et al., 2011; SATHYAPRIYA et al., 2012).In the environment, plant roots interact with soil microorganisms, including metal-resistant microbes, that can promote plant growth by facilitating the solubilisation or mobilisation of soil metals (BURD et al., 1998; MADHAIYAN et al., 2007). The same is valid for a group of soil and rhizospheric fungi and bacteria that are able to colonize plant roots and provide benefits to their hosts including the alleviation of abiotic stresses (ZAWOZNIK et al., 2011; GROPPA et al., 2012). ANDRADE et al. (2009) reported that arbuscular mycorrhizal fungi enhance Zn uptake by jack bean plants, a process that can improve the phytoremediation process. By facilitating these processes, symbiotic microbes can improve crop protection against biotic and abiotic stresses (ANDREWS et al., 2011; KHAN et al., 2011; SALDAJENO; HYAKUMACHI, 2011; TIKHONOVICH; PROVOROV, 2011), and this beneficial symbiosis may be effectively used to improve plant tolerance (GRATÃO et al., 2005a).

The *Burkholderia* genus has been reported to be beneficial to agriculture because these bacteria compete with phytopathogens and promote plant growth by establishing symbiotic relationships in the rhizosphere, fixing nitrogen (MINERDI et al., 2001; PAYNE et al., 2006), solubilising phosphate (GORIS et al., 2004; PAYNE et al., 2006; LUVIZOTTO et al., 2010)

and degrading xenobiotic compounds. These organisms have potential as bioremediators, and their metabolic diversity allows them to use herbicides and hydrocarbons as carbon and energy sources (COENYE et al., 2001).

Taken together, these data suggest that phytoremediation techniques can be further improved, and the phytoremediation potential of certain plants be increased through an association with microorganisms, as observed by Luo et al. (2011). Therefore, in this report we investigated the effects of *Burkholderia* sp. SCMS54, a Cd-tolerant bacterial strain isolated from a coffee plantation field that was contaminated with high concentrations of Cd (TEZOTTO et al., 2012), on the growth, pattern of Cd uptake and Cd distribution in tomato plants exposed to $CdCl_2$.

4.2 Materials and Methods

4.2.1 Bacterial strain and tomato plants

The bacterial strain used were tolerant to Cd, Ni and Zn and classified as *Burkholderia* sp. strain SCMS54 isolated from Cd contaminated soil (described in Chapter 2). In the green house studies we used sensitive tomato plants (*Solanum lycopersicum* L. cultivar Calabash Rouge) selected in previous study (PIOTTO, 2012).

4.2.2 Physiological characterisation of SCMS54

Indole-acetic acid (IAA) production was evaluated using a modified version of the qualitative method developed by Bricet al. (1991). First, the bacterial isolate was inoculated in a 5% tryptone soy broth (TSB) medium supplemented with L-tryptophan (5 mM) and incubated with shaking (150 rpm) at 28ºC for 72 h in the dark. The cultures were then centrifuged (5 min, 7000 x g, room temperature), and 400 µL of Salkowski reagent was added to 900 µL of the supernatant. This mixture was incubated for 30 min at room temperature and analysed in a spectrophotometer (530 nm). The obtained absorbance values were interpolated in a standard curve to determine the IAA concentration.

The ability of the *Burkholderia* sp. strain to solubilise inorganic phosphate was shown by a halo obtained after the cultivation of the isolate in culture medium supplemented with inorganic phosphate $Ca_3(PO_4)_2$ at 28ºC for 72 h, as reported by Vermaet al. (2001). The results were quantified by estimating the halo size (cm) and dividing it by the colony size (cm) to generate a solubilisation index (SI).

Siderophore production was evaluated using a standard method on CAS-Agar medium (SCHWYN; NEILANDS, 1987). The isolate was cultivated on CAS-Agar plates at 28ºC for

72 h, and the production of siderophores was confirmed by the presence of a yellow or orange halo around the bacterial colony.

4.2.3Transformation of the *Burkholderia* sp. strain SCMS54 with pCM88for gfp labeling

The plasmid pCM88 carrying a *gfp* genewas kindly provided by Dr. Christopher James Marx and Dr. Mary Lidstrom (University of Washington). A DH5-α (TOP-10-Invitrogen) strain was used as the host cell in the cloning experiment. The plasmid extraction was performed using the miniprep kit (Quiagen, Valencia, CA, USA) according to the manufacturer's recommendations.

For electroporation, the competent cells were obtained according to a modified protocolreported by Ferreira et al. (2008). Briefly, bacterial cells were grown for 3 d at 28^{o}C on solid LB (SAMBROOK; RUSSEL, 2001) medium. One loop of bacterial cells was inoculated into 5 mL of LB liquid medium and incubated at 28^{o}C with shaking until the culture reached an OD_{600nm} 0.1 - 0.2. The culture was transferred into 50 mL of liquid medium and grown to mid-exponential growth phase (OD 1.0) at 28^{o}C. Cells were collected by centrifugation (3000 x g) at 4^{o}C for 10 min. The cell pellets were washed a total of three times (twice with chilled, sterile water and once with 10 % glycerol at 4^{o}C) and were then resuspended in 10% glycerol to a concentration of approximately 10^{9} cellsper mL. Aliquots of 100 µL of cells were frozen in liquid nitrogenand then stored at –80^{o}C.

Burkholderia sp. SCMS54 was electroporated (Gene Pulser, BioRad - 2.5 kV, 25 µF, 100, 200 or 400 Ω, 0.2 cm cuvettes) with 50 ng of pCM88 plasmid. After incubation at 28ºC (150 rpm) for 1 h, the transformed cells were plated onLB medium supplemented with tetracycline. One colony was randomly selected, stored at -80ºC and named SCMS54:*gfp* infurther studies.

4.2.4 Plant-bacteria interaction bioassay

4.2.4.1 Experiment 1 – Hydropony assay

An*in vivo*hydropony bioassay was performed to quantify the effect of strain SCMS54:*gfp*on the Cd tolerance and phytoextraction of tomato plants (*Solanum lycopersicum* L. cultivar Calabash Rouge). Tomato seeds were surface-disinfected with 70% ethanol for 1 min, 2.5% sodium hypochlorite for 2 min, 70% ethanol for 1 min and at least two subsequent rinses in sterile distilled water. The seeds were then inoculated with strain SCMS54:*gfp*, incubated at room temperature for 1 h in sterile distilled water (control) or in a bacterial suspension(10^{8} CFU.mL^{-1}) of strain SCMS54:*gfp*. Then both inoculated and non-inoculated

seeds were sown in a vermiculite substrate and maintained in a greenhouse. During the experiment, the tomato plants were grown under natural light with a solar irradiance of 17.50 ± 5.54 $MJ.m^{-2}.d^{-1}$, a temperature of 21.29 ± 2.65°C and a relative humidity of 86.40 ± 6.65%.

After 30 d in vermiculite, the tomato plants (with and without SCMS54 seed inoculation) were transferred to 12-L trays containing Hoagland and Arnon (1950) nutrient solution at 10% ionic strength and a pH of 6. The nutrient concentrations were increased daily for up to 3 d to allow the plants to adapt. On the fourth day, the solution was changed to 50% ionic strength and supplemented with 0 or 30 μM $CdCl_2$. Resulting in four treatments: (i)-SCMS54:*gfp* –Cd; (ii) -SCMS54:*gfp* +Cd; (iii) +SCMS54:*gfp* –Cdand (iv) +SCMS54:*gfp* +Cd. Eight days after Cd addition, three plants from each treatment were sampled and divided into root, stemand leaf fractions.

Biomass measurement

The plant tissues were dried at 55°C for 5 d to reach a constant weight and then weighed. Plant samples were also collected for dry mass measurements at the time of Cd addition. The initial plant dry mass values(control and under Cd exposure) were subtracted from the final plant dry mass measurements, and values were expressed as the growth rate during 8 d.

Cd accumulation

The Cd concentrations in tomato roots, leaves and stems were determined by ICP-OES after the plants were dried and subjected to an overnight digestion in a solution of nitric acid:perchloric acid (3:1) with heating.

Bacterial re-isolation

Burkholderia sp. strain SCMS54:*gfp* was isolated from leaves, stems, roots and the hydroponic solution 8 d after Cd addition, according to the procedure reported by Araújo et al. (2002). The plant tissues (from inoculated and non-inoculated seeds) were weighed and macerated into 2 mL of PBS buffer, and appropriate dilutions were plated onto Nutrient Agar medium supplemented with 100 $\mu g.mL^{-1}$of the antibiotics kanamycin (natural resistance) and tetracycline (plasmid resistance). After the plates were incubated for 3 d at 28ºC, they were exposed to UV light, and the number of CFU (colony forming units) was determined to estimate the population density of only the fluorescent bacteria.

Fluorescence microscopy

The roots of the plants from each treatment (+SCMS54:*gfp*+Cd; +SCMS54:*gfp*-Cd; -SCMS54:*gfp*+Cd +SCMS54:*gfp*-Cd) were removed, cut longitudinally, arranged on amicroscope slide with waterand immediately observed. Fluorescence microscopy was

performing using a Zeiss Axiophot-2 epifluorescence microscope with the recommended filter for GFP analysis. The GFP-tagged bacterial cells were excited with the use of a 490-ηm filter, and the images were captured with a video camera using the ISIS software (Meta Systems, Germany).

Hydrogen peroxide contents

Hydrogen peroxide (H_2O_2) contents were determined according to the method of Alexieva et al. (2001). Three samples (roots and leaves) from each treatment group were macerated in liquid nitrogen and homogenised in 0.1% (w:v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12100 x g for 15 min at 4ºC, and 200 μL of the supernatant was added to 200 μL of 100 mM potassium phosphate buffer (pH 7.0) and 800 μL of 1 M KI.The reaction medium was left in the dark in an ice bath for 1 h. The absorbance was then read at 390 nm. The H_2O_2 contents for all samples were determined using H_2O_2 as a standard.

Chlorophyll contents

The chlorophyll contents were indirectly measured in all four treatments using a SPAD 502 chlorophyll meter (Minolta Corp. Ramsey, NJ, USA) (GRATÃO et al., 2012). The lateral border of the central leaflet from the fifth completely expanded leaf was used for this evaluation, and each leaf was measured three times. The data are presented in SPAD index units.

4.2.4.2 Experiment 2 – Pot assay

Plant-bacteria interaction pot bioassay

An *in vivo* bioassay was performed to verify the effect of strain SCMS54:*gfp* on the Cd tolerance and phytoextraction by Cd-sensitive tomato plants (*Solanum lycopersicum* L. cultivar Calabash Rouge). Tomato seeds were surface-disinfected similar as reported in hydroponic assay.

After 30 d in vermiculite, the tomato plants were transferred to 2-L pots containing sandy soil previously contaminated with 0 or 2 $mg.kg^{-1}$ of $CdCl_2$ (still allowed in crop soils, the limit detrminated by CETESB (2005) is 3 $mg.kg^{-1}$). In total, the present experiment presented four treatments: 1. -SCMS54:*gfp* –Cd (0 μM); 2. -SCMS54:*gfp* +Cd (2$mg.kg^{-1}$); 3. +SCMS54:*gfp* –Cd (0 μM); 4. +SCMS54:*gfp* +Cd (2$mg.kg^{-1}$). Fertlizer Peters Professional® 20-20-20water soluble fertilizer (Produquímica) adding macro and micronutrientes every four days. Experimental design was performed completely random with four biological repetition each tretament. After 26 days of Cd exposure, plants were colected, washed and divided into root, stem and leaf fractions.

Biomass measurement

The plant tissues were dried at 55°C for 5 d to reach a constant weight and then weighted. Three repetitions were also collected for dry mass measurements at the time of Cd addition. The initial plant dry mass values (control and under Cd exposure) were subtracted from the final plant dry mass measurements, and values were expressed as the growth rate during 26 d.

Nutrient analysis and Cd accumulation in tomato plants and soil

Four plants were randomly picked for the P, S, Mg, Ca, Fe, Cu, Zn, Mn and Cd concentrations measurementsin tomato roots, leaves and stems were determined by ICP-OES after the plants were dried and subjected to an overnight digestion in a solution of nitric acid:perchloric acid (3:1) with heating according to Malavota, Vitti and Oliveira (1997). Nitrogen quantification was performed according to Kjeldahl (1883). Cd were extracted by DTPA (MANN; RITCHIE, 1993). The soil Cd contents were also determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).

Bacterial re-isolation

Burkholderia sp. strain SCMS54:*gfp* was isolated from leaves, stems, roots and soil 26 d after Cd exposure according to the procedure described in hydropony assay.

Chlorophyll contents

The chlorophyll contents were indirectly measured in all three treatments using a SPAD 502 chlorophyll meter (Minolta Corp. Ramsey, NJ, USA) described previously in the hydroponic assay.

Total RNA isolation and cDNA synthesis

Plant roots were collected from all treatment describe above, and the RNA was isolated using the Ambion Purelink kit (Life Technology). The RNA was ressuspended in 30 μL of dietyl-pirocarbonate (DEPC) treated water and stored at -80^{o}C. The integrity and amount of extracted RNA was verified in a denaturant agarose gel 1.2% in FA 1X (MOPS 200 mM, sodium acetate 50 mM and EDTA 10 mM), containing formaldehyde (0.7%) and ethidium bromide (0.3 g.mL^{-1}). The RNA was quantified by O.D.$_{260}$, measured in spectrophotometer Quantifluor-ST Fluorometer (Promega, Madison, USA). All material used obtain and treat the RNA was sterilized and/or treated with DEPC to eliminated RNAse. Total RNA (1 μg) was reverse-transcribed into cDNA using dT primers (Invitrogen) and 200 U SuperscriptII Rnase H- reverse transcriptase (Invitrogen) according to the procedure supplied with the enzyme.

qPCR analysis

All the amplification reactions by qPCR used the thermocycler StepOne (Applied Biossystems) programmed to a initial denaturation of 5 minutes at 94°C, followed by 30 cycles of 15 seconds at 94°C and 1 minute at 60°C. The specificity of qPCR primer sets were evaluated by the melting curve with gradient from 60 to 96°C ranging 1°C each 30 seconds. Each amplification reaction used 2 µL of cDNA (30 ng), 10 µM of each primer and the Ambion SYBR Green qPCR. We used the following primers: I. Heat shock gene, LeHsp90F: GAGAATCATGAAGGCACAAGCTCTC, LeHsp90R: CTTATCAGCATCAGCTCTCTTCCTG, II. Metallothionein gene, LeMTF: GCTGTGGATCTAGCTGCAAGTGCG and LeMTR: AAGGGTTGCACTTGCAGTCAGATCC (GOUPIL et al., 2009; TOMBULOGLU et al., 2012), III. broad range heavy metal transporter gene, NrmR3: TGT GCT ATC GTC CCA ACT CTG AT and NrmR33: TGG ACA CAC TGA AGA ACG TTG AG (OUZIAD et al., 2005).

The selection of endogenous gene, to be used as a normalizer was made by testing the Ct values for different described endogenous genes. Thereby, the actin gene (ActinF: GGGATGGAGAAGTTTGGTGGTGG and ActinR: CTTCGACCAAGGGATGGTGTAGC) (TOMBULOGLU et al., 2012) presented the lowest deltaCt and the higher amplification efficiency, resulting in its selection as the normalizer for gene expression analysis.

4.2.5 Experimental design and statistical analysis

For plant dry mass measurement there were 3 repetition (with two plants per repetition) of each treatment, for plant Cd content and SPAD measurement there were 4 repetition (1 plant per repetition) of each treatment and all other analysis (bacterial reisolation, bacterial growth curve, bacteria Cd content determinations, physiological characterisation analysis,fluorescence microscopy analysis and hydrogen peroxide content) there were 3 repetition of each treatment. The experimental design was performed completely random, and all the samples were measured in a random order. The significance of the observed differences was verified using a one-way analysis of variance (ANOVA, using R software version 2.15.1) followed by Duncan's test, with a significant cut off of $p < 0.05$. In gene expression data, we performed the test of student ($\alpha=0.05$).

4.3 Results

4.3.1 Physiological characterisation of the SCMS54 isolate

The physiological characterisation of *Burkholderia* sp. SCMS54 demonstrated that this bacterium produces 20 µg of Indole-Acetic Acid (IAA), solubilises inorganic phosphate with a SI > 1.8 and produces siderophores resulting in a significant orange halo around the bacterial colony.

4.3.2 Tomato hydropony bioassay with SCMS54:*gfp* interaction (Experiment 1)

SCMS*54:gfp* was inoculated on tomato seeds before they were sown in vermiculite. Prior to the transplant, the fluorescent SCMS*54:gfp* was only able to be re-isolated from inoculated seedlings, mainly in the roots. This bacterium (SCMS54:*gfp*) was not observed in non-inoculated tomato plants. Additional data were collected 8 d after the addition of Cd, and, at that point, the fluorescent SCMS*54:gfp* was re-isolated from both shoots and roots of inoculated plants (Table 4.1) that were growing in the hydroponic solution under Cdstress. The greatest number of SCMS*54:gfp* CFUs was found in the roots, and the presence of Cd further increased the number of SCMS*54:gfp* CFUs (Table 4.1). Fluorescence microscopy analysis supported these findings, as more fluorescent bacteria were observed in the roots with Cd than in those without Cd. SCMS*54:gfp* was observed mainly in the apoplast of tomato plants (Figure 4.1). The plants inoculated with SCMS54:*gfp* bacteria exhibited better growth in Cd treatments than did the non-inoculated plants. The chlorosis levels were lower in plants inoculated with *Burkholderia* sp., and this result was independent of the Cd supply (Figure 4.2 D). Although Cd induced chlorosis in tomato plants, its effect was attenuated by the presence of *Burkholderia* sp. (Figure 4.2 D).

Table 4.1- Density of SCMS54:*gfp* bacteria re-isolated from tomato in hydropony. The abundance data, in CFU/g of tissue, were log-transformed to stabilise the variance. The results are the means ± SE (n = 3)

Treatments	Bacterial density			
	Leaves		Roots	
	-Cd	+Cd	-Cd	+Cd
-SCMS54:*gfp*	nd*	nd	nd	nd
+SCMS54:*gfp*	2.98 ± 0.26 a	3.27 ± 0.29 a	4.14 ± 0.13 B	5.00 ± 0.10 A

*nd = not detected. Means of three replicates followed of standard error. Different letters in the same row indicate statistically different means ($P< 0.05$).

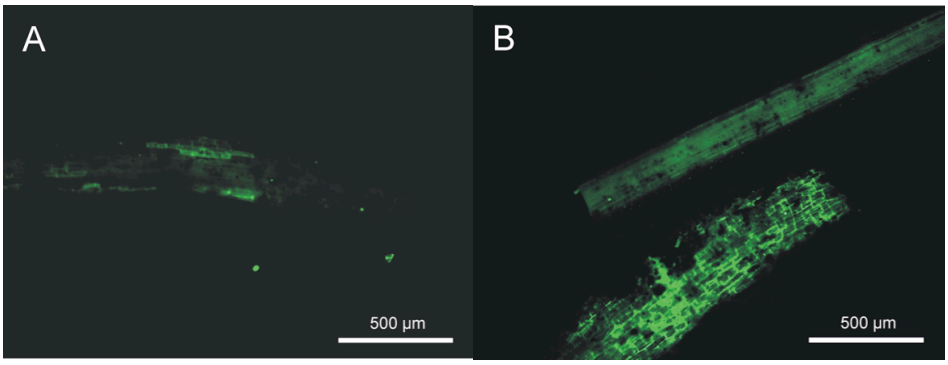

Figure 4.1- Fluorescence of tomato root surface biofilm formed by SCMS54:*gfp*. (A) Low bacterial cell density during root interaction of tomato without $CdCl_2$ supplement. (B) High concentration of SCMS54:*gfp* on a tomato root after 30 µM $CdCl_2$ supplement. The samples were collected at 42 d after inoculation. The microscopy magnification is 200 x in both images

The dry biomass measurements of shoots, roots and total plants were higher in Cd-stressed plants inoculated with SCMS54:*gfp* than in non-inoculated plants (Figure 4.2 A-C). SCMS54:*gfp* inoculated plants exhibited a 50% higher shoot and dry biomass than did non-inoculated plants in the presence of Cd (Figure 4.2 A and C). In roots, the bacterial inoculation increased the root biomass independent of the presence of Cd in the growth solution.

Cd accumulated predominantly in the roots in Cd treatments. However, SCMS54:*gfp*-inoculated plants exhibited less Cd accumulation in the roots than did the non-inoculated (Figure 4.2 E).The inoculated plants also showed a 14% lower level of total Cd when compared to non-inoculated plants (Table 4.2).

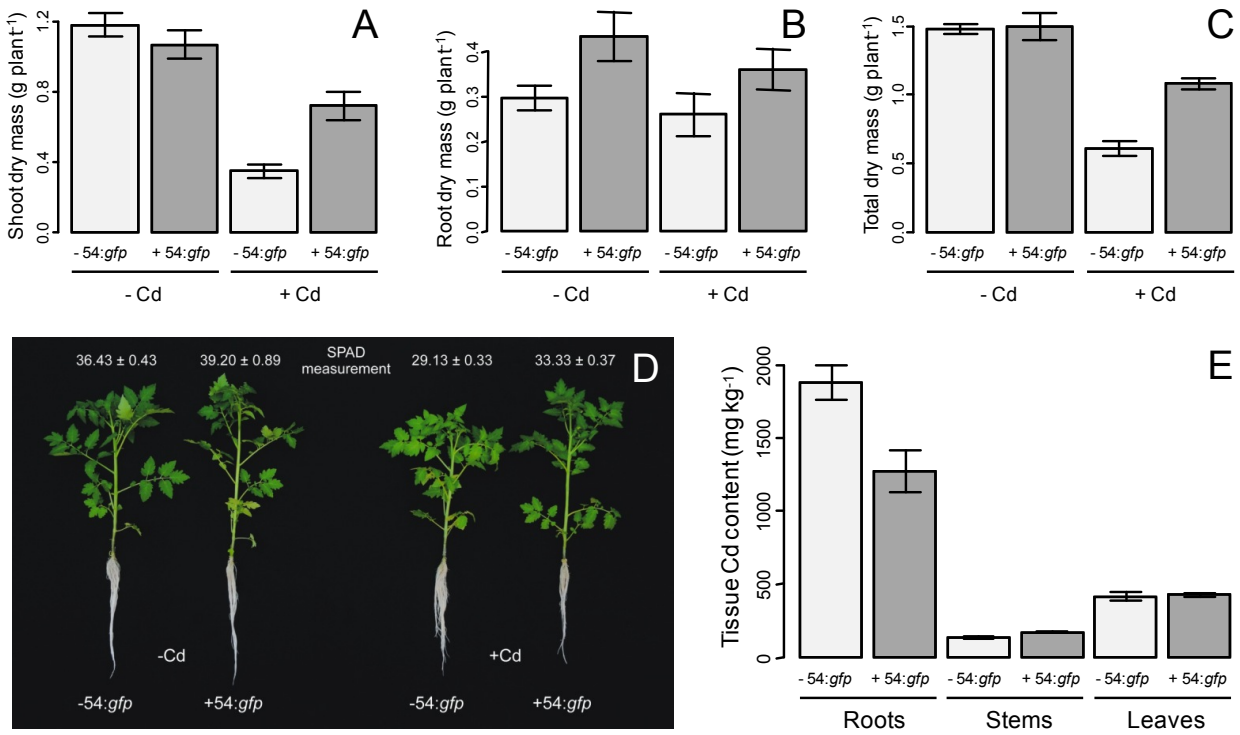

Figure 4.2- The enhancement of hydropony tomato plant growth by *Burkholderia* sp. SCMS54:*gfp*. The strain was inoculated on tomato seeds (*Solanum lycopersicum* L., cultivar Calabash Rouge). After a 30-d period, seedlings of inoculated and non-inoculated tomato plants were transferred to a hydroponic system with and without 30 µM $CdCl_2$. Six plants were sampled 8 d after Cd supplementation. The presented data are the average dry weights of (A) shoots, (B) roots (C) and entire plants. The bars represent the standard error of each treatment. (D) Tomato without (left) and with (right) Cd supplementation, both with and without SCMS54:*gfp* inoculation. (E) Cd bioaccumulation in tomato plants with and without SCMS54:*gfp* inoculation. The tissue Cd content was measured in leaves, stems and roots using inductively coupled plasma-optical emission spectrometry (ICP-OES). The results are the means of three replicates for each sample. The bars represent the standard error for each treatment

Table 4.2- Total Cd bioaccumulation in tomato tissue in hydropony. The Cd accumulation levels were measured in mg Cd/tissue. The results are the means ± SE (n = 3)

Tissue	**- SCMS54:*gfp***	**+ SCMS54:*gfp***
Roots	711.12 ± 89.54 Aa	611.01 ± 57.40 Aa
Steams	81.10 ± 1.94 Cb	126.19 ± 4.91 Ba
Leaves	449.82 ± 10.78 Bb	561.76 ± 21.86 Aa
Total	**1242.03 ± 87.30 a**	**1298.95 ± 34.32 a**
Translocation	**0.43 ± 0.03 a**	**0.53 ± 0.03 a**

Means of three replicates followed of standard error. Different uppercase letters in the same column indicate statistically different means ($P< 0.05$). Different lowercase letters in the same row indicate statistically different means ($P< 0.05$).

The peroxide contents did not vary in the leaves of tomato plants, independent of the treatment group (Table 4.3). However, roots of all plants inoculated with *Burkholderia* sp. SCMS54:*gfp* exhibited lower peroxide contents (14% without Cd and 33% in the presence of Cd - Table 4.3).

Table 4.3- Tomato response under Cd stress conditions in hydropony. Peroxide (H_2O_2) quantification ($\mu mol.g^{-1}$ of tissue) was used as an indicator of stress. The presented results are the means ± SE (n = 3)

Treatments	**Peroxide quantification**			
	Leaves		**Roots**	
	-Cd	**+Cd**	**-Cd**	**+Cd**
- SCMS54:*gfp*	8.48 ± 0.84 Aa	9.69 ± 0.87 Aa	4.22 ± 0.04 Ab	5.21 ± 0.17 Aa
+SCMS54:*gfp*	9.25 ± 0.35 Aa	9.72 ± 1.72 Aa	3.62 ± 0.13 Ba	3.48 ± 0.17 Ba

Means of three replicates followed of standard error. Different uppercase letters in the same column indicate statistically different means ($P< 0.05$). Different lowercase letters in the same row indicate statistically different means ($P< 0.05$).

4.3.3 Tomato - SCMS54:*gfp* pot interaction assay (Experiment 2)

The dry biomass measurements of shoots and roots were higher in Cd-stressed plants inoculated with SCMS54:*gfp* than in non-inoculated plants (Figure 4.3). SCMS54:*gfp* inoculated plants exhibited a 78% higher shoot than did non-inoculated plants in the presence

of Cd (Figure 4.3 A). In roots, the bacterial inoculation also doubles root biomass in the presence of Cd.Cd induced chlorosis in tomato plants, although the chlorosis levels were attenuated in plants inoculated with *Burkholderia* sp. (Figure 4.3 C).

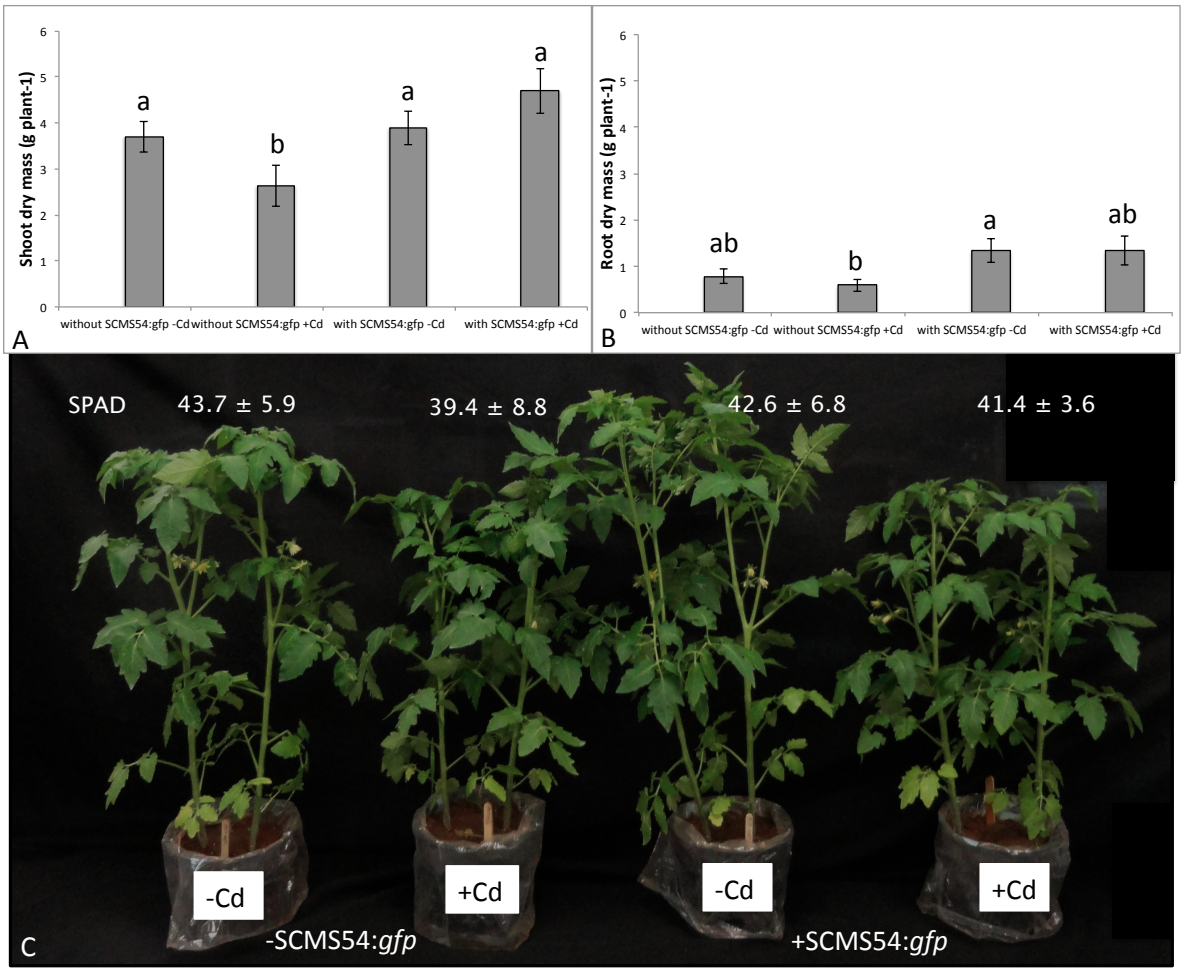

Figure 4.3- The enhancement of tomato plant growth by *Burkholderia* sp. SCMS54:*gfp* in pot. The strain was inoculated on tomato seeds (*Solanum lycopersicum* L. cv Calabash Rouge). After a 30-d period, seedlings of inoculated and non-inoculated tomato plants were transferred to pots with and without 2 $mg.kg^{-1}$ $CdCl_2$. Six plants were sampled 26 d after transplanted. The presented data are the average dry weights of (A) shoots, (B) roots. The bars represent the standard error of each treatment. (C) Tomato without and with Cd supplementation, both with and without SCMS54:*gfp* inoculation

Cd accumulated predominantly in the shoot in Cd treatments. However, SCMS54:*gfp*-inoculated plants exhibited less Cd accumulation in shoots and roots than did the non-inoculated. The inoculated plants also showed a 38% and 23% lower level of Cd in leaves and roots, respectively, when compared to non-inoculated plants (Table 4.5). Cd concentration in soil was not detected in non contaminated soil, and 1.56 and 1.65 $mg.kg^{-1}$ in treatments +SCMS54:*gfp*-Cd and +SCMS54:*gfp*+Cd, respectivilly, which represents around 80% of the applied Cd (2 $mg.kg^{-1}$).

Prior to the transplant, the fluorescent SCMS54:*gfp* was only able to be re-isolated from inoculated seedlings. This fluorescent bacterium was not observed in non-inoculated tomato plants. SCMS54:*gfp* CFUs was found in the shoot, roots and rizosphere.The presence of Cd, SCMS54:*gfp* were more abundant in roots, while without Cd SCMS54:*gfp*colonized mainly shoots (Table 4.4), the presence of Cd in the rizosphere benefits SCMS54:*gfp*growth.

Table 4.4-Density of SCMS54:*gfp* bacteria re-isolated from tomato in pots. The abundance data, in CFU/g of tissue, were log-transformed to stabilise the variance. The results are the means ± SE (n = 3)

Treatment	Leave	Root	Rizosphere
-SCMS54:*gfp*-Cd	nd*	nd	nd
-SCMS54:*gfp*+Cd	nd	nd	nd
+SCMS54:*gfp*-Cd	5.35±0.01 Aa	3.45±0.10 Bb	2.38±0.01 Bc
+SCMS54:*gfp*+Cd	4.84±0.00 Bb	5.55±0.00 Aa	3.12±0.07 Ac

*nd = not detected. Means of three replicates followed of standard error. Different uppercase letters in the same collum indicate statistically different means ($P< 0.05$). Different lowercase letters in the same row indicate statistically different means ($P< 0.05$).

SCMS54:*gfp* inoculation changes nutrient balance in tomato plants. Plants inoculated with *Burkholderia* sp. (treatment +SCMS54+Cd) decrease the absorption of most nutrients: N, P, K, S, Mg, Ca, Mn and Zn, while treatment with only Cd did not cause a great unbalance in nutrients absorption. On the other hand, Fe content was increased mainly in roots and P content was increased in shoot in the presence of the inoculated bacteria (Table 4.5). The presence of only Cd increased Cu, S and P content.

Table 4.5– Nutrient balance in inoculated and non inoculated plants with and without Cd

Treatment	N	P	K	Ca	S	Mg
			g.Kg-1			
			Leave			
-SCMS54-Cd	38.64 ± 3.9 ab	3.99 ± 0.85 b	19.12 ± 2.29 ab	12.01 ± 1.02 a	6.02 ± 0.16 b	7.65 ± 0.72 ab
+SCMS54-Cd	36.05 ± 4.63 ab	5.51 ± 0.65 a	22.95 ± 0.93 a	9.68 ± 0.39 b	6.40 ± 0.09 a	8.1 ± 0.65 a
-SCMS54+Cd	39.26 ± 4.38 a	4.59 ± 0.49 ab	15.87 ± 1.36 b	12.04 ± 0.72 a	6.54 ± 0.06 a	7.23 ± 0.41 ab
+SCMS54+Cd	33.80 ± 3.55 b	4.68 ± 0.45 ab	17.02 ± 5.17 b	8.75 ± 1.71 b	5.82 ± 0.13 b	7.20 ± 0.41 b
			Steam			
-SCMS54-Cd	16.60 ± 4.18 a	2.06 ± 0.27 b	26.39 ± 2.56 b	5.38 ± 1.55 a	2.40 ± 0.53 ab	5.05 ± 1.10 a
+SCMS54-Cd	14.71 ± 1.41 ab	3.45 ± 0.55 a	26.97 ± 1.13 ab	4.38 ± 0.80 ab	2.25 ± 0.21 ab	4.38 ± 0.40 ab
-SCMS54+Cd	16.07 ± 1.80 ab	2.86 ± 0.35 a	30.41 ± 3.80 a	4.66 ± 0.73 ab	2.75 ± 0.38 a	5.05 ± 0.93 ab
+SCMS54+Cd	12.78 ± 0.96 b	3.11 ± 0.43 a	26.39 ± 3.64 ab	3.99 ± 0.71 b	1.88 ± 0.22 b	3.85 ± 0.74 b
			Root			
-SCMS54-Cd	34.69 ± 15.02 a	1.30 ± 0.70 a	6.38 ± 3.52 a	2.38 ± 1.04 a	2.24 ± 0.22 ab	11.10 ± 5.18 b
+SCMS54-Cd	13.49 ± 6.31 b	0.94 ± 0.41 b	4.40 ± 0.45 b	1.80 ± 0.91 ab	2.58 ± 0.47 a	14.17 ± 6.14 a
-SCMS54+Cd	13.83 ± 7.63 b	1.24 ± 0.77 ab	5.70 ± 2.78 ab	2.03 ± 0.89 ab	1.99 ± 0.32 b	2.80 ± 5.75 ab
+SCMS54+Cd	8.96 ± 0.43 b	0.98 ± 0.41 ab	4.93 ± 0.64 ab	1.77 ± 0.12 b	2.23 ± 0.25 ab	3.23 ± 1.52 ab

	Cu	Fe	Mn	Zn	Cd
			mg.kg-1		
			Leave		
-SCMS54-Cd	10.75 ± 0.55 ab	451.25 ± 44.35 ab	806.25 ± 86.99 bc	58.88 ± 9.16 ab	0.16 ± 0.04 c
+SCMS54-Cd	12.13 ± 1.71 a	266.25 ± 180 b	716.25 ± 274.7 a	61.75 ± 4.85 a	0.0 ± 0.04 c
-SCMS54+Cd	12.00 ± 0.70 a	430.00 ± 40.77 ab	957.50 ± 96.47 b	52.88 ± 6.83 b	23.27 ± 3.95 a
+SCMS54+Cd	9.25 ± 0.55 b	468.75 ± 77.73 a	640.00 ± 126.93 c	54.38 ± 10.34 ab	14.5 ± 2.40 b
			Steam		
-SCMS54-Cd	3.50 ± 0.35 b	68.75 ± 9.6 ab	270.50 ± 41.24 ab	80.13 ± 17.65 b	0.0 ± 0.04 c
+SCMS54-Cd	3.88 ± 0.41 ab	71.25 ± 8.19 a	237.88 ± 27.16 ab	94.00 ± 8.14 a	0.0 ± 0.04 c
-SCMS54+Cd	4.25 ± 0.56 a	57.50 ± 7.50 b	292.63 ± 57.48 a	83.00 ± 6.68 ab	17.13 ± 2.33 a
+SCMS54+Cd	3.75 ± 0.56 ab	67.50 ± 5.59 ab	200.13 ± 39.07 b	80.75 ± 11.16 ab	12.77 ± 2.3 b
			Root		
-SCMS54-Cd	13.83 ± 6.43 b	11103.33 ± 8399 b	400.00 ± 182,79 a	98.50 ± 45.88 a	0.28 ± 0.13 b
+SCMS54-Cd	16.00 ± 5.76 ab	20216.67 ± 4842 a	263.50 ± 41.84 b	74.67 ± 15.23 ab	0.0 ± 0.04 b
-SCMS54+Cd	19.67 ± 11.60 a	3663.33 ± 8649 ab	418.33 ± 181.98 a	96.00 ± 42.28 ab	11.55 ± 3.86 a
+SCMS54+Cd	17.83 ± 5.21 ab	8116.67 ± 6441 ab	283.33 ± 27.46 b	74.83 ± 4.74 b	8.96 ± 0.39 a

The presence of the bacteria in tomato plants induce the expression of *hsp90* and *nrmr3* genes, a chaperon and a broad range heavy metal transporter, respectively. Moreover, in the presence of Cd, only in the presence of the inoculated bacteria (SCMS54:*gfp*) metallothionein gene is induced (Figure 4.4).

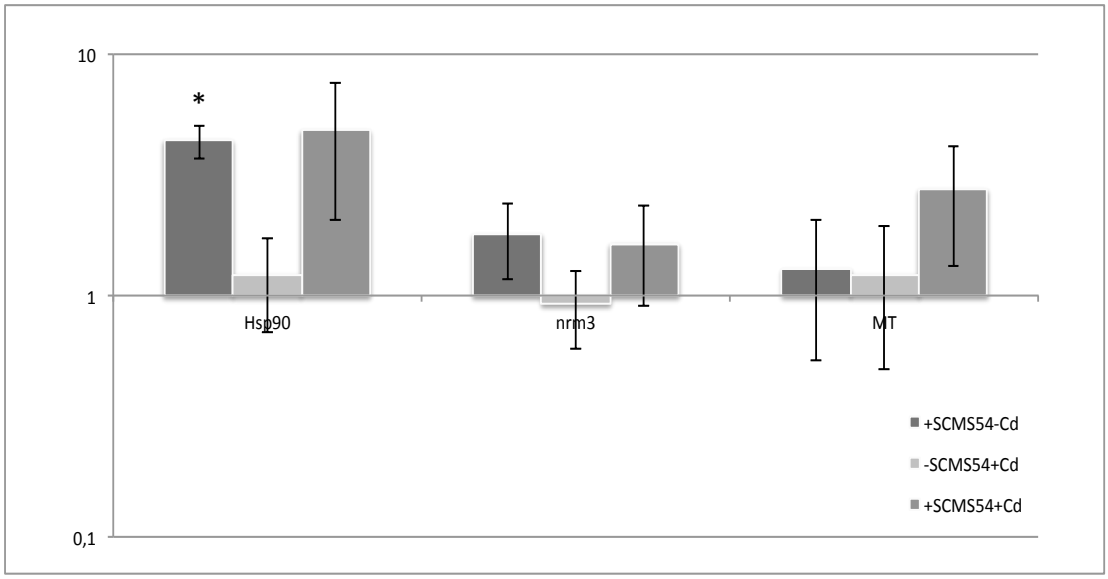

Figure 4.4- Relative effects of +SCMS54-Cd, -SCMS54+Cd and +SCMS54+Cd treatments, compaired to control (-SCMS54-Cd) on tomato plants gene expression: Hsp90 (chaperone), Nrm3 (heavy metal transporter) and MT (metallothionein). The actin as a reference gene. The results are means +/- standard errors of three replicates. Values of asterisks (*) differ statistically of control treatment (α= 0.05) according to t test of Student

4.4 Discussion

Cd toxicity is a worldwide agricultural and environmental issue (GRATÃO et al., 2005a; AZEVEDO; LEA, 2011), and the introduction of Cd into the environment is increasing due to inappropriate industrial residue management. Further research is needed to understand the genetics and physiology of microorganisms under HM stress and to develop and optimise bioremediation techniques to minimise soil and water contamination (AL HASIN et al., 2010). Several studies have indicated that plants may stabilise HM more effectively when they are inoculated with plant growth-promoting bacteria (PGPB) due to induced plant-HM tolerance, growth and survival (PETRISOR et al., 2004; WU et al., 2006; GRANDLIC et al., 2008).There are several mechanisms by which tolerance can be achieved, and it appears that this selected microorganism (SCMS54) may be tolerant to different HM due to different tolerance mechanisms, such as detoxification, exclusion, extracellular precipitation, membrane-associated metal pumps and intracellular Cd input (ROANE; PEPPER, 2000; SCHWAGER et al., 2012).

Members of the genus *Burkholderia*are commonly found in the rhizosphere and in soil microbiota (VALVERDE et al., 2006)is one of the most versatile and adaptable genus (COMPANT et al., 2008), including species that are tolerant to HMs, some of which have previously been shown to be able to grow at up to 2.5 mM of Cd (ABOU-SHANAB et al.,

2007). Previous work and our data suggest that this genus may be particularly promising for use in the bioremediation of HM-contaminated soils. To assess the potential of this isolate to increase plant tolerance to Cd and, indirectly, its potential as a bioremediator, we analysed a serie of parameters of *Burkholderia* sp. strain SCMS54. Initially, Cd tolerance was investigated based on bacterial growth in the presence of Cd and the bioaccumulation of this metal in the bacteria. A high accumulation of Cd was observed in the bacterial pellet for both studied concentrations (244.36 $\mu g.g^{-1}$ and 679.57 $\mu g.g^{-1}$ at 0.3 mM and 1.28 mM Cd, respectively) (chapter 2). The Cd concentrations detected in the bacteria were shown to be higher than those observed in *Brevibacillus brevi*(VIVAS et al., 2005), which accumulated 8.4 $\mu g.g^{-1}$, and lower than in *Methylobacterium oryzae*(MADHAIYAN et al., 2007), which accumulated 3372 $\mu g.g^{-1}$ (30 $nmol.mg^{-1}$) Cd. Our results demonstrating that *Burkholderia* sp. is more tolerant and able to accumulate higher Cd amounts than other bacteria suggest that its use in association with plant species that are not Cd tolerant may be an alternative to improve plant tolerance. For example, Vivas et al. (2003) demonstrated that arbuscular mycorrhizal (AM) strain *Glomus mosseae* and *Brevibacillus* sp., also isolated from Cd contaminated soil, increased *Trifolium* plant biomass. This characteristic is of great importance when considering the growth performance of plants under Cd stress because once the bacteria have immobilised Cd, its potential toxicity with respect to root development is decreased, favouring a better nutrient uptake by the plant and resulting in normal or even superior growth performance.

In addition to its high tolerance to Cd, this bacterium (SCMS54:*gfp*) in hydroponic system, was shown to be able to predominantly colonise the roots of the Calabash Rouge tomato cultivar, which was previously characterised as a Cd sensitive tomato (PIOTTO, 2012), while in pot assay it colonizes both shoot and root, probably in pot assay, the tomato plants were older, and the bacteria had more time to colonize shoot. Because we used a Cd sensitive tomato as a model, the results of the plant-bacteria interaction and their impact on growth rates and chlorosis were easily measured. Interestingly, a higher number of SCMS54:*gfp*cells were observed in both roots and leaves of tomato plants grown in the presence of Cd. Based on these data, it is possible that the presence of Cd confers an advantage regarding the growth of *Burkholderia* sp., a behaviour that has been previously reported by Lazzaro et al. (2008). Moreover, it is also possible that the lower concentration of Cd inside the plant (due to the presence of *Burkholderia*sp. strain) makes the root niche a less stressful environment, stimulating bacterial colonization. These authors used real time PCR to show that *Burkholderia*sp. target sequences were more abundant in Cd treated soil. In addition, Singh et al. (2009) observed that *Burkholderia* sp. colonizes rice seedling more

effectively than *Rhizobium*, thus concluding that *Burkholderia* sp. was a more aggressive colonizer. Nevertheless, it is possible that the predominance of *Burkholderia* sp. may have been a result of Cd toxicity towards other bacteria. In this study, the abundance of *Burkholderia* sp. in the presence of Cd was confirmed by fluorescence microscopy. The fluorescence microscopy analysis also confirmed that the SCMS54:*gfp* bacteria mainly colonised the intercellular spaces, a strategy also adopted by *Burkholderia* sp. RRE3 and *Rhizobium* sp. RRE6 during the colonisation of rice plants (SINGH et al., 2009) and by *Methylobacterium mesophilicum* SR1.6/6 during sugarcane colonisation (ROSSETTO et al., 2011).

The observed reduction in growth is a common response in Cd-treated, non-inoculated plants, and similar results have been reported by other authors (GRATÃO et al., 2008a; GARG; AGGARWAL, 2011; SHANMUGARAJ et al., 2013). However, certain microorganism-inoculated plants have been shown to acquire a higher tolerance for growth in environments contaminated with heavy metals. For example, De Souza et al. (2012) demonstrated that lead tolerance in *Calopogonium mucunoides* can be improved by the presence of arbuscular mycorrhiza fungi in the soil. Our study (in both experiments: hydropony and pot) demonstrated that the bacteria-inoculated tomato plants exhibit normal growth rates as compared to the un-inoculated control. Moreover, when the tomato plants were cultivated in the presence of $CdCl_2$, the effects of Cd toxicity (growth reduction and chlorosis) were clearly attenuated in the inoculated sample. This type of positive interaction may result from the adsorption of Cd to the bacterial cell wall, as was observed in similar findings reported for *Burkholderia* sp. (JIANG et al., 2008) and *Methylobacterium* (MADHAIYAN et al., 2007) in association with maize and tomato plants, respectively. Nevertheless more research is still needed because it is still difficult to address in detail the performance of the strain in the soil environment. As reported and discussed in detail by Kuffner et al. (2010), microbes may have a mobilizing or stabilizing effects, which will greatly influence the Cd uptake (See also Compant et al. (2008) for a review on plant growth promoting *Burkholderia* sp.).

In our study, tomato plants (from hydropony or pot) inoculated with *Burkholderia* sp. accumulated less Cd than did the treatement with only Cd, which is an important characteristic since we used a soil contamination (2 $mg.kg^{-1}$) allowed by CETESB (2005). These may have occurred because a portion of the available Cd in the soil has been adsorbed to the bacterial cell wall, thereby reducing the amount of Cd that is bioavailable to the plant root system. A similar scenario was previously reported by Pabst et al. (2010), who measured

the distribution of Cdions in *Pseudomonas putida*and showed that Cd was mainly found on the bacterial cell surface. Moreover, Guelfi et al. (2003), working with *Aspergillus nidulans*grown in the presence of $CdCl_2$, reported that Cd accumulated rapidly and to high concentrations in the mycelium even at the lowest Cd concentration used in the study.

Although we did not analyse Cd adsorption in the cell wall, we did show that the bacterial SCMS54 isolate, in addition to the ability to tolerate Cd and possibly due to the selective action of the Cd contaminated soil from which it was isolated, was able to accumulate Cd, presumably as its mechanism for Cd tolerance. This finding was further confirmed by the reduced Cd concentration observed in the medium where the bacteria had been grown. Therefore, regardless of where the Cd accumulates in the bacterial cell, we assume that the association between the tomato plant and bacteria was beneficial; i.e., the interaction resulted in normal tomato growth under high $CdCl_2$ concentrations, which was likely due to a reduction in Cd available to the plant root system. Moreover, it is well known that Cd can compete with other nutrients, and thus, the proportion between Cd and other nutrients under the bacterial association favoured other essential nutrients, allowing the tomato plants to grow normally.

Additional data on chlorosis further support the mechanistic approach presented here. For example, Cd has been shown to cause chlorosis in leaves (GRATÃO et al., 2008a; MONTEIRO et al., 2011); however, the SCMS54 inoculated tomato plants exhibited reduced chlorosis in the presence of $CdCl_2$, indicating that the inoculated bacterium had a clear, positive effect.The inoculated plants may instead have induced a mechanism to protect chlorophyll, which ultimately can increase photosynthetic activity. This effect was demonstrated by Shahabivand et al. (2012), who showed that plants inoculated with root endophyte and arbuscular mycorrhizal fungi (*Piriformospora indica* and *Glomus mosseae*) exhibited higher photosynthetic indices (PI) than did non-inoculated plants. Moreover, Cd has been reported to induce stress and increase the amount of peroxide in Citrumelo (PODAZZA et al., 2012), sweet potato (KIM et al., 2010) and tomato (MONTEIRO et al., 2011) roots, whereas our current data revealed that the presence of SCMS54:*gfp* in roots decreased the peroxide content. This result indicates that the tomato plant, although cultivated in the presence of $CdCl_2$, did not show any indication (such as chlorosis) of stressful conditions. These physiological and biochemical responses contributed to the normal or improved growth performance of the tomato plants.

A complementary explanation for the increased plant growth and tolerance is the physiological characteristics of the bacteria that are also involved in plant growth, such as the

production of IAA (DODD et al., 2010), siderophores and solubilised inorganic phosphate (JIANG et al., 2008; LUVIZOTTO et al., 2010), which were detected in *Burkholderia* sp. SCMS54 and confirmed by nutrients quantification in pot assay, showing that inoculated plants increase phosphor in shoot due to the phosphate solubilisation by the bacterium as well as iron (mainly in root) due to the production of siderophore by SCMS54:*gfp*. Previous studies have shown that the phosphate-solubilising bacteria *Bacillus megaterium* enhanced Cd bioavailability in the soil and also promoted Cd phytoextraction by *Brassica juncea* (JEONG et al., 2012). Moreover, siderophores are reportedly able to bind to Cd in *Streptomyces tenda* F4 (DIMKPA et al., 2008). Therefore, all of these characteristics support the potential of *Burkholderia* sp. SCMS54 for promoting plant growth and increasing plant tolerance to Cd.Yet, other factors, such as phytohormones, ethylene production as well as ACC deaminase production, which were not evaluated in this study, can also play a role in *Burkholderia* sp.-plant interaction (BELIMOV et al., 2009; COMPANT et al., 2008).

Moreover, genes related to stress conditions such as chaperones, heavy metal transporterand metallothionein can also be involved in Cd tolerance. Chaperones, such as Hps90, are responsible for protein folding, translocation and degradation in different cellular process. Activation in plants was reported in several stress condition, including boron (TOMBULOGLU et al., 2012), Cd (MILIONI; HATZOPOULOS, 1997), arsenic and chromium (GOUPIL et al., 2009). In the present study, the presence of the bacteria (SCMS54), not the presence of Cd induced chaperon gene expression, the same expression pattern occurred with nrm3 gene, nramp genes encode a broad-range of heavy metals transporters, however it was not induced in tomato in the presence of Cd in this work and in Ouziadet al. (2005) report. The activation of genes due to the presence of the bacteria occurs because bacterial colonisation can trigger global defence mechanisms in the host plant (SATHYAPRIYA et al., 2012), and increase plant tolerance to stress. Metallothionein (MT) is expressed in stress conditions, it can scavenging ROS and bind to both toxic and essential metals (including Cd) through their cysteine residue thiol groups (TOMBULOGLU et al., 2012) in plant cytoplasm and sequester in a vacuole or out of the cells (GOUPIL et al., 2009), and play important roles in detoxification, metal ion homeostasis, and metal transport adjustment (GUO et al., 2013), and increase plant tolerance to Cd (KIM et al., 2011).There are more than one type of metallothionein, Wang et al. (2011) analysed two different MT, one was constitutively expressed while the other was responsive to Cd. Therefore,the presence of Cd can also not influence MT expression, this was described in tomato (present work and OUZIAD et al., 2005) and sugarcane (GUO et al., 2013), only the combination

+SCMS54:*gfp*+Cd induced MT expression, corroborating with the hypothesis that bacterial colonisation can trigger global defence and showing that the response is different in stressed and non stressed environment. We propose this interaction as a possible enhancer for plant Cd tolerance.

Similar to stress genes expression, the presence of the bacterium (SCMS54:*gfp*) influences more the nutrient balance than the presence of Cd, in treatment with *Burkholderia* sp. and Cd several nutrient concentrations decrease Ca, K, Mg, Mn and Zn. Different from the present work, previous report shows that Ca, K, Zn and Mn concentration decrease in tomato plants with different Cd concentrations (CARVALHO-BERTOLI et al., 2012). In the treatment with only Cd (of the present work) we observed an increase in S concentrations in leaves, S is part of metallothienein (MT) (GUO et al., 2013), glutathione and phytochelatin (RAUSCH; WACHTER, 2005), molecules described to induce Cd tolerance, despite of MT gene expression indicate that MT is not induced by Cd, S can be used to produce phytochelatin or glutathione. In addition, the treatment with only Cd also increased Cu content in roots, the same result was reported by Carvalho-Bertoli et al. (2012) and Obata and Umebayashi (1997), Cu can bind to the radical –SH, present in metallothionein and phytochelatin, which can be induced in the presence of Cd.

Nitrogen is an essential macronutrient, responsible for the biosynthesis of all aminoacid, protein and enzymes, despite of the decrease in nitrogen content in the presence of the bacteria, inoculated plants grew more, presenting a higher biomass, and consequently a total protein and nitrogen higher than non inoculated plants. Hu et al. (2013) reported that nitrate (not ammonium) induce Cd and Zn uptake (increasing fitoextraction). In the pot assay of the present work, we used a fertilizer with nitrate, enhancing Cd uptake, finding a high concentration of Cd in leaves, the addition of SCMS54:*gfp*decreased Cd absorption, decreasing phytoextraction potential, but on the other hand, increasing plant tolerance.

It is clear that further studies are necessary, and on-going research is being performed in our laboratory. For example, the interaction of *Burkholderia* sp. SCMS54 with other plant species, such as peppers, and other tomato lines that distinctly respond to Cd and other metals and a detailed analysis of soil nutrients and plant nutrient uptake, among other avenues of investigation, will help further our understanding of this beneficial plant-bacteria interaction, the mechanisms involvedand the specific conditions for which this isolated bacterium can be used.

In conclusion, our results suggest that the interaction between tomato plants and *Burkholderia* sp. in soil contaminated with Cd enhances plant growth both in controlled

(hydroponic system) and soil (pot) conditions. Importantly, the interaction also alleviates Cd stress and should be tested in contaminated field trials. Finally,seed treatment by inoculation with *Burkholderia* sp. SCMS54 can be used to promote plant growth and increase plant tolerance to Cd in contaminated environments.

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5 SEEKING FOR A NOVEL RUBISCO ENZYME USING CULTIVABLE AND NON-CULTIVABLE APPROACHES

Abstract

Global food production needs to rise fast to reach increasing food demand. Enhancing photosynthesis is one strategy to improve crop yield. RubisCO is found in diverse organisms that must fix CO_2 under a wide variety of environmental conditions.With the diversity of known RubisCO genes in the environment, it is desirable to learn how nature has already genetically engineered this enzyme, modifying protein properties in order to provide the necessary carbon to support CO_2-dependent growth. Clearly, the discovery of a functional RubisCO with novel properties in nature would be advantageous. Alternatively, many effortshave been made to genetically engineer RubisCO with desired properties.The current study is directed at systems that might be used to efficiently isolate genes encoding any form of RubisCO from the environment, using both cultivable methods and a non-cultivable metagenomic approach. The cultivable methods were used to isolate a subgroup of form IV RubisCO that uses methyl-thioadenosine (MTA) for their metabolism.Using this approach, eight different bacterial strains were isolated from Olentangy River water, some of which already had well characterized RubisCO (e.g. *Rhodopseudomonas*). Others, however, did not have any form of RubisCO (e.g. *Herbaspirillum seropedicae*), while three strains (*Hydrogenophaga, Acinetobacter* and *Rubrivivax*) had only the form I RubisCO previously described but are able to grow on MTA as the sole sulfur source. The metagenomics approach, by contrast, works reasonably wellonly with already characterized RubisCO sequences. The *Rodobacter capsulatas* Δ*cbbL*/*cbbM*selection system, however, still needs to be improved in order to select novel sequences of RubisCO.

Keywords: Metagenomic; RubisCO; Methythioadenosine (MTA)

5.1 Introduction

Crop productivity has improved through traditional plant breeding, however as arable land becomes scarcer, it will be unable to sustain an increasing global population. Global food production will need to increase more than 50% before 2050 to meet the increasing demand. Therefore, it is critical to search for new strategies to improve crop yield. Increasing photosynthesis efficiency is one of the most promising possibilities (WHITNEY; HOUTZ; ALONSO, 2011).

There are six known metabolic routes by which microorganisms and plants assimilate carbon dioxide into organic matter: the Calvin-Benson-Bassham (CBB or reductive pentose phosphate pathway), the reductive tricarboxylic acid cycle, the Wood-Ljungdahl acetyl

coenzyme A pathway, the hydroxypropionate (3-HP) bicycle, the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) pathway, and the dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycles (BERG et al., 2007; HUBER et al., 2008; TABITA et al., 2008). Plants, however, can use only the first pathway (CBB).

Ribulose bisphosphate carboxylase/oxygenase (RubisCO) (EC 4.1.1.39) is the key enzyme of the CBB reductive pentose phosphate pathway. The sugar ribulose 1,5-bisphosphate (RuBP) accepts a molecule of CO_2, with the enzyme RubisCOcatalyzing this CO_2 fixation reaction (Figure 5.1) (TABITA et al., 2008). Along with the carboxylation of RuBP, RubisCO also can catalyze the oxygenation of RuBP. The carboxylation of RuBP generates 2 molecules of 3-phosphoglycerate (3PGA), while oxygenation of RuBP generates one molecule of 3PGA and one molecule of 2-phosphoglycolate (2PG) (TABITA et al., 2008).

Ribulose-1,5-bisphosphate carboxylase oxygenase(RubisCO) (EC 4.1.1.39)is an ancient enzyme. Earth's atmosphere was high in CO_2content and low in molecular oxygen (O_2) around 3.5 billion years ago (Figure 5.2) showing perfect conditions for RubisCO activity. Nowadays, RubisCO is an enzyme with a great diversity of sequence and function. There are four known forms or types of RubisCO found in nature, forms I, II, III and IV (TABITA, 1999; TABITA et al., 2007), each of which is placed in a separate category based on differences in primary sequence. Phylogenetic analysis shows the existence of three different clades of functional RubisCO (forms I, II and III), with the more diverse RLP (or form IV RubisCO) (Figure 5.3), probably share a common ancestor: a form III from a methanogenic Archaea (TABITA et al., 2008). Phylogenetic analysis of RubisCO and RLP amino acid sequences indicate that there are three distinct lineages of bona fide RubisCO (forms I, II and III) and six distinct clades of RLP molecules (form IV) which are unable to catalyze CO_2 fixation due to substitutions on several active site residues (Figure 5.3) (HANSON; TABITA, 2001).

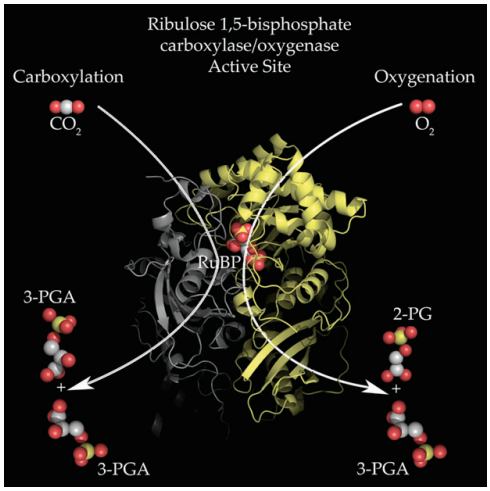


Figure 5.1- The basic catalytic unit of all RubisCO molecules (TABITA et al., 2008)

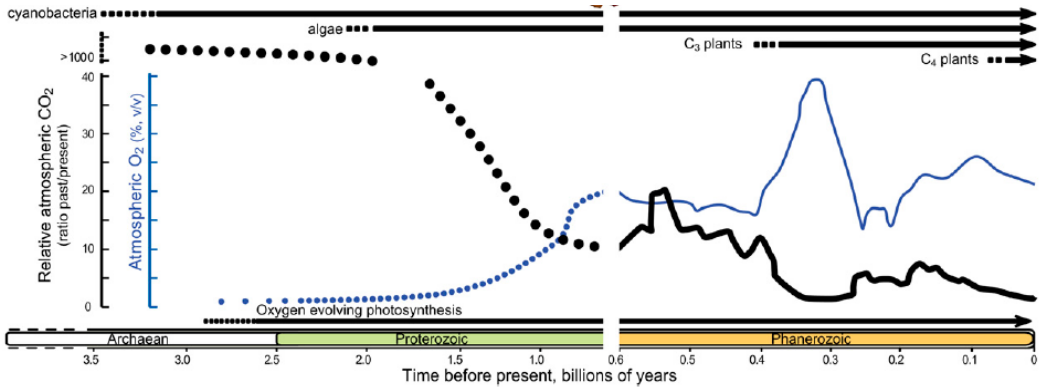


Figure 5.2- The evolutionary timelines of different photosynthetic organisms and variation in atmospheric CO_2 (thicker line) and O_2 levels during earth's history. Hypothetical atmospheric are represented by dotted lines (WHITNEY; HOUTZ; ALONSO, 2011)

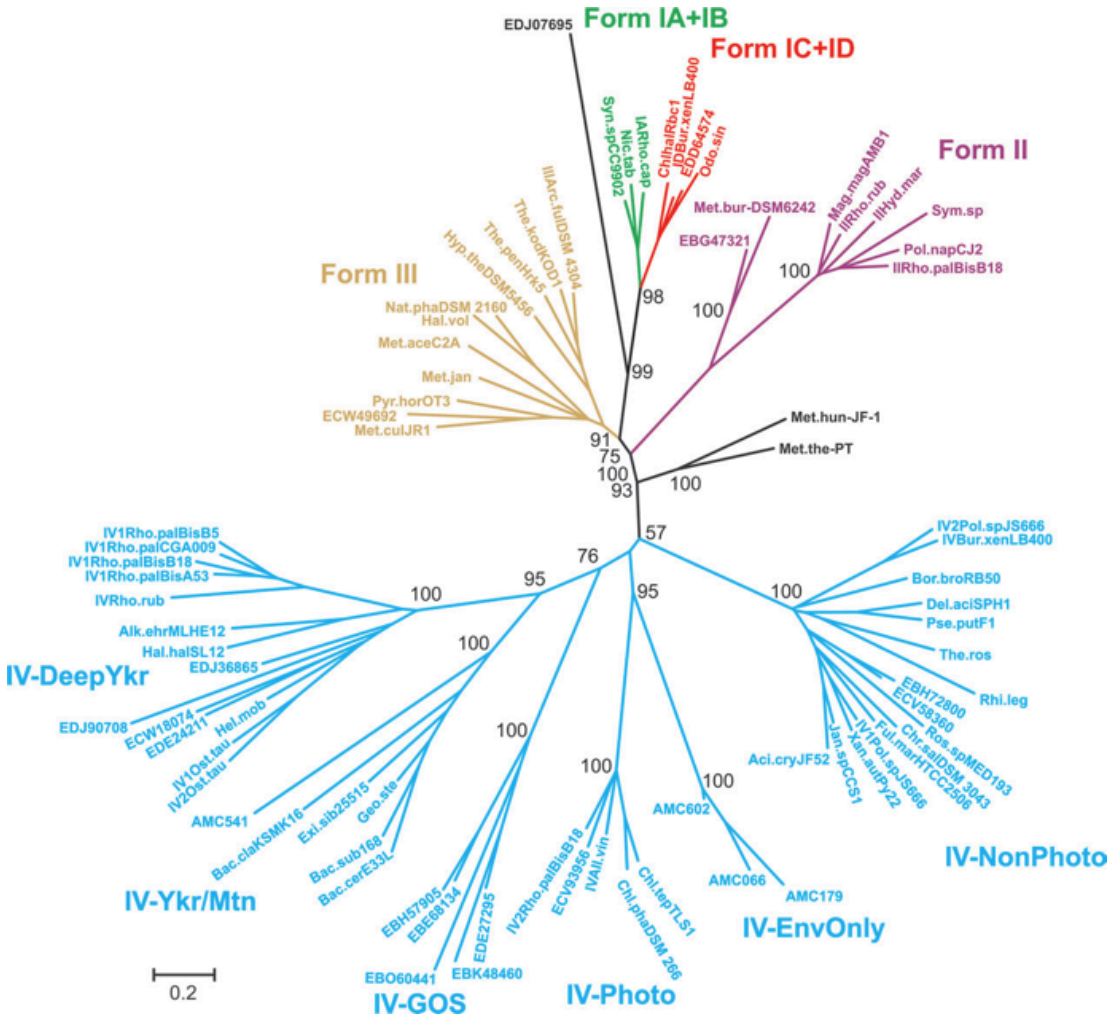
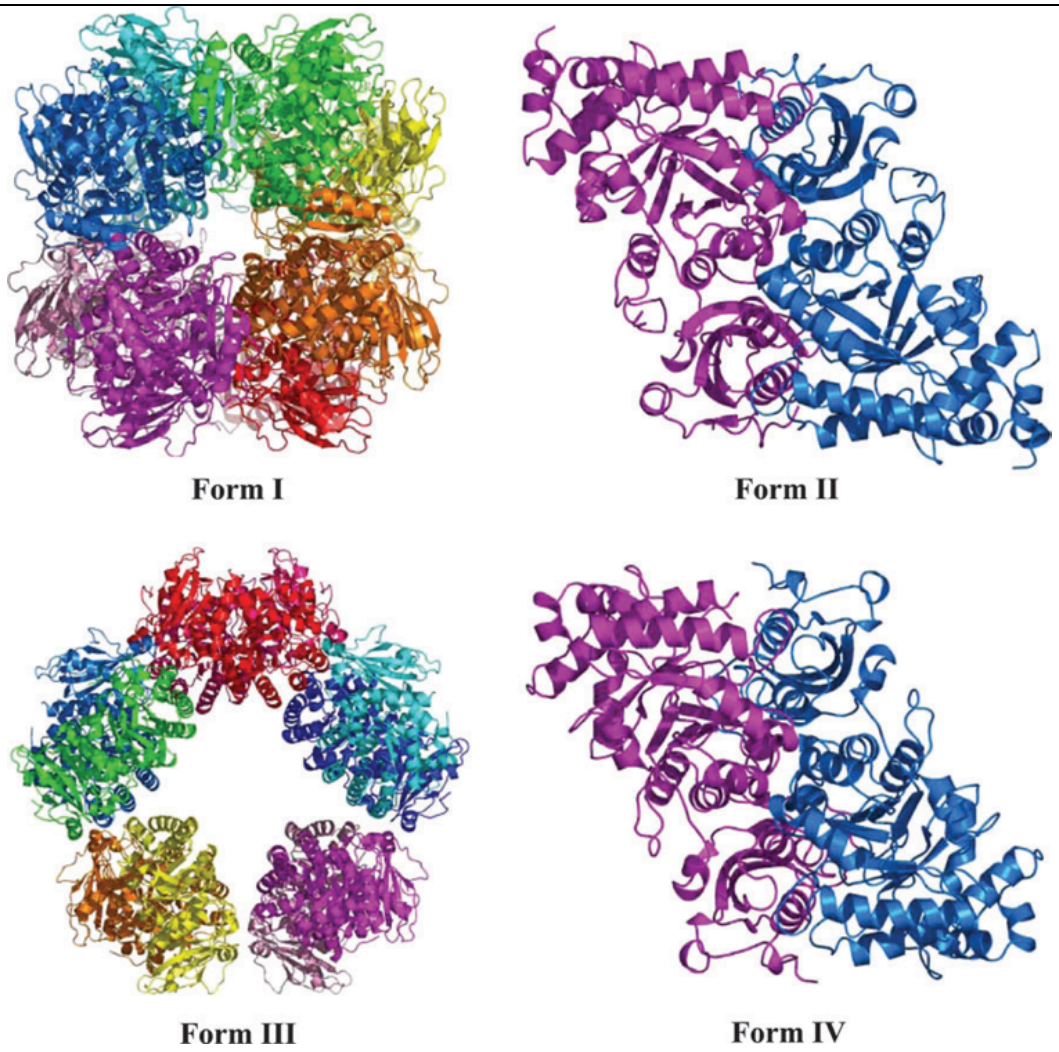


Figure 5.3- Phylogenetic tree illustrating the three classes of RubisCO and the six clades of RLP. The tree was produced by Neighbor-Joining in the MEGA 4.0 software, bootstrap of 1000 (TABITA et al., 2008)

There are four different RubisCO holoenzyme forms that are structurally unique.The large (catalytic) subunit dimer is common to all forms. Form I is the most abundant; it is found in eukaryotes and bacteria, it is composed of eight large subunits and eight small subunits (L_8S_8) (Figure 5.4). Form I can be sub-divided into the green (plants, cyanobacteria and some facultative autotrophic bacteria) and the red group (purple bacteria and eukaryotic marine algae). These subgroups can be further subdivided into forms IA and IB (green group) and forms IC and ID (red group) (TABITA et al., 2008; WATSON; TABITA, 1996).

A



B

Lineage	Subunit composition	Rubisco activity	Rubisco active site	Phylogenetic distribution
I-A	L_8S_8	+	+	α, β, γ-Proteobacteria, Cyanobacteria, Prochlorales, Sargasso Sea metagenome, GOS metagenome
I-B	L_8S_8	+	+	Cyanobacteria, Prochlorales, Eukaryotes-Viridiplantae (Streptophyta, Chlorophyta), Euglenozoa, Sargasso Sea metagenome
I-C	L_8S_8	+	+	α, β -Proteobacteria, Chloroflexi
I-D	L_8S_8	+	+	α, β, γ-Proteobacteria, Eukaryotes-stramenopiles, Rhodophyta, Haptophyceae
II	L_2 and L_n	+	+	α, β, γ-Proteobacteria, Eukaryotes-Alveolata (Dinophyceae)
III	L_2 and $(L_2)_5$ (L_n?)	+	+	Methanogenic and thermophilic crenarchaeota, thermophilic and halophilic euryarchaeota
IV-NonPhoto	?	?	-	α, β, γ-Proteobacteria, Chloroflexi
IV-DeepYkr	?	-	-	α-Proteobacteria, Clostridia, Non-methanogenic euryarchaeota, Eukaryotes-*Ostreococcus tauri*
IV-AMC	?	?	-	Acid mine drainage microbial consortium
IV-GOS	?	?	-	GOS sequence collection
IV-Photo	L_2	-	-	α, β -Proteobacteria, Chlorobia
IV-YkrW	L_2	-	-	Firmicutes, acid mine drainage microbial consortium

Figure 5.4- (A) Structures of different forms of RubisCO. Form I: L_8S_8, Form II: $L_{2\text{-}8}$, Form III: L_2 or $(L_2)_5$, Form IV (RubisCO-like Protein or RLP): appears thus far always to have an L_2 structure (TABITA et al., 2008); (B) Properties and distribution of RubisCO (TABITA et al., 2008)

The structure of form II RubisCO is similar to basic dimer of the form I RubisCO because it occurs as just the dimer of large subunits (Figure 5.4) (TABITA et al., 2008). Form II RubisCO is found exclusively in bacteria and in the plastids of dinoflagellates (TABITA;

MCFADDEN, 1974). The large subunit (RbcL) is definitely the catalytic subunit. It is functional without the small subunit (RbcS), but RbcS affects activity by influencing the correct conformation of the catalytic core of RbcL.

Many Archaea were shown to contain a separate class of RubisCO, called form III (TABITA, 1999). All known form III RubisCO is found exclusively in Achaea. Form III RubisCO is similar to form II, in that it occurs as large subunits only (L_2) (Figure 5.4) (FINN; TABITA, 2003). The main difference is that organism containing Form III RubisCO does not have phosphoribulokinase (PRK). Instead, they use 1 5-phosphoribose-D-1-pyrophosphate (PRPP) to generate RuBP (TABITA et al., 2007). Phylogenetic studies, together with other structural homology and gene conservation studies, show that *Methanomicrobia* form III RubisCO can potentially be the common ancestor of all other RubisCO proteins (TABITA et al., 2008).

RubisCO catalysis efficiency is dependent on the inherent ability of the enzyme to discriminate between CO_2 and O_2(TABITA et al., 2008). The RubisCO family shows significant kinetic variability (comparing form I, II and III) despite sharing the same catalytic chemistry (Figure 5.5). Improvements in CO_2 fixation rate for forms I and II RubisCO generally come at the expense of lower affinity for CO_2. Therefore, some photosynthetic organisms that live under low CO_2 and high O_2 have evolved mechanisms of CO_2 concentration around RubisCO (e.g. C4 plants, algae and cyanobacteria). In addition, in these organisms RubisCO has a lower CO_2 affinity (higher Km for CO_2), but higher carboxylation rates (vCO_2) (Figure 5.5-in light green). The elevated CO_2 carboxylation compensates for the low CO_2 affinity. The opposite occurs in C3 plants (most crops) and algae (Figure 5.5-in dark green) which lack a CO_2 concentrating mechanism but present a higher CO_2 affinities (lower K_m) and better CO_2 specificity over O_2(Figure 5.5) (WHITNEY; HOUTZ; ALONSO, 2011). Thus, the goal is to obtain the most efficient RubisCO enzyme, that would have high carboxylation rates (V_{CO2}), high oxygen tolerance, along with high CO_2 specificity (high $S_{C/O}$) and a high affinity for CO_2 (low Km).

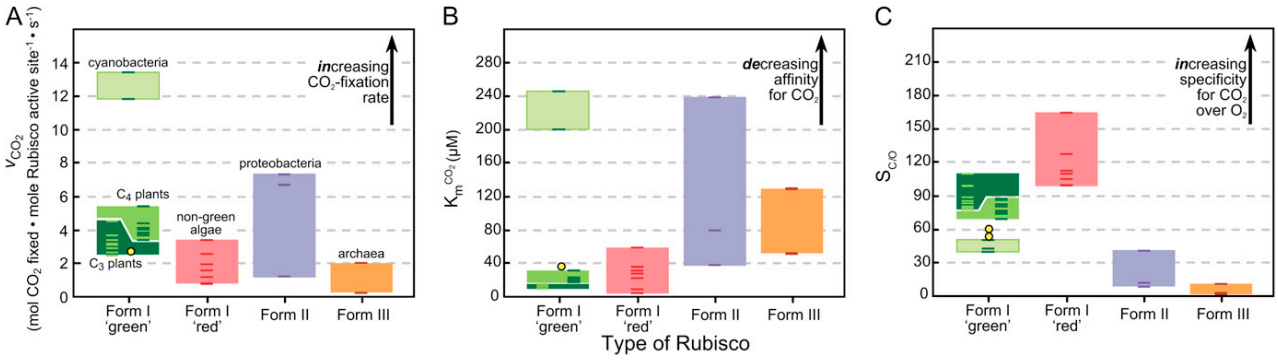


Figure 5.5- Comparative catalytic features of different RubisCO forms. Individual dashes in each column represent separate catalytic measurements for each RubisCO form (A) V_{CO2}: CO_2 carboxylation rates; (B) K_m: CO_2 affinity; (C) $S_{C/O}$: CO_2 specificity (WHITNEY; HOUTZ; ALONSO, 2011)

The sequences found most recently were classified as form IV RubisCO (HANSON; TABITA, 2001). Form IV proteins are not capable of catalyzing RuBP-dependent CO_2 fixation. For this reason they are called RubisCO-like-proteins (RLPs), to differentiate from bona fide RubisCO (forms I, II and III). They are also comprised of large subunits only (Figure 5.4). It was shown that*Bacillus subtilis* RLP participates in a methionine salvage pathway by catalyzing the enolization of an RuBP analogue: 2,3-diketo-5-methylthiopentyl-1-P (ASHIDA et al., 2003). RLPs are found in proteobacteria, cyanobacteria, Archaea, and algae. RLPs based on amino acid sequence similarities are divided into six different subgroups: IV-Photo, IV-Nonphoto, IV-YkrW, IV-DeepYrkW, IV-GOS (Global Ocean Sequencing), and IV-AMC (Acid Mine Consortium) (TABITA et al., 2007).

5.1.1 Form IV RubisCO: MTA metabolizing group

RLP are involved in some aspects of sulfur metabolism, described in different form IV groups. In the IV-Photo group *Chlorobaculum tepidum* RLP was described (HANSON; TABITA, 2001, 2003) while in the IV-YkrW group *Bacillus subtilis* (ASHIDA et al., 2003), *Microcystis aeruginosa* (CARRÉ-MLOUKA et al., 2006) and *Geobacillus kaustophilus*(IMKER et al., 2007) were described. The RLP proteins catalyze a tautomerase/enolase reaction of a methionine salvage pathway (MSP) in which the substrate 2,3-diketo-5-methylthiopentanyl-1-phosphate (DKMTP1P) is converted to 2-hydroxy-3-keto-5-thiomethylpent-1-ene 1-phosphate (HK-MTP 1P) (Figure 5.6 and 5.7) (TABITA et al., 2007).*B. subtilis* mutant lacking YkrW/MtnW has a relatively constrainedphenotype that is manifested only under severe sulfurstarvation conditions (SEKOWSKA; DANCHIN, 2002; TABITA et al., 2007).

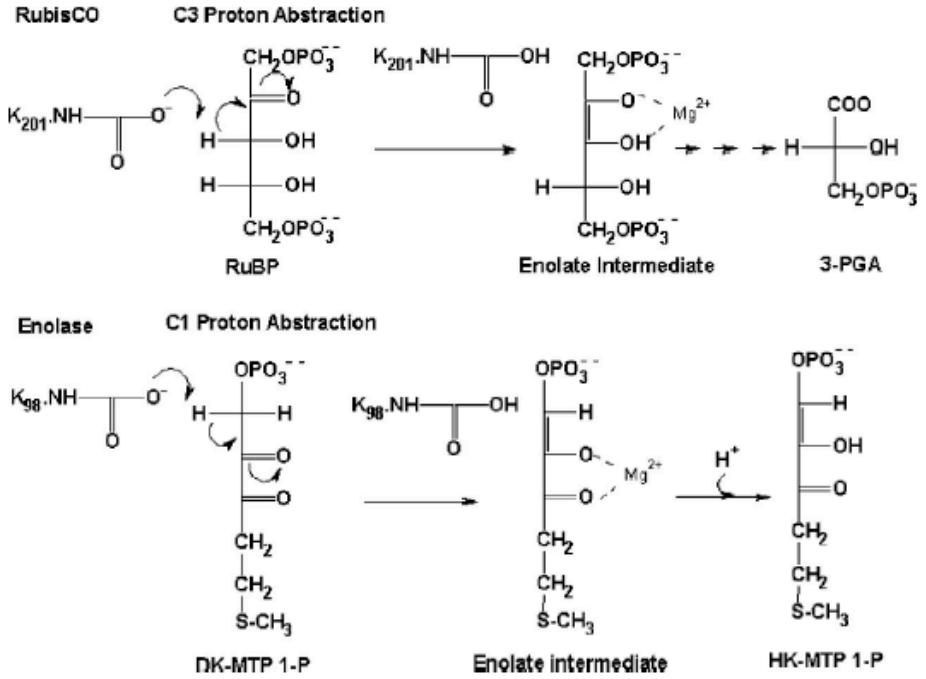


Figure 5.6-RubisCO and *Bacillus* RLP catalyze reactions using analogous substrates (TABITA et al., 2007)

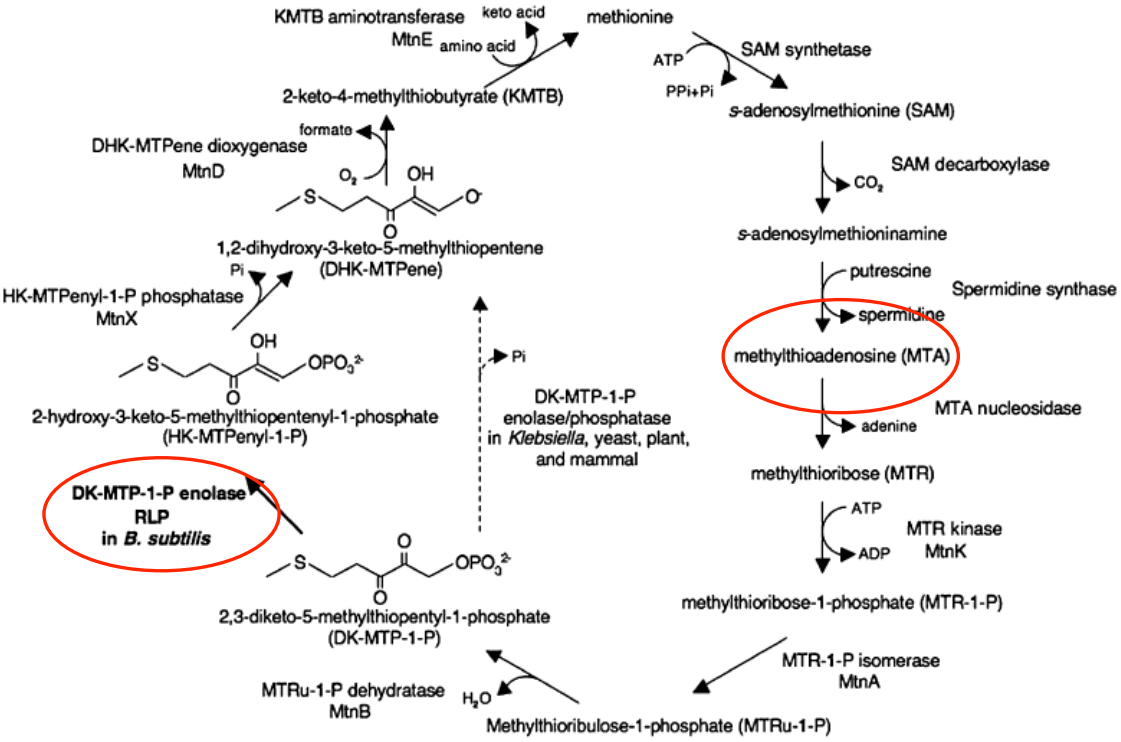


Figure 5.7- The methionine salvage pathway in *B. subtilis* (ASHIDA et al., 2008)

However, in *R. rubrum,* RLP was shown to catalyze a distinct reaction that uses 5-methylthioribulose-1-phosphate (MTR-1-P) as the substrate as part of novel methionine

salvage pathway (ERB et al., 2012; IMKER et al., 2008; SINGH; TABITA, 2010) since it was reported that it can not catalyze either the DK-MTP 1-P enolaseor the MTRu 1-P 1,3-isomerase reaction (WARLICK et al., 2012).

R. rubrum RLP uses a different substrate and was shown to catalyze a novel isomerization reaction where 5-methylthio-D-ribulose 1-phosphate is converted to a 3:1 mixture of 1-methylthio-xylulose 5-phosphate and 1-methylthioribulose 5-phosphate, suggesting that both RLP and RubisCO in *R. rubrum* can present different physiological roles related to Methythioadenosine (MTA) metabolism (SINGH; TABITA, 2010). Both RubisCO and some RLPs do possess functional similarities in that both proteins catalyze reactions using analogous substrates in both cases via an initial enolization-type reaction (Figure 5.6) (TABITA et al., 2007).

MTA may serve as the sole sulfur source under both aerobic and anaerobic growth conditions. In Methionine Salvage Pathway (MSP) schemes, oxygen is required in a dioxygenase step, however under anaerobic growth the pathway is not described (Figure5.7). Singh and Tabita (2010) show that in *R. rubrum* RubisCO can catalyze two separate reactions in carbon (via the CBB CO_2 assimilatory cycle) and sulfur (via an MSP) metabolism. Using MTA as the sole sulfur source, in aerobic growth conditions RLP is required, while in the absence of oxygen*R. rubrum* needs RubisCO to grow.

5.1.2 Seeking unknown functional RubisCO molecules

Most metagenomic studies are based on high throughput sequencing and are mainly focused on questions in microbial ecology. They describe the organisms found in a particular environment and their functions based on predicted protein sequences (FERRER et al., 2012; STEWART, 2011). Even single-cell sequencing (MARTINEZ-GARCIA et al., 2012) has been used. However, few metagenomic studies focus exclusively on RubisCO enzyme (WITTE et al., 2010), which could be important for finding unknown but functional RubisCO in Nature.

The isolation and characterization of RubisCO genes one by one would be very time-consuming and laborious. Besides that, the prediction of structure-function is limited when using only molecular techniques. Moreover, most common RubisCO has already been identified. That is the reason why it is important to develop a system that randomly selects an enzyme from the environment. It is important toremember that some environmental organisms (abundant or not) are not necessarily cultivable, highlighting the importance of non-culture methods to study this enzyme (WITTE, 2012).

A different approach to assess RubisCO's environmental diversity is a sequence-based method that uses PCR to amplify segments of RubisCO genes from metagenomic samples (ALFREIDER et al., 2009; SWAN et al., 2011). This is possible since some regions of RubisCO protein are highly conserved. Alfreider et al. (2009) developed primers with little degeneracy that amplifies portions of the coding region of the large subunit of RubisCO from form I or form II enzymes, however only a portion of the gene is amplified and sequenced, which is not enough to study enzyme function or obtain enzyme structure (HORKEN; TABITA, 1999). Moreover, distantly related organisms (by 16S rRNA) present similar RubisCO protein sequences, such as *Synechococcus* PCC6301 RbcL and *Rhodobacter capsulatus* SB1003 CbbL that are 72% identical (ALTSCHUL et al., 1997; WITTE, 2012).

Witte et al. (2010) used RubisCO open reading frames (ORF) of metagenomic libraries to access the diversity of "wild" RubisCO. In order to examine biochemical information of uncultivated RubisCO diversity, this research group adapted an extant gene expression system. A RubisCO deletion strain of *Rhodobacter capsulatus* (strain SBI/II$^-$) was developed as a means of selecting for positive or negative mutations in prokaryotic RubisCO genes based on the ability or inability to restore autotrophic growth in strain SBI/II$^-$. *Rhodobacter* (a purple non-sulphur alphaproteobacterium) is able to support high levels of expression of functional RubisCO in many modes of growth (excluding or including oxygen) (SMITH; TABITA, 2003). There are several advantages to using this expression system: the growth of the host organism indicates if the recombinant gene encodes for a functional RubisCO. Besides that, assay-based screens of multiple clones for RubisCO activity are time consuming and the method requires $^{14}CO_2$ radioactive.

Therefore, the present study sought to isolate previously undescribed RubisCO molecules (forms I, II and IV) using cultivable (seeking form IV) and uncultivable techniques (selecting forms I, II or III). Using cultivable methods, form IV RubisCO was isolated by selecting bacteria that grew under various conditions (photoautotrophic, photoheterotrophic, chemoautotrophic and chemoheterotrophic) using MTA as sole sulfur source. For metagenomic (uncultivable) selectionthe *Rhodobacter* strain SBI/II$^-$ expression system was used to search for non-described expressed bona fide (that can fix CO_2) RubisCO sequences (including forms I, II and III).

5.2 Materials and Methods

5.2.1 Isolating bacteria that metabolize MTA

Water samples from the Olentangy River (Columbus, Ohio, USA) were taken. 1mL of this sampled water was inoculated under different growth conditions: (i) photoautotrophic (with light and H_2 and CO_2); (ii) photoheterotrophic (with light, CO_2 and malate); and (iii) chemoautotrophic (in the dark with H_2, CO_2 and O_2). In all conditions, 20mL of Ormerod's minimal media were used with MTA as sole source of sulfur (S). For the positive control malate was added to Ormerod's minimal media allowing any microorganism that uses malate to grow (there are many described bacteria that use malate as carbon source), while for the negative control, we did not use any source of S (without MTA or malate) in the media, disabling any microorganism to grow unless there is S contamination in any of the reagents or the glass tubes.

In the photoautotrophic (without malate) and photoheterotrophic (with malate) cultures, gas consisting of 20% CO_2/balance H_2 was constantly bubbled through the media. The gas was anaerobic (no O_2) and the cultures were exposed to 24 hour incandescent light (Table 5.1). Chemoautotrophic cultures were bubbled with 10% CO_2/ 40% H_2/ 50% air in darkness (Table 5.1). The enrichment cultures grew for 7 days until an O.D. 600~1. MTA minimal media cultures from the three enrichment conditions (photoautotrophic, photoheterotrophic and chemoautotrophic) were diluted ($1:10^5$ times) and 100 μL was spread on Ormerod's minimal media-agar plates. One set of plates were placed in the dark (with CO_2, H_2 and O_2). Another set of plates were placed in light (with CO_2 and H_2, but no O_2) (Figure 5.8).

Single colonies with different color and morphology were selected and streaked out on a new plate with the same medium. This procedure was repeated three times in sequence in order to obtained pure colonies. After isolation, each strain was tested again for growth on liquid media with MTA as only source of S.

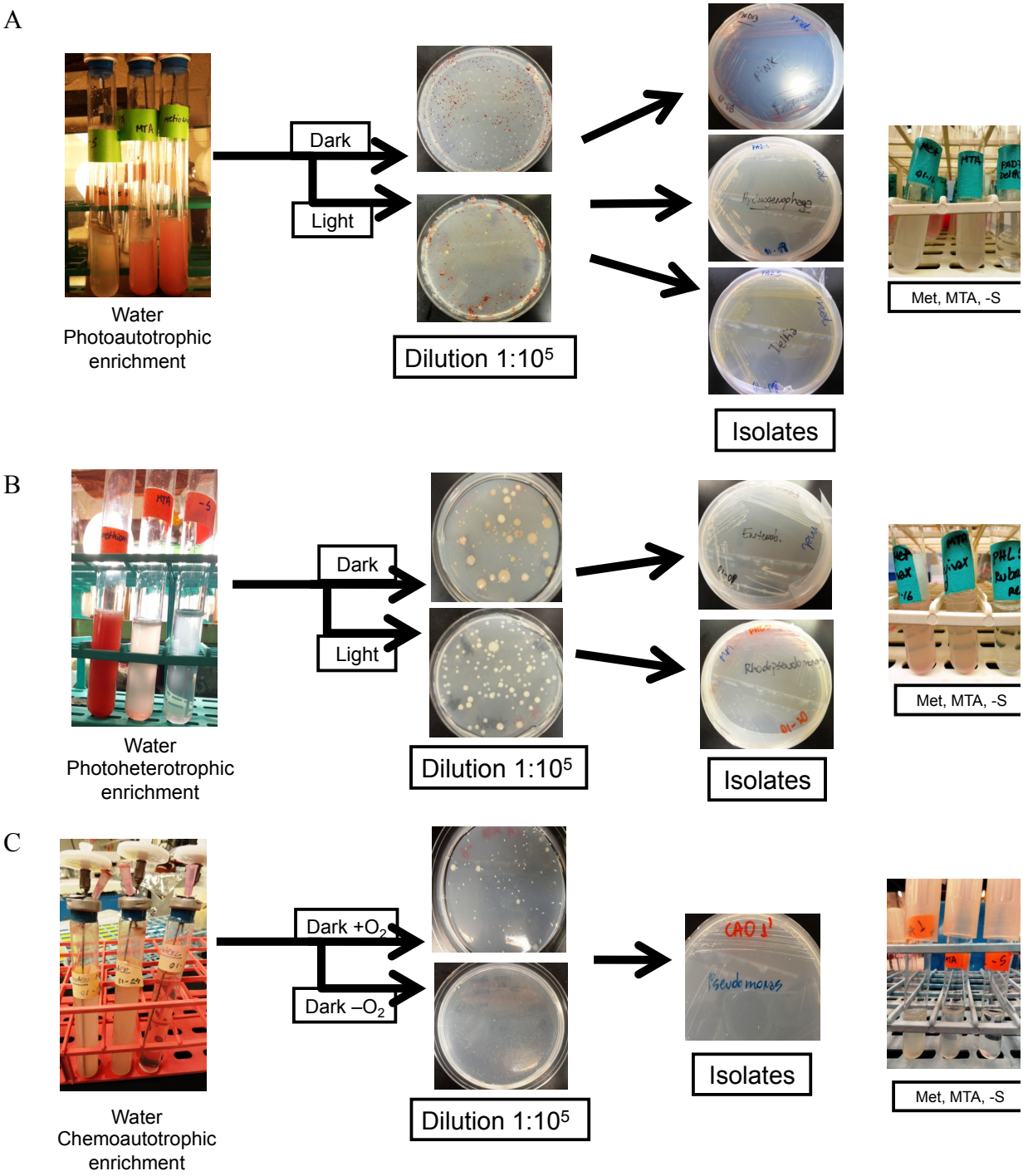


Figure 5.8- (A) Photoautotrophic, (B) Photoheterotrophic and (C) Chemoautotrophic enrichments were separately plated in solid Ormerod's minimal media, single colonies were picked and streaked out. After three times streaking out single colonies, we obtained isolated colonies. Each isolate was tested again to be sure it could grow on MTA as only source of S

Table 5.1- Characteristic of all enrichment conditions used

Enrichment condition	Gas tank	Light presence	Carbon source	O_2 presence
Photoautotrophic	20% of CO_2/H_2	Light	CO_2	Anaerobic
Photoheterotrophic	20% of CO_2/H_2	Light	Malate and CO_2	Anaerobic
Chemoautotrophic	20% of $CO_2/H_2/O_2$	Dark	CO_2	Aerobic

5.2.2 Recovery of Sequences from the Environment that Encode Functional RubisCO

5.2.2.1 Environmental DNA

Water samples were collected from Olentangy River, Columbus, Ohio. An aliquot of these samples was inoculated in bottles with 350 mL of Ormerod's minimal media (no malate), with 20% of CO_2/H_2 gas in light (photoautotrophic conditions) or 10% CO_2/ 40% H_2/ 50% Air in darkness (chemoautotrophic conditions). The enrichment culture grew for 7 days until O.D. 600~1, and 10 mL was transferred to a fresh 350 mL bottle for a second autotrophic selection.High molecular weight genomic DNA (gDNA) was obtained from these enrichment cultures with Quiagen Genomic Tips Kit.

To check the presence of part of RubisCO sequence, a 600 bp fragment ofform I RubisCO genes was amplified with conserved-site primers from Alfreider et al. (2003). The reaction conditions included1X Invitrogen Taq reaction buffer, 1mM $MgCl_2$, 0.2 mM of each primer (FIAif forward 5'- CGGCAC(GC)TGGACCAC(GC)GT(GC)TGGAC- 3` and reverse 5`-GTA(AG)TCGTGCATGATGAT(GC)GG-3`), 0.2 mM of dNTP, 1U of Taq Polymerase and 100 ng of template (gDNA). The cycle was 94^{o}C for 2 min, 25 cycles of (94^{o}C for 20 s, 55^{o}C for 20 s, 72^{o}C for 50 s), and the final extension 72^{o}C for 10 min. *Synechococcus 6301 rbcL* gene was used as the positive control and reaction mixture without template was used as the negative control.

5.2.2.2 Functional metagenomic selection

To isolate metagenomic RubisCO sequences that encode functional proteins a similar strategy to previous studies (WITTE et al., 2010) was used.

The gDNA was partially digested with *Rsa*I (NEB) restriction enzyme for 2 min at 37^{o}C, it was used 1U of enzyme per 1 μg of DNA, the reaction was stopped with 1 μL of EDTA (0.5M). This digested gDNA was run in a 0.8% agarose gel that was used to select fragments between 2 and 6 kb by using a Qiagen gel extraction kit. After that, the fragments were given a single A-tail using Taq DNA polymerase enzyme at 72^{o}C for 15 min with dATP as the only nucleotide.

The A-tailed DNA was cloned into the pCR8-GW/TOPO vector (Invitrogen) (Figure 5.9) using a topoisomerase-based reaction, incubated for 1 hour at room temperature to ligate, then transformed into chemically competent Top10 *E. coli*cells. Positive transformants were then selected by incubating the transformed cells in 100 mL of liquid LB with 100 μg.mL^{-1} spectinomycin. This should have built a small-insert environmental metagenomic library in

the TOPO vector. The pCR8-GW/TOPO cloning region is flanked by unique sites which allows for downstream cloning using phage λ recombinase Gateway technology (Invitrogen).

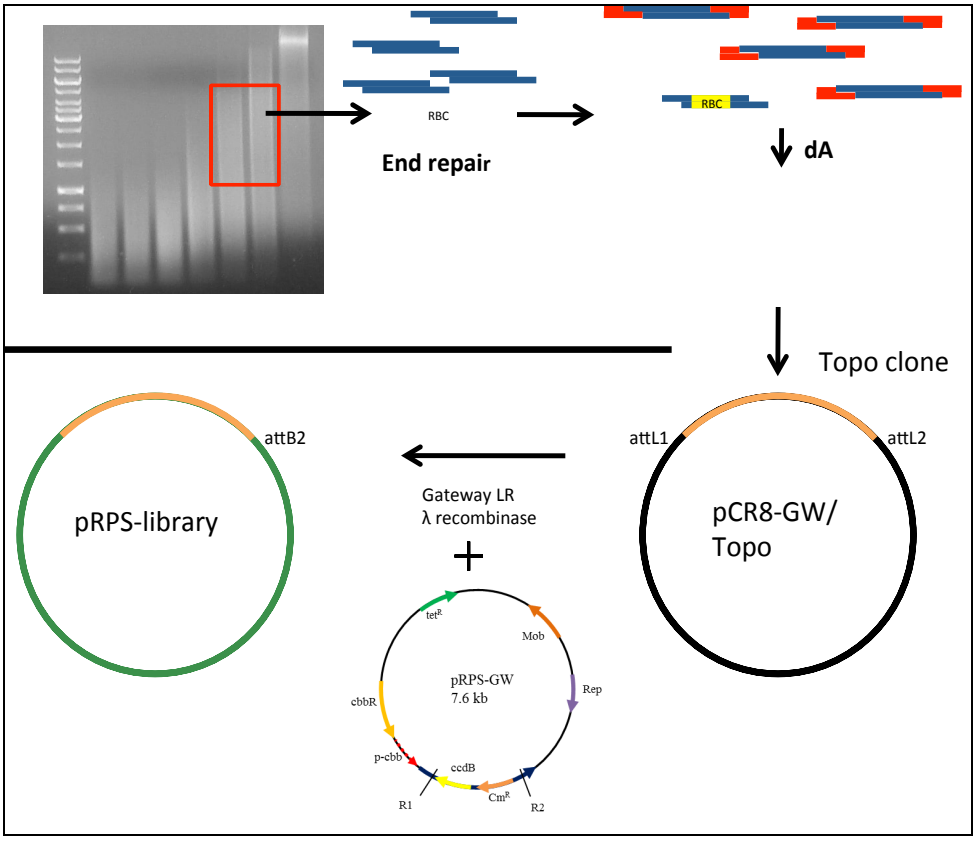


Figure 5.9- Construction of environmental enriched library (WITTE, 2012)

The gDNA library was transferred from the pCR8 vector library into a library based on the pRPS-GW vector (Figure 5.10). pRPS-GW is derived from pRPS-MCS3, a broad host-range plasmid that contains an MOB site such that the library can be maintained in *E. coli*, but transferred to an expression strain such as *Rhodobacter capsulatus* SBI/II$^-$ via conjugation (WITTE et al., 2010). The conjugation occurred by overnight incubation of *E. coli* hosting the pRPS-GW environmental library and *R. capsulatus* SBI/II in solid PYE medium. After that it was transferred to PYE liquid medium with antibiotics that select for *Rhodobacter capsulatus* SBI/II$^-$ (rifampicin 1 mg.mL^{-1}) and the library plasmid pRPS-GW (tetracycline 12.5 µg.mL^{-1}).

The library (maintained in *E. coli*) can then be shuttled to the RubisCO-deletion SBI/II$^-$ strain of *R. capsulatus*. Plasmids containing an expressible RubisCO gene (using either the upstream *cbbM* promoter on pRPS or an endogenous promoter) should complement growth of SBI/II$^-$ under autotrophic growth conditions (Ormerod's minimal media with 20% of CO_2/H_2, with light and no oxygen).

5.3 Results and Discussion

5.3.1 Isolates that metabolize MTA

After plating the $1:10^5$ dilution of the MTA enrichment cultures, each isolated colony was restreaked onto a new minimal medium agar plate, and restreaked three more times to obtain isolated colonies. After that, within the enrichment conditions all similar colonies were grouped according to their colors and morphology, and at least three colonies of each group were selected to sequence part of the 16S rRNA gene in order to identify the studied isolates. A total of 71 isolates were sequenced and compared with NCBI database by BLAST tool. The frequency and classification of all isolated bacteria are described in Table 5.2.

Subsequently, the isolated bacteria were re-inoculated in different growth conditions: chemoautotrophic (with and without O_2), photoautotrophic, photoheterotrophic (Table 5.4) to verify if it could grow alone without the interaction of other microorganisms in these conditions.

Table 5.2- Number of sequenced isolates from the different enrichment conditions Photoautotrophic, Photoheterotrophic and Chemoautotrophic

Enrichment	Plated in MM	Isolated bacteria	Number of sequenced Isolate
Photoautotrophic	light	*Rhodopseudomonas sp.*	10
		Rubrivivax gelatinosus	3
	dark	*Rhodopseudomonas sp.*	3
		Rubrivivax gelatinosus	2
		Hydrogenophaga sp.	1
		Delftia tsuruhatensis	4
Photoheterotrophic	light	*Rhodopseudomonas sp.*	10
		Rubrivivax gelatinosus	4
		Enterobacter sp.	1
	dark	*Rhodopseudomonas sp.*	3
		Rubrivivax gelatinosus	8
		Enterobacter sp.	2
Chemoautotrophic	with O_2	*Rhodopseudomonas sp.*	5
		Rubrivivax gelatinosus	2
		Pseudomonas sp.	1
		Herbaspirilum seropedicae	2
	without O_2	*Rubrivivax gelatinosus*	5
		Pseudomonas sp.	4
		Acinetobacter sp.	1
Total			**71**

Table 5.3- Characteristics of obtained isolates (available in literature)

Enrichment	Isolates	Class	MTA genes*	Rubisco genes**
PA	*Delftia*	*Betaproteobacteria*	+	Form IV
PA	*Hydrogenophaga*	*Betaproteobacteria*	-	Form I
PH	*Enterobacter*	*Gammaproteobacteria*	+	-
CA	*Pseudomonas sp*	*Gammaproteobacteria*	+	Form IV
CA	*Herbaspirilum seropedicae*	*Betaproteobacteria*	+	-
CA	*Acinetobacter sp*	*Gammaproteobacteria*	+	Form I
PH and PA and CA	*Rhodopseudomonas*	*Alphaproteobacteria*	+	Form I, II and IV
PH and PA and CA	*Rubrivivax*	*Betaproteobacteria*	+	Form I

* MTA nucleosidase or phosphorylase genes were found in Kegg pathway database (Figure 5.10) or NCBI database
** Genes encoding form I, II, III, IV RubisCO were found in the articles cited in the text

The most frequently isolated bacteria were *Rhodopseudomonas*spp. and *Rubrivivax gelatinosus*, they were isolated from all enrichment cultures (photoautotrophic, photoheterotrophic and chemoautotrophic) (Tables 5.2 and 5.3). These two isolates are from Alpha and Betaproteobacteria Class, respectively. There are several genes of MTA pathway deposited in different databases (NCBI- National Center for Biotechnology Information and Kegg pathway database). It is well described that *Rhodopseudomonas* has genes encoding RubisCO Form I, II and IV showing a great plasticity (LARIMER et al., 2004), that is why it was frequently isolated from all enrichment conditions. As for purple bacterium *Rubrivivax gelatinosus*, its genome encodes for only Form I RubisCO (ASHIDA et al., 2008) however there is no study of RubisCO of this bacterium species, the genome of the two different strains were sequenced in 2012 (HU et al., 2012; NAGASHIMA et al., 2012), and there is only a total of three genomes sequenced for this species (NCBI database).

All other isolates were exclusive to each enrichment condition, *Delftia* and *Hydrogenophaga* genus (both Betaproteobacteria) are exclusively from photoautotrophic enrichment (Tables 5.2 and 5.3). *Delftia* has RubisCO form IV genes (ASHIDA et al., 2008) but without any study focusing on RubisCO genes or protein. However, this isolate is able to grow with CO_2 as the only carbon source. It grows photoautotrophically, as well as photoheterotrophically (anaerobic) and chemoautotrophically (with O_2). *Delftia* is described as promoting bioremediation (UBALDE et al., 2012) as well as causing human infection (SHIN; CHOI; KO, 2012). The colonies present a yellowish color. However, this organism needs to be further investigatedto check if it has an undescribed form of RubisCO that is able to use CO_2. As for *Hydrogenophaga,* it has a form I RubisCO described (ALFREIDER et al., 2003; YOUSUF et al., 2012) and it is able to grow in all tested conditions (Chemoautotrophic - with and without O_2, Photoautotrophic, Photoheterotrophic) which shows the versatility of its metabolism. This Gram-negative bacteriumis a hydrogen-oxidizing bacteria, isolated from

water, mud and soil, and previously classified as *Pseudomonas* genus (KIM et al., 2010). It has been studied mainly due its ability to accumulate polyhydroxyalkanoates (PHA) from different substrates (POVOLO et al., 2012), besides that enzymes responsible for the oxidation of CO to carbon dioxide (carbon monoxide dehydrogenases (CO-DH) were described in *H. pseudoflava* (KANG; KIM, 1999), which confirms that this bacterium needs RubisCO enzyme to use CO_2 as carbon source.

The only isolate exclusively found on photoheterotrophic enrichment is *Enterobacter* sp. (Table 5.3) that grows only in this condition (Table 5.4) despite of the presence of several genes responsible for MTA metabolism (Figure 5.10 and Table 5.3). It has the highest number of genes described in the methionine salvage pathway (Figure 5.10) indicating a high plasticity. However, it does not have any form of RubisCO and does not grow under any autotrophic conditions (photoautotrophic and chemoautotrophic) (Table 5.4).

Pseudomonas sp., *Herbaspirillum seropedicae* (both with O_2) and *Acinetobacter* sp. (without O_2) were isolated exclusively from chemoautotrophic enrichment. Tests were not performed to check after isolation if they grew chemoautotrophically or photoautotrophically, but they did grow with malate and light (photoheterotrophic), malate and dark (chemoheterotrophic) and probably CO_2 and dark (chemoautotrophic) from where it was isolated (data not tested). *Pseudomonas* sp. is a gamma proteobacteria and an ubiquitous environmental bacterium (STOVER et al., 2000) whose genome encodes for a form IV RubisCO (ASHIDA et al., 2008) and some other MTA metabolism related genes (Table 5.3 and Figure 5.10). Probably that is why it was isolated from chemoautotrophic condition, while *Herbaspirillum seropedicae* (Betaproteobacteria) is described as a endophyte and diazotrophic (MARIN et al., 2013) but also as a rice pathogen (YE et al., 2012; ZHU et al., 2012). *H. serpedicae* has no RubisCO gene described (Table 5.3) despite of having its genome sequenced (YE et al., 2012; ZHU et al., 2012). However, it does possess the enzyme that catalyzes MTA (Figure 5.10) therefore it can have a form IV RubisCO. *Acinetobacter* sp. (Gammaproteobacteria) was described to grow facultative chemolithoautotrophicly (carboxydobacteria) and methylotrophically (using both methanol and methylamine as carbon source) as well as presenting RubisCO activity (RO et al., 1997) (Table 5.3) and also genes for MTA metabolism (Table 5.3 and Figure 5.10).

Table 5.4- Isolates were tested in different growth conditions: chemoautotrophic, photoautotrophic, photoheterotrophic

Enrichment	Isolates	Growth on			
		CA (with O_2)	CA (without O_2)	PA	PH
PA	*Delftia*	+	-	+	+
PA	*Hydrogenophaga*	++	+	++	+
PH	*Enterobacter*	-	-	-	+
CA	*Pseudomonas sp*	nt	nt	nt	+
CA	*Herbaspirilum seropedicae*	nt	nt	nt	+
CA	*Acinetobacter sp*	nt	nt	nt	+
PH and PA and CA	*Rhodopseudomonas*	+	±	++	+
PH and PA and CA	*Rubrivivax*	++	±	+++	+

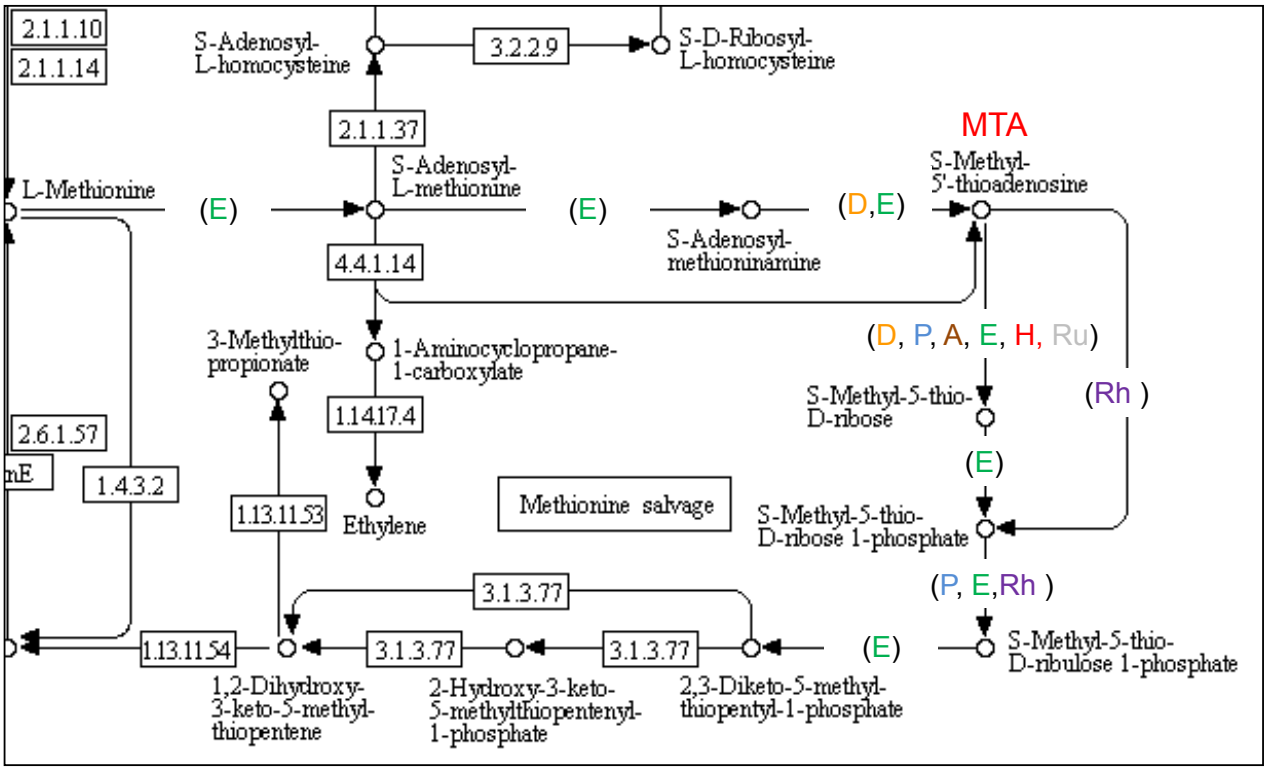


Figure 5.10 - Methionine salvage pathway using Kegg pathway database (http://www.genome.jp/kegg/pathway.html). All enzymes from the different isolated bacteria E= *Enterobacter,* D= *Delftia,* P= *Pseudomonas,* A=*Acinetobacter,* Rh= *Rhodopseudomonas,* Ru= *Rubrivivax,* H=*Herbaspirillum* that were present in Kegg database was marked in the pathway

Despite of the focus of this cultivable approach being to find an undescribed Form IV RubisCO, it is also possible to describe an unknown form I or II RubisCO in *Delftia* or *Rubrivivax.* Form III RubisCO was not accessed in the cultivable assay since Archea need special conditions to grow in Petri plates. *Delftia*has no form I or II RubisCO described, despite growing under photoautotrophic conditions, which indicates a possible undescribed bona fide RubisCO or a different CO_2 fixation pathway. Furthermore, *Rubrivivax* genera is still not well studied, it has a form I RubisCO described, but due its versatility, since it grew

in all tested conditions, this genera may can have another form of RubisCO or may also use other CO_2 fixation routes.

Most of the isolates had RubisCO genes (forms I, II or IV) and genes involved in the methionine pathway that uses MTA (Table 5.3 and Figure 5.10). However, two isolates *Enterobacter* spp. and *Herbaspirilum seropedicae*did not have any RubisCO (forms I, II or IV) genes described; and *Hydrogenophaga* does not have any of the genes of MTA metabolism annotated in its genome. Thereby, only three generaisolated (*Delftia, Pseudomonas and Rhodopseudomonas*) have form IV RubisCO described, while *Hydrogenophaga, Herbaspirillum, Acinetobacter, Rubrivivax*and *Enterobacter*generado not have a form IV described in the literature. These isolates can be further investigated to check for the presence of a form IV RubisCO or a similar protein that plays the same role in MSP, since all use MTA as S source.

5.3.2 Tested strategies to select Functional Metagenomic RubisCO

Genomic DNA (gDNA) was isolated from enriched cultures (photoautotrophic and chemoautotrophic grown) and before any analysis small fragments (600 bp) of form I RubisCO gene (*cbbL* or *rbcL*) were amplified using conserved-site primers (ALFREIDER et al., 2003) (Figure 5.11). It was not possible to design conserved-site primers to amplify the entire gene, since the ends of the RubisCO gene sequences are not conserved.

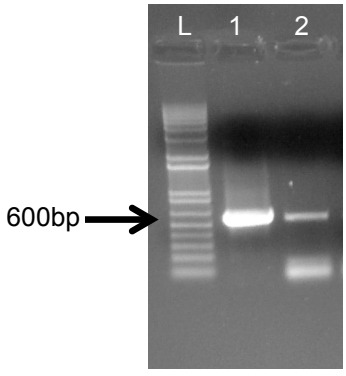


Figure 5.11- Amplification of form I RubisCO gene (*cbbL/rbcL*) using conserved-site primers only to check the presence of the enzyme (ALFREIDER et al., 2003). L: Invitrogen 1Kb ladder, 1. *Synechococcus* 6301 *rbcL* gene (positive control); 2. Environmental DNA sample

To check if amplified fragments were really RubisCO, the PCR products were cloned into a vector to build a library. A few clones were sequenced from each library. Sequences corresponding to form I RubisCO of *Rhodobacter blasticus* (photoautotrophic enrichment),

Rhodospirilum centenum (photoautotrophic enrichment) and *Hydrogenophaga pseudoflava*(chemoautotrophic enrichment) were found. Therefore, despite the full RubisCO large subunit gene being around 1000 bp, it was confirmed that at least 600bp of different *cbbL*/*rbc*L RubisCO genes were present in the gDNA sample.

Curiously, *H. pseudoflava*was isolated in the cultivable assay in a photoautotrophic condition, and grew in all tested conditions (photoautotrophic, chemoautotrophic and heteroautotrophic), indicating a great plasticity, but the type strain of this organism has no MTA genes described. On the other hand, this organism has a form I RubisCO described, agreeing with the results reported here (form I amplification). This indicates that different methods can achieve the same goal; e.g., finding undescribed RubisCO.

The pRPS-MCS3 plasmid used for the library includes a strong RubisCO promoter in the pRPS plasmid (Figure 5.12), therefore the host strain can grow under autotrophic conditions if this plasmid allows expression exogenous RubisCO sequences of interest.This system had previously been developed by Smith and Tabita (2003) and applied by Witte et al. (2010) for ocean metagenomic library sequences. The present study was aimed to test strategies to directly select functional metagenomic RubisCO from the water environment in order to find RubisCO with characteristics of interest such as high oxygen tolerance (high specificity) or high affinity for CO_2. To approach this goal, each step was analyzed, and besides the protocols described in Material and Methods, a number of additional protocols were tested and described hereafter.

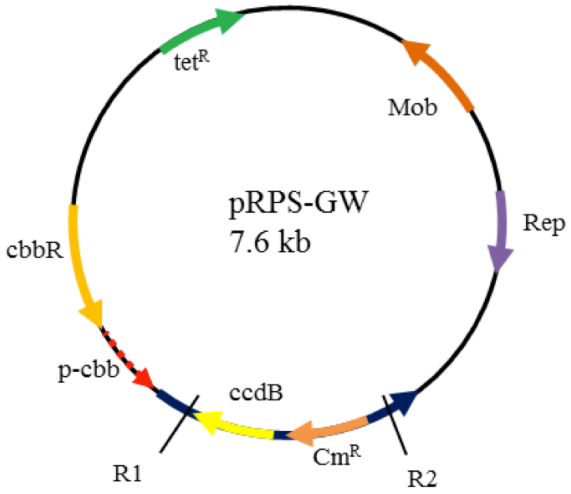


Figure 5.12- pRPS-GW expression. GW designation indicates compatibility of the vector with the Gateway cloning system (Invitrogen). The Plasmid contained a tetracycline resistance gene (Tet), a *lacZ* gene with multiple cloning site, and the *R. rubrum* RubisCO promoter region (*pcbb*M) and its cognate transcriptional activator gene *cbb*R (WITTE, 2012)

Test 1- TOPO plasmid digested with *rsa*I enzyme

Initially the environmental library was prepared from the photoautotrophic enrichment; the gDNA was digested for 2 min using 1U of enzyme for each 1 µg of DNA. The *Rsa*I enzyme recognizes GTAC and leaves blunt ends (not needing end repair) leaving a thymine in the 3' end of the sequence which is ideal for the next step (A tailing).

The digested gDNA was size selected (1 to 6 kb) by gel purification, after that the A-tailing reaction was performed, and adenine was added using TAQ polymerase at 72^{o}C for 15min.

This A-tailed, digested and size selected environmental DNA was then ligated into the TOPO vector and transformed into chemically competent Top10 cells. Witte (2012) tried to clone digested environmental DNA directly into pRPS plasmid several times with no positive results. A MIDIprep of the environmental library was prepared and the amplification of form I RubisCO gene (*cbbL/rbcL*) was performedto check the presence of a RubisCO gene. Only the positive samples proceeded to the next step.

The TOPO library was then transferred to pRPS-GW using the LR clonase II kit (Invitrogen). The cloning region of pRPS-GW is flanked by a RubisCO operon promoter whereby transcription can be initiated in *Rhodobacter* under autotrophic growth conditions. A *ccdB* gene (lethal in *E. coli* strains lacking *ccdA*) is strategically located in pRPS-GW so that

when recombination occurs, the *ccdB* gene is lost, thus ensuring that *E. coli* cells transformed with unreacted pRPS-GW can not propagate. The library was checked with the conserved-site *cbbL/rbcL* primers to test for the presence of RubisCO genes.The positive libraries were used for mattings in order to transfer the library from *E. coli* to *Rodobacter* SBI/II- with an overnight incubation on non-selective media. After 24h the matting mixture was transferred to PYE medium with rifampicin (100 $\mu g.mL^{-1}$) and spectinomycin (10 $\mu g.mL^{-1}$) (to select for *Rhodobacter*), and tetracycline (12.5 $\mu g.mL^{-1}$) (to select for the pRPS plasmid). After selection on PYE, SBI/II- containing the plasmid library was inoculated in Ormerod's minimal media with 20% of CO_2/ 80% H_2 (CO_2 is sole source of carbon), in the presence of light but no oxygen. However none of the *Rodobacter* SBI/II- samples complemented with environmental DNA grew.

In order to find what step went wrong, the TOPO library (where the environmental DNA was initially cloned) was plated. Individual isolates were grown overnight in LB before isolating each plasmid. Upon digestion with *Eco*RI, most of the clones did not have an insert despite the positive RubisCO PCR results (Figure 5.13). This showed that probably the environmental DNA did not ligate properly, and that the pieces that did ligate were probably very small fragments.

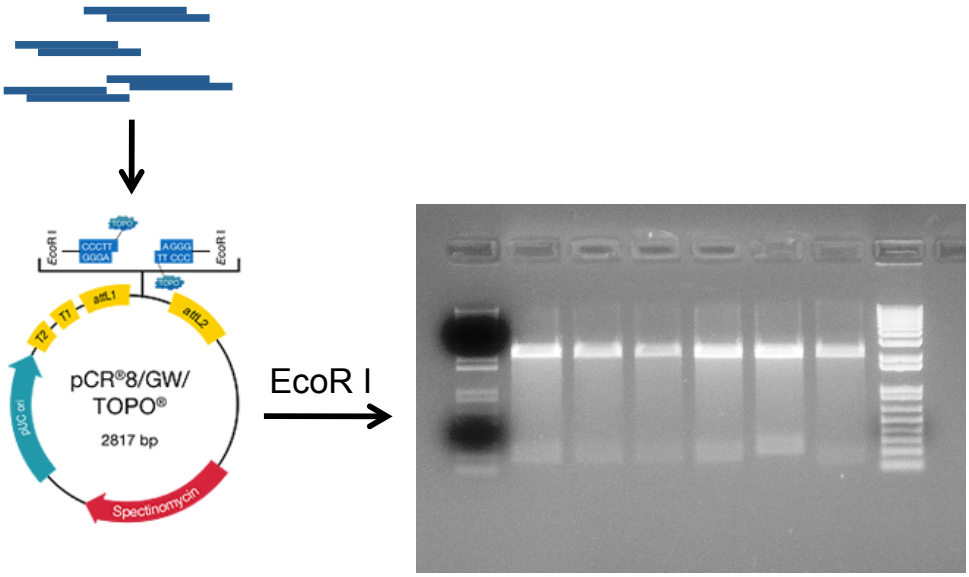


Figure 5.13 - TOPO vector digested with *Eco*RI enzyme, all clones had **no** insert

5.3.2Adjusting TOPO vector protocol: A-tailing for 1.5 h

In an attempt to increase the ligation efficiency of environmental inserts with the TOPO plasmid, we used a higher incubation time of 1.5 hour (Figure 5.14) instead of 15 min, of A-tailing. This resulted in a more efficient ligation, with more clones with inserts after EcoRI digestion. Then, the TOPO library was transferred to the pRPS expression vector. A form I PCR was perform to check the presence of RubisCO gene inserts, with the pRPS library amplified with 600 bp of form I by PCR. Next, this pRPS library was transferred (by mating) from *E. coli* to *Rhodobacter* I/II$^-$. However, this approach also did not achieve the final result: a complementary sequence that could complement CO_2 fixation in *Rhodobacter* SBI/II$^-$.

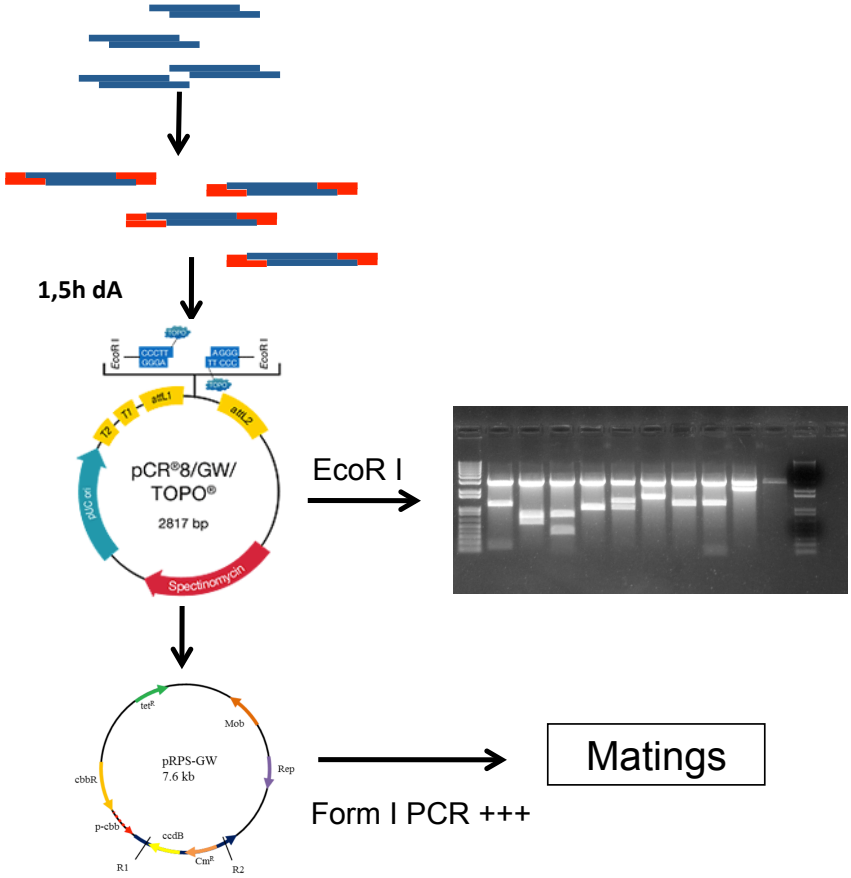


Figure 5.14- TOPO vector with 1.5 h A-tailing increased plasmid-environmental insert ligation. After that, the TOPO library was transferred to the pRPS expression vector (with a positive form I PCR) and then transferred to the pRPS-library by mating to *Rhodobacter* I/II$^-$

Test 2 -Testing another commercial plasmid: the pENTR plasmid

Since the TOPO plasmid did not present many inserts, and it did not work even when increasing A-tailing time, we tried to use another vector. Perhaps 1.5 h at 72^{o}C somehow degraded the environmental DNA, possibly cutting and inactivating the RubisCO gene.

The new vector was a commercial plasmid, pENTR, that was chosen because it also had an *att* region to transfer the library to pRPS using lambda recombinase. Besides that, pENTR had the *ccdB* gene (lethal in *E. coli* strains lacking *ccdA*), allowing selection for plasmids containing an insert.

The same protocol was used, we only changed the TOPO plasmid to the pENTR plasmid (Figure 5.15). To check for the presence and size of inserts, pENTR was digested for 1h at 37^{o}C with *Bam*HI, presenting several insert sizes bigger than 1000 bp, and a positive form I PCR result, indicating the presence of part of a RubisCO sequence. With that, the library was transferred to pRPS (with a positive form I PCR) and then transferred to *Rhodobacter* I/II^{-}. Despite all the positive controls, this library also did result in growth by complementing carbon fixation in *Rhodobacter* SBI/II^{-} and the organism did not grow autotrophically in the presence of the environmental library.

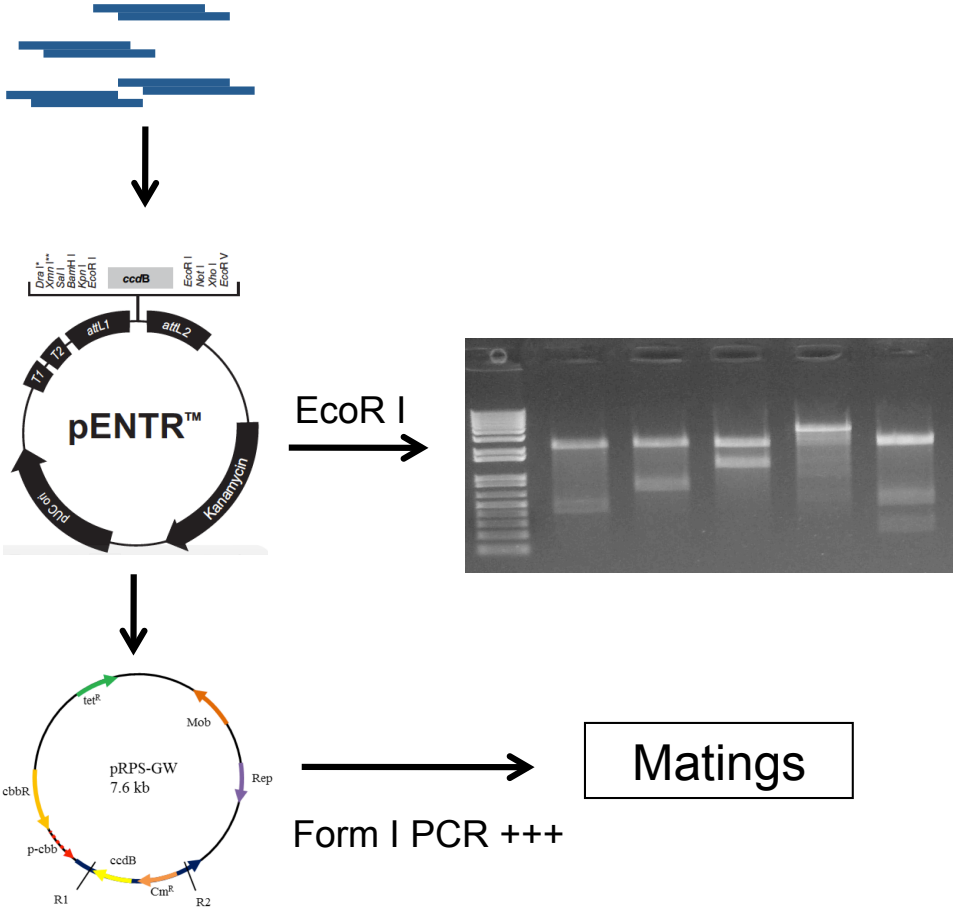


Figure 5.15- Environmental DNA digested with *rsa*I, cloned into *pENTR* plasmid (digested with *Eco*RI to check inserts) and transferred to expression plasmid (pRPS) amplifying 600 bp of form I RubisCO, proceeded to the mattings transferring pRPS-library into *Rhodobacter* I/II^{-}

Test 3 - Attempts to optimize the ligation (insert-plasmid) by addinglinkers and amplifying the environmental DNA (insert)

In order to test the ligation (with the insert-plasmid) so that insert quantity is not an issue, another approach was attemped, using Sau3AI-digested genomic DNA ligated to a 15 bp linker containing a *not*I restriction sequence (WITTE, 2012) and insert PCR amplification. The *not*I sequence in the linker allowed ligation to the complementary 5' unique overhang in the pENTR vector. The linker also provided a target for subsequently used PCR primers. PCR can be used to amplify the linker-ligated gDNA and the resulting fragments could be cloned into both TOPO and pENTR vectors. After doing this, some clones with environmental DNA in the TOPO and pENTR vector were selected and digested with *Eco*RI to check the size and presence of the inserts. Both plasmids contained inserts of variable size (Figure 5.16).

Inserts from both vectors were transferred to the pRPS expression vector, and PCR was performed to check for the presence of a form I RubisCO gene. Libraries containing

RubisCO genes (as determined by PCR) were transferred to *Rhodobacter* I/II$^-$ by mating. However, once again, no culture tubes showed growth.

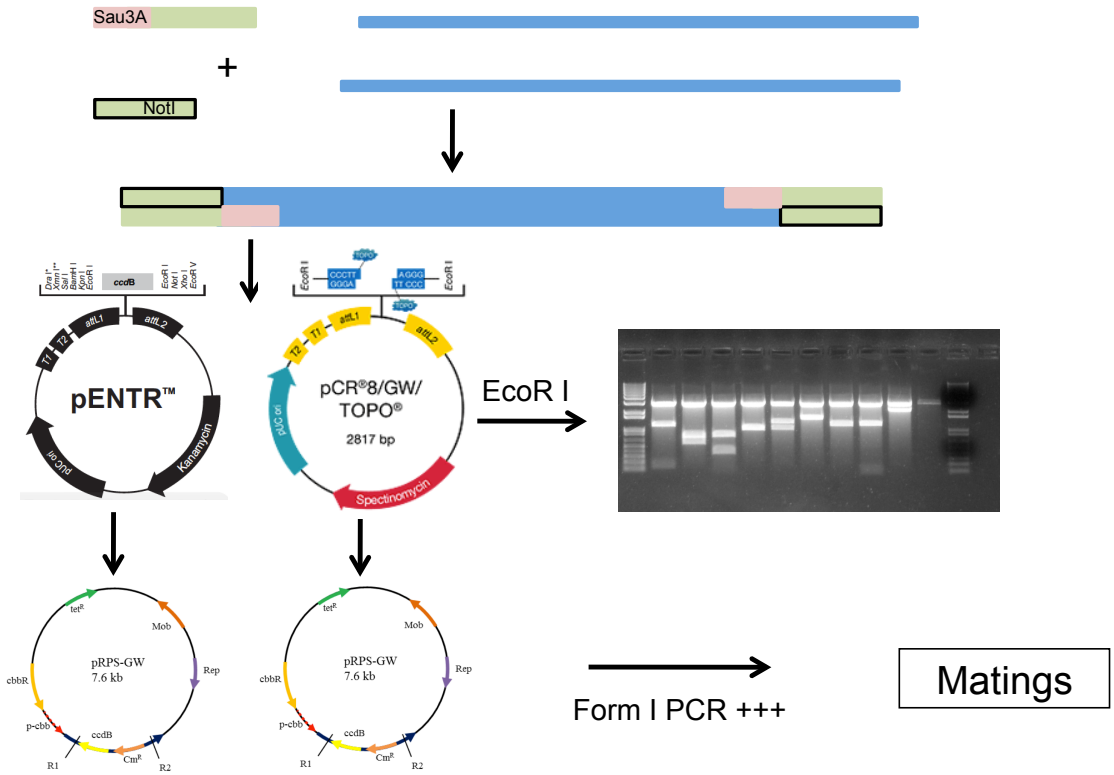


Figure 5.16- Environmental DNA digested and ligated with linkers, amplified, cloned into the TOPO and *pENTR* plasmid and transferred to expression plasmid (pRPS) and thenmated to *Rhodobacter* I/II$^-$

Why did all of the above attempts with pRPS fail? There are several possible explanations: First,the many steps employed, from the DNA extraction until the *Rhodobacter* I/II$^-$ expression, might create problems. During these many steps it is possible that some of these steps resulted in physical fragmentation of the DNA sequences, so even if a RubisCO sequence was present it was not able to be transcribed into RNA or translated into protein due to this damage to the DNA sequence. A second possibility:the pRPS plasmid on average presents inserts of 1000bp. This is a little less than the size of a form I RubisCO large subunit gene, however the environmental DNA sequence needs to have around 1500 bp, and for form I RubisCO must include the small subunit gene. Thus, the entire RubisCO (*cbbLS/rbcLS*) gene sequence needs to be within this 1500 bp sequence, and the chances of that happening are low; Thirdly, it is conceivable that the environmental RubisCO sequences in our samples encode structurally different RubisCO that is so unusual that *Rhodobacter* I II$^-$ cannot express these RubisCO genes due to codon bias or failure of the I/II- chaperones to recognize the

translated products. This explanation is probably the most unlikely reason for the inability to achieve complementation, based on the sequences that we have seen.

Test 4 - Using a Bacterial Artificial Chromosome (pGNS-BAC) in order to insert larger inserts

The last test was performed with pGNS-BAC (KAKIRDE et al., 2011), previously used for heterologous expression of metagenomic libraries. pGNSis 16.5 kbp with the ability to accept large inserts (up to 80 kbp) (KAKIRDE et al., 2011). This BAC had already been investigated by Witte (2012). *Rhodobacter capsulatus* I II$^-$ is naturally resistant to gentamycin antibiotic, which was the original selection used for pGNS-BAC. Thus, the first step was to change the gentamycin cassette to tetracycline resistance (the same antibiotic resistance as pRPS vector).

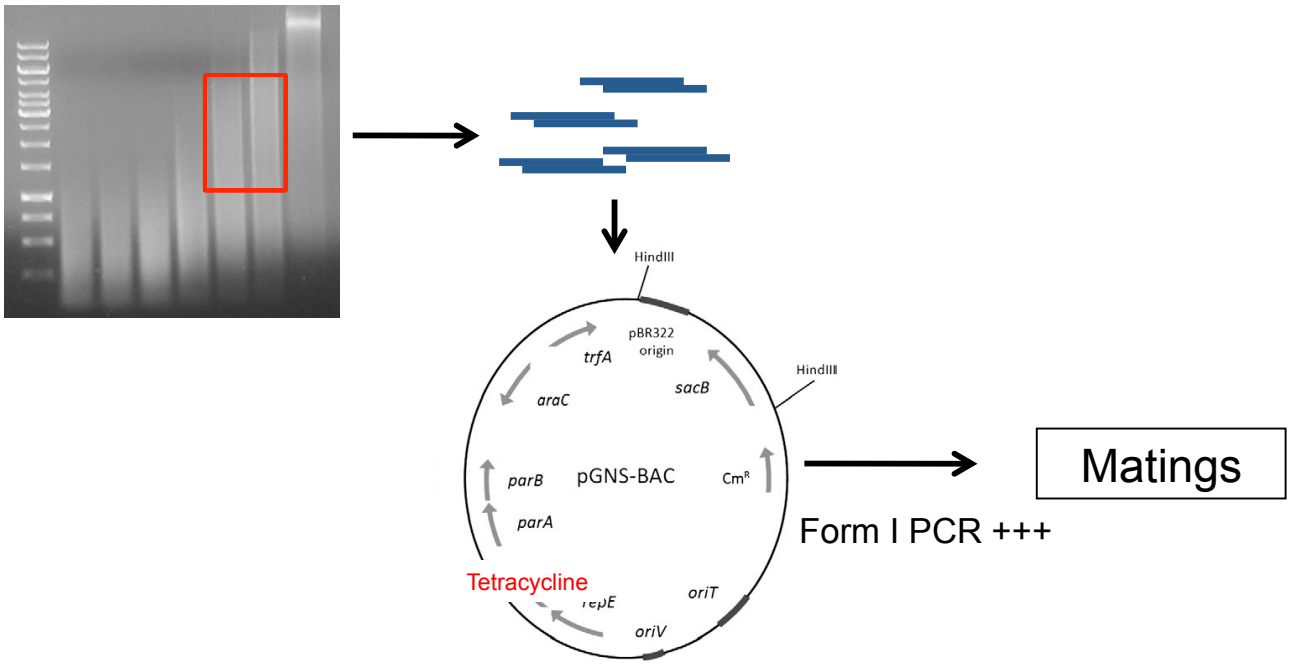


Figure 5.17- Environmental DNA digested cloned into pGNS BAC (in *E. coli*) transferred by mating to *Rhodobacter* I/II$^-$

DNA isolated from organisms from environmental samples was digested with *rsa*I (1U for 1 µg of DNA) for 2 min. The digested DNA was size selected (1 to 6 Kb) by gel purification, purified, and then ligated using 60 ng of insert into 100 ng of pGNS-BAC for 1 hour at room temperature. The library of pGNS-BAC was first selected for inserts in *E. coli*, and then transferred into *Rhodobacter capsulatus* I II$^-$ via conjugation. Once again, there was

no evidence of complementation or growth under autotrophic (RubisCO-requiring) conditions (Figure 5.17).

The matting efficiency of pGNS between *E. coli* and I/II- is not known. Because the inserts would be quite large, expression of environmental RubisCO from pGNS would require *R. capsulatus* I/II$^-$ to recognize the native promoter of the environmental RubisCO gene. It may be that there was one or more complete RubisCO genes in the pGNS library that could not be expressed simply because I/II$^-$ could not recognize the upstream promoter.

Another thing that can happen is that form I PCR amplifies even if there are only few copies of the gene, and the inoculum is a sample of the entire flask, so if there are very few copies of RubisCO there is less chance to go through the end of the process.

5.4 Conclusions and future research

Different protocols to isolate any form of RubisCO genes from the environment were tested. The cultivable methods are more laborious and time consuming. However, when isolating an organism with a non-described RubisCO, it is possible to check what other genes (besides RubisCO) are involved in the process of MTA growth and the location of these genes. This method selected bacteria capable of growing in MTA as sole S source while searching for different forms of form IV RubisCO. Although, *Hydrogenophaga, Acinetobacter* and *Rubrivivax* had only a form I RubisCO described since these organisms could use MTA there may be a form IV RubisCO present in their genomes, however one that has not yet been described. On the other hand, *Delftia*has only a form IV RubisCO described (and it doesn't fix CO_2), but it is able to grow photoautotrophicly.What is fixing CO_2? Is it a Form IV with as-yet undescribed functionality, or is it a not-yet described Form I or II RubisCO or other CO_2 fixation pathway involved? In contrast, *H. seropedicae* has no RubisCO described, thus photoautotrophic growth should be tested and chemoautotrophic growth re-checked.

Isolation results are the first steps towards finding new RubisCO enzymes that potentially possess new catalytic properties (higher specificity, faster carboxylation, lower Km for CO_2).There are many possibilities if we seek a versatile bacterium that can grow in different environments, with different conditions: chemoautotrophic, chemoheterotrophic, photoautotrophic and photoheterotrophic (similar to *Rhodopseudomonas*) and are abundant in the environment. If only versatility (not abundance) is necessary, *Delftia* is a great option. On the other hand, if the aim is finding different form IV RubisCO, *Hydrogenophaga and*

Acinetobacter should be investigated. Thus, this study opened different pathways where research may proceed.

The other tested way of isolating different RubisCO genes from the environment was via the metagenomics approach, which is less time consuming and can use uncultivable organisms. Using the *R. capsulatus* SBI/II$^-$ selection system, it should be possible to check if environmental sequences (from non-cultivated bacteria) encode a RubisCO protein, and to study all of the enzyme properties (oxygen tolerance or high affinity for CO_2) without needing to cultivate the original organisms. However, the direct selection of environmental DNA sequences, without previous sequencing, not knowing where the genes start or end, or what sequences are being cloned into *E. coli* and transferred into *Rhodobacter capsulatas* Δ*cbbL*/*cbbM* still needs to be optimized.

We tested different methods of inserting fragmented environmental DNA into the pRPS vectors. This vector has a promoter that can drive expression in the host organism (I/II$^-$). It may be, however, that this promoter does not work with the new sequences, due to codon bias, a lack of a suitable ribosome binding site on the transcript, or another unidentified factor. Larger inserts (like those in pGNS) would only magnify the problem since they would not even have the I/II$^-$ compatible promoter.

Each of these techniques has benefits. The metagenomic approach scans enormous amount of data (sequences) in a short period of time. However, there are more chances to find an undescribed enzyme if an efficient system is established. On the other hand, if you have the organism, different interacting experiments can be performed, so it is interesting to invest in both techniques, choosing for one or another depending on the objective of your research.

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6 FINAL CONSIDERATIONS

These research contributed to elucidate how redox balance influences Cd and Ni tolerance mechanisms in two tolerant bacteria isolated from Cd contaminated soil and non contaminated soil. Most of tested antioxidant enzymes are important to Cd tolerance contributing to cell redox balance, since all enzyme activities was increased with Cd. Besides that, the isolate from contaminated soil were able to accumulate more Cd, expressed more isoforms of all tested enzymes, presenting a higher metabolic diversity and plasticity. Despite of the different tolerance mechanisms to each metal, most isoenzyme expression present similar response to Cd and Ni (except for GST enzyme).

We also characterized a Cd tolerant bacteria (SCMS54) able to promote a beneficial interaction with tomato plants (both in hydroponic system and green house pots), enhancing plant growth and reducing Cd absorption, resulting in an increase in plant tolerance to high concentrations of this toxic heavy metal. Bacteria inoculation decrease plant peroxide concentration and chlorosis levels, as well as an increase in stress genes (chaperones, heavy metal transporter and metallothionein). There are a several factors that can contribute to the increase plant growth and Cd tolerance: production of indole-acetic acid (IAA), solubilization of inorganic phosphate (increasing P uptake), production of siderophore (increasing Fe uptake) as well as the temporally adsorption of Cd by the bacteria. The increase in plant tolerance to Cd allows farmers to plant productive crops in soils with Cd concentration permitted by CETESB (lower than 3 $mg.kg^{-1}$).

Burkholderia sp. SCMS54 inoculation is a cheap technique that does not produce residue that contaminates the environment, and is able to promote tomato growth in the presence of Cd as a result of a bacterial-plant root beneficial interaction by a mechanism that appears to trigger a global response in plants and decrease Cd concentration in roots.

RubisCO research contributed to help Prof. Tabita's research group to design a metagenomic system to find environmental RubisCO (or any other environmental enzymes) with non described characteristics, helped also the characterization of several cultured (non model) bacterial that were able to grow photoautotrophicly, increasing the number of organism that this RubisCO lab can work with.