FRERES MENTOURI CONSTANTINE 1 UNIVERSITY
Faculty of Natural Sciences and Life
Molecular and Cellular Biology laboratory
And
AIX-MARSEILLE UNIVERSITY
Faculty of Life and Health Sciences
National Center for Scientific Research
Bioenergetics and Protein Engineering laboratory

A Thesis
Submitted in fulfillment of the requirements for the degree of
Doctor in Microbiology

Presented and publicly defended by

Hiba BAAZIZ

10 July 2018

Isolation of *Shewanella* sp. from Algeria and characterization of a system involved in detoxification of chromate

Jury

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Rabah ARHAB	**Professor, Larbi Ben M'Hidi University**	**Reviewer**
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Patricia BONIN	**Research Director, MIO, Marseille**	**Examiner**
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Preface and acknowledgement

I submit my heartiest gratitude to the members of my dissertation committee: ARHAB Rabah, BOURSOLLE Véronique, MIHOUBI Ilham and BONIN Patricia for granting me honor of reading and evaluating my work.

A Doctoral Journey

« Ladies and gentlemen, this is your captain speaking, welcome to Marseille-Provence Airport. Local time is 11:55 for your safety and comfort, please remain seated with your seat belt fastened, we will be landing momentarily»

I could see Marseille, a foreign city, ghosts of Constantine cross my mind and I already feel homesick.

I left the airport to find Michel, my advisor, I expected a chubby old man with a strict face only to find a tall good looking one wearing a welcoming smirk on his face, after a few minutes I found myself greeting foreign lab mates.

Three years later…

I just finished my thesis and I have acquired knowledge and gained a new family. I feel sincerely grateful to have an advisor like Michel, who continuously supported my Ph.D. study, who had the patience, and always radiated me with motivation and immense knowledge, whose special sense of humor made me laugh all the time. Radia, whom I am deeply indebted to; without her support I would not join the BIP3 lab and acquire this amazing experience.

Vincent, the head of BIP3, who provided me the opportunity to join their team and steered me in the right direction through my PhD, his warm hospitality and constant humoring vibe made me always thankful to have him by my side; Cécile, who was a mother figure in the lab, the door to her office was always open whenever I ran into a trouble spot or had questions about my research or writing, which I am forever grateful for; Chantal, Olive, and Sandra, whom I will treasure their stimulating discussions and invaluable help; Amine and Ahmed, who were a scent from home and continuously helped me during my thesis.

My fellow lab mates more like my family! Whose presence, scientific help and the unforgettable funny moments I am forever grateful for. The brilliant Zitoun, who was my personal Google Scholar I am grateful for his advices and for dealing kindly with my endless bothering questions; Baptist, who I cannot imagine going to USA without; Natou, Sophie and Flora whom am forever grateful to their kindness and immense support; Cyril and Anne, my siblings, the little shrimp who was a pain in the neck but I am deeply thankful to him for being the first to welcome me in the team; Annouchka, who had a great impact on both myself and my research and who took it upon herself to hear my constant nagging.

Aside from my lab, I have never felt this lucky to have people back home who stood by me and cared for my wellbeing like the Algerian lab team in Constantine, who supported me throughout my first year and showered me with love when I was leaving to France. My parents, whom I revere the patronage and moral support extended with love, whose financial support and passionate encouragement made it possible for me to complete this PhD thesis. As my joy knows no bounds in expressing how grateful and lucky I am to have crazy sisters that supported me in every step in my journey.

This may be the end to this journey, but certainly, the beginning of another unexpected one is waiting around the corner…

Abbreviations

ALI	Air Liquid Interface
CFU	Colony-Forming Unit
CRP	Cylic-AMP Receptor Protein
DMSO	Dimethyl Sulfoxide
DPC	S-diphenylcarbazide
EPSs	Extracellular Polymeric Substances
EPA	Eicosapentaenoic Acid
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
LB	Luria Broth
MCP	Methyl-accepting Chemotaxis Protein
MOPS	Morpholino Propanesulfonic acid
Mtr	Metal Reducing
ROS	Reactive Oxygen Species
TMA	Trimethylamine
TMAO	Trimethylamine N-oxyde
Q	Quinone
QH_2	Quinol

Summary

The widespread use of the toxic heavy metal chromium (Cr) in industrial applications resulted in large quantities of Cr being discharged into the environment, causing severe contamination of global soil and water systems. Cr primarily exists in two stable forms, Cr(III) and Cr(VI). The latter is highly toxic due to its strong oxidizing nature and its high solubility. The model organism for bioremediation *Shewanella oneidensis* MR1 has evolved diverse resistance mechanisms to cope with chromate toxicity.

The first aim of the present thesis was to study the chromate resistance and reduction mechanisms of this bacterium under semi-aerobic conditions. We showed that *chrA$_{SO}$* gene is induced by chromate and its deletion impairs the chromate resistance and reduction capacity of MR-1 strain, we confirmed that its product functions as an efflux pump to extrude chromate ions from the cytoplasm protecting cells from chromate toxicity. With *cymA*-deletion mutants, we revealed the involvement of the c-type cytochrome CymA in chromate resistance and reduction. We also identified a potential chromate reductase DmsA2, as well as two other proteins Fdh and DmsA1 that are potentially involved in chromate resistance and reduction in MR-1 strain.

In the second part of this work, we isolated, identified and characterized two novel Mediterranean *Shewanella* sp. strains, *S. fidelis* H76 and *S. algidipiscicola* H111. Both strains are characterized by their great chromate resistance and their ability to reduce it efficiently even at high concentrations. Although the small size of its genome and the absence of several genes encoding enzymes known to play a role in chromate resistance and reduction, the H111 strain is the best chromate resistant strain. Interestingly, the air liquid interface biofilm (Pellicles) of both strains reduce more efficiently chromate than their free-swimming cells. Moreover, they can accumulate a significant amount of its reduced forms. These characteristics make those strains, in particular H111 strain, suitable candidates for chromate bioremediation.

Résumé

L'utilisation répandue du chrome (Cr) dans les applications industrielles a entraîné le rejet de grandes quantités de ce métal lourd toxique Cr dans l'environnement, causant une sévère contamination des sols et des systèmes hydrologiques globaux. Le Cr est principalement rencontré sous deux formes stables, Cr(III) et Cr(VI). Ce dernier est hautement toxique en raison de sa forte nature oxydante et de sa grande solubilité. L'organisme modèle pour la bioremédiation, *Shewanella oneidensis* MR1, a développé divers mécanismes de résistance pour faire face à la toxicité du chromate.

Le premier objectif de cette thèse était d'étudier les mécanismes de résistance au chromate et de sa réduction chez cette bactérie dans des conditions semi-aérobies. Nous avons a montré que le gène *chrA$_{SO}$* est induit par le chromate et que sa délétion altère la capacité de la souche MR-1 de résister au chromate et de le réduire. Nous avons confirmé que ChrA$_{SO}$ fonctionne comme une pompe d'efflux pour extruder les ions chromate du cytoplasme. En délétant le gène *cymA*, nous avons révélé son implication dans la résistance au chromate ainsi que dans sa réduction. Nous avons également identifié une potentielle chromate réductase DmsA2, ainsi que deux autres protéines Fdh et DmsA1 potentiellement impliquées dans la résistance au chromate et dans sa réduction chez la souche MR-1.

Dans la deuxième partie de ce travail, nous vous on a isolé, identifié et caractérisé deux nouvelles souches de *Shewanella*, *S. fidelis* H76 et *S. algidipiscicola* H111. Les deux souches se caractérisent par leur grande résistance au chromate et leur capacité à le réduire efficacement même à des concentrations élevées. En dépit de la petite taille de son génome et l'absence de plusieurs gènes codant des enzymes connues pour jouer un rôle dans la résistance au chromate ainsi que dans sa réduction, la souche H111 est la plus efficace. De façon intéressante, les biofilms de l'interface liquide-air (Pellicules) des deux souches réduisent plus efficacement le chromate que leurs cellules planctoniques. De plus, les pellicules des souches H76 and H111 peuvent accumuler une quantité importante de formes réduites du chromate. Ces caractéristiques montrent que ces souches, en particulier la souche H111, sont des candidats appropriés pour la bioremédiation du chromate.

الملخص

يؤدي الاستعمال الواسع للمعدن الثقيل السام الكروميوم (Cr) في التطبيقات الصناعية الى تفريغ كميات كبيرة منه في المحيط، مما يؤدي الى تلوث شديد للأنظمة البيئية اليابسة والمائية. يتواجد Cr اساسا على صورتين ثابتتين هما (Cr(III و Cr (VI) حيث أن الأخيرة عالية السمية بسبب طبيعتها التأكسدية القوية و درجة ذوبانها الكبيرة. لقد طورت Shewanella oneidensis MR1، كأنموذج لكائن حي للمعالجة البيولوجية، آليات مقاومة مختلفة للتعامل مع سمية الكرومات.

تمثل الجزء الأول من هذه الدراسة في دراسة مقاومة الكرومات و آليات اختزاله عند هذا البكتريوم تحت ظروف نصف-هوائية، حيث أظهرت الدراسة أن الجين *chrA_{SO}* يتم تحفيزه بالكرومات و أن سحبه يؤدي إلى تعطيل مقاومة لكرومات و قدرة اختزاله عند السلالة MR-1، كما تم تأكيد أن البروتين الناتج عن هذا الجين يعمل كمضخة دفق خارجي لطرد ايونات الكرومات من السيتوبلازما مما يؤدي إلى حماية الخلايا من التسمم بالكرومات. تم إظهار مشاركة cytochrome CymA من النوع c في مقاومة و اختزال الكرومات؛ كما تم التعرف على إنزيم اختزال محتمل للكرومات DmsA2 وكذلك على بروتينين أخرين هما Fdh وDmsA1 اللذين يحتمل مشاركتهما في مقاومة و اختزال الكرومات عند السلالة MR-1.

في الجزء الثاني من الدراسة، تم العزل و التعرف و التمييز لسلالتين جديدتين من جنس *Shewanella*. معزولتان من البحر الأبيض المتوسط و هما *S. fidelis* H76 و *S. algidipiscicola* H111. تميزت هاتين السلالتين بمقاومتهما الكبيرة للكرومات وقدرتهما لاختزاله بفعالية حتى عند التراكيز العالية. إن السلالة H111 هي السلالة الأحسن في مقاومة الكرومات بالرغم من صغر حجم جينومها وغياب العديد من الجينات المشفرة لإنزيمات معروفة بدورها في مقاومة واختزال الكرومات. ومن المُثير للاهتِمام، ان البيو فيلم الطافي لكلا السلالتين يختزل الكرومات بفعالية مقارنة بالخلايا السابحة الحرة؛ كما يمكنه أن يراكم كمية معتبرة من الصور المختزلة للكرومات. تجعل هذه المميزات السلالتين وبالأخص السلالة H111 مرشحتين ملائمتين للمعالجة البيولوجية للكرومات.

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INTRODUCTION

CHAPTER I:

CHROMIUM

1. Heavy metals

Heavy metals can be defined by various criteria including density, atomic weight, atomic number and chemical properties. However density is the main aspect to be considered as the defining feature (Prabhakaran *et al*, 2016). The "heavy metals" term refers from a purely physical point of view to an intrinsic property of elements: the density. According to Alloway & Ayres, 1997 designating natural metallic elements, metals and in some cases metalloid elements (about 65 elements) are characterized by a high density greater than 5 to 6 and an atomic number above 20.

From a biological point of view, two types of heavy metals can be distinguished according to their physiological and/or toxic effects: essential metals and toxic metals. The former are crucial elements in a trace state for many cellular processes and are found in a very low amount in biological tissues (Singh *et al*, 2011). They become toxic when their concentration exceeds a certain threshold. This is the case of copper (Cu), nickel (Ni), zinc (Zn) and iron (Fe). However, toxic metals have a polluting feature with toxic effects to living organisms even at low concentration and they have no known beneficial effects for the cell. This is the case for lead (Pb), mercury (Hg), cadmium (Cd) and chromium (Cr) (Figure 1) (Summers, 2009).

Unlike most organic contaminants, heavy metals are natural constituents in rocks and mineral deposits. Thus, these elements are normally present at low concentrations (in a trace state, less than 0.1%) in soils, sediments, surface waters and living organisms (Alloway & Ayres, 1997; Callender, 2003). Accumulation of heavy metals above the threshold level is mainly due to anthropogenic activities including mining, chemical manufacturing, agriculture, hospital wastewater and electronic waste. Heavy metals can pose cytotoxic, carcinogenic and mutagenic effects (Tóth *et al*, 2016).

2. Chromium features

The name "chromium" is derived from the Greek word "chroma" meaning "color" which refers to the multiple colored compounds that contain chromium such as $Na_2Cr_2O_4$ (bright orange), Cr_2O_3 (green) and the Zn and Pb salts of CrO_4^{2-} (bright yellow) (Lear, 2016). It was

Figure 1. Periodic table of metals and toxic metalloids: Elements Figuring in blue are toxic at high concentrations and the yellow colored elements are toxic and have no biological role in most organisms. The transition metals are shown in the red frame and the elements framed by the green are the metalloids. Figure adapted from (Summers, 2009).

Figure 2. Chromium traces in gemstones: A. Rubies found in marble deposits (https://www.gia.edu/ruby-description) **B.** Emerald crystals in mica schist (http://geology.com/gemstones/emerald/).

named by the French pharmacist and chemist Louis Nicolas Vauquelin who published in 1809 his discovery of the yellow pigment of chromium (Aharchaou, 2017).

The chromium (Cr) element belongs to the group of transition metals and it is the 24th element of the Mendeleev periodic table, located between vanadium and manganese. It has an atomic weight of 51.996, atomic number 24, a density of 7.14, a melting point of 1900°C, a moderate thermal expansion and a stable cubic crystal structure. In terms of abundance, chromium is the seventh element on Earth and the 21st in the Earth's crust, with an average concentration of 125 mg.kg^{-1} (Cervantes *et al*, 2001) (Figure 1).

3. Natural distribution of chromium

Chromium is a naturally occurring element that is ubiquitous in the environment. It is found in all compartments of the environment, in water, air, soil and also in living organisms. It is widely present in igneous rocks where it can substitute for Fe^{3+} and for Al^{3+} in other minerals such as tourmalines, micas and garnets (Guertin *et al*, 2016). Chromium traces are often responsible for the color of these minerals such as the red of the ruby and the green of the emerald (Bunn *et al*, 2013) (Figure 2). Rock alteration and erosion is an important source of chromium release to the environment (Nriagu, 1989).

4. Industrial use

Because of its hardness, gloss, high melting point and anti-corrosive properties, chromium is used in various industrial activities. It is extracted as chromite ore ($FeCr_2O_4$). From total chrome ore production, 90% is used in metallurgical industries for steel, alloy and nonferrous alloy production, 5% in refractory (cement, glass, ceramics and machinery) and the remaining 5% in chemical industries such as leather tanning, electroplating, wood preservation, pigment production and it can be used as an oxidizing agent (Dhal *et al*, 2013). As consequence of its extensive anthropogenic use, chromium is present in effluents originated from the different activities and represents a serious pollutant of sediments, soil, water and air (Focardi *et al*, 2013).

5. The environmental aspects and speciation of chromium

In the environment, chromium exists in different oxidation states ranging from -2 to +6. The stable forms, commonly occur in the pH and redox potential values found in the environment, are the trivalent Cr(III) which naturally predominates in the environment, and the hexavalent Cr(VI) which is rarely naturally occurring and is introduced in the environment mainly by anthropogenic activities (Focardi *et al*, 2013). On the other hand, Cr(IV) and Cr(V) are unstable intermediate forms of reactions between oxidizing and reducing agents of Cr(III) and Cr(VI) (Aharchaou, 2017).

The speciation of Cr(III) and (VI) depends on several parameters such as pH and their concentration in the environment. In natural media, major part of Cr(III) is included in hydroxides or in complexes with organic ligands while Cr(VI) occurs mainly in the form CrO_4^{2-} (Hossain *et al*, 2005).

5.1. Trivalent chromium speciation

Cr(III) is the most stable form of chromium, it is considered to be relatively immobile, sparsely soluble and of limited ecotoxicological interest (Gonzalez *et al*, 2003). However, at high concentration it becomes toxic, carcinogenic and teratogenic (Ahmad *et al*, 2009).

Cr(III) has little affinity for oxygen but has a greater affinity toward organic and inorganic ligands (Zayed & Terry, 2003). It forms insoluble complexes that precipitate as oxides, hydroxides or sulfates (Focardi *et al*, 2013). Under normal environmental conditions, Cr(III) is found in aqueous solution in the form of Cr^{3+}, $Cr(OH)^{2+}$, $Cr(OH)^{4-}$ and $Cr(OH)_3^0$. The latter form presents the most frequently encountered solid form. It is known to have a very low solubility at natural pH, which makes Cr(III) less toxic (Ramírez-Díaz *et al*, 2008). Indeed, the internalization mechanisms of Cr(III) remain poorly known. Some studies consider that it is unable to pass through cell membranes which would explain its relatively poor toxicity (Francisco R. *et al*, 2002).
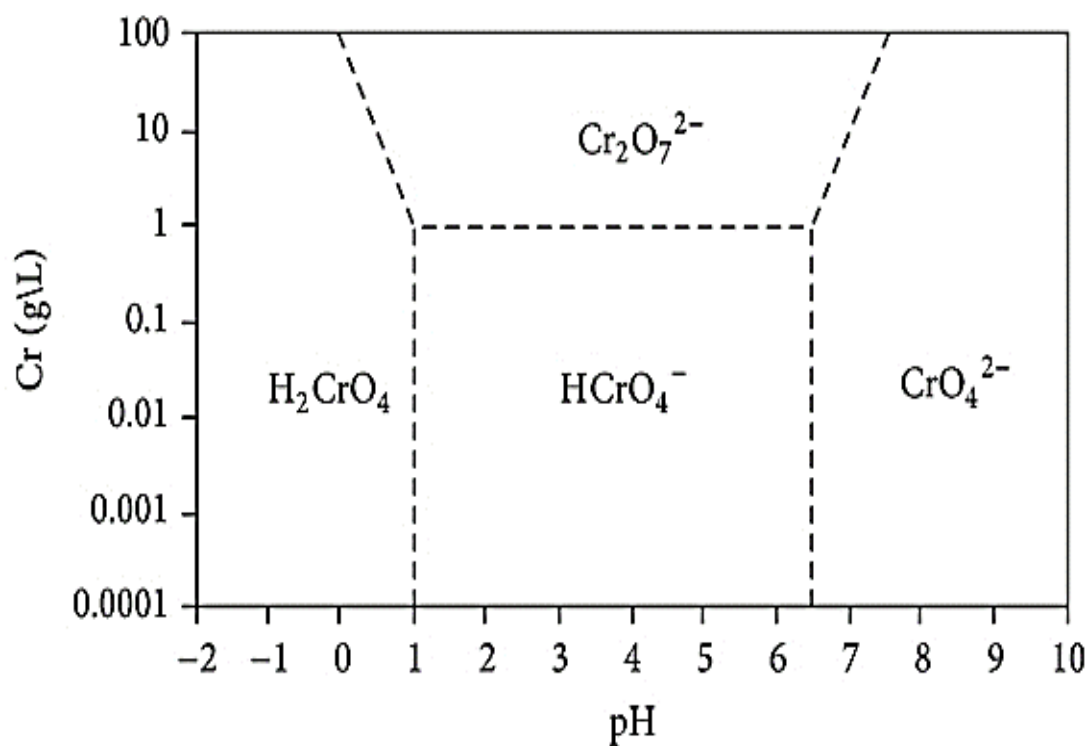
Figure 3. Cr(VI) Speciation diagram depending on pH. The dominant Cr(VI) species at pH lower than 0 is chromic acid (H_2CrO_4). Predominant Cr(VI) species at pH between 1.0 and 6.5 is hydrogen chromate ion ($HCrO_4^-$) while CrO_4^{2-} ions are dominant above pH 6.5. Dichromate anions ($Cr_2O_7^{2-}$) exist only at high concentrations about more than 1 g/L (Duranoğlu & Beker, 2015).

5.2. Hexavalent chromium speciation

Cr(VI) is a powerful oxidant found in oxyanions form which are very soluble in water (Nriagu & Nieboer, 1988). It is characterized by a much greater solubility, mobility and bioavailability than other forms of chromium including Cr(III) (Focardi *et al*, 2013). Its solubility depends on the cation to which it is associated. For example, potassium chromate K_2CrO_4 has a solubility of 38,96 g.L^{-1} at 20°C whereas the $PbCrO_4$, $CaCrO_4$ and $BaCrO_4$ complexes exhibit much less solubility (Hall, 1957).

Cr(VI) forms several species, the relative proportions of which depend on both the pH and the total Cr(VI) concentration. At low pH, close to 0, the chromic acid H_2CrO_4 which belongs to strong acids is the dominant species whereas between about 0.6 and 6, the hydrogen chromate $HCrO_4^-$ is the predominant form. In pH > 6, it is the chromate ion CrO_4^{2-} that prevails, above pH 7, only chromate ions CrO_4^{2-} exists in solution for all concentrations. Since no pH near 0 is found in the environmental matrices, only $HCrO_4^-$ et CrO_4^{2-} are present in the natural systems. Above a concentration of 1 g.L^{-1} of Cr (VI), the dimerization of chromate CrO_4^{2-} to dichromate $Cr_2O_7^{2-}$ is observed (Hall, 1957; Duranoğlu & Beker, 2015) (Figure 3). Under the conditions generally encountered in chromium-polluted waters, the chromate ion is the predominant form (Callender, 2003).

6. Abiotic chromium reactions in the environment

Chromium is known to interfere in various chemical and biological reactions, such as Cr(III) oxidation or Cr(VI) reduction reactions, that can modify its speciation and consequently its behavior in the environment.

6.1. Trivalent chromium reactions

In the environment, Cr(III) can be oxidized. However, this process is sparsely common since its oxidation requires the presence of a higher redox potential pair than that of the Cr(VI)/Cr(III) pair. In addition, the concentrations of Cr^{3+} in solution are almost nonexistent under the usual environmental conditions because Cr(III) precipitates almost completely in the form of $Cr(OH)_3$ or CrOOH. The trivalent chromium thus immobilized physically, on the

matrix of the soil or sedimented in a liquid media, is then protected from oxidation (Ahmad *et al*, 2009). It have been shown that only oxides of manganese (MnOOH) and molecular oxygen are capable of oxidizing Cr(III) to Cr(VI) (Fendorf, 1995).

6.2. Hexavalent chromium reactions

Cr(VI) is a powerful oxidant and can be readily reduced in the presence of several reducing agents. Fe(II) appears to be the most important possible reductant of Cr(VI) in the environment. Studies on the reduction of Cr(VI) by Fe(II) in solution have shown that, in addition to the respective concentrations of both species, temperature and pH influence the speed of reaction. The latter increases by the increasing of the temperature. The fastest reaction kinetics are observed in pHs between 6 and 8. Sulphides are also potential candidates for Cr(VI) reduction. They may be combined with Fe(II) in the form of ferrous sulfide, or with other divalent cation metals such as Mg^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, $Ni2^+$ and Mn^{2+}, or may be alone in the form of H_2S (Pettine *et al*, 1998; Sedlak & Chan, 1997). Natural organic matter, humic acids or fulvic acids, in soils or waters is also likely to reduce Cr(VI) (Bartlett & James, 1979; Alloway & Ayres, 1997).

Cr(VI) can also be reduced photochemically. Studies on photo-reduction of Cr(VI) in natural media have shown that the mechanism is indirect and needs the Fe(II)/Fe(III) pair to transfer electrons from the organic ligands to Cr(VI). The organic Fe(III)-ligand complexes absorb light and produce Fe(II). The latter in its turn reduces Cr(VI) to Cr(V) then to Cr(IV) and finally to Cr(III). At each step, Fe(II) is reoxidized to Fe(III) which can again be complexed with organic ligands and thus resume the cycle (Gaberell *et al*, 2003).

7. Chromium toxicity

7.1. Toxicity of chromium in humans

Cr(III) has long been considered to be an essential micronutrient in animal and human alimentation, since it seems to participate in the metabolism of glucose and lipids (Anderson, 1997). Nevertheless, this role as an essential element has been recently debated, some authors do not recognize Cr(III) as a nutrient for human health and reported that exposure to high levels

via inhalation, ingestion or dermal contact may causes some adverse health effects (Di Bona *et al*, 2011).

It is well known that Cr(VI) is carcinogenic at high doses and presents a greatest health risk (Keegan *et al*, 2008). Cr(VI) is generally considered more toxic than Cr(III) and its high toxicity comes from its great facility of crossing biological membranes and its properties of powerful oxidant (Katz S & Salem H, 1993). Cr(VI) enters the body by three routes of exposure: inhalation, absorption through the skin or ingestion. For occupational exposure, the airways and skin are the primary routes of uptake (De Flora, 2000). Breathing high levels of Cr(VI) can cause irritation to the nasal cavity, breathing difficulty (asthma and cough), severe ulcers and perforations of the nasal septum and it may also cause respiratory tract cancer. Skin contact with certain Cr(VI) compounds can cause skin allergies and skin ulcers. Its ingestion can lead to disturbances in the storage organs causing damage to the DNA which leads to mutations and possible carcinogenicity. Concentrations about 100 $mg.kg^{-1}$ of body mass are lethal to humans (Jomova & Valko, 2011).

7.2. Toxicity of chromium in bacteria

Cr(VI) is highly toxic for bacteria because of its rapid entry to the cytoplasm where it may exert its toxic effects. Its toxicity is mainly related to the process of its reduction to lower oxidation states. During the stepwise Cr(VI) reduction, a whole spectrum of reactive oxygen species (ROS) are formed, which exert deleterious effects on cells (Cabiscol Català *et al*, 2000; Cheung & Gu, 2007; Thatoi *et al*, 2014).

Once inside the bacterial cell, Cr(VI) can be primarily reduced to highly cytotoxic Cr(V) by certain reductants such as ascorbic acid, glutathione (GSH), cysteine, hydrogen peroxide (H_2O_2,) and flavoenzymes such as glutathione reductase (GR). During this process, molecular oxygen is reduced to $O_2^{-\bullet}$, which generates H_2O_2, via dismutation. The resultant reactive intermediate Cr(V) species are quickly reoxidized to Cr(VI) by reacting with H_2O_2, which generates OH • radical via Fenton reaction (reaction 1). The reactive intermediates Cr(IV) and the final product Cr(III) resulting from Cr(VI) reduction, can also generate harmful ROS effectively through Fenton reaction during their re-oxidation processes (reactions 2 and 3,

$O_2 \xrightarrow[-0.16\ V]{e^-} O_2^- \xrightarrow[+0.94\ V]{e^-,\ 2H^+} H_2O_2 \xrightarrow[+0.38\ V]{e^-,\ H^+} HO^\bullet \xrightarrow[+2.33\ V]{e^-,\ H^+} H_2O$

(with H_2O released above the $HO^\bullet$ step)

$\cdot\ddot{O}\!:\!\ddot{O}\cdot$ $\cdot\ddot{O}\!:\!\ddot{O}\!:$ $H\!:\!\ddot{O}\!:\!\ddot{O}\!:\!H$ $\cdot\ddot{O}\!:\!H$

Oxygen   Superoxide anion   Hydrogen Peroxide   Hydroxyl radical

Reactive Oxygen Species (ROS)

DNA   **Lipids**   **Proteins**

Oxidation of nucleic acids   **Lipid peroxidation**   **Oxidation of protein residues**

Aldehyde production

Gene alteration   **Disturbance of membrane integrity**   **Protein alteration**

Cell damage

Figure 4. ROS effect on bacterial cell.

respectively). The reaction products of partial Cr(VI) reduction include H_2O_2, superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical (OH •), which are primarily responsible for the cyto- and genotoxicity of Cr(VI) (Poljsak $et\ al$, 2010).

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + OH^- + OH \bullet \quad \textbf{(Reaction 1)}$$

$$Cr(IV) + H_2O_2 \rightarrow Cr(V) + OH^- + OH \bullet \quad \textbf{(Reaction 2)}$$

$$Cr(III) + H_2O_2 \rightarrow Cr(IV) + OH^- + OH \bullet \quad \textbf{(Reaction 3)}$$
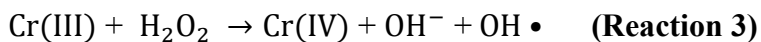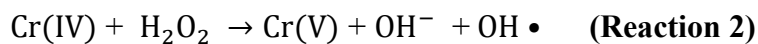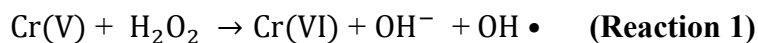
DNA is a main target of these ROS, which attack both the base and the sugar moieties producing single- and double-strand breaks in the backbone, adducts of base and sugar groups, and lesions that block replication (Cabiscol Català $et\ al$, 2000). ROS also attack proteins; the oxidation of certain residues causes the appearance of carbonyl groups and cleavage of peptide chains. Most damages are irreparable and lead to significant functional changes (non-recognition of a receptor by a ligand, loss of enzymatic activity) (Haleng $et\ al$, 2007), which destabilize and inactivate proteins and may even lead to cell death (Ezraty $et\ al$, 2017). Lipids are also major targets during chromium induced oxidative stress. Free radicals can attack directly polyunsaturated fatty acids in membranes and initiate lipid peroxidation leading to a decrease in membrane fluidity, which alters membrane properties and disrupts membrane-bound proteins. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products. Some of them, such as aldehydes, are very reactive and can damage other molecules like proteins (Figure 4) (Cabiscol Català $et\ al$, 2000).

In addition to generating ROS, the final product Cr(III) in itself can interact with negatively charged phosphate in DNA and inhibit DNA replication (Bencheikh-Latmani $et\ al$, 2007; Ramírez-Díaz $et\ al$, 2008). Furthermore, Cr(III) can also alter the structure and activity of the enzymes by reacting with their carboxyl and thiol groups (Cervantes $et\ al$, 2001).

CHAPTER II:

BACTERIAL CHROMIUM

RESISTANCE MECHANISMS
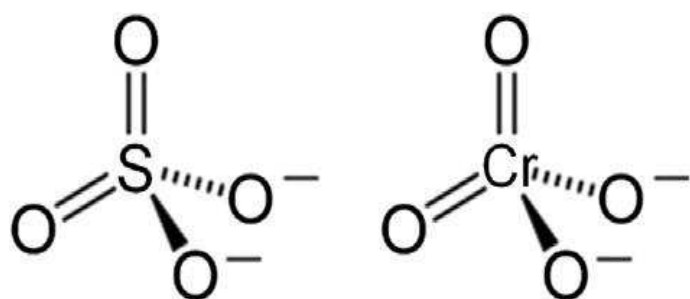
Figure 5. Structural similarity of sulfate and chromate ions.

1. Bacterial chromium resistance mechanisms

The majority of microbial species are sensitive to Cr(VI), but some species are resistant and can tolerate high levels of it. According to Gadd G.M., 1992, the resistance is "the ability of a microorganism to survive toxic effects of metal exposure by means of a detoxification mechanism produced in direct response to the metal species concerned". Bacteria have selected different resistance mechanisms to overcome Cr(VI)-stress. They primarily respond by using mechanisms that directly target Cr(VI) (Brown *et al*, 2006), which comprise:

- The Cr(VI) regulation uptake.

- The efflux of chromate ions from the cell cytoplasm and the reduction of Cr(VI) into Cr(III).

In addition, bacteria can undertake simultaneously other resistance strategies to prevent cell components from Cr(VI) toxicity and to repair the damages induced by Cr(VI)-stress (Brown *et al*, 2006; Ramírez-Díaz *et al*, 2008). The main mechanisms include:

- The activation of ROS scavenging enzymes for offsetting Cr(VI)-induced oxidative stress.

- Iron homeostasis to prevent the production of hydroxyl radicals through the Fenton reaction.

- The induction of the SOS response enzymes to counter DNA damage.

1.1. Cr(VI) uptake

One of common bacterial mechanisms to deal with Cr(VI) is the regulation of sulfate uptake shuttle system that is involved in initial Cr(VI) cellular accumulation (Brown *et al*, 2006). As mentioned previously, chromium exists mainly as the tetrahedral chromate ions CrO_4^{2-}, this form is chemically analogous to biologically important inorganic anions such as SO_4^{2-}. The structural similarity of chromate to sulfate (Figure 5) most likely constitutes the basis for its active transport across cell membranes via the sulfate ABC transporters which
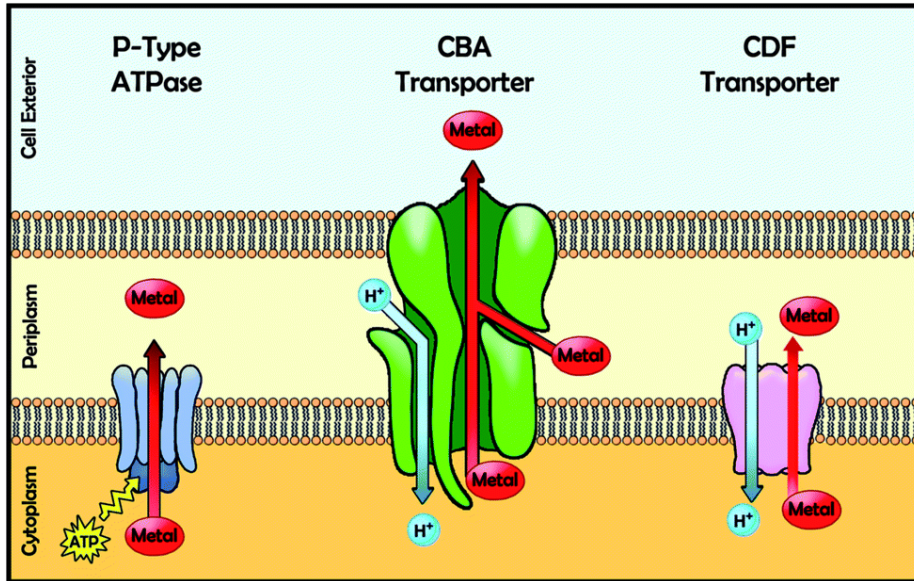
Figure 6. Major transporter families taking part in heavy metal resistance.
P-type ATPase transporter, CBA transporter and the cation diffusion facilitator
(CDF) (Prabhakaran *et al*, 2016).

belong to the sulfate/tungstate uptake transporter (SulT) family of the ABC superfamily of transporters (Cervantes *et al*, 2001; Aguilar-Barajas *et al*, 2011).

Some bacteria species such as *Caulobacter crescentus* down-regulate the sulfate ABC transporter in Cr(VI) exposure to reduce the entry of chromate into the cells and thus its intracellular accumulation (Hu *et al*, 2005). In contrast, it has been shown that *Pseudomonas putida* F1, *Cupriavidus metallidurans* CH34 and *Arthrobacter* sp. FB24 respond differently to chromate stress by up-regulating the sulfate ABC transporter coding genes as well as genes involved in sulfur metabolism (Brown *et al*, 2006; Thompson *et al*, 2007; Henne *et al*, 2009; Monsieurs *et al*, 2011). This overexpression suggests that chromate induces sulfur starvation in cells resulting whether from competition between sulfate and chromate for the transport which reduces the bacterial capability to uptake sulfate, or from the oxidative stress induced by Cr(VI) which decreases sulfur availability in cells (Brown *et al*, 2006).

1.2. Chromate ions efflux

1.2.1. The heavy metal efflux system

Active transport, also called efflux system, is the most important category of metal resistance systems. Bacteria use active transport mechanisms to export toxic metals from the cytoplasm to the extracellular medium in order to reduce their intracellular accumulation (Nies & Silver, 1995). This mechanism is mainly provided by primary transporters P-type ATPases which span the inner membrane and use ATP energy to pump metal ions from the cytoplasm to the periplasm, their substrates are inorganic cations such as H^+, Na^+, K^+, $Mg^{2+,}$ Ca^{2+}, Cu^+, Ag^+, Zn^{2+}, Cd^{2+}, Co^{2+} and Pb^{2+}. And it is also provided by secondary active transporters where the passage through the membrane occurs by utilizing an electrochemical gradient (Nies, 2003; Prabhakaran *et al*, 2016). This type of transporters comprises the cation diffusion facilitator (CDF) family transporters, which act as chemiosmotic ion-proton exchangers driven by a proton motive force and they export metal ions such as Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} (Grass *et al*, 2001), and CBA (resistance-nodulation-cell division proteins family) transporters which are three-component transenvelope pumps that act as chemiosmotic antiporters. They expel ions such as Cd^{2+}, Zn^{2+} and Co^{2+} from cyto- and periplasm to outside using a chemiosmotic gradient (Nies & Silver, 1995). CBA transporters
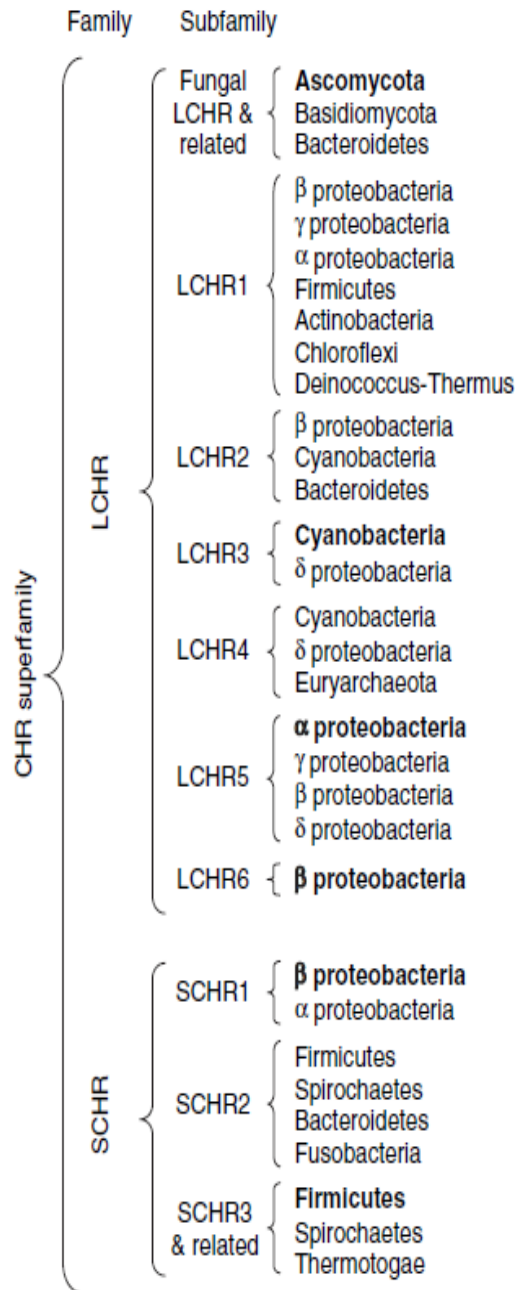
Figure 7. Taxonomy of the CHR superfamily. CHR proteins two families and 10 subfamilies. The group(s) of organism(s) in which these proteins were found are indicated on the right (Díaz-Pérez *et al*, 2007).

further remove periplasmic ions transported by ATPases or CDF transporters (Scherer Judith & Nies Dietrich H., 2009) (Figure 6).

None of the abovementioned transporters seem to be implicated in chromate ion efflux. However, a distinct transporter family called CHR superfamily have been reported. It encloses chromate efflux specific transporters.

1.2.2. The CHR superfamily

Chromate resistance determinants have been identified in Archaea, Bacteria and Eukarya (Nies *et al*, 1998; Flores-Alvarez *et al*, 2012). They consist of genes belonging to the chromate ion transport (CHR) superfamily (Ramírez-Díaz *et al*, 2008; Viti *et al*, 2014); which is classified as TC # 2.A.51 and includes CHR proteins encoded in chromosomes and in plasmids (Cervantes & Campos-Garcia, 2007). Their members belong to two protein families (LCHR and SCHR) and 10 different subfamilies. These proteins possess differences in membrane topology orientation as well as in their genomic context, probably evolved diverse physiological functions in addition to chromate transport (Diaz-Pérez *et al*, 2007).

Based on the CHR proteins sizes, they have been classified into two families (Figure 7):

SCHR family: It comprises monodomain proteins with a primary structure length of 123–234 amino acids, called short-chain CHR (SCHR) where bacterial SCHR protein pairs were clustered into three subfamilies (SCHR1–SCHR3) (Cervantes & Campos-Garcia, 2007; Diaz-Pérez *et al*, 2007).

LCHR family: It comprises bidomain proteins (homologous duplicated domains) with a primary structure length of 345–495 amino acids, called long-chain CHR (LCHR), where the proteins are clustered into six main subfamilies (LCHR1–LCHR6) (Nies *et al*, 1998; Cervantes & Campos-Garcia, 2007; Díaz-Pérez *et al*, 2007).

There are striking differences in the distribution of SCHR and LCHR subfamilies into bacterial taxa. LCHR1 subfamily is considered to possess the widest distribution inside bacterial taxa. Different subfamilies of CHR can be found in a single bacterial taxon. For example, β-proteobacteria possess CHR proteins from five different subfamilies (SCHR1,

LCHR1, LCHR2, LCHR5 and LCHR6), whereas γ-proteobacteria possess CHR proteins from only two different subfamilies (LCHR1 and LCHR5) and none from SCHR subfamilies (Figure 7). It is interesting to note that one bacterial species can harbor a large number of CHR proteins from the two subfamilies. Examples include the chromate-resistant bacterium *C. metallidurans* that possesses three LCHRs and one pair of SCHRs (Diaz-Pérez *et al*, 2007).

The genomic context suggests that CHR proteins possess other functions in addition to chromate transport. CHR genes clusters from different sources differ in their possession of associated potential regulatory genes. For example, genes coding for LCHR2 and LCHR5 members can be variably associated with a *chrC* gene encoding a probable superoxide dismutase (SOD) and *chrB* and *chrF* genes coding for regulators of *chrA* gene that codes for a chromate efflux pump. By contrast LCHR1, LCHR3, LCHR4, LCHR6 and all three SCHR subfamilies members possess a diverse genomic context and are not associated with any genes related to chromate resistance (Diaz-Pérez *et al*, 2007).
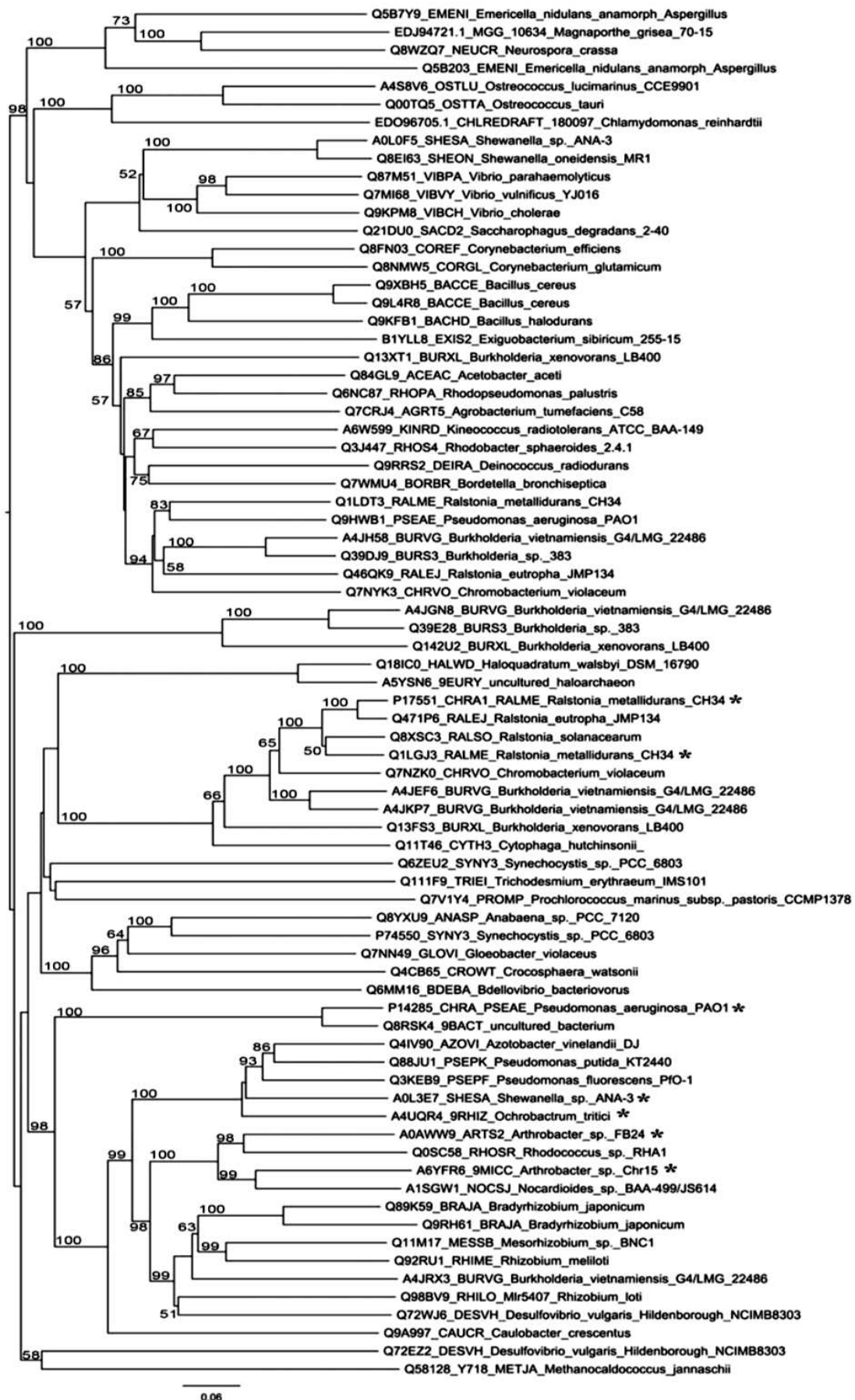
Figure 8. Phylogenetic tree of ChrA Orthologs (Henne *et al*, 2009).

1.2.3. The chromate efflux pump ChrA

The efflux of chromate is a resistance mechanism in bacteria generally conferred by chromate resistance determinants which include the *chrA* gene that encodes a hydrophobic inner membrane chromate efflux protein (Pimentel *et al*, 2002). ChrA functions as a chemiosmotic pump that extrudes chromate from the cytoplasm using the proton motive force (Ramírez-Díaz *et al*, 2008; Viti *et al*, 2014). Chromate efflux pumps are believed to be responsible for Cr(VI) resistance in bacteria (Diaz-Pérez *et al*, 2007; Ramírez-Díaz *et al*, 2008).

A ChrA protein has been characterized in detail in *C. metallidurans* CH34 (Nies *et al*, 1990) and *Pseudomonas aeruginosa* (Cervantes *et al*, 1990) and numerous putative ChrA homologs have been identified after the sequencing of bacterial genomes (Saier, 2003; Henne *et al*, 2009) (Figure 8).

In bacteria, the *chrA* genes can be located on plasmid and/or chromosomal DNA and they generally belong to operons with other chr genes (Juhnke *et al*, 2002; Viti *et al*, 2014). *C. metallidurans* CH34 (previously *Alcaligenes eutrophus* and *Ralstonia metallidurans*) (Cervantes *et al*, 1990; Cervantes & Campos-Garcia, 2007) harbors two chromate resistance determinants, the chr(1) cluster (*chrIB1A1CEF1*) present on the megaplasmid pMOL28 (Figure 9.A) and chr(2) cluster (*chrB2A2F2*) present on the chromosome (Figure 9.B) (Branco *et al*, 2008). ChrI, ChrB and ChrF are proposed to play regulatory roles for the expression of *chrA* with, respectively, ChrB as an activator and ChrF and ChrI as repressors. The ChrE protein is thought to be involved in the cleavage of chrome-gluthation complex (Juhnke *et al*, 2002; Cervantes & Campos-Garcia, 2007). The chromate-resistance determinant of *P. aeruginos*a belongs to the LCHR5 family. It is located on the pUM505 plasmid and organized in a putative *chrBAC* operon (Figure 9.C) (Ramírez-Díaz *et al*, 2011). ChrB plays a regulatory role for expression of *chrA* and *chrC* gene encoding a truncated protein which is probably not functional (Tauch *et al*, 2003).

The two ChrA homologs, encoded by genes carried on plasmids pMOL28 from *C. metallidurans* and pUM505 of *P. aeruginosa* are 29% identical and they are reported to display a different topology, 10 transmembrane segments for the *C. metallidurans* protein
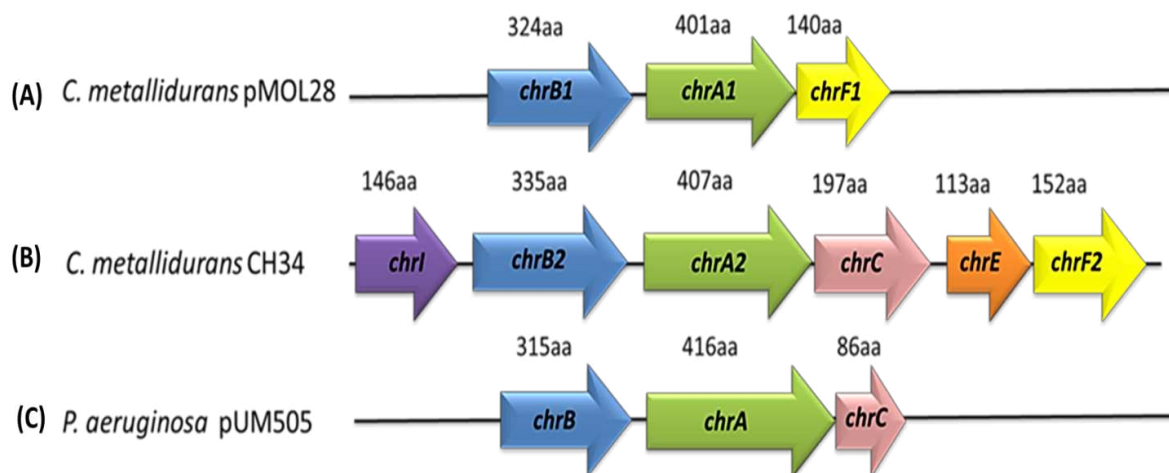
Figure 9. Schematic representation of the genomic context of the *chr* genes. *chrA* encodes a chromate efflux pump. *chrI*, *chrB* and *chrF* encode proteins that play regulatory roles for the expression of *chrA*. The *chrE* encodes a protein involved in the cleavage of chrome-gluthation complex. Figure adapted from Viti *et al*, 2014.

(Nies *et al*, 1998) and 13 transmembrane segments for that of *P. aeruginosa* (Jiménez-Meji-a *et al*, 2006).

1.3. Cr(VI) reduction

Bacterial reduction of Cr(VI) to Cr(III) is one of the main chromate resistance mechanisms. Since the first report of anaerobic Cr(VI) reduction by Romanenko VI, Koren'kov VN, 1977 in uncharacterized *Pseudomonas* sp., worldwide researchers have isolated both aerobic and anaerobic Cr(VI)-reducing bacteria belonging to a wide range of genera from diverse environments. Cr(VI) reduction can be achieved either non-enzymatically or enzymatically under aerobic and/or anaerobic conditions depending on the bacterial species (Ahemad, 2014). On one hand, non-enzymatic-reduction may take place by chemical reactions associated with intra/extracellular compounds produced during microbial metabolism, including amino acids, nucleotides, sugars, vitamins, organic acids, glutathione, sulfite and thiosulfates (Cervantes *et al*, 2001; Donati *et al*, 2003; Dhal *et al*, 2013; Joutey *et al*, 2015; Gutiérrez-Corona *et al*, 2016). Moreover, Cr(VI) can be reduced by Fe(II) and H_2S, the anaerobic metabolic end products of iron and sulphate-reducing bacteria (Somasundaram *et al*, 2009).

On the other hand, numerous bacterial genera, including *Pseudomonas, Bacillus* and *Arthrobacter* have been widely reported to reduce Cr(VI) using an enzymatic process (Thatoi *et al*, 2014; Viti *et al*, 2014). The ability to reduce Cr(VI) can be a secondary function for Cr(VI) reducing enzymes, which have a different primary role other than Cr(VI) reduction. For example, the nitroreductases NfsA/NfsB from *Vibrio harveyi* possess a nitrofurazone nitroreductase as primary activity and a Cr(VI) reductase activity as a secondary function (Kwak *et al*, 2003). Similarly, ferric reductase FerB from *Paracoccus denitrificans* uses both Fe(III)-nitrilotriacetate and Cr(VI) as substrates (Mazoch Jiří *et al*, 2004). These secondary functions may be related to bacterial enzymatic adaptation as a result of the relatively increase of Cr(VI) content in the environment owing to anthropogenic activities (Silver & Phung, 1996).

The enzymatic reduction can be partial, where Cr(VI) gets reduced to the highly unstable Cr(V) intermediate, which can get oxidized back to Cr(VI) in a redox cycle, giving its
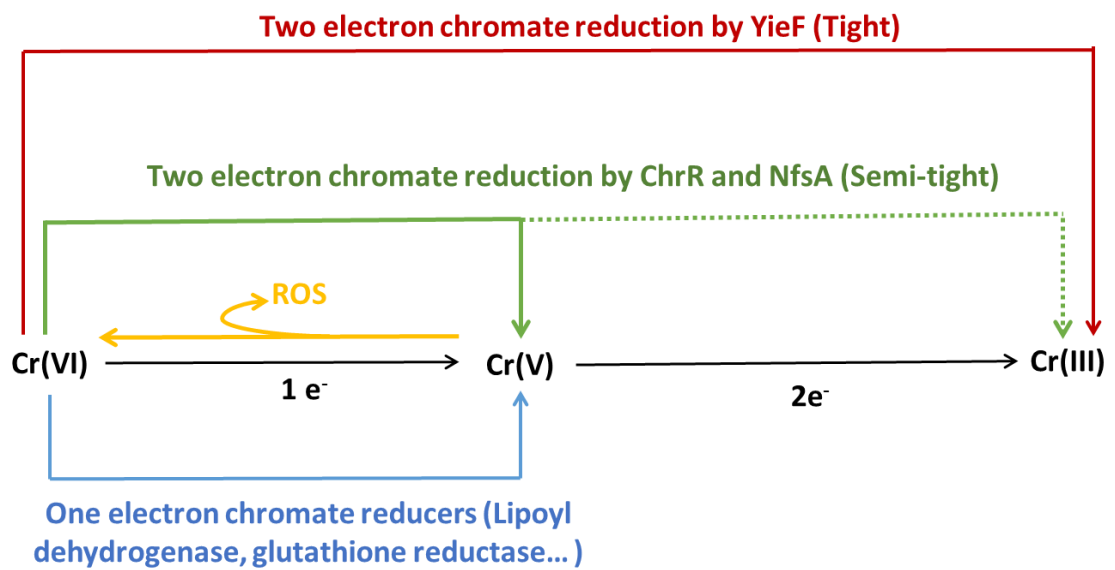
Figure 10. Enzymatic Cr(VI) reduction.

electrons to molecular oxygen and thus generating a large amount of ROS. This type of reduction is performed by chromate reducing enzymes belonging to one electron reducers which are flavin dependent. Such as lipoyl dehydrogenase, cytochrome c, glutathione reductase and ferridoxin-NAD (Shi & Dalal, 1990; Ackerley *et al*, 2004). However, complete reduction of Cr(VI) to Cr(III) is proceed by enzymes that belong to two electron reducers which are NAD(P) dependent enzymes. This type of enzymes can either proceed a tight or a semi tight chromate reduction (Ackerley *et al*, 2004a). In tight chromate reduction, Cr(VI) is directly reduced to Cr(III) in only one step by transferring four electrons, in which three electrons are consumed in reducing Cr(VI) and the fourth one is transferred to molecular oxygen generating ROS (superoxide). In this case, no Cr(V) intermediate is involved hence no redox cycle occurs. As a result, much lesser amount of ROS is generated during this process. YieF from *E. coli* present an example of two-electron chromate reducers that proceeds a tight chromate reduction (Figure 10). In the semi-tight chromate reduction, the enzymes catalyze a combination of one- and two-electron transfers to Cr(VI) with the formation of the unstable species Cr(V) transiently before further reduction to Cr(III) which minimizes partially the production of harmful radicals resulting from Cr(V) reoxidation (Figure 10). Hence, the semi-tight chromate reduction generates more ROS than the tight chromate reduction, but much lesser than the chromate reduction catalyzed by one-electron reducers. Examples of two-electron chromate reducers that proceed a tight chromate reduction include the cytoplasmic flavoproteins ChrR from *P. putida* and NfsA protein of *E. coli* (Figure 10) (Thatoi *et al*, 2014).

Based on sequence homologies, Cr(VI) reductases enzymes were broadly classified into two classes: Class I and Class II. Two of the most commonly studied Class I enzymes include the ChrR of *P. putida* and YieF of *E. coli* (Thatoi *et al*, 2014). Both enzymes are NAD(P)H-dependent homodimeric enzymes belonging to the NADH-dh2 family possess non-covalently bound flavin mononucleotide (FMN) (Ahemad, 2014). ChrR has a broad substrate specificity permitting the reduction of quinones, prodrugs, U(VI) and Cr(VI) ions (Barak *et al*, 2006). It has also a quinone reductase activity during Cr(VI) reduction. Thus, quinols produced by quinone reduction confer tolerance to ROS generated during Cr(VI) by neutralizing them (Ackerley *et al*, 2004; Cheung & Gu, 2007; Ramírez-Díaz *et al*, 2008). Although, YieF shares

sequence homology with ChrR (Ackerley *et al*, 2004), it reduces differently the chromate as explain above, and contrary to ChrR, YieF does not show semiquinone flavoprotein generation during chromate reduction (Viti *et al*, 2014; Thatoi *et al*, 2014).

Class II chromate reductases that bear no homology to the class I enzymes, possess nitroreductase activity. They reduce quinones and nitrocompounds effectively and vary in their ability to transform chromate. There are two members of the class II family, namely NfsA protein of *E. coli* and the ChfN protein of *B. subtilis* that possess also a chromate reductase activity (Park *et al*, 2000).

1.3.1. Cr(VI) Reduction pathways

Cr(VI) reduction can occur inside and/or outside the cell under aerobic and/or anaerobic conditions through Cr(VI) reductases either localized in the membrane fraction (Wang *et al*, 1991; Cheung & Gu, 2007) or in the cytosolic fractions (Suzuki *et al*, 1992; Park *et al*, 2000; Bae *et al*, 2005) of the Cr(VI) reducing bacteria.

1.3.2. Cr(VI) reduction localization

In intracellular processes, Cr(VI) is reduced in the cytosol using cytoplasmic soluble reductase enzymes. These enzymes play an intermediate role between associated biological electron donors involved in this process such as NADH and NADPH. This reduction process is not energy consuming but will directly affect the cell, since most of intracellular proteins catalyze a one-electron reduction from Cr(VI) to Cr(V) (Joutey *et al*, 2015). Many bacteria are known to participate in the intracellular reduction of Cr(VI) like the Gram negative bacteria *P. aeruginosa*, *E. coli* ATCC 33456 and *Enterobacter*. In addition, *B. subtilis* was also reported to carry out intracellular reduction of Cr(VI) (Thatoi *et al*, 2014).

In contrast, extracellular Cr(VI) reduction is beneficial to the cell since it does not require transport mechanisms to carry the chromate into the cell, and to later expel the Cr(III). Hence, extracellular reduction of Cr(VI) protects the cell from ROS effects resulting from Cr(VI) reduction. This process can be meditated by membrane bound reductases that can reduce Cr(VI) extracellularly as an electron acceptor by using electron shuttling compounds coupled to membrane reduction (Joutey *et al*, 2015). In some cases, soluble Cr(VI) reducing enzymes

such as flavin reductases, nitrate reductases, flavin proteins and ferrireductases, produced in the cytoplasm are exported into the media to extracellularly reduce Cr(VI), as reported in *P. putida* (Cheung & Gu, 2007).

1.3.3. Aerobic/Anaerobic Cr(VI) Reduction

Bacterial Cr(VI) reduction in the presence of oxygen occurs as a two or three step process, with Cr(VI) initially reduced to the short-lived intermediates Cr(V) and/or Cr(IV) before being further reduced to Cr(III). NADH, NADPH and electrons from the endogenous reserve are implicated as electron donors in the Cr(VI) reduction process. Aerobic Cr(VI) reduction is generally associated with soluble proteins which are localized as cytosolic proteins. Which is the case in aerobes like *P. putida* (Ishibashi *et al*, 1990). As exceptions, *P. maltophilia* O-2 and *Bacillus megaterium* TKW3 were found to utilize membrane-associated reductases for Cr(VI) reduction, in spite of being aerobes (Cheung & Gu, 2007).

In anoxic conditions, Cr(VI) can serve as a terminal electron acceptor in the respiratory chain for a large array of electron donors, including carbohydrates, proteins, fats, hydrogen, NAD(P)H and endogenous electron reserves. The Cr(VI)-reducing activities of anaerobes are associated with their electron transfer systems catalyzing the electron shuttle along the respiratory chain. Furthermore, the cytochrome family is frequently involved in enzymatic anaerobic Cr(VI) reduction (Mary Mangaiyarkarasi *et al*, 2011). Both membrane-associated and soluble enzymes mediate the process of Cr(VI) reduction under anaerobic conditions (Cheung & Gu, 2007). Reduction involving membrane-associated reductase has been reported in some chromate-reducing bacteria which utilized H_2 as electron donor and Cr(VI) as an electron acceptor (QuiIntana *et al*, 2001). In addition, some soluble enzymes have been also found to mediate this process, such as the soluble cytochrome c3 in *Desulfovibrio vulgaris* (Barrera-Díaz *et al*, 2012).

Some bacteria are capable of reducing chromate under both aerobic and anaerobic conditions. Examples include, *P. fluorescens* LB300 (Bopp & Ehrlich, 1988), *Achromobacter* sp. (Ma *et al*, 2007), *E. coli*, *P. ambigua* (McLean & Beveridge, 2001), *P. putida* (Barak *et al*, 2006) and *Bacillus* sp. (Liu *et al*, 2006), although, their rate of reduction can vary widely between the two conditions. For example, faster reduction rate of Cr(VI) under aerobic

conditions than anaerobic conditions has been reported in the case of *E. coli* ATCC 33456 (Shen & Wang, 1993).

1.4. Indirect Cr(VI) resistance mechanisms

The generation of ROS during partial Cr(VI) reduction causes oxidative stress in bacteria. The participation of bacterial proteins in the defense against stress induced by Cr(VI) represents one of the main mechanisms of Cr(VI) resistance that does not deal directly with Cr(VI) ions but rather protects cells from their damaging effects (Ramírez-Díaz *et al*, 2008). As a strategy for scavenging ROS, bacteria commonly modulate gene expression by inducing genes encoding antioxidant enzymes and proteins that can directly decompose the oxidant such as superoxide dismutase and catalase (Ackerley *et al*, 2004a), alkylhydroperoxide reductase (Ahp) and various peroxidases (Shi *et al*, 2015). And also induces genes encoding non-enzymatic antioxidant such as NADPH and NADH pools, glutathione/glutaredoxin, thioredoxins and ascorbic acid to restrain intracellular concentrations of ROS in order to prevent the damages beyond unmanageable. These antioxidants are not only known to diffuse free radicals and limit the risk of oxidative stress but they can also chelate the metal ions responsible for generating ROS (Mishra & Imlay, 2012).

Expression of these genes is typically regulated by OxyR, the primary regulator of oxidative stress response. It directly controls over 20 genes, including genes involved in ROS detoxification and genes with other roles in oxidative stress defense such as genes maintaining iron homeostasis (Li *et al*, 2014). Since free iron in its reduced form Fe^{2+} can generate ROS converting the less reactive hydrogen peroxide to the more reactive oxygen species, hydroxyl radical (Reaction 4), intracellular levels of iron must be carefully controlled to meet the metabolic needs of the cell while limiting cellular damage due to iron overload (Yang *et al*, 2009).

$$Fe^{2+} \ + \ H_2O_2 \ \rightarrow Fe^{3+} \ + \ OH^- + OH \bullet \quad \textbf{(Reaction. 4)}$$
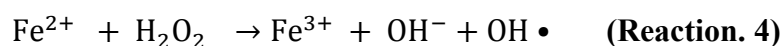
To cope with all protein and DNA damages caused by Cr(VI), bacteria possess a repair system acting as another defensive shield against Cr(VI)-induced oxidative stress. They primarily remove the damaged biomolecules before they accumulate and result in altered cell

metabolism or viability. For example, oxidized proteins are removed by proteolytic systems. Furthermore, they repair oxidatively damaged nucleic acids by specific enzymes belonging to SOS repair system (Poljsak *et al*, 2010).

CHAPTER III:

Shewanella oneidensis

MR-1

1. *Shewanella* genus

Shewanella is a genus of Gamma proteobacteria (Dikow, 2011) belonging to the *Alteromonadales* Order and the Family of *Alteromonadacea* (Vogel *et al*, 2005). Like many contemporary genera, the *Shewanella* have experienced a rocky road to their present status, as evidenced by the species *S. putrefaciens*. Originally isolated as an active agent in food spoilage (Derby & Hammer, 1931), this organism was first called "*Achromobacter putrefaciens*", then *Pseudomonas putrefaciens* (Shewan *et al*, 1960), *Alteromonas putrefaciens* (Lee *et al*, 2016) and finally, based on 5S rRNA sequences, the species was renamed *Shewanella putrefaciens* (MacDonell & Colwell, 1985). The genus *Shewanella*, named after James Shewan for his work in fisheries microbiology (Vogel *et al*, 2005), has only been recognized with its present name since 1985 and no further reclassifications at the level of the genus have been made to date (Dikow, 2011).

In general, members of *Shewanella* genus compose a diverse group of facultative anaerobic bacteria that are gram-negative rods 2–3 μm in length, 0.4–0.7 μm in diameter and motile due to a single polar flagellum. Most can easily grow on usual laboratory culture media after enrichment from environmental samples. The hallmark of many shewanellae is the ability to utilize a diverse array of final electron acceptors in the absence of oxygen, and many have capitalized on this ability that allows their wide distribution in nature by surviving in diverse habitats (Hau & Gralnick, 2007).

Today, this genus comprises 66 recognized species (http://www.bacterio.cict.net). Most of them, have been isolated from marine environments including seawater, sediment, tidal flats, marine invertebrates and fish, Antarctic sea ice, clinical samples and some species are found in activated sludge (Lee *et al*, 2016). Forty-one *Shewanella* species were proposed from 2002 to 2010 with a peak number of species described in 2006 (Janda & Abbott, 2014). Among the species proposed during this time period, two *Shewanella* species were described, *Shewanella fidelis* KMM 3582 [T] in 2003 and *Shewanella algidipiscicola* in 2007, isolated respectively from sediments of the South China Sea and from marine fish (cod and plaice) caught in the Baltic Sea (Ivanova, 2003; Satomi *et al*, 2007).
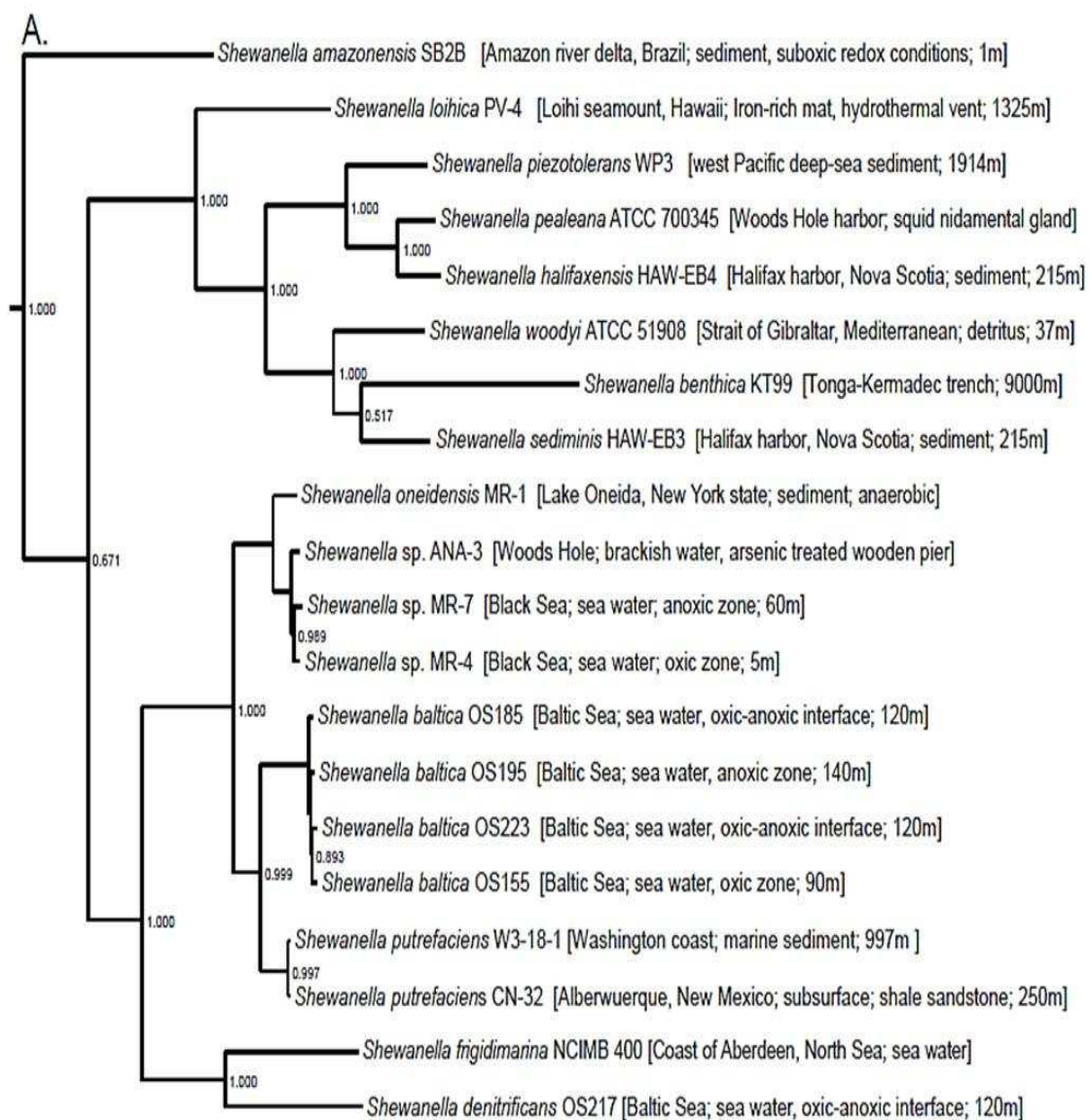
Figure 11. Phylogenetic tree, isolation site characteristics and the geographic origin of some *Shewanella* species with available genomes (Rodionov *et al,* 2011).

Shewanella genus species are of great interest for environmental clean-up due to their ability to convert heavy metals and toxic substances (e.g. cadmium, uranium) into less toxic products by using them as electron acceptors in certain respiratory situations(Dikow, 2011). In order to understand the underlying mechanisms of this ability of biotechnical interest, the genome of more than 32 *Shewanella* species have been fully sequenced and deposited in GenBank. Twenty of them are represented in a phylogenetic tree in Figure 11 (Rodionov *et al*, 2011).

A 16S rRNA gene-based phylogenetic reconstruction has revealed two major groups in the *Shewanella* genus. Usually, group 1 includes species that are piezotolerant and psychrotolerant such as *Shewanella violacea*, which has been isolated from the deep sea. Group 2 species are usually pressure sensitive and mesophilic and include *Shewanella oneidensis*, *Shewanella baltica* and *Shewanella putreficans* which have been isolated from a variety of environments, including fresh water lakes and spoiled meat products (Kato & Nogi, 2001). Overall, *Shewanella* is phylogenetically most closely related to the genera *Pseudoalteromonas, Alteromonas, Moritella, Ferrimonas* and *Colwellia*, all of which are members of the family *Alteromonadaceae* (Figure 12) (Bowman, 2015; Dufault-Thompson *et al*, 2017)

2. *Shewanella* genus characteristics

2.1. Phenotypic characteristics

Shewanella colonies on complex nutrient media typically have a pale tan to pink-orange or salmon color, which is due to strong accumulation of cytochrome proteins. However, this tendency is less pronounced in some species such as *S. hanedai* and *S. benthica, S. colwelliana* and *S. hanedai* (Bowman, 2015).

Unlike some *Shewanella* species that require Na^+ or seawater media for growth, *S. algae, S. frigidimarina, S. oneidensis*, *S. fidelis* and *S. algidipiscicola* do not require Na^+ for growth. However, they are capable of growing on certain NaCl concentrations. Some species like *S. colwelliana, S. hanedai* and *S. benthica* require amino acids and/or vitamins for growth. In
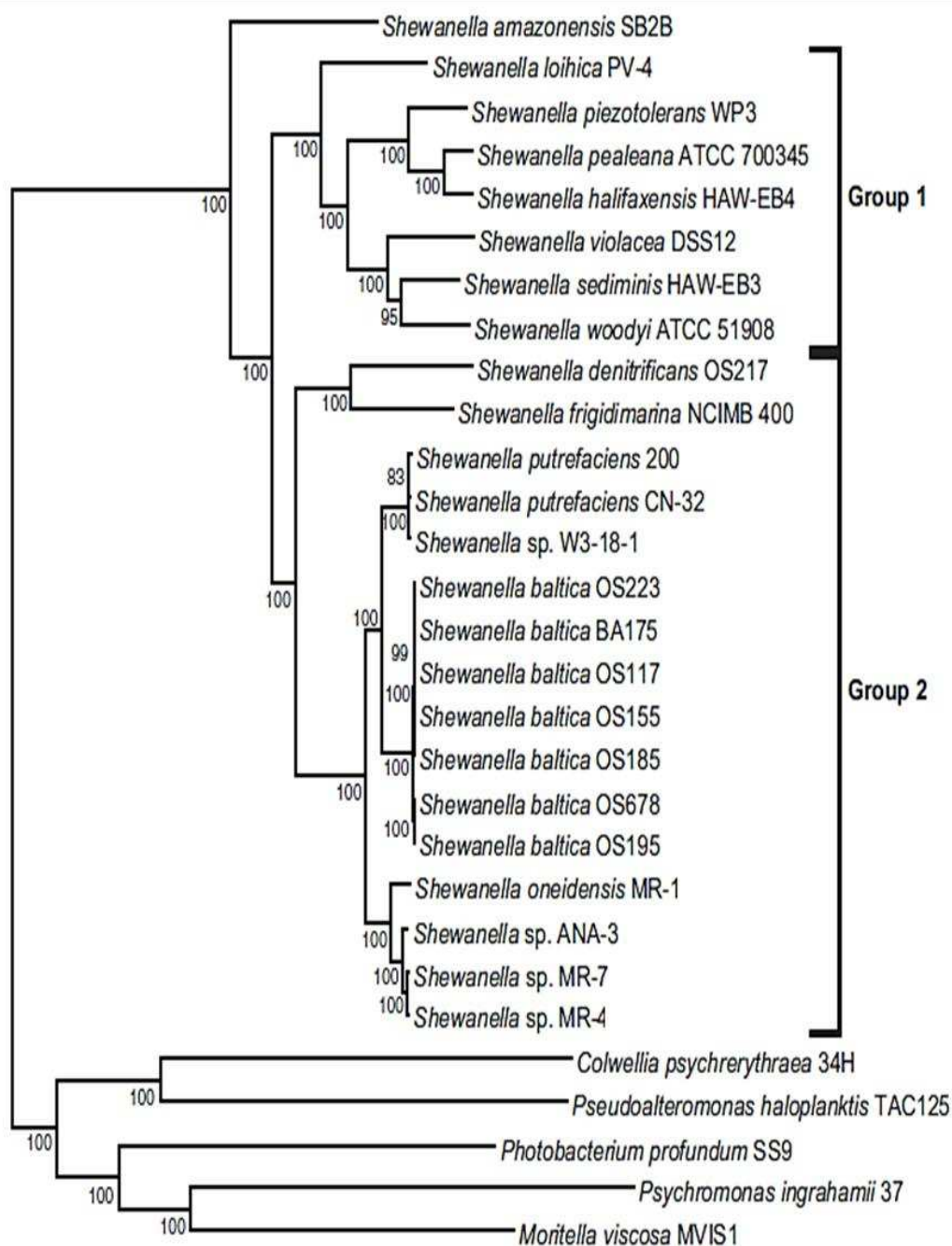
Figure 12. Phylogenetic reconstruction of the *Shewanella* genus showing the two groups: Group 1 containing piezotolerant and psychrotolerant species and group 2 enclosing pressure sensitive and mesophilic species. It shows also five outgroup species to which *Shewanella* is phylogenetically most closely related (Dufault-Thompson *et al*, 2017).

defined media, such as basal medium agar, these requirements can be met by adding yeast extract at concentrations of 0.05–0.1% (w/v) (Bowman, 2015).

Regarding the fatty acid profile, *Shewanella* species are rich in branched and odd-chain-length fatty acids. Psychrotolerant and psychrophilic *Shewanella* species have the unusual ability to synthesize eicosapentaenoic acid (EPA) which is essential for their survival at low temperatures, the levels of which range from 2–22%. Nevertheless, mesophilic species do not produce EPA (Bowman, 2015).

2.2. Pathogenicity

Usually, bacteria of the genus *Shewanella* are not pathogenic. However, *Shewanella putrefaciens*, has been associated with serious health disorders in freshwater fish. Therefore, it has been described as a new aetiological agent of a disease, named shewanellosis which causes skin disorders and haemorrhages in internal organs. It should be noted that *S. putrefaciens* could also be associated with different infections in humans, such as skin and tissue infections, bacteraemia and otitis. Enzymatic activity, cytotoxin secretion, adhesion ability, lipopolysaccharide and the presence of siderophores are potential virulence factors of *S. putrefaciens* (Paździor, 2016). Recent studies suggest that *S. algae* may in fact be more pathogenic than *S. putrefaciens* (Janda, 2014) and consider it as an emerging pathogen of skin and soft tissue infections mainly in patients with chronic ulcers and at times be multidrug resistant (Jampala, 2015). Its hemolysin production is believed to be an important factor of its pathogenicity (Khashe & Janda, 1998).

2.3. Energy metabolism

Shewanella species are chemoheterotrophic facultative anaerobes, with anaerobic growth typically of a respiratory nature. However, some species can also grow fermentatively; these include *S. frigidimarina* and *S. benthica*, both of which can ferment D-glucose, as well as *S. gelidimarina* which can ferment *N*-acetylglucosamine and chitin, but not D-glucose (MacDonell & Colwell, 1985; Bowman, 2015). In anaerobiosis, *Shewanella* species can use nitrate as an electron acceptor for growth. Moreover, Trimethylamine-*N*-oxide (TMAO) is also a common terminal electron acceptor among *Shewanella* species; its reduction to

trimethylamine (TMA) is usually responsible for the odors associated with *Shewanella* food spoilage (Shewan *et al*, 1972). They can also grow anaerobically by reduction of various sulfur compounds to H_2S including thiosulfate and sulfite or via reduction of fumarate to succinate, coupled to the oxidation of format (Bowman, 2015).

The majority of *Shewanella* species have the ability to facultatively reduce ferric iron, manganese and other metals, examples include *S. oneidensis* MR-1. They can grow anaerobically by coupling the oxidation of carbon compounds or H_2 to the reduction of Fe^{3+} to Fe^{2+} or of Mn^{4+} to insoluble Mn^{3+}. Their dissimilatory metal reduction is believed to be important in terms of metal cycling and mobilization in the environment (Bowman, 2015).

3. *Shewanella oneidensis* MR-1

Shewanella oneidensis MR-1 is an aquatic bacterium formerly known as *Alteromonas putrefaciens* MR1. Based on both 16SDNA and *gyrB* nucleotide sequences it was reclassified as a member of the genus *Shewanella*. It was isolated in 1988 from sediments of Oneida Lake at New York, whence its name. Like all *Shewanella* group 2 species, *S. oneidensis* MR-1 is able to grow at mesophilic temperatures (optimal growth was observed at 30°C) and it shows weak growth at NaCl concentrations above 3%. This bacterium exhibits cytochrome oxidase, catalase and gelatinase activities (Venkateswaran *et al*, 1999). Furthermore, it is capable of dissimilatory metabolism of manganese and iron oxides (MR-1 for <u>M</u>n-<u>R</u>educing bacterium) (Myers & Nealson, 1988; Venkateswaran *et al*, 1999).

In 2002, *S. oneidensis* MR-1 was the first of *Shewanella* spp whose genome have been fully sequenced and thus serves as the model organism for studying the functional repertoire of the *Shewanella* genus (Yang *et al*, 2015). It has a 5.13Mb genome containing 4590 genes, including 184 on a mega-plasmid (Heidelberg *et al*, 2002). *S. oneidensis* MR-1 possesses genes encoding the synthetic pathways of all amino acids and phenotypic analyzes have shown it to be prototrophic (Serres & Riley, 2006). It can therefore develop in a minimum medium with a single element as a source of carbon and electrons. It uses N-acetyl-glucosamine (chitin monomer), inosine as well as amino acids as a carbon source (Ringo *et al*, 1984; Yang *et al*, 2006; Driscoll *et al*, 2007). However, *S. oneidensis* MR-1 is unable to

use glucose naturally, but a simple pre-exposure of 24 hours to glucose allows the appearance of mutants able to use this substrate as a sole carbon source (Howard *et al*, 2011). This means that all genetic elements necessary for glucose metabolism are present but cannot be used by the wild-type strain.

S. oneidensis MR-1 harbors also genes coding for cytochromes and hydrogenases, which are integral members of the electron transport system and the reason of its great respiratory flexibility (see Respiratory capacities). Moreover, its genome contains many genes dedicated to mobility and chemotaxis, including 70 genes encoding components of the flagellum, as well as genes encoding two motor systems and three clusters of genes encoding components of the chemosensing (Heidelberg *et al*, 2002).

3.1. Chemotaxis

Bacterial survival depends on the ability to respond and adapt to changing environmental conditions, among the many challenges faced are scarcity of nutrients and the accumulation of toxic substances (Porter *et al*, 2011). Bacteria such as *S. oneidensis* MR-1 respond to these changes by altering their motile behavior in response to signal molecules. This behavior, called chemotaxis. It allows bacteria migration under the influence of a chemical gradient (Pandey & Jain, 2002) in order to find better environments by moving, through changes in the rotary behavior of the flagellum, toward areas where the concentration of favorable chemicals (attractants) is high. Or away from an area when they perceive an increase of unfavorable chemicals (repellents) to find more suitable area where the repellent is in lower concentration (Porter *et al*, 2011). Flagellar rotation is controlled by a chemotaxis pathway, where signals are transduced from chemoreceptor proteins, called methyl accepting chemotaxis proteins (MCPs), through a histidine protein kinase (CheA) and a response regulator (CheY) to the flagellar motor (Porter *et al*, 2011).

The chemotaxis ability of *S. oneidensis* MR-1 as well as all *Shewanella* species may provide a competitive advantage, allowing cells to move into more favorable environments which increases their chances of survival and growth. This ability, coupled with their wide range of potential electron acceptors, may help explain their nearly ubiquitous presence in widely disparate environmental niches (Li *et al*, 2010).

S. oneidensis MR-1 was shown to be chemotactic only toward some compounds, mainly corresponding to electron acceptors including nitrate, nitrite, TMAO, DMSO, fumarate, thiosulphate, Mn(III) and Fe(III) (Bencharit & Ward, 2005; Baraquet *et al*, 2009). Recently, other signals generating a chemotactic response in *S. oneidensis* MR-1 were identified. These includes the L-malate that was identified as attractants, as well as four new metals: Nickel and cobalt which were identified as repellents, whereas copper and chromate were identified as attractants (Armitano *et al*, 2011). Recent studies on *S. oneidensis* MR-1, confirmed that its taxis towards many, if not all, exogenous electron acceptors occurs via an energy taxis mechanism (Bencharit & Ward, 2005; Baraquet *et al*, 2009), where the signal is not the electron acceptor itself but it originates from its metabolism (Alexandre Gladys *et al*, 2004; Schweinitzer & Josenhans, 2010). Therefore, cells move towards an environment that is optimal for metabolic activity rather than one with the maximal attractant concentration (Porter *et al*, 2011).

S. oneidensis MR-1 is predicted to have a complex chemotaxis system that includes 27 MCPs genes and 3 clusters of chemosensing genes (che) (Fredrickson *et al*, 2008). Among the 11 MCPs containing a known detection domain in *S. oneidensis* MR-1, 5 MCPs (SO2240, SO3282, SO3642, SO3890 and SO4454) were shown to be involved in energy mechanism (Baraquet *et al*, 2009). Further, Armitano showed that MCP SO0987 is one of the MCPs involved in chromate detection (Armitano *et al*, 2011). Among the 3 clusters of chemosensing genes, the Che3 cluster that contains genes encoding CheA3, CheY3, CheW3, CheZ, CheR3, CheD3 and CheB3, is the essential one for chemotaxis (Li Jun *et al*, 2007; Armitano *et al*, 2013). So far, CheA3 the chemotaxis histidine protein kinase is the only one of three putative CheA that has been shown to be necessary for behavioral responses to anaerobic electron acceptors (Fredrickson *et al*, 2008). Additionally, the deletion of its response regulator CheY3 gene has also been shown to abolish the chemotactic capacities of *S. oneidensis* MR-1 (Armitano *et al*, 2013).
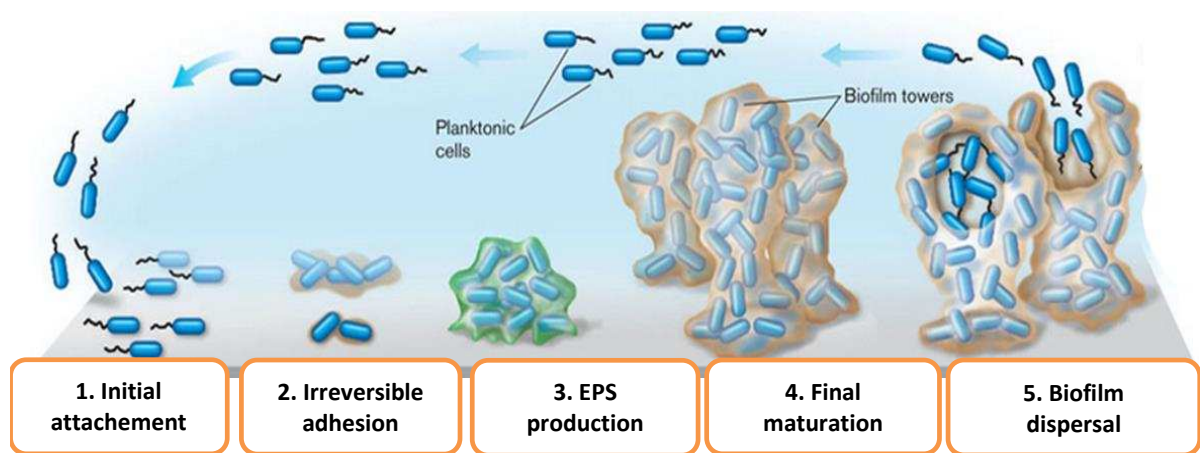
| 1. Initial attachement | 2. Irreversible adhesion | 3. EPS production | 4. Final maturation | 5. Biofilm dispersal |

Figure 13. Surface biofilm development stages in *Pseudomonas.*

Figure adapted from Foster & Slonczewski, 2017.

3.2. Biofilms

The theory of the lifestyle of bacteria not free but adhered to a surface was advanced by Henrici in 1933. He showed that the bacteria were predominantly sessile rather than planktonic. The term "biofilm" was introduced for the first time in 1978 by Costerton. Until 1987, the biofilm was perceived as a "slab" of matrix in which the sessile bacteria were integrated. Further, the development of optical then confocal microscopy, showed that biofilms have complex structures organized in three dimensions composed of aggregates of microorganisms, separated by free spaces called channels where the structure of the matrix is less dense allowing the supply of bacteria with nutrients and oxygen, the diffusion of communications signals and the elimination of their degradation products.

Today, we consider that the main lifestyle of bacteria is not planktonic but in the form of a multicellular community called biofilm. Ninety to 99.9% of the bacteria live in a biofilm (Pantaleon, 2015). The definition of a biofilm, according to (Hall-Stoodley *et al*, 2004) is a bacterial community adhered to an interface (air-liquid or solid-liquid) and living in a matrix composed essentially of water (up to 97%), polysaccharidic polymers secreted by microorganisms, degradation products and substances from the external environment and other components such as DNA, RNA and lipids. The biofilm matrix protects bacteria from many stresses, such as UV, free radicals, pH variations, detergents or dehydration and from the penetration of certain antibiotics. It also helps to concentrate nutrients, metals or useful ions close to cells (Davey & O'toole, 2000). Biofilm would be a strategic lifestyle for micro-organisms since it represents a protected environment for the growth in a hostile environment.

3.2.1. Surface biofilm in *S. oneidensis*

The ability of *S. oneidensis* to form surface biofilms has been extensively studied. It is capable of forming biofilms on mineral surfaces but also on aerobic glass (Lower *et al*, 2001; Thormann *et al*, 2004). Confocal microscopy studies have shown that the formation of biofilm by *S. oneidensis* is similar to that seen for *Pseudomonas aeruginosa* (Tolker-Nielsen *et al*, 2000; Foster & Slonczewski, 2017) (Figure 13). It begins with a reversible attachment of individual cells to a solid surface which will then multiply to form microcolonies, thus the adhesion becomes irreversible. The biofilm can then thicken by forming three-dimensional
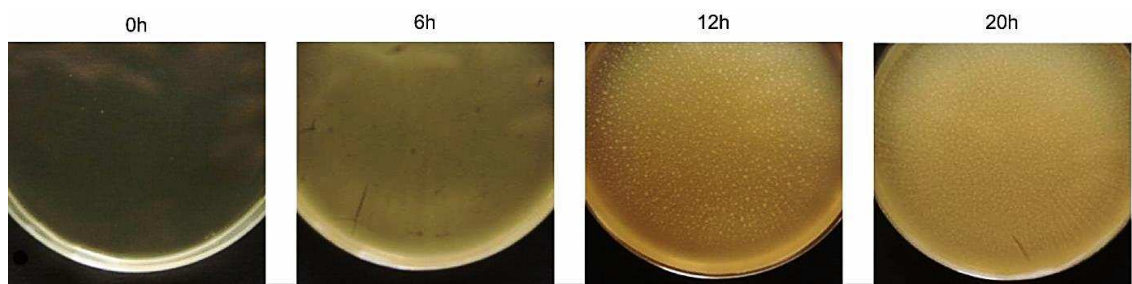
Figure 14. Pellicle formation in *S. oneidensis* MR-1 over time (Armitano *et al*, 2013).

structures (Thormann *et al*, 2004), through production of extracellular polysaccharides, nucleic acids, proteins and additional factors, which together compose the matrix of the mature biofilm (O'Toole & Ha, 2015). Studies showed that the flagellum in *S. oneidensis* plays an important role in the transition from a flat to a structured biofilm (Thormann *et al*, 2004; Armitano *et al*, 2013). Some bacterial cells may detach from the mature biofilm and enter the dissemination phase, which permits the colonization of new surfaces. Biofilm dispersal can be the result of several cues, such as alterations in nutrient availability, oxygen fluctuations and increase of toxic products or other stress-inducing conditions (Figure 13) (O'Toole & Ha, 2015).

3.2.2. Pellicle development in *S. oneidensis*

When grown in a liquid under aerobic and static conditions, some bacteria such as *S. oneidensis* MR1, *Bacillus subtilis, E. coli*, some *Pseudomonas, Salmonella* and *Vibrio* species exploit the air liquid interface (ALI) to build a floating biofilm, this structure is called pellicle, 'floating' biofilm or ALI biofilm (Armitano *et al*, 2014). The ALI is a favorable ecological niche because it provides access to high concentrations of oxygen originating from the air in addition to the nutrients present in the liquid. This interface is defined by opposing gradients: a downward-diffusing O_2 and upward-diffusing nutrients.

As previously said, *S. oneidensis* MR1 is capable of forming pellicles in a static condition and in the presence of oxygen (Liang *et al*, 2010), which is required to initiate pellicle formation and also for pellicle maturation steps. Pellicle cannot be formed under anaerobic conditions, even in the presence of alternative electron acceptors. Armitano (2013) have proposed that aerotaxis, which is the mechanism allowing the active localization of cells at the ALI, is involved in the pellicle formation in *S. oneidensis* MR-1. Pellicle initiates in the absence of growth and it occurs in three steps: rapid formation of a thin pellicle followed by the random aggregation of cells in the nascent pellicle and finally, the establishment of a thick mature and homogeneous pellicle (Figure 14). *S. oneidensis* MR1 pellicle formation requires the presence of a functional flagellum since it allows an active localization of the cells at the ALI. As already mentioned above, the flagellum rotation is under the control of the chemotaxis system (Che3) which is also essential for pellicle formation. Armitano showed

that the deletion of the histidine kinase CheA3 or its response regulator CheY3 gene leads to a heterogeneous pellicle or to the absence of pellicle, respectively, which means that the detection of chemotactic signals is important for pellicle development in *S. oneidensis* MR1 (Armitano *et al*, 2013).

The matrix of the *S. oneidensis* MR1 pellicle is mainly composed of exopolysaccharides, probably rich in glucose, but unlike surface biofilm, DNA does not appear to be a major component (Liang *et al*, 2010). Transcriptomic analyzes and analyzes of deletion mutants have shown that iron import mechanisms and cytochromes are important for pellicle development in *S. oneidensis* MR1 (Liang *et al*, 2012). It has also been shown that certain cations (Ca^{2+}, Mn^{2+}, Cu^{2+} or Zn^{2+}) are required for the formation of pellicle (Liang *et al*, 2010).

3.3. *S. oneidensis* MR-1 respiratory capacities

S. oneidensis MR-1 has remarkably diverse respiratory capacities as mentioned above. It uses oxygen as the terminal electron acceptor during aerobic respiration. However, under anaerobic conditions, it undertakes respiration by reducing more than 10 alternative terminal electron acceptors such as oxidized metals (including Mn(III) and (IV), Fe(III), U(VI)), fumarate, nitrate, TMAO, dimethyl sulfoxide (DMSO), sulfite, thiosulfate and elemental sulfur. Whereas organic molecules are taken into the cell for oxidation within the cytoplasm, the reduction of terminal electron acceptors occurs outside the cell or within the periplasm (Venkateswaran *et al*, 1999; Heidelberg *et al*, 2002; Fredrickson *et al*, 2008; Breuer *et al*, 2015). Such plasticity in alternative electron acceptors has not been observed in any other organism and that is what makes *S. oneidensis* MR-1 the most metabolically diverse species of its genus, which allows its competitiveness in complex aquatic and sedimentary systems and underpins its biotechnological contributions (Cordova *et al*, 2011). We can cite as an example for that, the large amounts of sulfide produced by *S. oneidensis* MR-1 from thiosulfate and sulfur that allow toxic metals to be immobilized as insoluble metal sulfides, as well as its ability to reduce soluble U(VI) and Cr(VI) to insoluble precipitates, which helps to remove soluble metal pollutants from the environment (Breuer *et al*, 2015).

Utilization of a wide range of electron acceptors is mediated by a complex respiratory network. The latter extends electron transfer from the cytoplasmic membrane to periplasm and outer membrane, and includes 42 putative *c*-type cytochromes reduced by quinols (Cordova *et al*, 2011; Breuer *et al*, 2015). *S. oneidensis* MR-1 depends on *c*-type cytochromes for the maintenance of respiratory versatility, as mutants lacking functional cytochrome *c* maturation genes are completely compromised in their ability to respire anaerobically (Fredrickson *et al*, 2008).

Regardless of the nature and location of electron acceptors, the core of anaerobic electron transport chain in *S. oneidensis* MR-1 is the redox cycling of quinone (Q) and its reduced form quinol (QH_2) (Marritt *et al*, 2012a, 2012b). Electrons from the cytoplasmic oxidation of organic electron donors drive the reduction of Q to QH_2 at the inner membrane (IM) level, by a membrane-associated NADH dehydrogenase, after which the resulting electrons are directed to periplasmic redox partners through the action of quinol dehydrogenases such as TorC and CymA. Which are penta-haem and tetra-haem c-type cytochromes, respectively, anchored to the IM by a single α-helix and contain periplasmic domains that catalyze QH_2 oxidation (Cordova *et al*, 2011; Marritt *et al*, 2012a; Breuer *et al*, 2015). It should be noted that TorC has a single redox partner, the TMAO reductase TorA, in contrast, CymA is able to transfer electrons to several partners (explained in more details below). This difference in the biochemistry of TorC and CymA is reflected at the genome level. TorC and TorA are produced from the same operon. Whereas, CymA is produced from an orphan gene that is expressed constitutively which allows its synthesis to be regulated independently from that of its redox partners (Breuer *et al*, 2015). Moreover, recent study showed that its expression can increase during anaerobiosis (Marritt *et al*, 2012b, 2012a).

CymA belongs to the NapC/NirT family of quinol dehydrogenases. It is considered a central element of many electron acceptors reduction pathways (Marritt *et al*, 2012a), since it acts as a common branching point in the electron transport chain by delivering electrons from the quinone pool to multiple terminal reductases as described above (Schwalb *et al*, 2003). Although, CymA possesses the common structural features of its family, which are an N-terminal transmembrane α-helical anchor and a globular tetraheme periplasmic domain, it
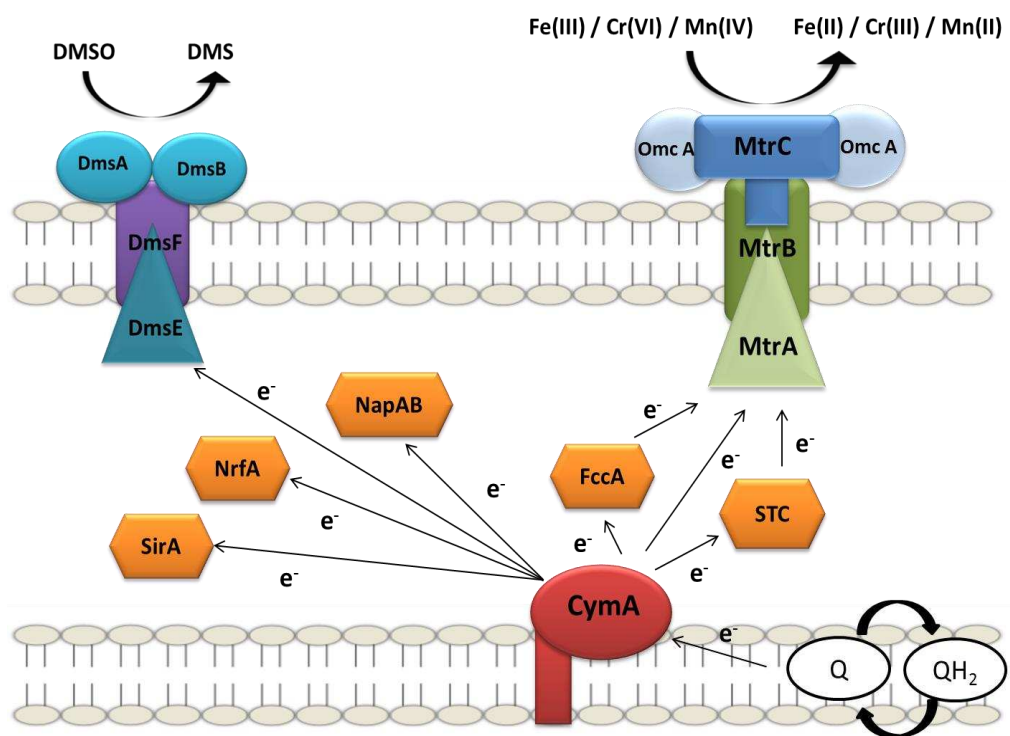
Figure 15. Electron transfer in *S. oneidensis* MR-1.

lacks specificity contrary to other family members (Schwalb *et al*, 2003). This allows its involvement in electron transfer to multiple terminal reductases comprising the CymA-dependent reductases that include the nitrate, nitrite, DMSO, Fe(III) and fumarate reductases, that appear to be located in the periplasm or outer membrane of *S. oneidensis* MR-1. Nevertheless, CymA is not involved in electron transfer to inner membrane anchored enzymes such as the TMAO, thiosulfate and sulfite reductases (Breuer *et al*, 2015; Kouzuma *et al*, 2015) (Figure 15).

3.3.1. Electron transfer in *S. oneidensis* MR-1

Among the various electron acceptors that *S. oneidensis* MR-1 reduces, sulfite, nitrite, nitrate and fumarate are able to cross the outer membrane for reduction in the periplasm. They are reduced respectively by SirA octahaem cytochrome, NrfA penta-haem cytochrome, NapAB nitrate reductase with a molybdenum catalytic cofactor and the fumarate reductases, FccA and IfcA, that cofactor is flavin adenine dinucleotide (FAD) (Figure 15) (Breuer *et al*, 2015).

Some electron acceptors as metal oxides need an extracellular electron transfer across the outer membrane to be reduced outside the cell. Three main mechanisms have been proposed to account for the respiratory reduction of extracellular electron acceptors by *S. oneidensis* MR-1. The first one is the direct electron transfer following physical contact of the extracellular substrate and outer membrane redox-active cytochromes. The two left mechanisms represent a mediated extracellular electrons transfer between the extracellular substrates and outer membrane cytochromes, either through flavins, also called electron shuttles (Figure 16), or along extracellular conductive appendages "nanopods" that can span distances greater than 100 Å to establish contact with solid substrates such as electrodes (Breuer *et al*, 2015).

Figure 16. Extracellular electron transfer (EET) pathways (Mtr pathway) in *S. oneidensis* MR-1 involved in direct EET (A) and mediated EET (B) (Kouzuma *et al*, 2015).
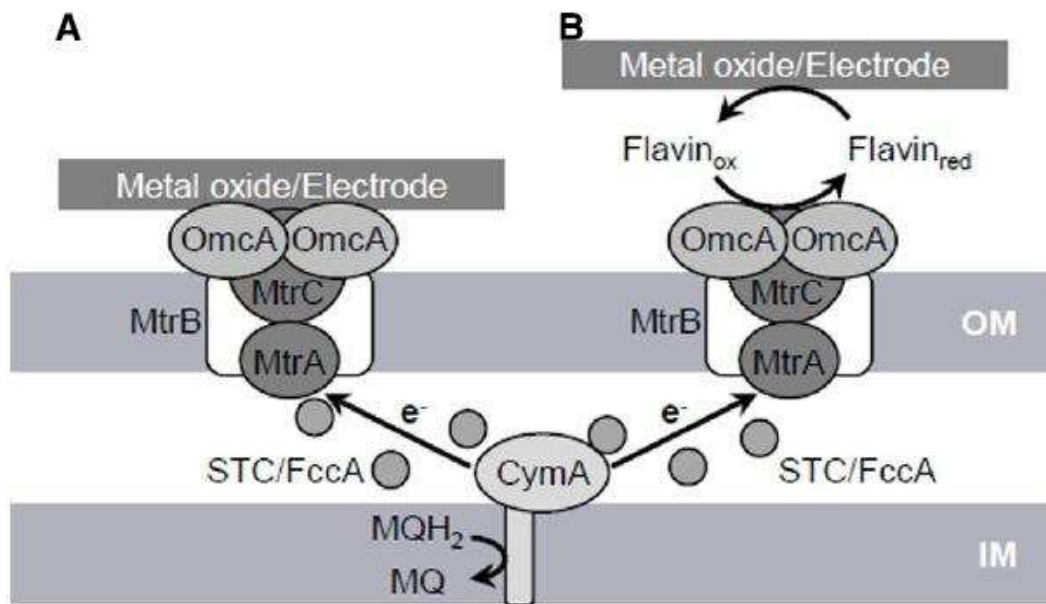
3.3.2. Direct electron transfer

The extracellular reduction of insoluble electron acceptors such as solid metals and electrodes by *S. oneidensis* MR-1 takes place via the metal reducing (Mtr) pathway which involves five proteins (Figures 15 and 16). These include CymA as well as the MtrABC complex and finally OmcA (Coursolle *et al*, 2010). The core of metal reduction components consists of the cytochrome Mtr complex which is located at the outer membrane and composed of three subunits, MtrA, MtrB and MtrC. These proteins are encoded by the *mtrCAB* operon that is expressed under microaerobic and anaerobic conditions, even in the absence of metal electron acceptors. Expression of this operon as well as *omcA*, that have role in metal reduction as described in more detail below, is controlled by the cAMP receptor protein CRP which regulates the expression of many anaerobic reductase genes in *S. oneidensis* MR-1 (Figures 15 and 16) (Saffarini, 2015).

MtrA is a 35 kDa decaheme c-type cytochrome which is associated with MtrCB with a 1:1:1 stoichiometry. It is regarded as a key protein for electron transfer to outer membrane c-type cytochromes (Kouzuma *et al*, 2015). It interacts with CymA which leads to MtrA reduction by electron transfer. Certain authors believe that the gap between CymA and MtrA is too wide to allow direct electron transfer between the two proteins. The possibility of an intermediate electron carrier that mediates electron transfer between the two proteins was then proposed. Thereby, two *c*-type cytochromes were suggested, FccA which is a flavocytochrome c with confirmed fumarate reductase activity, and STC also called CctA that is a small tetraheme c-type cytochrome whose function has not been determined yet (Kouzuma *et al*, 2015; Saffarini, 2015).

MtrA is embedded within the β-barrel protein MtrB, that forms a pore of 30–40 Å in diameter that can easily fit MtrA, forming a "porin cytochrome" electron transfer module (Richardson David J. *et al*, 2012). Some authors suggest that MtrB is required in its turn for the proper localization and insertion of the decaheme OmcA and MtrC in the outer membrane (Myers & Myers, 2000) by forming a stable complex with these outer membrane c-type cytochromes and supporting electron exchange between them (Kouzuma *et al*, 2015). Biochemical data indicate that OmcA and MtrC form a complex with a stoichiometry of
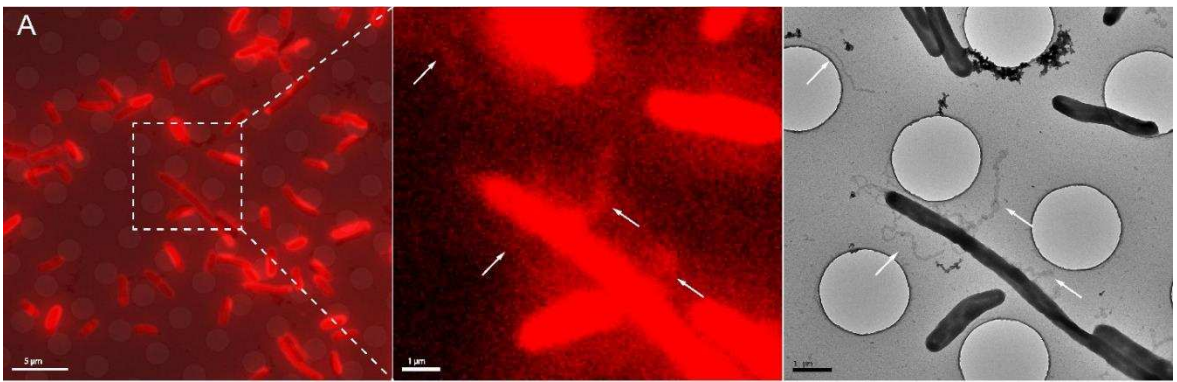
Figure 17. Microscopic image of nanopods synthesized by *S. oneidensis.* Left column: overlay of the fluorescence Light Microscopy (fLM) and Transmission Electron Microscopy (TEM) images. Middle and Right columns: Enlarged fLM and TEM views of nanopods. White arrows indicate the nanopods (Subramanian *et al*, 2017).

approximately 2:1 (Shi *et al*, 2006) and serve as the terminal reductases for extracellular electron acceptors in the Mtr pathway (Myers C.R. & Myers J.M., 2003, 2004).

In addition, to OmcA and MtrC, the *S. oneidensis* MR-1 genome encodes another decaheme *c*-type cytochrome designated MtrF which is an MtrC homologue whose genes is located upstream of the *mtrCAB* operon. MtrF is predicted be a component of the MtrDEF complex that is similar to MtrCAB but is postulated to have a function distinct from other outer membrane c-type cytochromes (Paquete *et al*, 2014). It is suggested that it plays a role in detoxification or reduction of radionuclides under aerobic conditions (McLean J. S. *et al*, 2008). Recent study has suggested the involvement of MtrF in metal reduction in *S. oneidensis* MR-1 (Coursolle & Gralnick, 2012).

3.3.3. Mediated electron transfer

Electron shuttles are soluble redox-active molecules that can mediate electron transfer between the cell surface and solid metal oxides or electrodes. In *S. oneidensis* MR-1 riboflavin (RF) (vitamin B_2) and flavin mononucleotide (FMN) have been identified as the electron shuttling molecules (Marsili *et al*, 2008; Von Canstein *et al*, 2008). These allow greater access to electron acceptors and can accelerate electron transfer to solid metals oxides through interaction with the outer membrane c-type cytochromes (Okamoto *et al*, 2013). Flavins bind specifically to outer membrane c-type cytochromes, FMN binds to MtrC while riboflavin is shown to associate with OmcA (Figure 16) (Okamoto *et al*, 2013, 2014).

In addition to flavins, the third mechanism proposed to support electron transfer from *S. oneidensis* MR-1 to solid surfaces involves nanopods to transfer electrons to metal oxides and electrodes (Barchinger *et al*, 2016). Nanopods, were detected in *S. oneidensis* MR-1 cells grown under limited oxygen conditions. They are not pilus-based structures, but rather, extensions of the outer membrane and periplasm containing outer membrane c-type cytochromes that are capable of extracellular electron transfer (Pirbadian *et al*, 2014). This type of mediated extracellular electron transfer mechanism requires the presence of outer membrane c-type cytochromes, as mutants lacking theses cytochromes are not only deficient in metal reduction, they also produced non-conductive nanowires (Figure 17) (Gorby *et al*, 2006; El-Naggar *et al*, 2010; Subramanian *et al*, 2017).

3.4. *S. oneidensis* MR-1 and chromate

3.4.1. Chromate reduction in *S. oneidensis* MR-1

S. oneidensis MR-1 have evolved diverse resistance mechanisms to cope with chromate toxicity. The main and the most studied Cr(VI)-stress response, that deals directly with chromate ions, in *S. oneidensis* MR-1 is the reduction of Cr(VI) to Cr(III).

It was shown that *S. oneidensis* MR-1 reduces Cr(VI) to Cr(III) as a terminal electron acceptor under anoxic conditions with outer membrane c-type cytochromes serving as terminal reductases for extracellular reduction (Myers C. R. *et al*, 2001; Belchik *et al*, 2011; Wang *et al*, 2013). Mutations of *mtrC*, or of the related genes coding MtrA, MtrB, CymA, alone or in combination, yield a partial decrease in the reduction of Cr(VI), but not complete inhibition, suggesting the existence of multiple Cr(VI) reduction pathways (Bencheikh-Latmani *et al*, 2005). Which is consistent with the existence in the *S. oneidensis* MR-1 genome of MtrABC paralog (Coursolle & Gralnick, 2012). Besides the direct electron transfer from c-type cytochromes to Cr(VI), the electron shuttle-mediated Cr(VI) reduction was also reported in *S. oneidensis* MR-1 (Han *et al*, 2016).

Recent study showed that the highest Cr(VI) reduction rates of *S. oneidensis* MR-1 in anaerobiosis are obtained at pH 7.0 and 30°C, with sodium lactate serving as an electron donor. However, the accumulation of reduction product, Cr(III), can substantially inhibit Cr(VI) reduction as well as cell growth. Moreover, the presence of O_2 strongly inhibits Cr(VI) reduction, suggesting that it might compete with Cr(VI) as an electron acceptor in cells (Han *et al*, 2016).

Although the extensive respiratory versatility of *S. oneidensis* MR-1 and its capacity to use Cr(VI) as terminal electron acceptor, to this day no study proved that this bacterium can use the Cr(VI) as respiratory substrate in anaerobic conditions.

3.4.2. *S. oneidensis* MR-1 response to chromate

Several transcriptome and proteome studies have analyzed the differential gene and protein expression profiles of *S. oneidensis* MR-1 in response to chromate stress (Brown *et al*, 2006; Thompson *et al*, 2007).The results reveal that some genes including those responsible for chemotaxis and motility are largely repressed under Cr(VI) exposure. However the main principal molecular response was the induction of various genes whose products are involved in general stress protection, in addition to genes with annotated functions in DNA metabolism, cell division and electron transport (eg., NADH dehydrogenase gene cluster and cytochrome c) (Chourey *et al*, 2006; Thompson, 2007).

Like *Pseudomonas putida* F1, *Cupriavidus metallidurans* CH34 and *Arthrobacter* sp. FB24, *S. oneidensis* MR-1, in response to Cr(VI), induces the sulfate ABC transporter genes (*sbp*, *cysW-2* and *cysA*) (Thompson *et al*, 2007) as well as genes coding for proteins involved in sulfur metabolism (*cysC*, *cysDN* and *cysIJ*) (Brown *et al*, 2006; Thompson *et al*, 2007; Henne *et al*, 2009; Monsieurs *et al*, 2011). The overexpression of the sulfate transporter under Cr(VI) exposure suggests that chromate induces sulfur starvation in cells. Two main factors have been evoked to explain the reason why chromate induces sulfur limitation. The first is competition between sulfate and chromate for the transport which reduces the bacterial capability to uptake sulfate, and the second possible reason is that oxidative stress induced by Cr(VI) decreases sulfur availability in cells (Figure 7) (Brown *et al*, 2006).

In addition, transcriptome and proteome analysis revealed also the up-regulation of the gene *SO3585* annotated as a putative azoreductase, as well as two downstream genes *SO3586* (glyoxalase family protein) and *SO3587* (hypothetical protein) in response to Cr(VI) exposure (Brown *et al*, 2006; Thompson *et al*, 2007). Further studies showed that *SO3585* gene is not critical for cell survival in the presence of chromate and does not participate in its enzymatic reduction. Thus, it was suggested that the product of *SO3585*, in conjunction with the glyoxalase family protein (*SO3586*) and hypothetical protein (*SO3587),* functions in cellular defense against natural thiol-reactive electrophiles as methylglyoxal, which is a by-product of glycolysis (Mugerfeld *et al*, 2009).

In contrast to Chr plasmidic and chromosomal clusters of *C. metallidurans*, that are up-regulated in the presence of chromate (Monsieurs *et al*, 2011; Viti *et al*, 2014), it was reported that the chromosomal gene *SO0986*, annotated as putative chromate transporter, was not upregulated in Cr(VI)-reducing conditions (Bencheikh-Latmani *et al*, 2005; Chourey *et al*, 2006; Viti *et al*, 2014). In fact, there is no expression evidence to indicate that active efflux of Cr(VI) is used as a mechanism involved in chromate toxicity response in *S. oneidensis* MR-1.

At the end of this introduction part, we can conclude that the interaction between *Shewanella oneidensis* MR-1 and chromate is extremely complicated, thus little is known about the underlying chromate resistance mechanisms of this bacterium. The objective of the first part of this thesis is to improve understanding about these mechanisms by deciphering the role of certain genes (*SO0986*, *fdh*, *dmsA1*, *dmsA2* and *cymA*) in *S. oneidensis* MR-1 resistance toward chromate. The aim of the second part of thesis is to search for new *Shewanella sp*. strains able to efficiently resist to and reduce various chromate concentrations under planktonic and pellicle conditions, and to carry out a comparative genomic analysis between the new isolated strains and the model strain, *S. oneidensis* MR-1, in pursuance of emerging common or specific genetic characteristics related to chromate resistance.

RESULTS

PART I:

S. oneidensis MR-1

CHROMATE RESISTANCE AND

REDUCTION

Article 1:

ChrA$_{SO}$, the chromate efflux pump of *Shewanella oneidensis*, improves chromate survival and reduction

Hiba Baaziz, Cyril Gambari, Anne Boyeldieu, Amine Ali Chaouche, Radia Alatou, Vincent Méjean, Cécile Jourlin-Castelli and Michel Fons
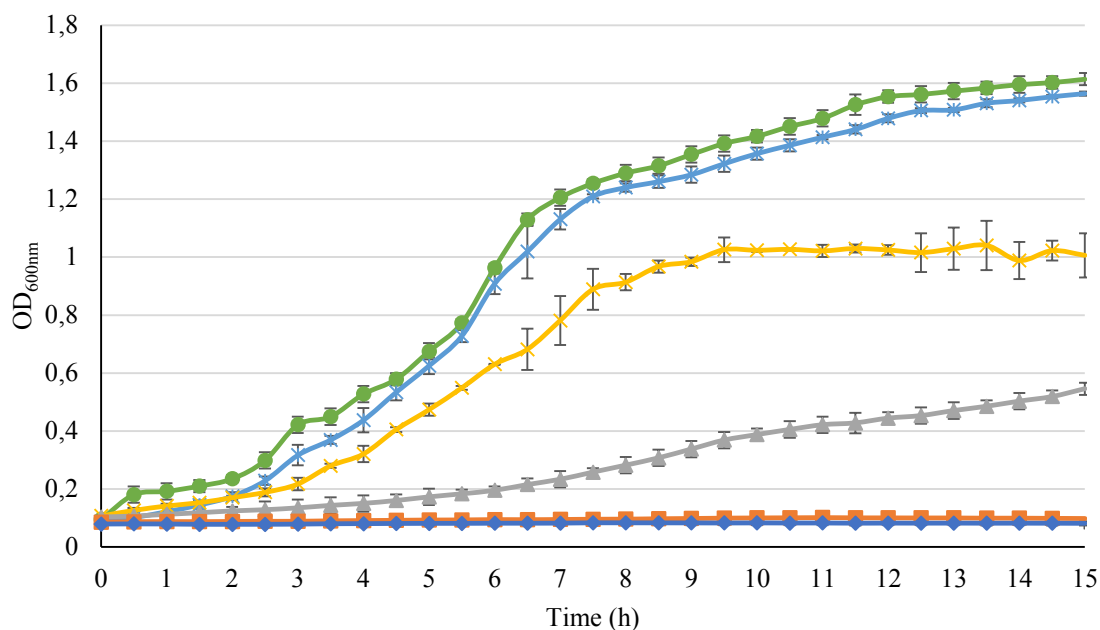
Figure 18. Growth kinetic of *S. oneidensis* MR-1 under semi-aerobic conditions in the presence of various chromate concentrations: 0mM (———), 0,2mM (———), 0,5mM (———), 1mM (———), 2mM (———) and 3mM (———).

Figure 19. Chromate reduction by *S. oneidensis* MR-1 after 24h (Bleu bars) and 48h (Red bars) of incubation at 28°C under semi-aerobic (A) and anaerobic (B) conditions, in the presence of various chromate concentrations: 0.2, 0.5, 1 and 2mM.

1. *S. oneidensis* MR-1 chromate resistance and reduction

As explained in the introduction part, *S. oneidensis* MR-1 is able to reduce Cr(VI). On the one hand, we investigated the chromate survival of *S. oneidensis* MR-1 when exposed to various chromate concentrations (0.2, 0.5, 1, 2 and 3mM) under semi-aerobiosis (10% of O_2, see material and methods chapter). Cells were cultured in LB medium at 28°C overnight. Growth kinetic was performed in 24 well plates after adding inoculum from overnight cultures, to reach an initial OD_{600nm} of 0.1, in LB supplemented or not with chromate. The OD at 600nm was monitored every 30 min in Tecan microplate reader at 28°C with shaking for 15 h.

As shown in Figure 18, *S. oneidensis* MR-1 growth slows down as chromate concentration increases. Cells can resist in the presence of 0.2, 0.5 and 1mM of chromate under semi-aerobic conditions. The initial chromate concentration of 0.2mM does not seems to highly alter the growth. However, growth in the presence of 0.5 and 1mM of chromate is 2-fold and 3-fold, respectively, lower than that in the absence of chromate. Moreover, results show that *S. oneidensis* MR-1 cells cannot resist in the presence of more than 2mM of chromate under semi-aerobic conditions.

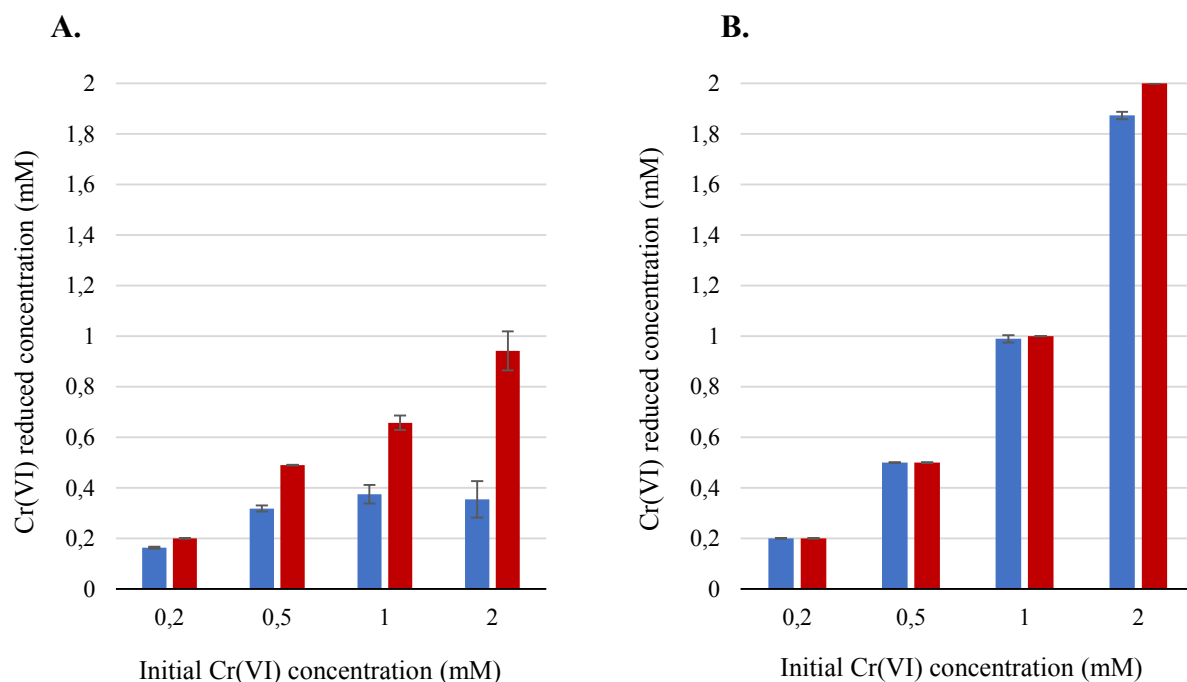On the other hand, we investigated the chromate reduction efficiency of *S. oneidensis* MR-1 toward various chromate concentrations (0.2, 0.5, 1 and 2mM) after 24h and 48h of incubation at 28°C under semi-aerobic and anaerobic conditions. Cells grown overnight were gently centrifuged and resuspended in LB medium supplemented with chromate to reach an initial OD_{600nm} of 2. After incubation, cultures were centrifuged to separate cells and supernatant. The chromate reduction activity was determined on both cellular and supernatant fractions by measuring colorimetrically with a spectrophotometer residual Cr(VI) concentration using the S-diphenylcarbazide (DPC) and total chromate concentration by ICP-OES. The concentration of reduced Cr(VI) was calculated as described in Material and Methods chapter.

As shown in Figure 19 A, the initial Cr(VI) concentrations of 0.2mM and 0.5mM are totally reduced within 48h of incubation in semi-aerobiosis. A significant amount of Cr(VI) is
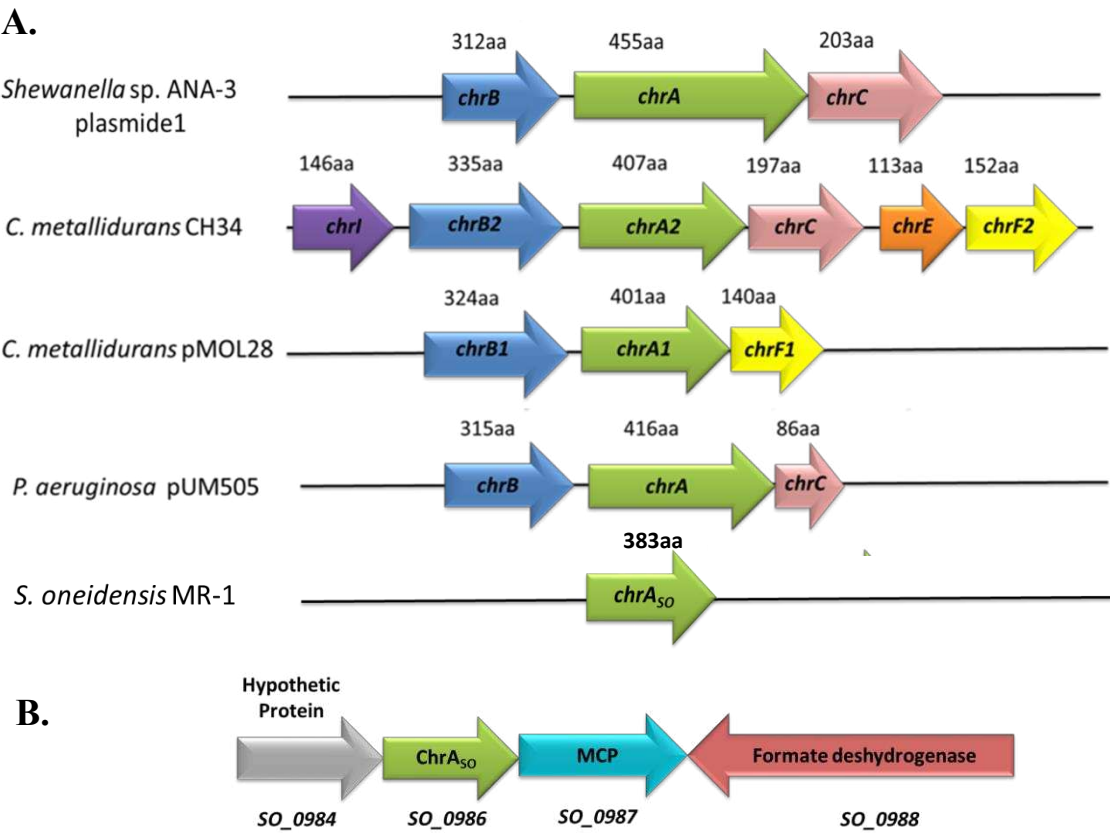
Figure 20. A. Schematic representation of *chr* genes genomic context. B. Schematic representation of *chrA$_{SO}$* genomic context.
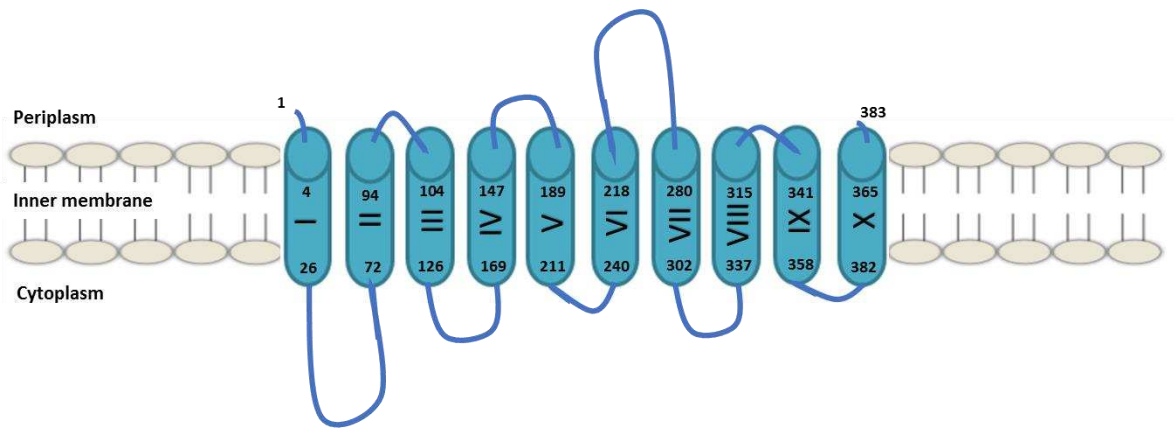

Figure 21. ChrA$_{SO}$ predicted topology. The topology of ChrA$_{SO}$ inside the cytoplasmic membrane is schematized as predicted with the TMHMM software. The amino acids are numbered from the N- to the C-terminus of the protein.

reduced at high initial chromate concentrations 1mM and 2mM. MR-1 shows the ability to reduce totally the initial chromate concentration of 0.2, 0.5 and 1mM within 24h of incubation in anaerobic conditions (Figure 19 B). The initial chromate concentration of 2mM is reduced at 91% within 24h of incubation, before being completely reduced after 48h of incubation in anoxic conditions (Figure 19 B).

Overall, these results show that in both semi-aerobic and anaerobic conditions the amount of reduced Cr(VI) increases with increasing initial chromate concentration, and the chromate reduction efficiency of MR-1 in anaerobic condition is higher than in semi-aerobiosis.

1.1. Chromate efflux pump of *S. oneidensis* MR-1

As previously mentioned, chromate efflux pumps (ChrA) can be responsible for chromate resistance (Díaz-Pérez *et al*, 2007; Ramírez-Díaz *et al*, 2011). Although, the numerous studies show *S. oneidensis* MR-1 ability to reduce Cr(VI) as a direct defense mechanism to cope with chromate toxicity, the involvement of a chromate efflux pump in Cr(VI) resistance behavior has never been reported.

We performed a Blastp search on *S. oneidensis* MR-1 using the well-known chromate ion transporters, such as the ChrA proteins of *Shewanella* sp. ANA3, *Cupriavidus metallidurans* and *Pseudomonas aeruginosa*. We only identified the SO0986 protein, encoded by a chromosomal gene, as a ChrA homolog. In contrast to the species cited above, *S. oneidensis* MR-1 does not contain homologs of the *chrB* and *chrF* genes that sometimes co-occur with *chrA* (Figure 20 A). The corresponding gene of SO0986 protein, that we decided to hereafter refer to as ChrA$_{SO}$ (SO for <u>S</u>hewanella <u>o</u>neidensis), has a different genomic context. *chrA$_{SO}$* is located upstream to *SO0987* gene coding for an MCP, which has been previously shown to be implicated in chromate detection in *S. oneidensis* MR-1 (Armitano *et al*, 2011), and to *SO0988* gene coding for a putative formate dehydrogenase (Figure 20 B).

ChrA$_{SO}$ contains 383 amino acids with an apparent molecular mass of 40.9 kDa. It includes two chromate transporter regions (PF02417) and therefore belongs to the LCHR subfamily of the chromate ion transporter (CHR) superfamily. The TMHMM program predicts that ChrA$_{SO}$ contains 10 transmembrane helices, which is usual for the proteins of the
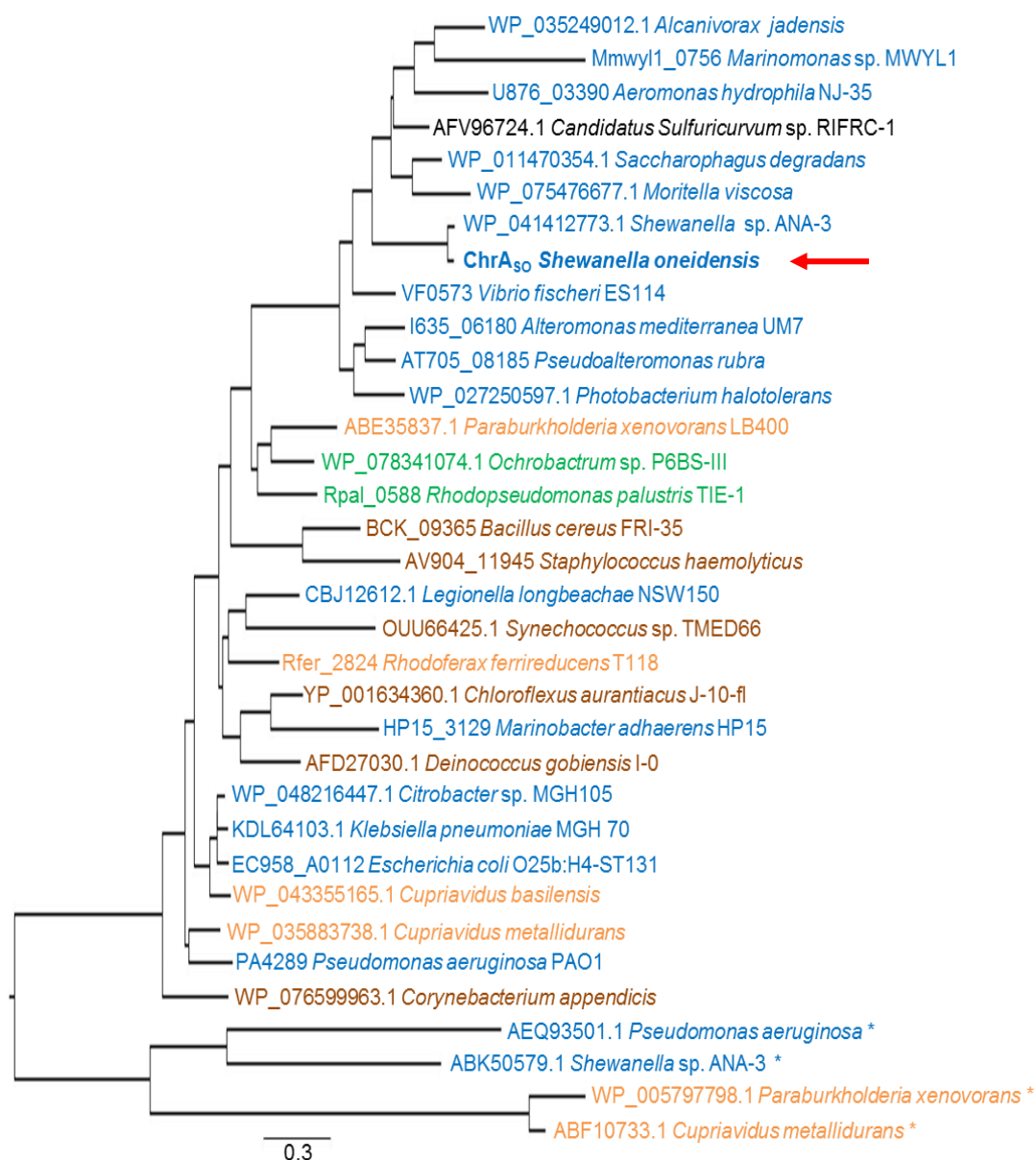
Figure 22. Phylogenetic tree of a subset of ChrA proteins. Sequence and species names are indicated. The names are colored according to their origin: α-Proteobacteria in green, β-Proteobacteria in orange, ε-Proteobacteria in black, γ-Proteobacteria in blue and Terrabacteria in brown. $ChrA_{SO}$ is indicated by a red arrow. The asterisks indicate plasmid-encoded ChrA proteins.

LCHR family. The ChrA$_{SO}$ protein is most likely inserted in the cytoplasmic membrane with its N-terminus oriented towards the periplasm, as schematized in Figure 21.

Furthermore, we performed a Blastp search using ChrA$_{SO}$ as query. Several hundreds of homologous putative proteins were identified in bacteria belonging for the vast majority of them to the Proteobacteria group, but also for some of them to the Terrabacteria group. The highest percentages of identities were found between ChrA$_{SO}$ and proteins from other *Shewanella* species. ChrA$_{SO}$ is also highly homologous to proteins belonging in particular to *Vibrio* and *Pseudoalteromonas* species. We constructed then a phylogenetic tree using a subset of proteins taken from species representative of the different taxonomic families (Figure 22). As expected, ChrA$_{SO}$ clusters with ChrA proteins of the γ-proteobacterial class but more particularly with those of the Aeromonadales, Alteromonadales and Vibrionales orders. Strinkigly, ChrA$_{SO}$ shows the highest percentage of homology with the chromosome-encoded ChrA proteins from *Shewanell*a sp. ANA-3, *C. metallidurans* and *P. aeruginosa* compared to their plasmid-encoded ChrA proteins, that were functionally characterized (Juhnke *et al*, 2002; Pimentel *et al*, 2002; Aguilar-Barajas *et al*, 2008). As an example, ChrA$_{SO}$ displays 89% sequence identity with the chromosome-encoded ChrA and only 26% with the plasmid-encoded ChrA proteins of *Shewanella* ANA-3.

1.1.1. The involvement of chromate efflux pump in chromate survival and reduction of *S. oneidensis* MR-1

We investigated the involvement of ChrA$_{SO}$ in chromate resistance and evaluated its impact on of *S. oneidensis* MR-1 ability to reduce chromate. Therefore, we constructed a strain deleted of *chrA$_{SO}$* and compared its capability to survive and reduce chromate to that of the wild-type. Our results as reported in detail in figure 2 and 3 of article 1, show that the *chrA$_{SO}$* deleted strain exhibits a chromate sensitive phenotype compared to the wild-type. The *chrA$_{SO}$* absence affects strongly the Cr(VI) reduction ability of *S. oneidensis* MR-1, where the amount of Cr(VI) reduced by the *chrA$_{SO}$* mutant is about one third of that reduced by the wild-type strain. Furthermore, we measured the *in vitro* chromate reductase activity on crude extracts from the wild-type and the *chrA$_{SO}$* mutant strains, to decipher whether ChrA$_{SO}$ possesses a chromate reductase activity. The results show that the specific activity of

chromate reductase is similar for both crude extracts, indicating that ChrA$_{SO}$ is not directly involved in Cr(VI)-reduction.

We also investigated the expression of *chrA$_{SO}$* under various chromate concentrations indirectly by measuring β-galactosidase activities of *S. oneidensis* MR-1 and *E. coli* strains containing a chrA$_{SO}$::lacZ transcriptional fusion. Our results show clearly that, although *chrA$_{SO}$* is expressed without chromate, its expression level increases when chromate is added and it can nearly be 2-fold higher in the presence of 0.2mM chromate than in its absence, which indicates that *chrA$_{SO}$* expression is induced by chromate in *S. oneidensis* MR-1 (Article 1, Fig 4).

Finally, after demonstrating that *chrA$_{SO}$* plays a role in chromate resistance and reduction in *S. oneidensis* MR-1, we wondered if it could play a similar role when heterologously expressed in *E. coli* under various chromate concentrations. Our results reveal that *chrA$_{SO}$* expression confers a chromate resistance to high chromate concentrations and improves the Cr(VI)-reduction ability of *E. coli* cells (Article 1, Fig 5).

Article 1:

ChrA$_{SO}$, the chromate efflux pump of *Shewanella oneidensis*, improves chromate survival and reduction

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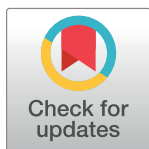
ChrA$_{SO}$, the chromate efflux pump of *Shewanella oneidensis*, improves chromate survival and reduction

Hiba Baaziz[1,2], Cyril Gambari[1], Anne Boyeldieu[1], Amine Ali Chaouche[1], Radia Alatou[2], Vincent Méjean[1], Cécile Jourlin-Castelli[1]*, Michel Fons[1]*

1 Unité de Bioénergétique et Ingénierie des Protéines, Institut de Microbiologie de la Méditerranée, Aix-Marseille Université, Centre National de la Recherche Scientifique, Marseille, France, 2 Laboratoire de Biologie Moléculaire et Cellulaire, Université des Frères Mentouri Constantine 1, Constantine, Algeria

* jourlin@imm.cnrs.fr (CJC); mfons@imm.cnrs.fr (MF)

Abstract

The chromate efflux pump encoding gene *chrA$_{SO}$* was identified on the chromosome of *Shewanella oneidensis* MR1. Although *chrA$_{SO}$* is expressed without chromate, its expression level increases when Cr(VI) is added. When deleted, the resulting mutant Δ*chrA$_{SO}$* exhibits a chromate sensitive phenotype compared to that of the wild-type strain. Interestingly, heterologous expression of *chrA$_{SO}$* in *E. coli* confers resistance to high chromate concentration. Moreover, expression of *chrA$_{SO}$* in *S. oneidensis* and *E. coli* significantly improves Cr(VI) reduction. This effect could result either from extracytoplasmic chromate reduction or from a better cell survival leading to enhanced Cr(VI) reduction.

Introduction

The transition metal chromium has different oxidation states, but only two of them are stable, namely the trivalent Cr(III) and the hexavalent Cr(VI) forms. The Cr(III) form which is relatively insoluble is considered to be poorly toxic since it can hardly penetrate the cells. The Cr(VI) form (chromate and dichromate) is soluble and can be conveyed through cell membranes by different transporters, making it a very toxic compound for both eukaryotic and prokaryotic cells [1].

Chromate, which is chemically and structurally similar to sulfate, is thought to enter the bacterial cells mainly via sulfate ABC transporters. Once inside the cells, Cr(VI) can be reduced, either enzymatically or not, giving rise to intermediates which are responsible for various damages to DNA and cellular components. Some of these alterations are due to the generation of reactive oxygen species (ROS). When present inside the cells, Cr(III), the final product of Cr(VI) reduction, causes toxic effects on both DNA and proteins by binding respectively to phosphate, and to carboxyl and thiol groups [2].

Bacteria have developed different strategies to resist chromate [2,3]. One strategy is to limit its cell entry by reducing Cr(VI) to Cr(III) extracellularly. Reduction can be achieved anaerobically by membrane bound reductases like flavin reductases, cytochromes and

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hydrogenases that are part of electron transport systems using chromate as electron acceptor. In sulfate-reducing bacteria for example, extracellular Cr(VI) reduction can be carried out both enzymatically and chemically by oxidation of the H_2S generated during anaerobic respiration [4,5].

A second strategy is to reduce aerobically the intracellular Cr(VI) [6]. The best known cytosolic proteins involved in this reduction are ChrR of *Pseudomonas putida* and YieF of *Escherichia coli* [7,8]. Both enzymes share sequence homologies and belong to the class I chromate reductases. Nevertheless their mechanisms of action are different. YieF directly reduces Cr(VI) to Cr(III) through a four-electron transfer reaction while ChrR proceeds by combining one- and two-electron transfer steps [7]. As a result, ChrR generates more ROS than YieF during chromate reduction. The class II chromate reductases could require NAD(P)H as an electron donor and generally possess a nitroreductase activity. One of them, NfsA from *E. coli*, was characterized and shown to increase chromate tolerance when overproduced [9]. Intracellular chromate reduction is often associated with mechanisms to detoxify the cell from the ROS and to repair the lesions, caused in particular to DNA [10,11].

A third strategy to deal with chromate toxicity is to rapidly extrude it from the cell cytoplasm by the mean of chromate efflux pump. The ChrA proteins of *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* were the first proteins proved to be involved in this process [12,13]. Thereafter, several ChrA homologues were identified based on genome sequence analysis and grouped into a large family, called the Chromate ion transporter (CHR) family [14]. This latter was further divided into two subfamilies on the basis of the protein length. One subfamily contains proteins with a sequence length of about 180 amino acids, named short-chain CHR (SCHR), and bearing only one chromate transporter domain. The proteins of the second subfamily, containing two chromate transporter domains, are larger (around 400 amino acids) and called long-chain CHR (LCHR). The ChrA proteins possess several transmembrane regions and are encoded by genes that can be found either on chromosome or on plasmid. Some bacteria possess several *chrA* genes associated or not with other genes involved in chromate resistance or regulation (like *chrB*, *chrC* or *chrF*, found for example in *C. metallidurans*, *Ochrobactrum tritici* and *Shewanella sp.* strain ANA-3) [14]. These examples are not exhaustive and different strategies can co-exist in a same bacterium.

Shewanella oneidensis is an aquatic bacterium that belongs to the ɣ-Proteobacteria class. It can utilize a wide range of terminal electron acceptors including fumarate, nitrate, TMAO, as well as oxidized metals including Fe(III) and Mn(IV). This respiratory versatility makes it a key player for bioremediation [15]. The capability of *S. oneidensis* MR-1 to resist to and/or reduce Cr(VI) has been explored in various conditions, pointing out the involvement of different mechanisms for these functions [16–18]. A transcriptomic analysis revealed that more than 80 genes were upregulated when strain MR-1 was under Cr(VI)-reduction condition [19]. Moreover, the response regulator SO2426 was shown to control the expression of genes involved in the relationship between Fe homeostasis and Cr(VI)-induced stress tolerance [20]. Concerning reduction, MtrC and OmcA, two extracellular decaheme cytochromes located in the outer membrane of the cells and previously known to be involved in Fe(III) reduction, were characterized as terminal Cr(VI) reductases *in vitro* as well as *in vivo* [18,21]. A protein homologous to ChrR of *P. putida* was also identified in *S. oneidensis* (SO3585), but its role in chromate resistance seems not to be crucial since only the initial rate of chromate disappearance is affected in the *so3585*-deleted strain [22].

The aim of this work was to confirm that resistance to chromate in *S. oneidensis* MR-1 involves a chromate efflux pump and to evaluate the impact of this efflux pump on the capability of this bacterium to reduce Cr(VI) to the less toxic form Cr(III).

Materials and methods

Strains, medium and growth conditions

The *E. coli* strains CC118 λpir and 1047/pRK2013 used for conjugation, and MC1061 derivatives used for chromate resistance and reduction assays were routinely grown in Lysogeny Broth (LB) medium at 37°C, or 30°C when specified [23,24].

All *S. oneidensis* strains used in this study are derivatives of strain MR1-R (referred as wild-type and used instead of MR-1 to allow counter-selection by rifampicin in the conjugation experiments) and were routinely grown at 28°C in LB under aerobic (agitation) or anaerobic (static) conditions. In the latter case, LB medium was supplemented with 20 mM trimethylamine oxide (TMAO) as electron acceptor. When growth was performed in the presence of chromate, disposable tubes were used and incubated either statically or under agitation. The dissolved oxygen (DO) of the latter condition measured by a Clark electrode was 10%. This condition was then referred as semi-aerobic. Chromate challenge was carried out by supplementing LB medium at the required final concentration with a filter-sterilized stock solution of potassium chromate (K_2CrO_4, Sigma-Aldrich).

If required, media were solidified by adding 17 g.L^{-1} agar. When needed, chloramphenicol (Cm) was used at 25 μg.mL^{-1}. Growth was determined spectrophotometrically by monitoring changes in optical density at 600 nm compared to the same medium without bacterium (OD_{600nm}).

Chromate resistance assays in *S. oneidensis*

S. oneidensis pre-cultures grown overnight on LB plates were suspended in LB medium and used to inoculate fresh LB medium to an initial $OD_{600nm} = 0.2$. Cells were then grown until an $OD_{600nm} = 0.5$ and submitted to two different assays.

For the spot assay, 10-fold serial dilutions of cell cultures were spotted on LB plates supplemented or not with 0.5 mM of chromate and incubated at 28°C. Plates were scanned after 4 days of incubation.

For the viability assay, chromate was added to the cell cultures ($OD_{600nm} = 0.5$) to a final concentration of 0.2 mM. After 5 hours, cells were appropriately diluted in LB and spread onto LB agar, and incubated at 28°C. The total number of viable cells was estimated based on the number of colony-forming unit (CFU). Results were expressed as the percentage of viable counts measured in these conditions compared to that of the same culture grown in the absence of chromate.

Cr(VI)-reduction assays in *S. oneidensis*

In vivo Cr(VI) measurements were carried out on both semi-aerobic and anaerobic cultures. When fresh cultures reached an $OD_{600nm} = 0.5$, chromate was added to a final concentration of 1 mM. After 2h of incubation at 28°C, cell cultures were centrifuged at 8,000 g for 5 min and residual Cr(VI) concentration present in the supernatant was determined using the S-diphenylcarbazide (DPC) method [25] slightly modified. Briefly, 10, 20, and 50 μL supernatant samples were added in a 2 mL tube containing 1 mL of H_3PO_4 0.5% and the volume of water necessary to obtain a 1960 μL final volume. Forty μL of DPC reagent (5 mg.mL^{-1} in 95% acetone and stored in dark at room temperature) were added, gently mixed and kept at room temperature for 5–10 min. Absorbance was measured at 540 nm. Cr(VI) concentration in the samples was calculated from a standard curve. These experiments were repeated three times.

In vitro measurements of chromate reductase activity were performed on crude extracts as described previously [26] with slight modifications. Briefly, wild-type and *chrA*$_{SO}$ deleted

mutant strains were grown overnight in LB medium at 28°C under agitation. The cells were then harvested, washed with sodium phosphate buffer (pH7) and resuspended in the same buffer before disruption by French press. The cell lysates were then centrifuged and the supernatants (crude extracts) recovered. Protein concentrations were measured using Protein Assay Dye Reagent (Bio-Rad), and bovine serum albumin as standard. Reaction mixtures (1 mL) containing 100 μM of K_2CrO_4 [Cr(VI)] and 1.5 mg of proteins (crude extracts) were incubated at 30°C for 30 min. The residual Cr(VI) concentration in the reaction mixture was estimated using the DPC method as described above.

Construction of plasmid p*chrA*$_{SO}$

To evaluate the impact of ChrA$_{SO}$ on chromate resistance and reduction in *E. coli*, the *chrA*$_{SO}$ open reading frame (*so0986*) was PCR-amplified from MR1-R chromosomal DNA, from upstream of the ATG start codon (with an optimized Shine Dalgarno) to the TAA stop codon, and cloned between the *Sac*I and the *Xba*I restriction sites of the pBAD33 vector, under the control of the inducible P$_{ara}$ promoter [27]. The resulting plasmid, called p*chrA*$_{SO}$, was introduced into strain MC1061 and controlled by DNA-sequencing (S1 Table).

Chromate resistance and reduction assays in *E. coli*

Pre-cultures of MC1061 derivatives containing either the plasmid p*chrA*$_{SO}$ or the pBAD33 vector were used to inoculate fresh LB medium to an initial OD$_{600nm}$ = 0.05. Cells were grown until an OD$_{600nm}$ = 0.2 prior to the addition of chromate at various final concentrations (0, 0.2, 0.4, 0.8 and 1.2 mM), then the cells were incubated at 30°C. The cultures were regularly sampled to measure the OD$_{600nm}$ and to quantify the chromate using the DPC method.

In vitro chromate reductase activities were measured on crude extracts from *E. coli* cells containing either the pBAD33 vector or the plasmid p*chrA*$_{SO}$ as described above for the measurements on *S. oneidensis* crude extracts.As ChrA$_{SO}$ is most probably inserted inside the cytoplasmic membrane, its overexpression is toxic for the host. Preliminary growth assays of the *E. coli* strain harboring p*chrA*$_{SO}$ were carried out at different temperatures (30°C or 37°C) in the presence of different concentrations of arabinose (0, 0.005 or 0.01%). We observed that a temperature of 30°C and the absence of inducer allowed a better growth of the strain, thus these parameters were used in further experiments.

Construction of a deletion mutant

The *chrA*$_{SO}$-deleted strain was constructed as described previously [28]. Briefly, upstream and downstream regions flanking the gene were amplified by PCR from a MR1-R strain (by using primers SO_0986 D2 to D5, S1 Table) and cloned into the suicide vector pKNG101 at the restriction sites *Bam*HI and *Spe*I. The ligation product was transformed into the *E. coli* CC118 λpir strain. The resulting plasmid was introduced into MR1-R strain by conjugation using the *E. coli* helper strain 1047/pRK2013 [29]. The plasmid was integrated in the MR1-R chromosome by a first recombination event and removed by a second recombination event in the presence of 6% sucrose. Deletion of *chrA*$_{SO}$ gene was confirmed by PCR.

Reporter gene assay

A putative promoter region was searched upstream of the start codon of *chrA*$_{SO}$ using the BPROM Softberry online software (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). A DNA fragment corresponding to the 400 bp upstream from the ATG was amplified by PCR using *S. oneidensis* MR1-R chromosomal DNA,

digested with *Xba*I and *Spe*I, and ligated upstream of the *lacZ* gene of vector pACYC184-lacZ, which was previously constructed by cloning the β-galactosidase-encoding gene *lacZ* from pGE593 into the vector pACYC184 (S1 Table) [30,31]. The ligation product was transformed into *E. coli* strain MC1061. The resulting plasmid called p*chrA*$_{SO}$::*lacZ* was introduced into *S. oneidensis* MR1-R by conjugation. Plasmid construction was checked by DNA sequencing. As a control, another fusion, named p*mxd*$_{450}$::*lacZ*, was similarly constructed using a DNA fragment corresponding to the 450 bp upstream from the ATG of *mxdA* [32]. *S. oneidensis* MR1-R strain containing p*chrA*$_{SO}$::*lacZ* or p*mxd*$_{450}$::*lacZ* was grown overnight on LB-agar plate. The overnight culture was diluted to an $OD_{600nm} = 0.1$ in fresh liquid LB medium containing various concentrations of chromate (0, 0.05, 0.1 and 0.2 mM) and incubated in semi-aerobiosis at 28˚C for 16 hours prior to β-galactosidase activity quantification. The β-galactosidase activity was determined by a Miller assay adapted for use with plate reader [33]. Briefly, cells were transferred into a microtiter plate and OD_{600nm} was measured using a Tecan Spark 10M microplate reader. Cells were then lysed using lysozyme and PopCulture reagent (Sigma-Aldrich) prior to incubation with Z buffer (62 mM Na_2HPO_4, 45 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). *O*-nitrophenyl-ß-D-galactopyranoside (ONPG) was added to the mix before kinetic quantifications of OD_{420nm}. Slope of the obtained curves were calculated and β-galactosidase activity (arbitrary units) was determined by the following equation:

$$(1000 \times slope)/(OD_{600nm} \times volume\,of\,reaction\,(\mu L)).$$

Bioinformatics analysis

The amino acid sequence of ChrA$_{SO}$ was retrieved from the MicroScope Microbial Genome Annotation and Analysis Platform (https://www.genoscope.cns.fr/agc/microscope/home/). Prediction of transmembrane helices was carried out using the TMHMM online software (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Proteins sharing homologies with ChrA$_{SO}$ were searched in the Bacteria kingdom using the BlastP software on the NCBI server (https://blast.ncbi.nlm.nih.gov). Representative sequences of different phyla were subsequently selected, as well as the sequences of four plasmid-encoded ChrA proteins that were previously characterized. For the phylogenetic analysis, we used the "Phylogeny.fr" software in the "one-click" mode, i.e. with the default parameters optimized by the authors (http://www.phylogeny.fr/). For the tree rendering step, we used the software "FigTree" version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) in which we entered the result in Netwick format obtained with "Phylogeny". The tree rooting was performed with the midpoint option.

Results

S. oneidensis chromosome contains a chromate efflux pump encoding gene

The *S. oneidensis* MR-1 genome contains only one gene (*so0986*) whose product is annotated as a putative chromate efflux pump. This gene is not located on the megaplasmid but on the chromosome. Accordingly, a Blastp search performed on *S. oneidensis* using well-known chromate ion transporters, such as the ChrA proteins of *P. aeruginosa* and *C. metallidurans*, only identified the SO0986 protein as a ChrA homolog. In contrast, *S. oneidensis* does not contain homologs of the *chrB* and *chrF* genes which sometimes co-occur with *chrA*.

For clarity, we will refer to the SO0986 protein as ChrA$_{SO}$. This protein of 383 amino acids with a molecular mass of 40.9 kDa contains two chromate transporter regions (PF02417) and

therefore belongs to the LCHR subfamily of the chromate ion transporter (CHR) superfamily. The TMHMM program predicted that $ChrA_{SO}$ contains 10 transmembrane helices, which is usual for the proteins of the LCHR family. The $ChrA_{SO}$ protein is most likely inserted in the cytoplasmic membrane with its N-terminus oriented towards the periplasm, as schematized in Fig 1A.

A Blastp search was performed using $ChrA_{SO}$ as query. Several hundreds of homologous proteins were identified in bacteria belonging for the vast majority of them to the Proteobacteria group, but also for some of them to the Terrabacteria group. As expected, the highest percentages of identities were found between $ChrA_{SO}$ and proteins from other *Shewanella* species. $ChrA_{SO}$ is also highly homologous to proteins belonging in particular to *Vibrio* and *Pseudoalteromonas* species. A phylogenetic tree was constructed using a subset of proteins taken from species representative of the different taxonomic families (Fig 1B). Not surprisingly, $ChrA_{SO}$ clusters with ChrA proteins of the γ-proteobacterial class but more particularly with those of the Aeromonadales, Alteromonadales and Vibrionales orders. Strinkigly, $ChrA_{SO}$ shows the highest percentage of homology with the chromosome-encoded ChrA proteins from *Shewanella* sp. ANA-3, *P. aeruginosa* and *C. metallidurans* compared with their plasmid-encoded ChrA proteins, that were functionally characterized [12,13,34]. As an example, $ChrA_{SO}$ displays 89% sequence identity with the chromosome-encoded ChrA and only 26% with the plasmid-encoded ChrA proteins of *Shewanella* ANA-3. Moreover *S. oneidensis* megaplasmid does not harbor *chrA* gene.

$ChrA_{SO}$ is involved in chromate resistance in *S. oneidensis*

Since $ChrA_{SO}$ clearly belongs to the chromate ion transporter family, we wondered if it could be involved in chromate resistance in *S. oneidensis*. We therefore constructed a strain deleted of the *chrA$_{SO}$* gene and compared its capability to survive in the presence of chromate to that of the wild-type strain.

Wild-type and mutant cells were first allowed to grow at 28˚C until an OD_{600} of 0.5. These pre-cultures were then submitted to two chromate resistance tests. In the first one, cells were allowed to grow for 5 hours after addition of 0.2 mM chromate in the pre-cultures. Afterwards, bacterial cells were diluted and plated on LB-agar to determine the percentage of survival after "colony forming unit" counting. The same experiment was carried out in the absence of chromate as a control. As observed on Fig 2A, the survival percentage is about 40% in the wild-type strain while it is only about 25% in the *chrA$_{SO}$* deleted strain. In the second test, serial 10-fold dilutions of the bacterial pre-cultures were performed and then spotted on plates containing either no chromate or 0.5 mM chromate. The plates were incubated at 28˚C to allow bacterial growth. In the absence of chromate, no growth difference is observed between the wild-type and the *chrA$_{SO}$* mutant strains (Fig 2B). The presence of chromate impairs the growth of both strains, but the effect is clearly more pronounced for the *chrA$_{SO}$* mutant strain (Fig 2B). These results strongly suggest that $ChrA_{SO}$ is involved in chromate resistance in *S. oneidensis*.

Chromate reduction is impaired in a *chrA$_{SO}$* deleted strain

The impact of $ChrA_{SO}$ on the capability of *S. oneidensis* to reduce Cr(VI) was evaluated by comparing the percentage of Cr(VI)-reduction obtained when the wild-type, the *chrA$_{SO}$* mutant and the *chrA$_{SO}$* mutant containing the plasmid p*chrA$_{SO}$* were exposed to 1 mM chromate either in semi-aerobic (as defined in the Materials and methods section) or anaerobic conditions during 2 hours. The concentration of Cr(VI) was evaluated by the DPC method. As shown in Fig 3, in both conditions (semi-aerobiosis and anaerobiosis), the deletion of *chrA$_{SO}$*
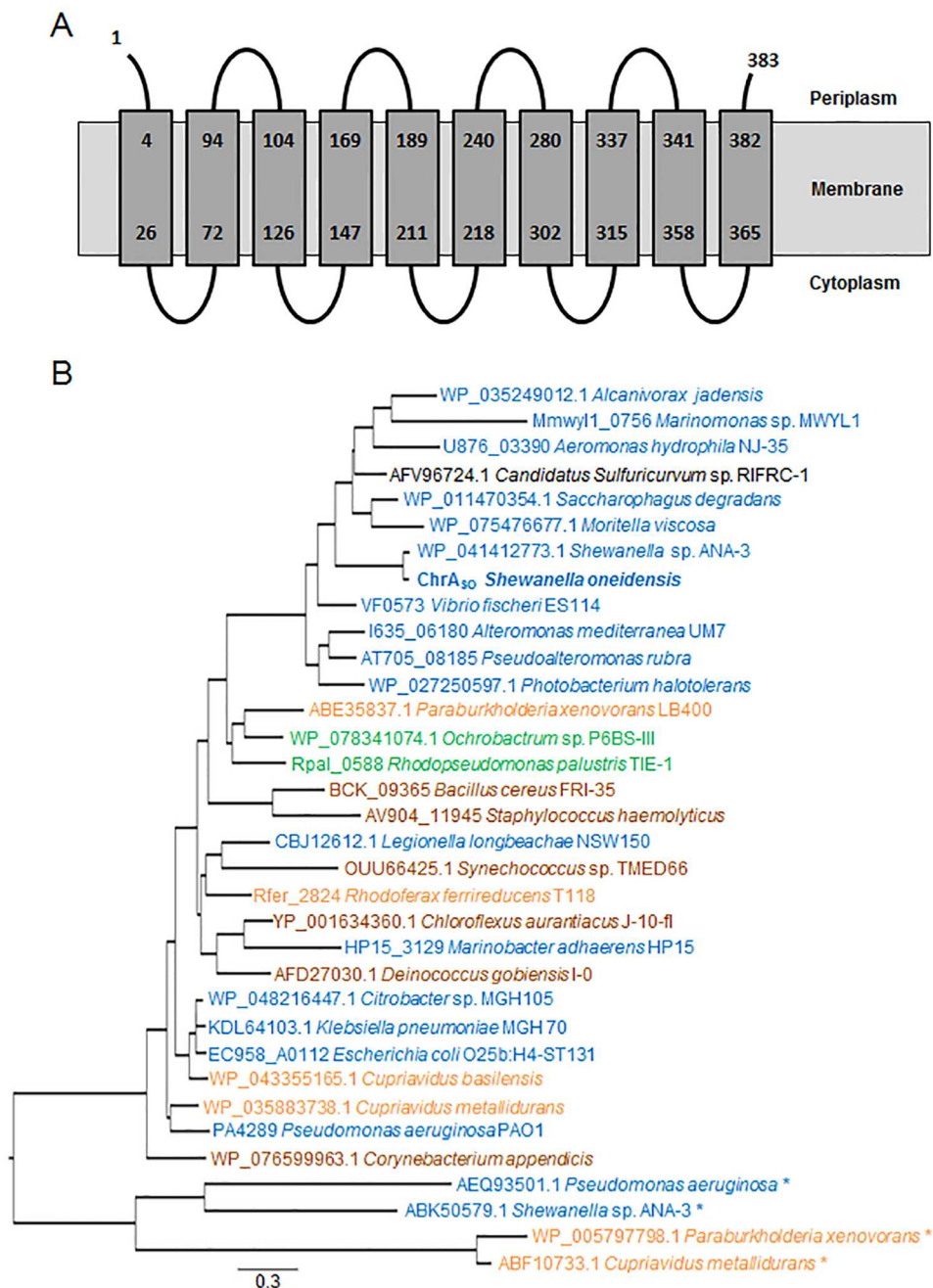
Fig 1. ChrA$_{SO}$ predicted topology and phylogenetic analysis of ChrA proteins. (A) The topology of ChrA$_{SO}$ inside the cytoplasmic membrane is schematized as predicted with the TMHMM software. The amino acids are numbered from the N- to the C-terminus of the protein. (B) Phylogenetic tree of a subset of ChrA proteins. Sequence and species names are indicated. The names are colored according to their origin: α-Proteobacteria in green, β-Proteobacteria in orange, ε-Proteobacteria in black, γ-Proteobacteria in blue and Terrabacteria in brown. ChrA$_{SO}$ is indicated in bold. The asterisks indicate plasmid-encoded ChrA proteins.

has a dramatic impact on Cr(VI)-reduction. Indeed, the amount of Cr(VI) reduced by the *chrA$_{SO}$* mutant is about one third of that reduced by the wild-type strain. As expected, the presence of p*chrA$_{SO}$* in the *chrA$_{SO}$* mutant restores a reduction efficiency comparable to that of the wild-type strain.

A
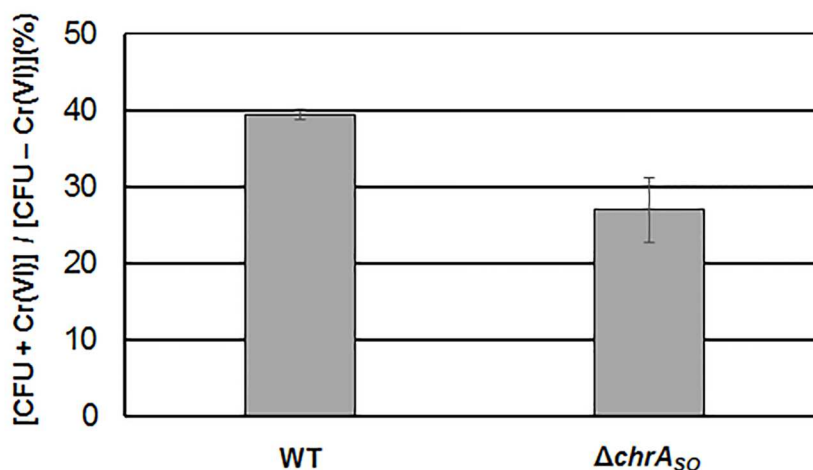
B
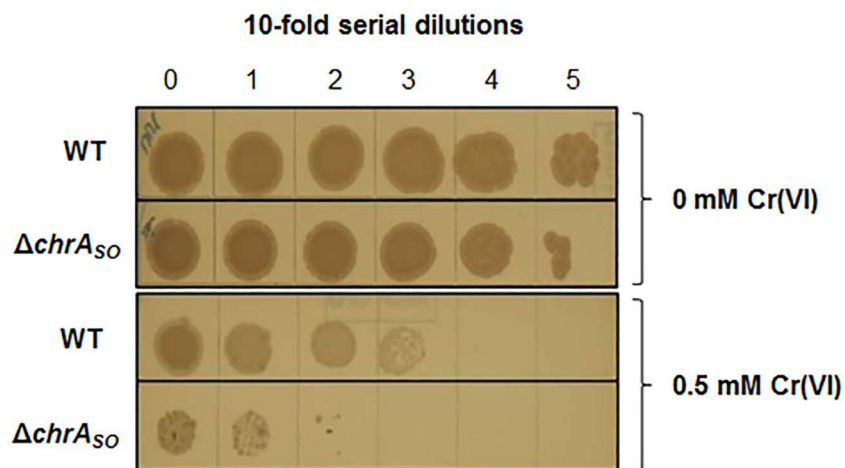
Fig 2. Effect of *chrA_{SO}* on chromate resistance in *S. oneidensis* strains. Two different assays were conducted to evaluate the impact of *chrA_{SO}* on chromate resistance. (A) In the first assay, [CFU + Cr(VI)]/ [CFU—Cr(VI)] (%) indicates the percentage of viable counts of the wild-type (WT) and the *chrA_{SO}* deleted mutant (Δ*chrA_{SO}*) after 5 hours of growth in semi-aerobiosis conditions and in the presence of 0.2 mM chromate, compared to that of the same strains grown in similar conditions without chromate. Values are means ± standard deviations (error bars) from at least two experiments. The mean absolute values of the number of CFU counted in the absence of chromate are $2.49 \times 10^9.mL^{-1}$ and $2.41 \times 10^9.mL^{-1}$ for the wild-type and the *chrA_{SO}* deleted mutant strains, respectively. (B) In the second assay, 10-fold serial dilutions of the wild-type (WT) and the *chrA_{SO}* deleted mutant (Δ*chrA_{SO}*) cultures were spotted on LB agar supplemented or not with 0.5 mM chromate before incubation at 28˚C during 4 days.

A Semi-aerobic conditions
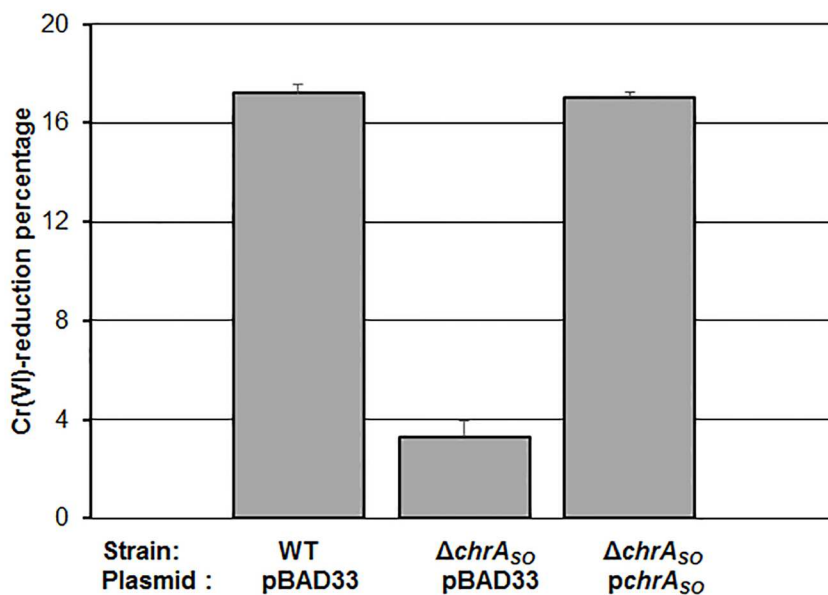
B Anaerobic conditions
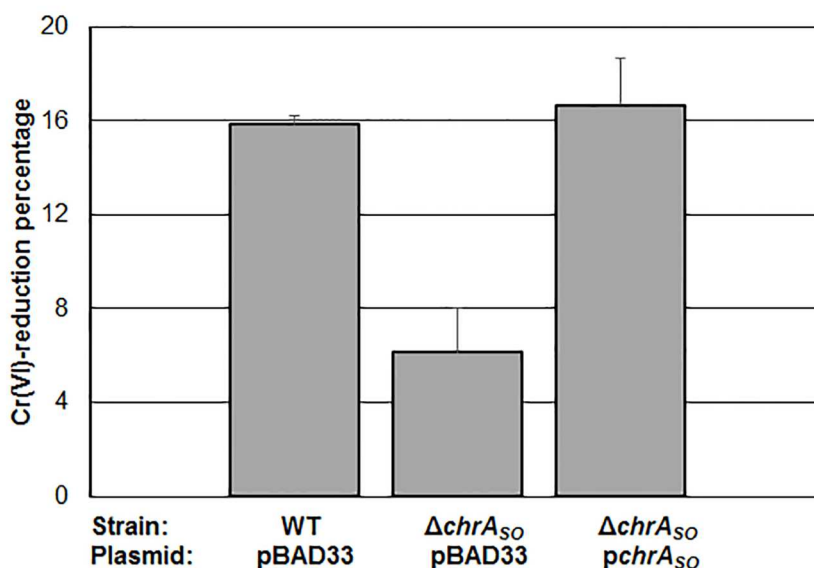
Fig 3. Impact of *chrA*SO on chromate reduction in *S. oneidensis* strains. The WT/pBAD33, Δ*chrA*SO/pBAD33 and Δ*chrA*SO/p*chrA*SO strains were challenged with 1 mM chromate before quantification of Cr(VI). Results were expressed as the percentage of chromate reduced after 2 hours of incubation in semi-aerobic (A) or anaerobic conditions (B). Values are means ± standard deviations (error bars) from at least three experiments.

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To decipher whether ChrA$_{SO}$ possesses a chromate reductase activity, we performed *in vitro* chromate reductase activity on crude extracts from the wild-type and the *chrA*$_{SO}$ mutant strains and the results show that the specific activity of chromate reductase is similar for both crude extracts (0.78 ± 0.1 and 0.88 ± 0.05 nmol reduced-Cr(VI).min^{-1}.mg^{-1}, respectively), indicating that ChrA$_{SO}$ is not directly involved in Cr(VI)-reduction.
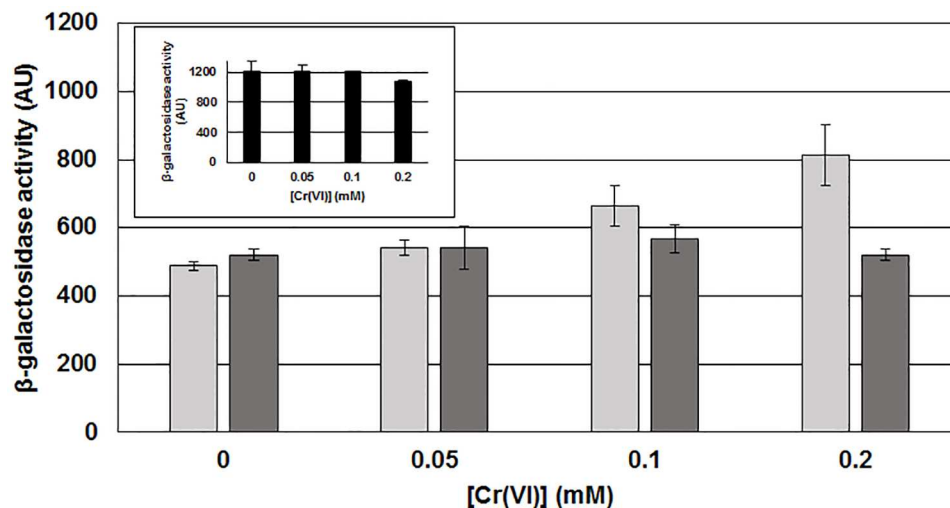
Fig 4. Expression of *lacZ* fusions in the presence of chromate. The wild-type *S. oneidensis* strain containing either the plasmid p*chrA*$_{SO}$::*lacZ* (transcriptional fusion between the promoter of *chrA*$_{SO}$ and the *lacZ* reporter gene; light grey bars) or the plasmid p*mxd*$_{450}$::*lacZ* (transcriptional fusion between the promoter of *mxdA* and the *lacZ* reporter gene; dark grey bars), used as a control, was grown during 16 hours in the presence of increasing concentrations of chromate (0, 0.05, 0.1 and 0.2 mM) before β-galactosidase activity was determined. The MC1061 *E. coli* strain containing the plasmid p*chrA*$_{SO}$::*lacZ* was grown in similar conditions and β-galactosidase activity was also determined (black bars in the insert). [Cr(VI)] indicates the concentration of chromate during growth. β-galactosidase activity is expressed as Miller arbitrary units (AU). Values are means ± standard deviations (error bars) from at least three experiments.

https://doi.org/10.1371/journal.pone.0188516.g004

The *chrA*$_{SO}$ expression is induced by chromate

Since *chrA*$_{SO}$ is involved in chromate resistance, we wondered if its expression could be regulated by chromate. We thus constructed a transcriptional fusion using the *lacZ* gene as a reporter. The sequence upstream of the start codon of *chrA*$_{SO}$ was first analyzed with Bprom software (Softberry) to search for a putative promoter. Sequences corresponding to putative -10 (`CATAAT`) and -35 (`TTTTCA`) boxes separated by 20-bp were identified respectively 33 and 59-bp upstream from the start codon. The 400-bp upstream from the start codon and containing these boxes were amplified and cloned upstream of the promoterless *lacZ* gene. The plasmid bearing this transcriptional fusion was introduced in *S. oneidensis*. β-galactosidase activities were then measured on the resulting strain grown with various concentrations of chromate. As shown in Fig 4, a significant level of β-galactosidase is observed when the strain is grown in the absence of chromate, meaning that the putative promoter of *chrA*$_{SO}$ is functional in this condition. Interestingly, β-galactosidase activity increases with rising concentrations of chromate. Indeed expression from the fusion is nearly 2-fold higher in the presence of 0.2 mM chromate than in its absence. As controls, β-galactosidase activities were measured on *E. coli* containing the *chrA*$_{SO}$::*lacZ* fusion and on *S. oneidensis* containing another transcriptional fusion (*mxd*A::*lacZ*). In both cases, the activities were similar whatever the growth conditions. These results indicate that *chrA*$_{SO}$ expression is induced by chromate in *S. oneidensis*.

Heterologous expression of *chrA*$_{SO}$ gene confers higher chromate resistance and reduction to *E. coli*

We demonstrated that *chrA*$_{SO}$ plays a key role in chromate resistance and reduction in *S. oneidensis* and we wondered if it could play a similar role when heterologously expressed. For this purpose, *E. coli* strains containing either the plasmid p*chrA*$_{SO}$ or the pBAD33 vector were

grown in the presence of various concentrations of chromate (0, 0.2, 0.4, 0.8 and 1.2 mM) and the time course of growth was followed by measuring the OD_{600} of the different cultures. To avoid toxicity due to $ChrA_{SO}$ overproduction, cultures were performed at 30˚C without the inducer arabinose (for details see the Materials and methods section). As shown in Fig 5A, when the initial concentration of chromate is 0.2 mM, growth of both strains is similar during the first 8 hours. Then, cells containing the p*chrA*$_{SO}$ continue to grow whereas cells containing only the vector stop growing. With higher initial concentrations of chromate, the growth remains possible when the p*chrA*$_{SO}$ is present, although it slows down as concentration in chromate increases. In contrast, higher concentrations of chromate have a dramatic impact on cells harboring only the vector. The residual concentration of Cr(VI) was evaluated after 2 and 7 hours of growth. As shown on Fig 5B, the percentage of Cr(VI)-reduction is quickly improved when the cells contain the p*chrA*$_{SO}$ compared to the ones with the vector. Reduction seems to be inefficient after 7 hours of incubation when the vector is present, whereas it is clearly improved in cells harboring the p*chrA*$_{SO}$. These results suggest that $ChrA_{SO}$ has also a positive effect on the reduction efficiency of Cr(VI) in *E. coli*.

We performed *in vitro* chromate reductase activity assays on crude extracts from *E. coli* strains containing either the pBAD33 vector or the p*chrA*$_{SO}$ plasmid and the results show that the specific activity of chromate reductase is similar for both crude extracts (1.1 ± 0.12 and 1.05 ± 0.06 nmol reduced-Cr(VI).min^{-1}.mg^{-1}, respectively), confirming that $ChrA_{SO}$ is not directly involved in Cr(VI)-reduction.

Discussion

A bioinformatics analysis revealed that $ChrA_{SO}$ (SO0986) belongs to the large family of chromate ion transporters. In agreement with this prediction, a *S. oneidensis* strain deleted of the *chrA*$_{SO}$ gene is less resistant to chromate than the wild-type strain. Strikingly, expression of *chrA*$_{SO}$ in *E. coli* makes the strain capable of growing in the presence of high chromate concentrations, as also observed for the plasmid-encoded ChrA of *Shewanella* ANA-3 and for several ChrA of *Burkholderia xenovorans* LB400 [34,35]. Therefore $ChrA_{SO}$ most probably functions as a chromate efflux pump and expulses chromate ions from the cytoplasm, as demonstrated for some members of the CHR family [13,34].

It is noteworthy that, in the presence of $ChrA_{SO}$, the percentage of Cr(VI) reduction is higher than in its absence in both *S. oneidensis* and *E. coli*. A direct role of $ChrA_{SO}$ in Cr(VI) reduction was ruled out, since the specific activity of chromate reductase measured *in vitro* on crude extracts from both *S. oneidensis* and *E. coli* strains is similar in the absence and the presence of $ChrA_{SO}$. One hypothesis to explain this phenomenon is that $ChrA_{SO}$ promotes Cr(VI) reduction by expulsing chromate outside the cells where it can then be reduced. In *S. oneidensis*, this scenario is worth considering as two outer membrane decaheme cytochromes, MtrC and OmcA, are known to be involved in extracellular chromate reduction [21]. Another hypothesis could be that the presence of $ChrA_{SO}$ improves cell survival by lowering intracellular Cr(VI) concentration and consequently Cr-induced damages inside the cells, which allows the cells to grow and reduce Cr(VI) over an extended period of time. This could be true for both *S. oneidensis* and *E. coli*.

This study also shows that, although *chrA*$_{SO}$ is expressed without chromate, the level of expression is higher in its presence. Two previous studies performed on *S. oneidensis* using transcriptomic approaches missed this regulation, probably because the applied cut-offs were quite stringent [19,36]. Indeed, these studies considered only the genes showing at least a 2-fold or a 3-fold change in expression, respectively. Moreover the experimental conditions used in both studies were different from ours, which can also explain this discrepancy. The fact that
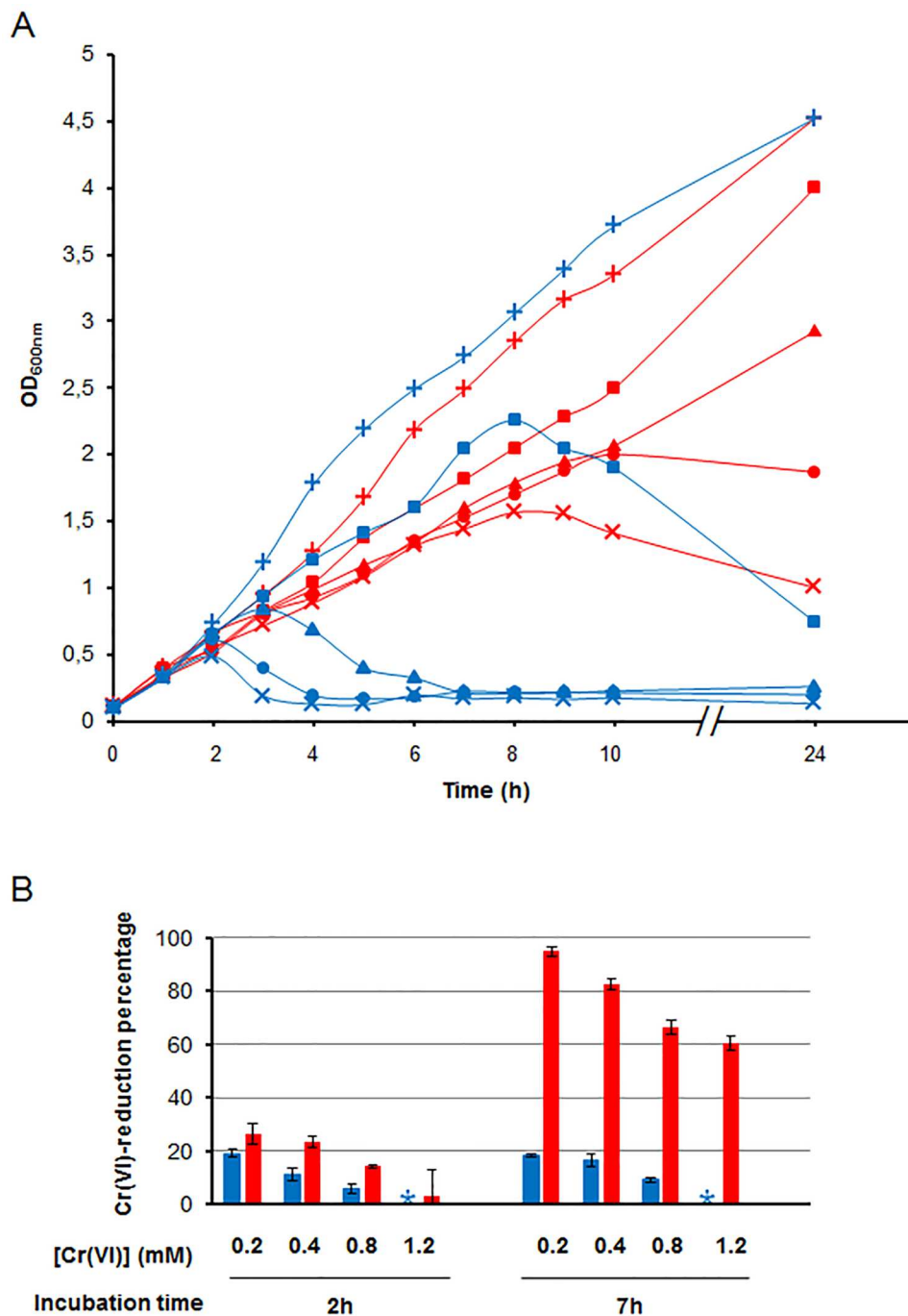
Fig 5. Chromate resistance and reduction by ChrA$_{SO}$ in *E. coli*. (A) Chromate resistance due to the expression of *chrA$_{SO}$* in *E. coli* was assessed by comparing the growth of MC1061/p*chrA$_{SO}$* (red lines) to that of MC1061/pBAD33 (blue lines) in the presence of various concentrations of chromate (+, 0 mM; ■, 0.2 mM; ▲, 0.4 mM; ●, 0.8 mM and ×, 1.2 mM) at 30˚C. Values are means from at least three experiments. (B) Chromate reduction by MC1061/p*chrA$_{SO}$* (red bars) and MC1061/pBAD33 (blue bars) was evaluated as the percentage of chromate reduced after 2 hours and 7 hours of challenge. [Cr(VI)], concentration of chromate added before growth expressed as mM; * indicates that the amount of chromate reduced is below detection level. Values are means ± standard deviations (error bars) from at least three experiments.

chrA$_{SO}$ expression is chromate-induced is reminiscent of what was observed for the expression of several *chr* genes in other bacteria. For example, in *C. metallidurans,* transcription of the plasmidic *chrA1* gene was shown to be up-regulated by chromate [12]. In *B. xenovorans* LB400, which possesses six genes encoding chromate ion transporters, only the *chrA2* gene present on a megaplasmid is subject to chromate-induced expression [35]. Interestingly, it was recently demonstrated that ChrA2 is the major determinant of chromate resistance in this organism [37]. The *chrBACF* operon of *Ochrobactrum tritici* 5bvl1, which is located in a chromosomally integrated transposon, proved also to be chromate-induced [38]. It was subsequently shown that the ChrB protein is a transcriptional regulator that binds to the *chrBACF* promoter region to regulate its expression [39]. As mentioned in the result section, there is no ChrB homolog in *S. oneidensis*, meaning that the regulation of *chrA*$_{SO}$ expression probably depends on a regulatory protein belonging to a family different from that of ChrB. It will be interesting to identify this protein, since no chromate-sensing regulator except from ChrB was previously described.

In conclusion, although ChrA$_{SO}$ plays a key role in chromate resistance in *S. oneidensis*, other transporters could be involved in this process. Of interest, genes encoding the three components of a putative heavy metal efflux pump (SO0518 to SO0520, CzcCBA) were observed to be up-regulated by chromate [19]. This suggests a role in chromate resistance, although this pump does not belong to the CHR family.

Supporting information

S1 Table. Plasmids and primers used in this study.
(TIF)

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Author Contributions

Conceptualization: Hiba Baaziz, Cyril Gambari, Anne Boyeldieu, Radia Alatou, Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

Data curation: Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

Formal analysis: Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

Funding acquisition: Radia Alatou, Vincent Méjean.

Investigation: Hiba Baaziz, Cyril Gambari, Anne Boyeldieu, Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

Methodology: Hiba Baaziz, Cyril Gambari, Anne Boyeldieu, Amine Ali Chaouche, Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

Project administration: Vincent Méjean.

Supervision: Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

Validation: Radia Alatou, Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

Writing – original draft: Hiba Baaziz, Cyril Gambari, Anne Boyeldieu, Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

Writing – review & editing: Vincent Méjean, Cécile Jourlin-Castelli, Michel Fons.

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A. Kinetic growth

B. Kinetic of chromate reduction

Figure 23. Growth and chromate reduction kinetics of $\Delta chrA_{SO}$ under semi-aerobiosis.
MR-1/pBAD33 (———), $\Delta chrA_{SO}$/pBAD33 (———), $\Delta\ chrA_{SO}$ /p$chrA_{SO}$ (– ▪ ·), MR-1/p$chrA_{SO}$ ($\cdots\times\cdots$). The black arrow indicates addition of 1mM final concentration of chromate.

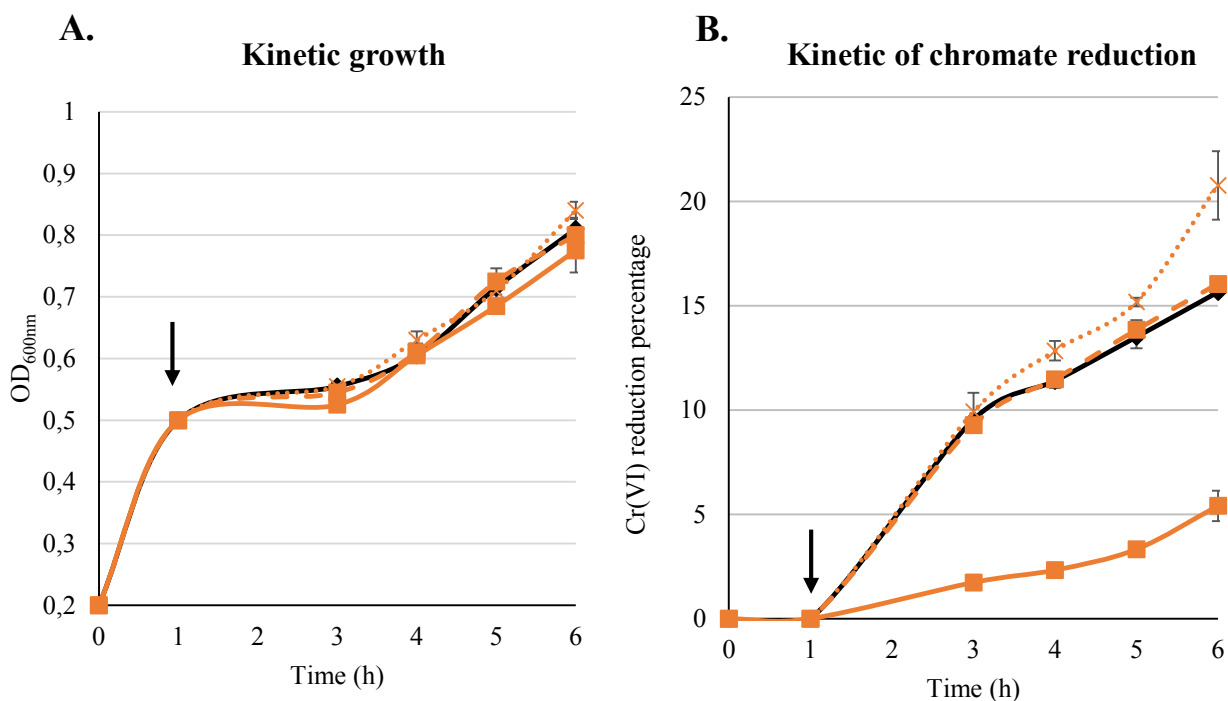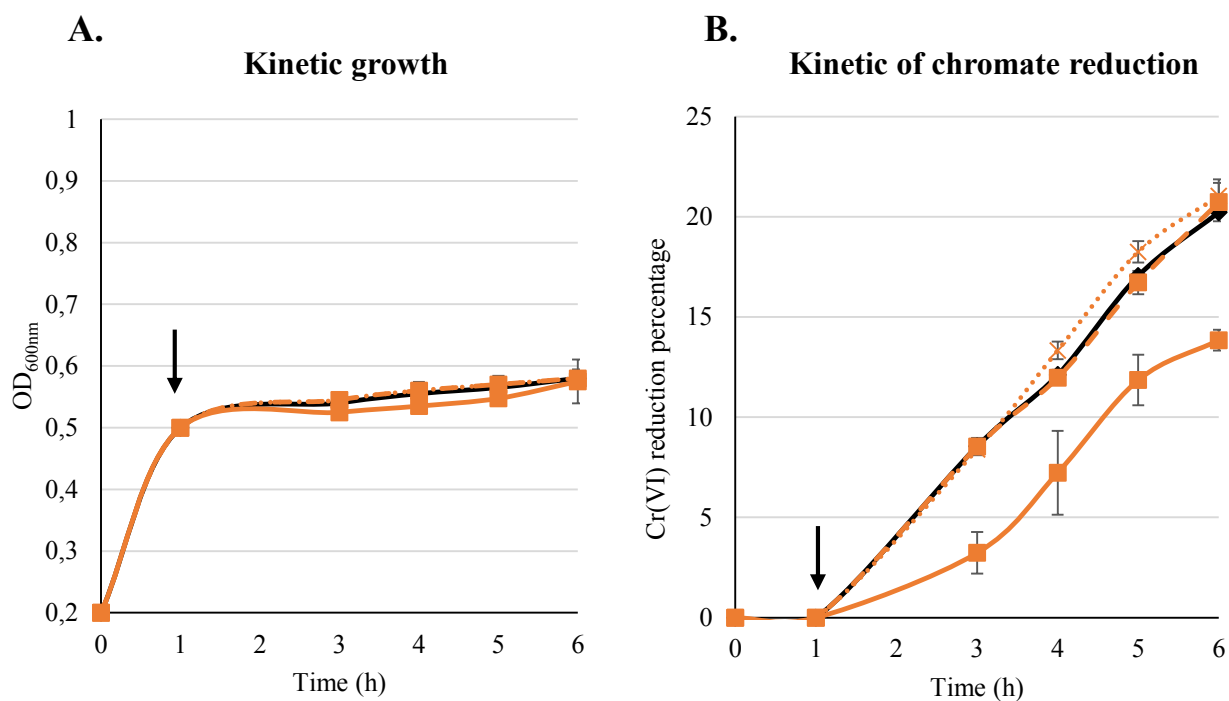**A.** Kinetic growth

B. Kinetic of chromate reduction

Figure 24. Growth and chromate reduction kinetics of $\Delta chrA_{SO}$ under anaerobiosis.
MR-1/pBAD33 (———), $\Delta chrA_{SO}$/pBAD33 (———), $\Delta\ chrA_{SO}$ /p$chrA_{SO}$ (– ▪ ·), MR-1/p$chrA_{SO}$ ($\cdots\times\cdots$). The black arrow indicates addition of 1mM final concentration of chromate.

1.2. Additional results

In addition to the results reported in article 1, we investigated the impact of *chrA_{SO}* deletion on the ability of *S. oneidensis* MR-1 to resist and reduce chromate over time, by using a similar method to that described in detail in Article 1. The wild-type strain (*S. oneidensis* MR-1/pBAD33), *chrA_{SO}* deleted strain (Δ*chrA_{SO}* /pBAD33) and *chrA_{SO}* mutant harboring p*chrA_{SO}* (Δ*chrA_{SO}*/p*chrA_{SO}*) were incubated in LB medium supplemented with 1mM of chromate under semi-aerobic and anaerobic conditions for 5 hours at 28°C. The time course of growth was followed by measuring the OD_{600nm} of cultures to investigate strains survival under chromate, while the chromate reduction ability was evaluated by measuring the residual Cr(VI) concentration each hour starting from two until five hours of incubation in the presence of chromate. The latter was added when cells reached an OD_{600nm} of 0.5.

During the first hours of incubation, the absence of *chrA_{SO}* does not seem to alter the growth in the presence of chromate under semi aerobic conditions. In contrast, the growth of both wild-type and mutant seems to be affected in the presence of chromate under anaerobic conditions (Figure 23A and 24A). Moreover, the ability of the *chrA_{SO}* deleted strain to reduce chromate remains dramatically impaired within 5h of incubation in both semi-aerobic and anaerobic conditions (Figure 23B and 24B). We noticed that the impact of the *chrA_{SO}* deletion is more pronounced in semi-aerobiosis, where the chromate reduction of *chrA_{SO}* deleted strain is about 5-fold lower than that of the wild-type (Figure 23B). However, in anaerobic conditions chromate reduction of the mutant strain is only 2-fold lower than that of the control strain (Figure 24B), which indicates that the chromate efflux pump ChrA_{SO} plays a major and indirect role in chromate reduction response of *S. oneidensis* MR-1 especially under semi-aerobic conditions.

As expected, the complemented mutant Δ*chrA_{SO}*/p*chrA_{SO}* is able to reduce efficiently chromate as well as the wild type strain under both incubation conditions. Moreover, the overexpression of *chrA_{SO}* in the wild-type strain seems to improve slightly the reduction efficiency of *S. oneidensis* MR-1 under both incubation conditions (Figure 23B and 24B).

A.

Kinetic growth

B.

Kinetic of chromate reduction
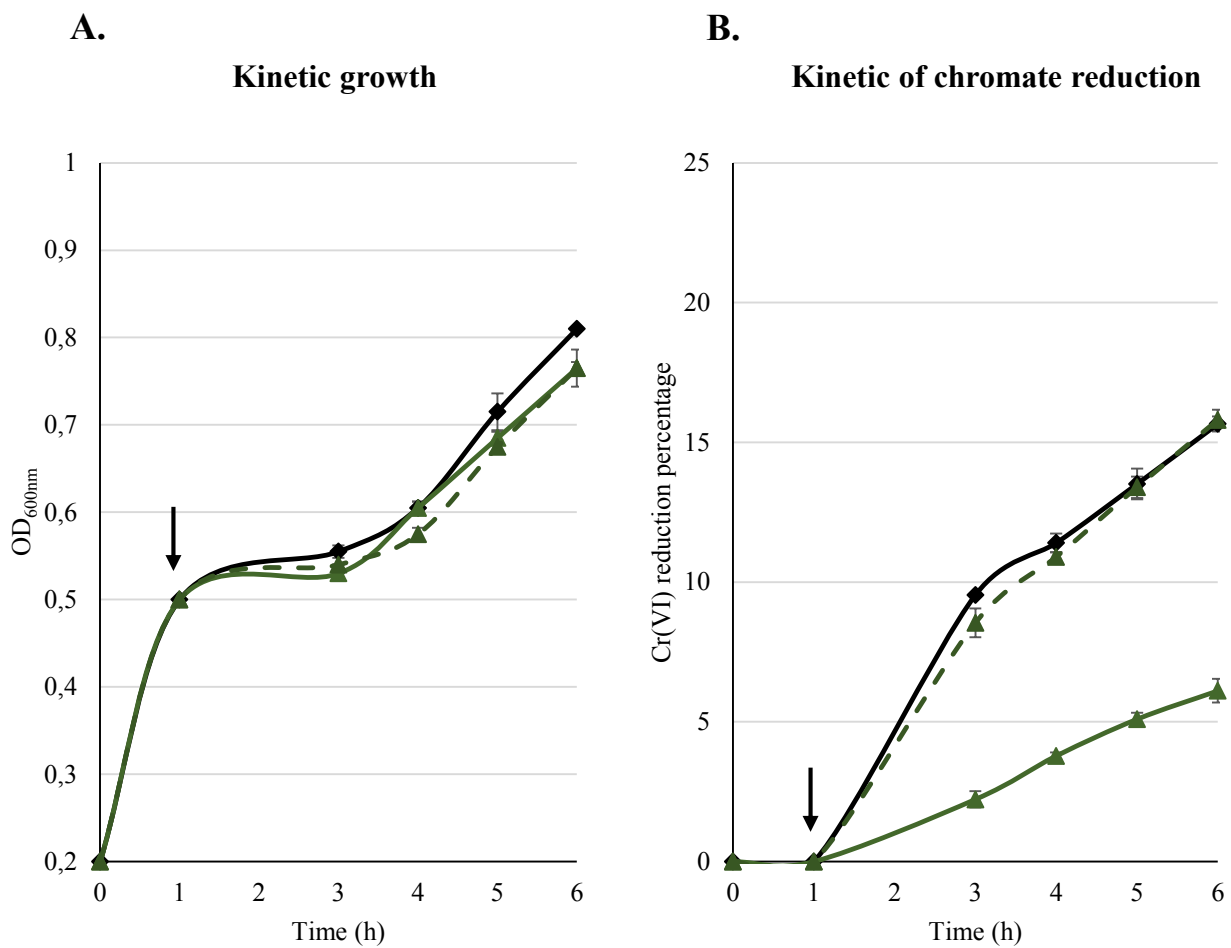
Figure 25. Growth and chromate reduction kinetics of Δ*fdh* under semi aerobiosis.
MR-1/pBAD33 (——◆——), Δ*fdh*/pBAD33(——▲——), Δ*fdh*/p*fdh* (– ▲ ·). The black arrow indicates addition of 1mM final concentration of chromate.

1.3. The involvement of a formate dehydrogenase in chromate reduction in *S. oneidensis* MR-1

The figure 19B shows that a putative formate dehydrogenase coding gene (*fdh*) is located close to the *chrA_SO* and to *SO0987* encoding an MCP known to detect chromate. We then wondered if the Fdh could play a role in chromate resistance and reduction. We therefore constructed a MR-1 mutant deleted of the *fdh* gene. Using a strategy similar to that developed when we studied the role of ChrA$_{SO}$, the *fdh* deleted strain and Δ*fdh* mutant harboring p*fdh* (Δ*fdh*/p*fdh*) were incubated with 1mM of chromate under semi-aerobic and anaerobic conditions for 5 hours at 28°C. The time course of growth was followed by measuring the OD$_{600nm}$ of the strains culture and the residual Cr(VI) concentration was measured each hour starting from two until five hours of incubation with chromate added when cells reached an OD$_{600nm}$ of 0.5.

The growth of the strains in the presence of chromate under semi-aerobic (Figure 25A) and anaerobic conditions was similar to that of the wild-type which indicates that the absence of the *fdh* gene does not affect the survival of *S. oneidensis* MR-1 during the first 5 hours of incubation in the presence of chromate. Under anaerobic conditions, Δ*fdh* seems to reduce chromate as efficiency as the wild-type strain indicating that the absence of *fdh* gene does not affect the chromate reduction ability of *S. oneidensis* MR-1 in anoxic conditions (Data not shown). Nevertheless, under semi-aerobiosis, the chromate reduction ability of Δ*fdh* is impaired. Δ*fdh* strain reduces chromate 3-fold lower than the wild-type, which shows the possible implication of the formate dehydrogenase in chromate reduction mechanisms of *S. oneidensis* MR-1 (Figure 25B).

1.4. The involvement of DMSO reductases DmsA1 and DmsA2 in chromate reduction of *S. oneidensis* MR-1

A transcriptomic analysis revealed that more than 80 genes are upregulated when *S. oneidensis* MR-1 is exposed to chromate in anaerobiosis, including the DMSO reductase cluster (*dmsAB-1*) (Bencheikh-Latmani *et al*, 2005). *S. oneidensis* MR-1 has a second gene cluster (*dmsAB*-2) predicted to encode a putative DMSO reductase. However, it was shown that the expression of both clusters is not induced by DMSO. We therefore wondered if the
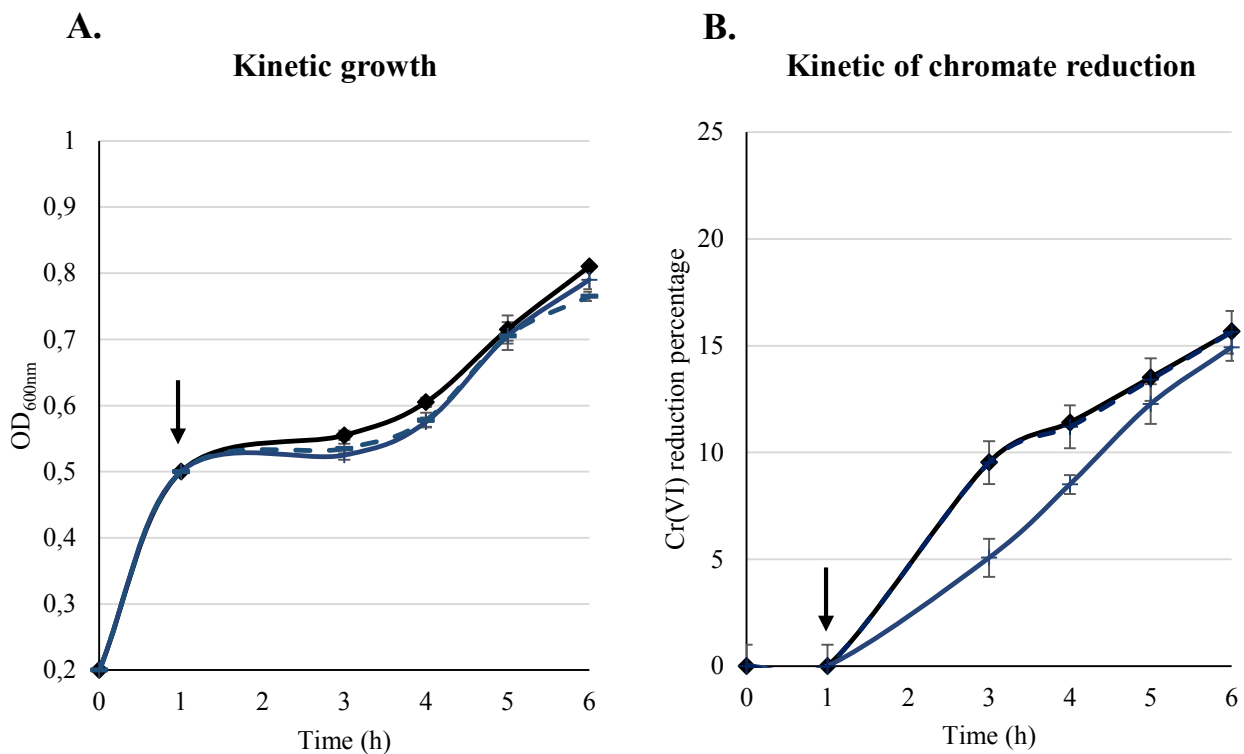
Figure 26. Growth and chromate reduction kinetics of Δ*dmsA1* under semi aerobiosis.
MR-1/pBAD33 (●──), Δ*dmsA1*/pBAD33 (┼──), Δ*dmsA1*/p*dmsA1* (─ ┼). The black arrow indicates addition of 1mM final concentration of chromate.
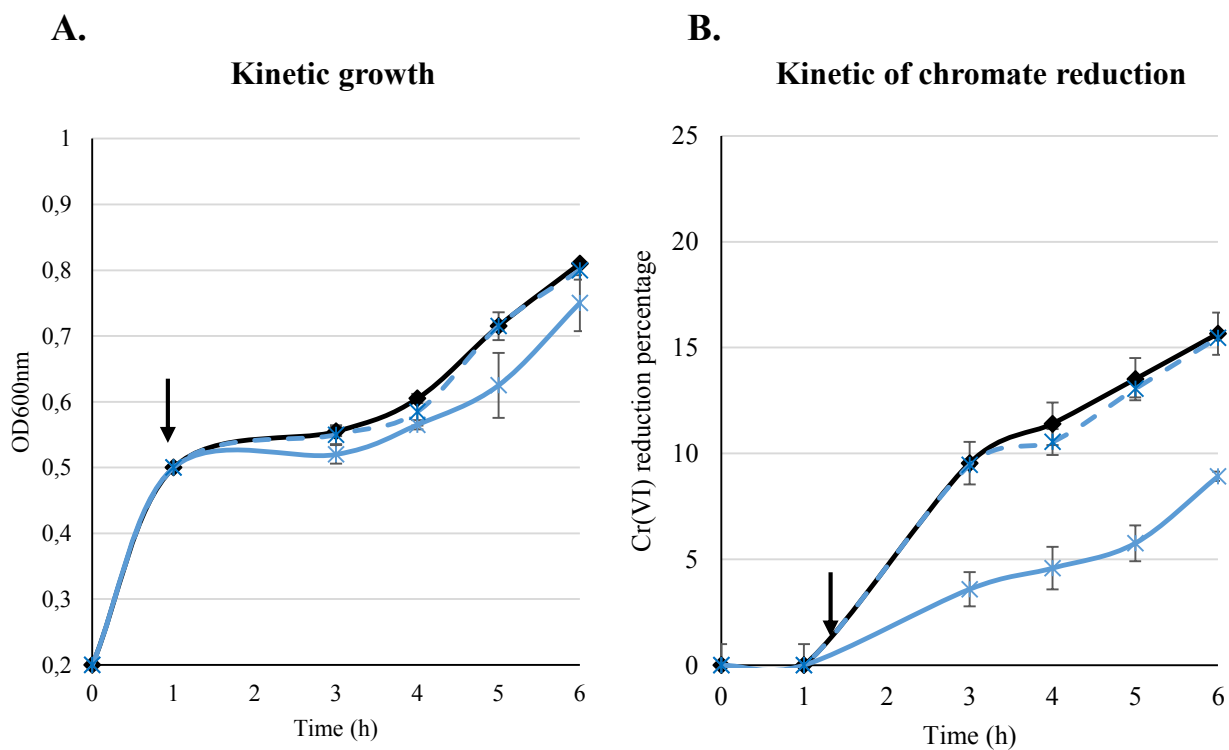
Figure 27. Growth and chromate reduction kinetics of Δ*dmsA2* under semi aerobiosis.
MR-1/pBAD33 (●──), Δ*dmsA2*/pBAD33 (✳──), Δ*dmsA2*/p*dmsA2* (─ ✳). The black arrow indicates addition of 1mM final concentration of chromate.

DmsA1 and DmsA2 play a role in chromate survival and reduction abilities of *S. oneidensis* MR-1. To answer these questions, *S. oneidensis* MR-1 strains deleted of *dmsA1* or *dmsA2* genes, formerly constructed in our laboratory, together with a double mutant Δ*dmsA1*Δ*dmsA2* that we constructed were incubated with 1mM chromate for 5 hours at 28°C under semi-aerobic and anaerobic conditions, in order to investigate their chromate survival and reduction abilities. As previous experiments, the time course of growth was followed by measuring the OD_{600nm} of the strains culture and the residual Cr(VI) concentration was measured each hour starting from two until five hours of incubation with chromate added when cells reached an OD_{600nm} of 0.5.

The results show that in both conditions the growth of Δ*dmsA1*, Δ*dmA2* as well as Δ*dmsA1*Δ*dmsA2* is similar to that of the wild-type strain, which means that absence of *dmsA1* and *dmsA2* has no effect on chromate survival of *S. oneidensis* MR-1 in semi-aerobic as well as in anaerobic conditions during the first hours of incubation (Results in anaerobiosis are not shown). Concerning the chromate reduction efficiency, in anaerobic conditions all mutants show a chromate reduction rate similar to that of the wild-type, which indicates that DmsA1 and DmsA2 are not directly involved in chromate reduction of *S. oneidensis* MR-1 in anaerobiosis. However, under semi-aerobic conditions, the chromate reduction ability of all mutants is affected. Δ*dmsA1* mutant seems to be transiently affected with a chromate reduction capacity of about one half of that of the wild-type strain 2 hours after the addition of Cr(VI) (Figure 26B). With regard to the Δ*dmsA2* mutant, the level of Cr(VI) reduction remains 3-fold lower than that of the control strain during the experiment (Figure 27B), which indicates the implication of the corresponding protein in chromate reduction mechanisms of *S. oneidensis* MR-1 during the first hours of incubation in semi-aerobiosis. These results show that DmsA2 seems to play a more important role of Cr(VI) reduction than DmsA1.

1.5. The involvement of cytochrome-c CymA in chromate reduction of *S. oneidensis* MR-1

As explained earlier in the introduction part, CymA is a central element of many electron acceptor reduction pathways. It transfers electrons to several reductases located in the periplasm and in the outer membrane such as DMSO reductases and Mtr complex. We

A.

Kinetic growth

B.

Kinetic of chromate reduction
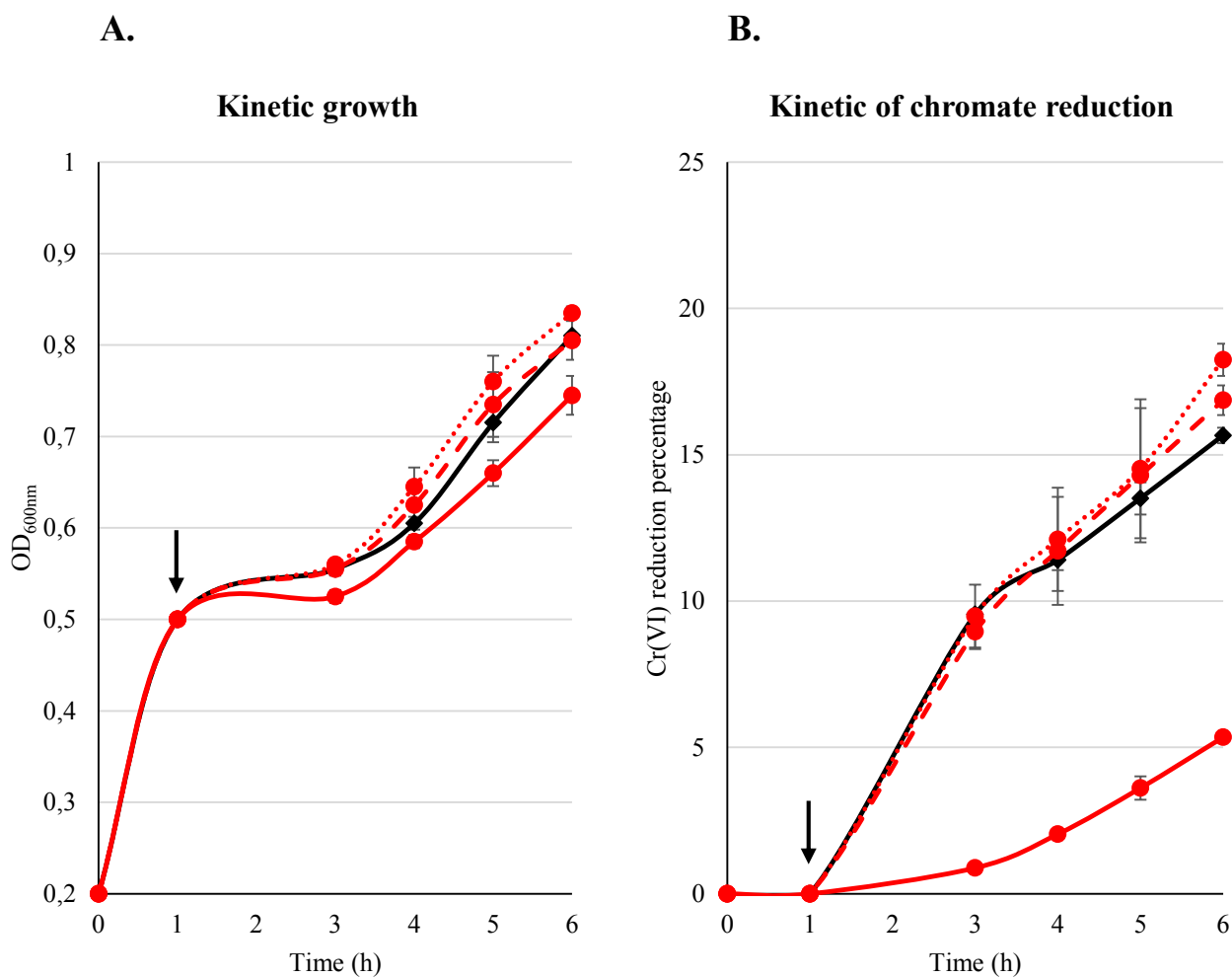
Figure 28. Growth and chromate reduction kinetics under semi aerobiosis.

MR-1/pBAD33 (—●—), Δ*cymA*/pBAD33 (—●—), Δ*cymA*/p*cymA* (– ● ·), MR-1/pcymA (···●···).
The black arrow indicates addition of 1mM final concentration of chromate.

investigated the effect of CymA absence on chromate survival and reduction of *S. oneidensis* MR-1. Therefore *S. oneidensis* MR-1 strain deleted of *cymA* previously constructed in our laboratory together with the mutant complemented strain that we constructed (Δ*cymA*/p*cymA*) were incubated under semi-aerobiosis and anaerobiosis with 1mM chromate added when cells reached OD_{600nm} of 0.5. Growth and chromate reduction kinetics were performed using the same techniques used previously.

Results show that in anaerobic conditions the growth of mutant and the complemented mutant strains is similar to that of the wild-type strain, which mean that the absence of *cymA* does not affect the chromate survival of *S. oneidensis* MR-1 under anoxic conditions (data not shown). However, we noticed that under semi-aerobiosis, the mutant Δ*cymA* shows a slight growth defect in the presence of chromate compared to the wild-type strain. This slight growth defect is restored to the wild-type growth levels when the mutant is complemented (Δ*cymA*/p*cymA*) (Figure 28A). This result indicates that CymA may play a partial role in chromate resistance of *S. oneidensis* MR-1 under semi-aerobic conditions during the first hours of incubation.

Within 5 hours of incubation with chromate, Δ*cymA* shows chromate reduction rates similar to that of the wild-type strain in anaerobiosis which indicates that the absence of *cymA* has no effect on chromate reduction ability of *S. oneidensis* MR-1 in anaerobiosis. Nevertheless, under semi-aerobic conditions, the chromate reduction ability of Δ*cymA* is strongly affected. Δ*cymA* reduces chromate 4-fold lower than the wild-type which reveals that *cymA* plays an important role in chromate reduction of *S. oneidensis* MR-1 in semi-aerobic conditions (Figure 28B). As expected, the complemented mutant is able to reduce chromate as efficiently as the wild-type strain. Moreover, the overexpression of *cymA* in the wild-type strain does not seem to highly improve the reduction efficiency of *S. oneidensis* MR-1. CymA may be probably involved in electron transfer to different terminal reductases playing a partial role in Cr(VI) reduction.

PART II:

ISOLATION AND

CHARACTERIZATION OF NEW

Shewanella STRAINS

Figure 29. Maps showing the location of Stora harbor (36°54'06.9"N 6°52'45.4"E) . It is situated in the northeast of Algeria in the Sekikda province. Pictures are taken from Google earth (2018).

1. Isolation and identification of new *Shewanella* species

Shewanella species can be found in different types of environments. Most of strains whose genome sequence is available are from marine environment but only *Shewanella woodyi* ATCC 51908 was originally isolated from the Mediterranean Sea. The aim of the second part of this thesis was to isolate and characterize new *Shewanella sp.* strains from the Algerian coast and investigate their chromate survival and reduction abilities. To this end, ten samples of seawater and sediments coming from different harbors and beaches were collected (Table 5, Material and Methods).

The strategy that we developed in order to isolate *Shewanella* cells present in samples, is to favorise their growth and to multiply their amount by performing successive enrichment cultures of samples in LB medium supplemented with TMAO and NaCl to mimic marine environment conditions. Colonies presenting similar phenotypic aspects of *Shewanella* species were selected and further subjected to PCR amplification using the primer set She211F and She1259R that specifically targets *Shewanella* 16SDNA. Among the numerous clones verified by PCR, only eight selected at the third culture enrichment of sediment water coming from Stora harbor (Figure 29), gave a 1kb amplicon that was further subjected to sequencing. Their analysis revealed that, six of them display high identity percentage with that of *Pseudomonas* species. The nucleotide sequence of 16SDNA of the two left isolates, H76 and H111, show the highest degree of identity with *Shewanella* species. The H76 isolate exhibits 99% identity with *Shewanella fidelis* Strain KMM 3582 (Gene Bank accession number: NR_025195.1) and *Shewanella schlegeliana* strain HRKA1 (NR_024792.1), respectively. With respect to the isolate H111, 99% identity is found with *Shewanella algidipiscicola* strain S13 (NR_041297.1), *Shewanella algidipiscicola* strain NBRC 102032 (NR_114023.1) and *Shewanella colwelliana* strain ATCC 39565 (NR_043074.1), respectively.

To confirm the identification of H76 and H111 strains as *Shewanella fidelis* and *Shewanella algidipiscicola* strains, respectively, the *gyr*B genes of both strains were partially amplified by PCR using the specific set of primers UP2R and UP1, prior to sequencing. The

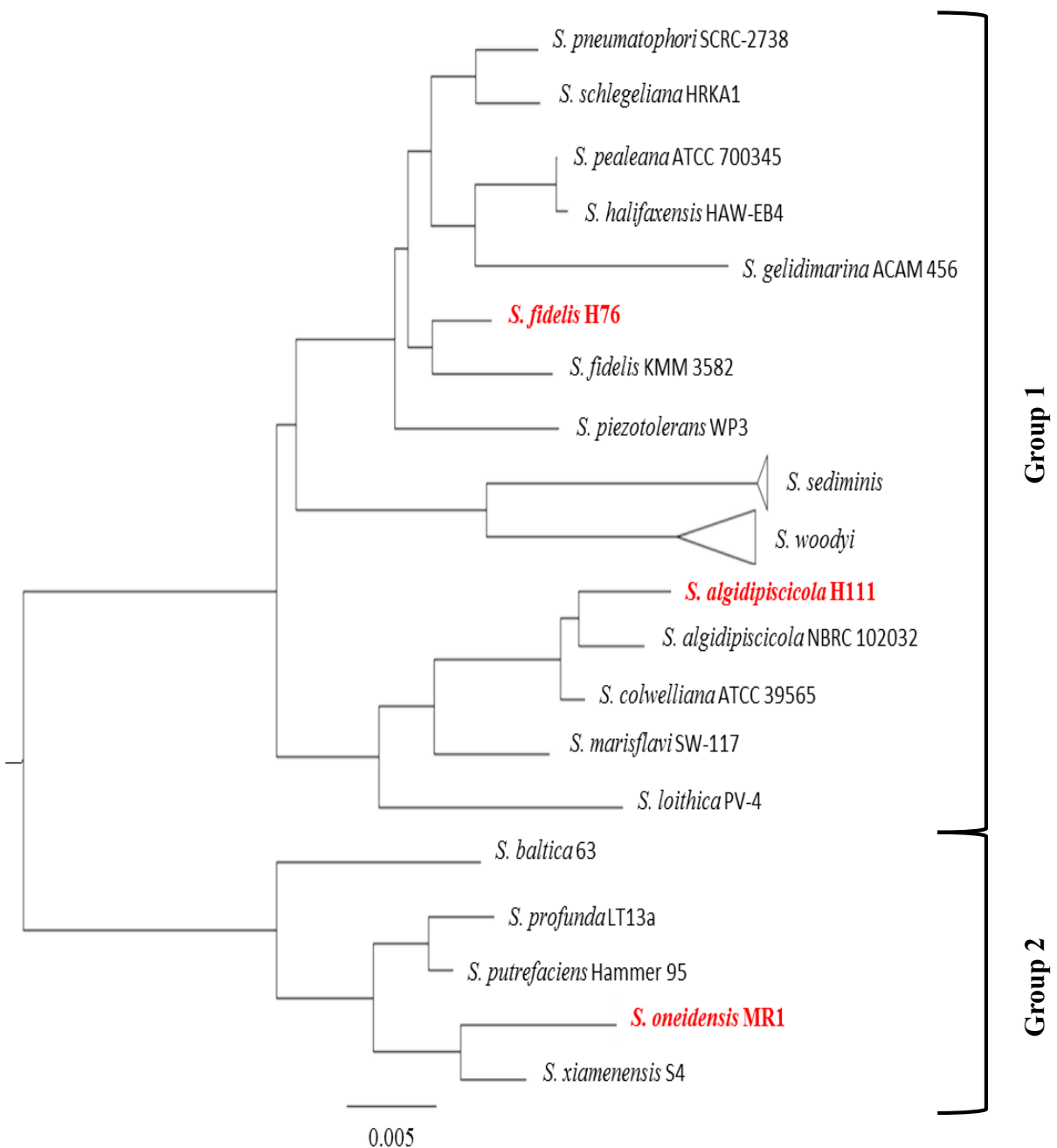**Figure 30. 16SDNA phylogenetic tree** showing the position of *S. fidelis* H76, *S. algidipiscicola* H111 and closely related *Shewanella* species including *S. oneidesnsis* MR-1. The group of *S. sediminis* includes HAW EB3 and DSM 17055 strains. The group of *S. woodyi* includes NR_074846, ATCC 51908 and MS32 strains. The group one and two represent the main *Shewanella* groups. The scale bar indicates a genetic distance of 0.005.

sequences obtained were compared with the NCBI gene bank database using the BlastP search program. The sequence homology search shows that, GyrB of H76 strain display the highest degree of homology (99%) with that of *Shewanella fidelis* Strain KMM 3582 (NR_025195.1) that was firstly isolated from sediments of the South China Sea in 2003 (Lee *et al*, 2016). The GyrB primary structure of H111 strain is identical to that of *Shewanella algidipiscicola* strain NBRC 102032 (NR_114023.1) previously isolated from marine fish (cod and plaice) caught in the Baltic Sea of Denmark in 2007 (Ivanova, 2003). The strains H76 and H111 were then identified as *Shewanella fidelis* and *Shewanella algidipiscicola*, respectively. These results were confirmed later when we could access to the draft genome of both strains.

The 16SDNA sequences of some of the nearest neighbours of H76 and H111 together with the reference strain MR-1 were used for the construction of the phylogenetic tree by MOLE BLAST (http://blast.ncbi.nlm.nih.gov/*moleblast*/*moleblast*.cgi) to evaluate their evolutionary origin and to show the genetic relatedness between H76 and H111. Unlike the reference strain MR-1 that is a member of the second group enclosing pressure sensitive and mesophilic species, both H76 and H111 strains belong to the first group of *Shewanella* that usually contains piezotolerant and psychrotolerant species. Furthermore, the two strains are present in different sub-groups, despite the fact that they were isolated from the same sample (Figure 30).

2. Growth characteristics

During the isolation procedure of the novel strains *S. fidelis* H76 and *S. algidipiscicola* H111, we noticed that both are not able to grow in regular LB medium. They require a final concentration of NaCl higher than 5g/l that is usually found in LB medium to grow. In order to determine the favorable growth conditions for *S. fidelis* H76 and *S. algidipiscicola* H111, cells of both strains were cultivated aerobically at 28°C in LB medium supplemented with different concentrations of NaCl (15, 20, 25 and 30g/l). The time course of growth was followed by measuring the OD_{600nm} of cultures.
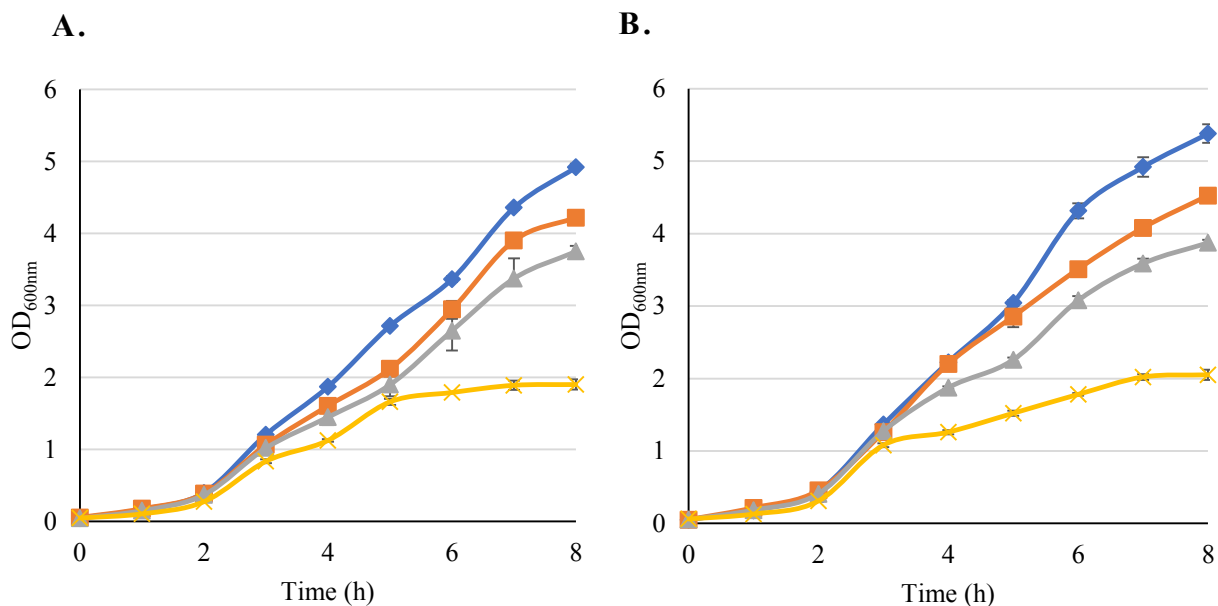
Figure 31: Growth kinetic of H76 (A) and H111(B) *Shewanella* strains at 28°C and under different NaCl concentrations at 28°C. 15g/l of NaCl ( ), 20g/l of NaCl ( ), 25g/l of NaCl ( ) and 30g/l of NaCl ( ).
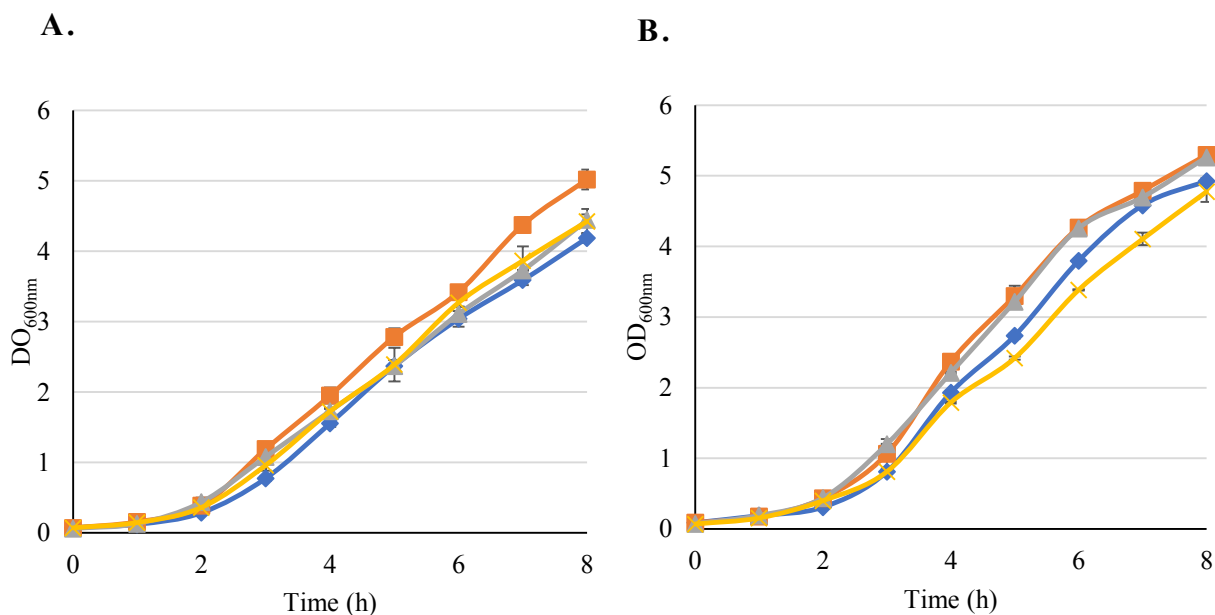
Figure 32: Growth kinetic of H76 (A) and H111(B) *Shewanella* strains at different temperatures, 25°C ( ), 28°C ( ), 30°C ( ) and 35°C ( ).

The kinetic of growth shows that both strains are able to grow in the presence of 20g/l, 25g/l and 30g/l of NaCl in culture medium with an optimum growth at 15g/l of NaCl. However, results reveal impairment in growth of both strains in the presence of 30g/l of NaCl (Figure 31).

We investigated also the temperature range of growth of *S. fidelis* H76 and *S. algidipiscicola* H111. Therefore, strains were cultured aerobically in liquid LB medium supplemented with NaCl (15g/l) at different temperatures 25°C, 28°C, 30°C and 35°C. The time course of growth was followed by measuring the OD_{600nm} of the strains culture. Results Show that *S. fidelis* H76 and *S. algidipiscicola* H111 are capable of growing in temperatures ranging from 25°C to 35°C (Figure 32).
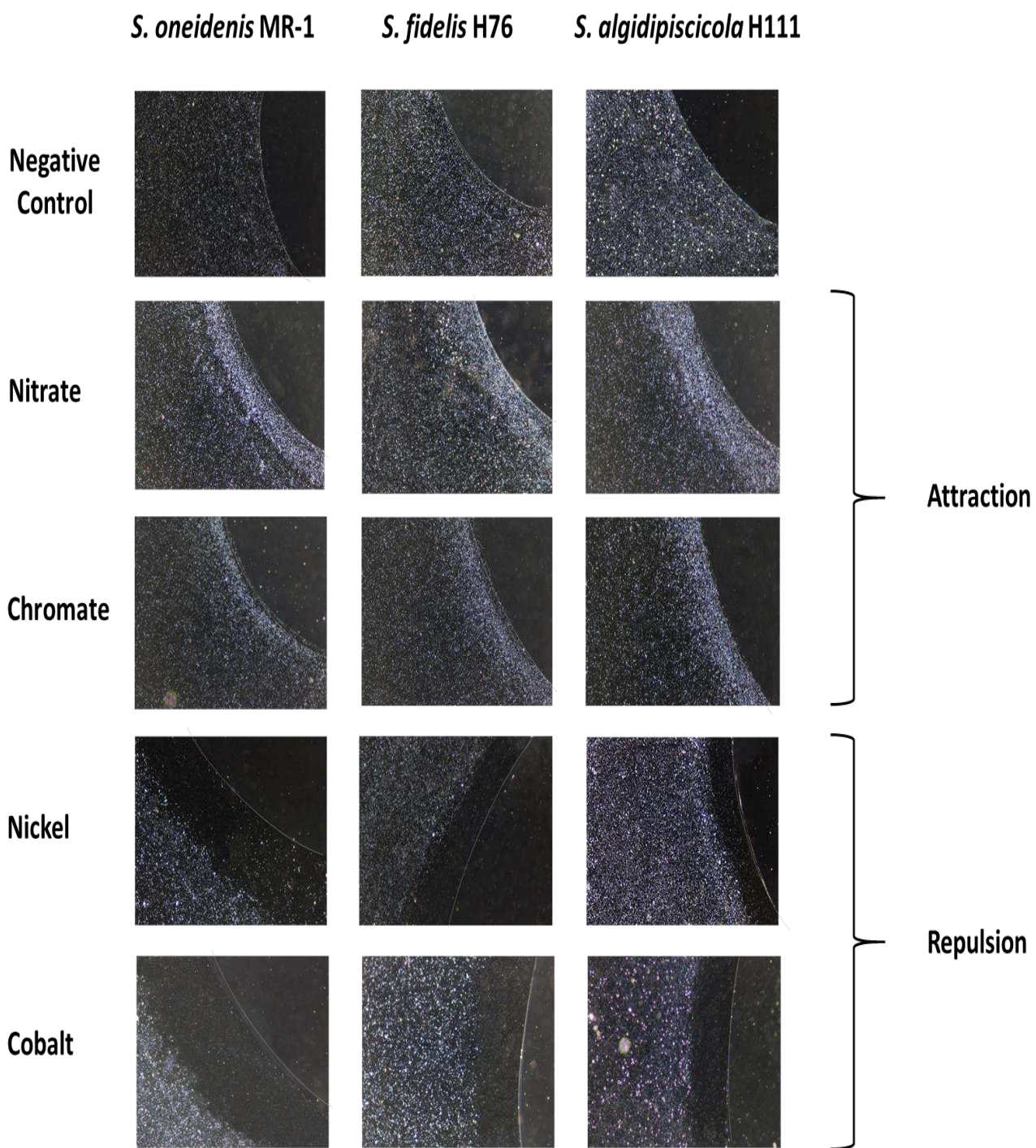
Figure 33: Chemotactic behavior of *S. oneidensis* MR-1, *S. fidelis* H76 and *S. algidipiscicola* H111 toward nitrate, chromate, nickel and cobalt.

3. Chemotaxis behavior of *S. fidelis* H76 and *S. algidipiscicola* H111

It is known that *S. oneidensis* MR-1 is chemotactic towards many compounds. We sought the chemotactic response of the two-novel isolated *Shewanella* strains *S. fidelis* H76 and *S. algidipiscicola* H111 towards nitrate, chromate, nickel and cobalt using the technique of microplug (μPlug) assay described in Material and Methods.

Results show that *S. fidelis* H76 and *S. algidipiscicola* H111 have a chemotactic behavior similar to that of *S. oneidensis* MR-1. For all strains, cells accumulate and appear as a white halo around the plugs containing nitrate and chromate indicating that *S. fidelis* H76 and *S. algidipiscicola* H111 as well as *S. oneidensnsis* MR-1 are attracted to nitrate and chromate. However, the halos around the plugs containing nitrate are slightly more intense than those observed around plugs containing chromate. In contrast, a repulsion zone surrounded by a faint accumulation of cells is observed around plugs containing nickel and cobalt indicating that both strains are repulsed by theses metals (Figure 33).

4. Chromate resistance of *S. fidelis* H76 and *S. algidipiscicola* H111

The main reason for isolating new *Shewanella* strains was to investigate their chromate resistance and reduction capacities. As a preliminary test to determine a range of Cr(VI) concentrations in which growth of *S. fidelis* H76 and *S. algidipiscicola* H111 could occur under semi-aerobic conditions, cells were cultured in LB medium supplemented with different chromate concentrations (0.2, 0.5, 1, 2 and 3mM).

As shown in Figure 34, *S. fidelis* H76 and *S. algidipiscicola* H111 growth that occurs at 0.2mM of chromate is similar to that of the chromate-free culture. Moreover, contrary to MR-1 that shows a 40% decrease in cells survival rate in the presence of 0.5mM of chromate, the growth and of H76 and H111 cells does not seem to be highly altered under semi-aerobiosis (Figure 34 and 35). These results indicate that H76 and H111 strains strongly resist to chromate concentrations lower than 0.5mM, better than MR-1 strain. MR-1, H76 and H111 strain in the presence of 1mM of chromate show a cell survival rates of 30%, 40% and 60%, respectively, which indicates that *S. algidipiscicola* H111 present a better growth in the presence of 1mM of chromate compared to *S. fidelis* H76 and to *S. oneidensis* MR-1 (Figure
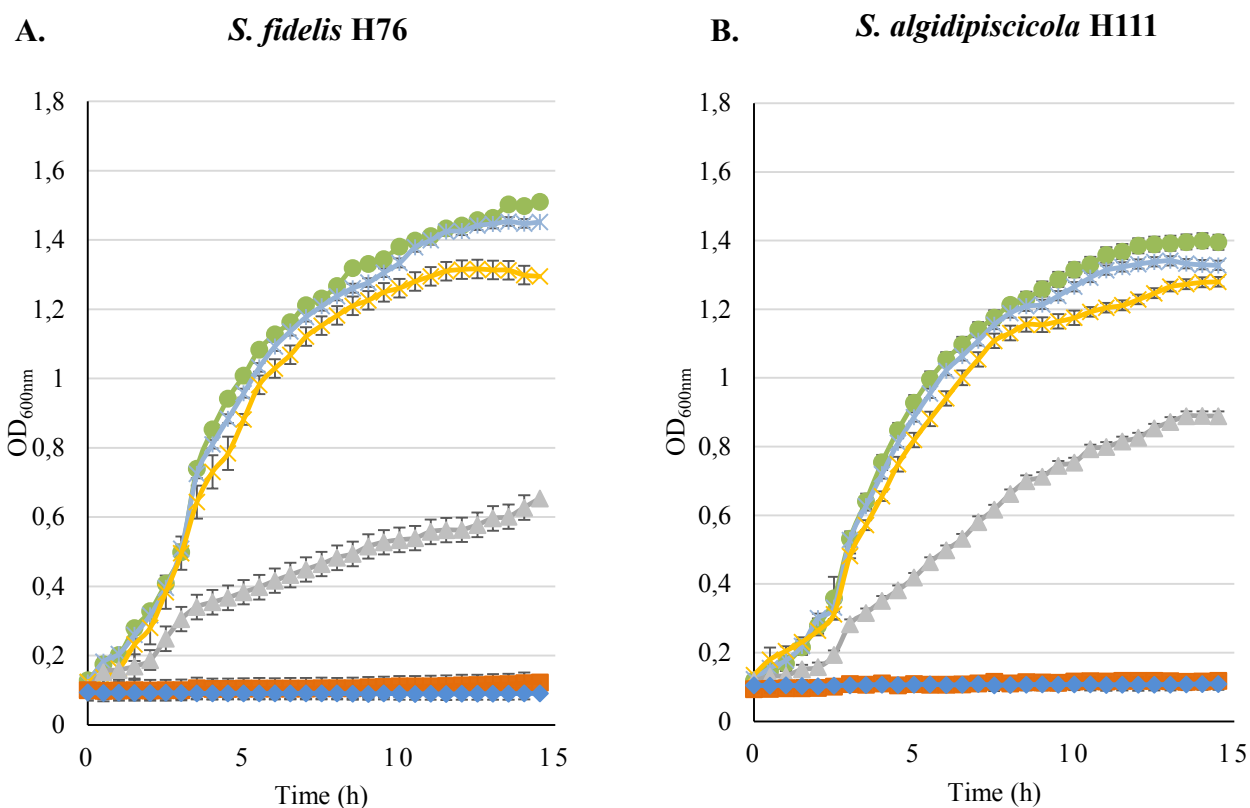
Figure 34. Growth kinetic of *S. fidelis* H76 (A), *S. algidipiscicola* H111 (B) under semi-aerobic conditions in the presence of increasing concentrations of chromate: 0mM (━●━), 0,2mM (━✳━), 0,5mM (━✕━), 1mM (━▲━), 2mM (━■━) and 3mM (━◆━).
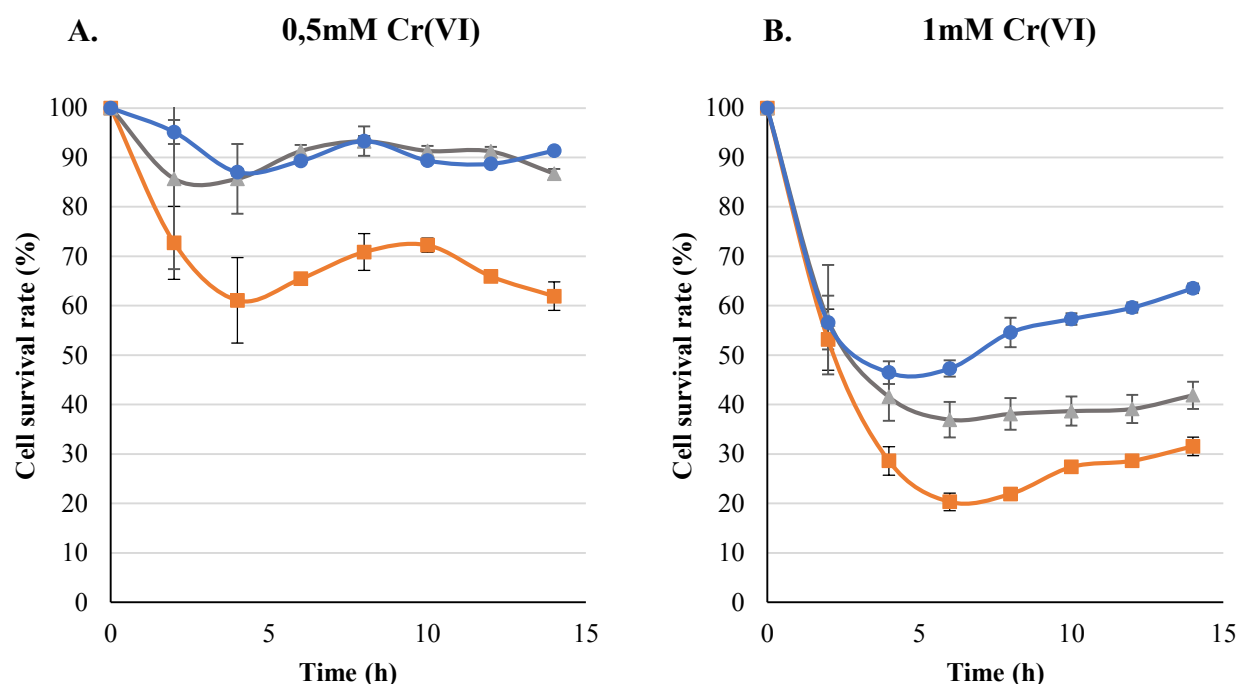
Figure 35. Survival rate of MR-1 (━■━), H76 (━▲━) and H111 (━●━) in the presence of 0,5mM (A) and 1mM (B) of chromate under semi-aerobic conditions. The percentage of cell survival represents the optical density of chromate free culture compared to that of culture in the presence of chromate: $(OD_{600nm} - Cr(VI)/ OD_{600nm} + Cr(VI))*100$.

35). Nevertheless, chromate concentrations higher than 2mM seem to highly alter the growth of H76 and H111 strains as well as MR-1 under semi-aerobic conditions (Figure 34).

Subsequently, we investigated the effect of chromate on cell survival of H76 and H111 strains as well as MR-1, in the presence of chromate. To this end, cells with an initial OD_{600nm} of 0.5 were incubated in the presence of various chromate concentrations (0.2, 0.5, 1, 1.5, 2 and 3mM) for 5 hours at 28°C under semi-aerobic conditions. Chromate free cultures of strains served as a negative control. Afterwards, 10-fold serial dilution of each culture was spotted on LB plates. The latter were then incubated 24h at 28°C to allow bacterial growth.

As shown in Figure 36, the viability of the reference strain MR-1 as well as H76 decreases with increasing chromate concentrations. Despite the fact that high chromate concentrations starting from 1mM seem to strongly alter the viability of MR-1 and H76 cells, the latter show a better chromate resistance compared to that of MR-1. Surprisingly, the viability rate in H111 cells is significantly greater than that of MR-1 and H76 cells. The high chromate concentrations starting from 1.5mM does not seem alter the viability of H111 cells, indicating that *S. algidipiscicola* H111 can resist and survive even at high chromate concentrations which strongly confirms our previous results.

S. oneidensis MR-1
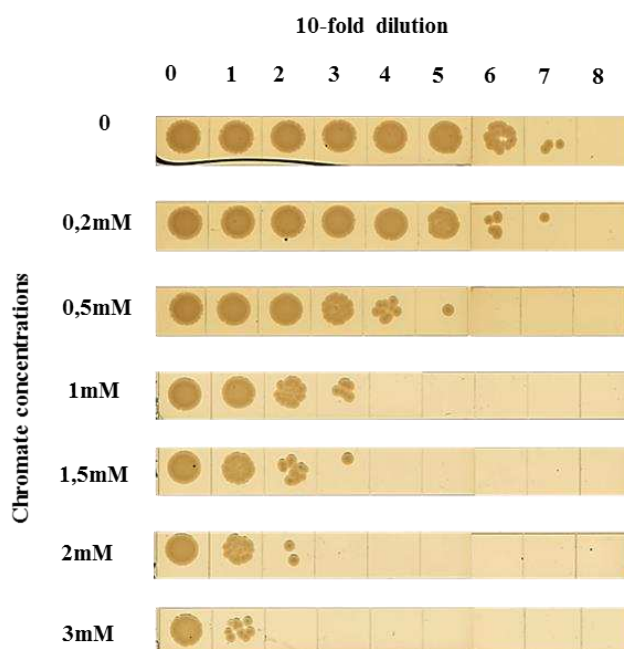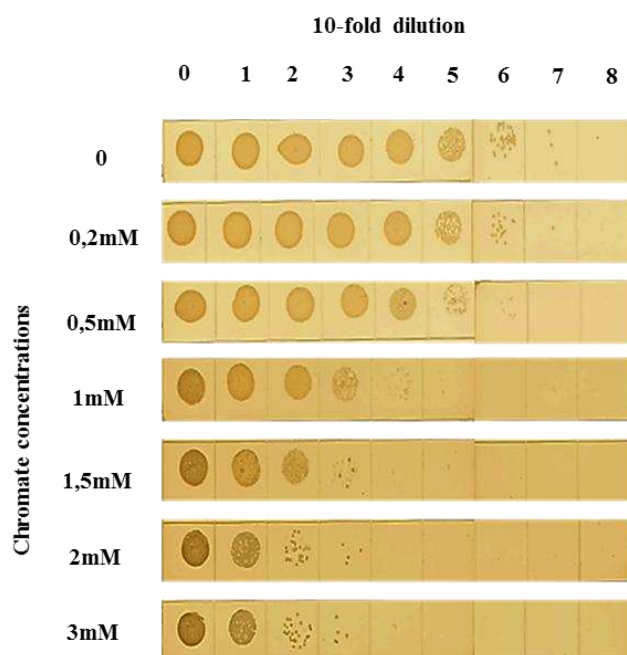
S. fidelis H76

S. algidipiscicola H111
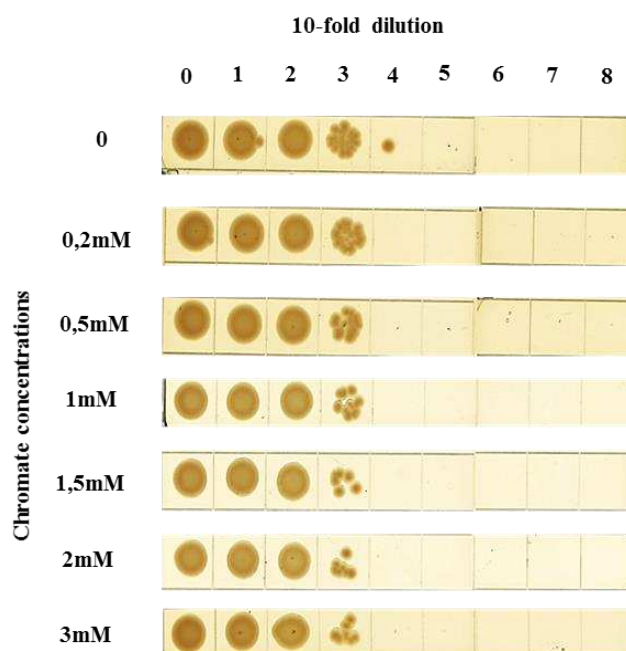
Figure 36. Chromate survival of *S. oneidensis* MR-1, *S. fidelis* H76, and *S. algidipiscicola* H111 after 5h of incubation at 28°C under semi-aerobic conditions, in the presence of various chromate concentrations: 0,2, 0,5, 1, 1.5, 2 and 3mM.

5. Genome analysis of *S. algidipiscicola* H111 and *S. fidelis* H76 strains

The uncommon capabilities of strains H76 and H111 to resist and reduce chromate, prompted us to investigate their genetic characteristics compared to the model strain MR-1. To this end, the whole genome of H111 and H76 strains was sequenced at the "Molecular Research LP (MR DNA) Laboratory" (USA).

As mentioned previously in the introduction, in aerobic conditions, resistance to chromate is mainly due to chromate efflux pumps (ChrA-like), and to type I (ChrR- and YieF-like) or type II (NfsA-like) chromate reductases. *S. oneidensis* strain MR-1 can express a ChrR-like protein corresponding to an azoreductase which does not seem to be crucial as only the initial rate of chromate disappearance is affected in the Δ*chrR* mutant (Mugerfeld *et al*, 2009a). We demonstrated in the first part of this thesis, that *chrA$_{SO}$* encodes a chromate efflux pump. We also showed that two other genes *fdhA* and *dmsA2* are involved in the chromate resistance phenotype of the strain MR1 when grown in semi-aerobic conditions. In contrary to the limited role of DmsA1.

We focused throughout the genome analysis on the presence of homologous genes (*chrA$_{so}$*, *chrR*, *NfsA*, *fdh*, *dmsA1* and *dmsA2*) that could code for homologous functions previously identified in strain MR-1 to be involved in chromate resistance and/or reduction. The analysis was carried out using the BlastP and the synteny programs of the Mage platform (http://www.genoscope.cns.fr/agc/microscope/home/index.php).

5.1. General features of *S. fidelis* H76 genome

Genome sequencing reveals that H76 genome consists of 4873,941 bases and contains 4,717 predicted genes with a mean G+C% value of 45.70. Among them, 4,552 are potential protein coding genes (CDS) without artefacts, 10 rRNAs and 118 tRNAs.

5.1.1. *chrA*- and *fdh*-like genes

Genomic analysis shows the presence of *chrA*-like (*SFH7-v1-11572*) and *fdh*-like (*SFH7-v1-11571*) genes on the chromosome. Their putative products share 61.46% and 69.18% of identity, respectively with the corresponding proteins of MR-1. As in MR-1, the *chrA*-and
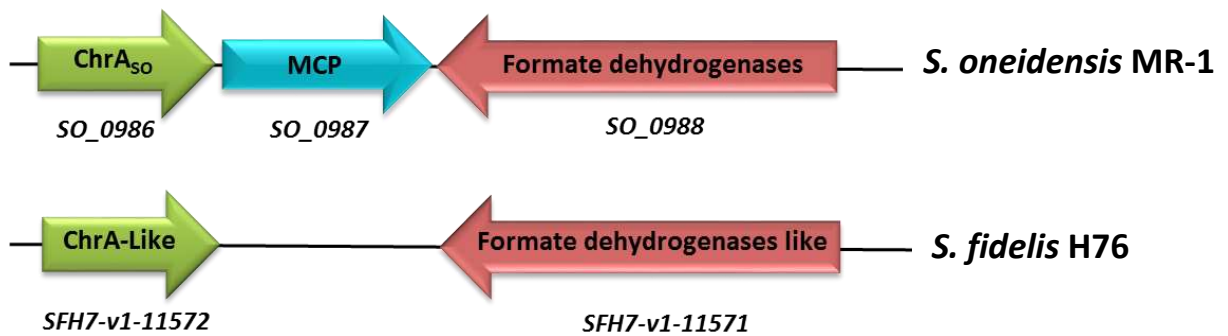
Figure 37. Schematic comparison of the organization of genes coding for *chrA*-like and *fdh*-like proteins in MR1 and H76. ChrA-like and Fdh-like proteins of H76 share 61.46 % and 69.18 % identity, respectively with the corresponding proteins of MR-1.

fdh-like genes are convergent. However, it is noticeable that the gene coding for the chromate sensing MCP is lacking (Figure 37).

5.1.2. *chrR*-like genes

The putative products of two genes, *SFH7_v1_11418* and *SFH7_v1_11419*, share significant identity of 69.48% and 59.82% with the N-terminal and the C-terminal parts of the ChrR-like azoreductase of the strain MR-1, respectively. This suggests that the genes *SFH7_v1_11418* and *SFH7_v1_11419* probably result from a fission event of an initially functional gene or from a sequence artifact. Synteny analysis shows that a part of genes surrounding *SO3585* are also conserved in H76.

5.1.3. *nfsA*-like genes

Despite the absence of the chromate reductase NfsA in the reference strain MR-1, we carried out a BlastP search using the sequence of the chromate reductase NfsA of *E. coli.* The results reveal the presence of the *SFH7_v1_11027* gene coding for a putative protein that presents 57.92% identity with NfsA from *E. coli*. The blastp search using the sequence of *SFH7_v1_11027*, show that the corresponding protein shares high levels of identity with potential gene products of *S. halifaxensis* (91.25%), *S. peleana* (83.33%) and *S. piezotolerans* (72.5%), 3 species phylogenetically close to *S. fidelis* (Figure 30).

5.1.4. *dmsA1*-like and *dmsA2*-like genes

The presence of gene *SFH7_v1_20363* encoding a DmsA1-Like protein that shares 63.46% of identity with that from MR-1 was revealed. Moreover, the synteny analysis shows that the entire region surrounding the *SFH7_v1_20363* gene is highly conserved.

Subsequently, a BlastP analysis was carried out with the *dmsA2* gene product as query. The same *SFH7_v1_20363* gene product was identified exhibiting only a low identity percentage of 32.93%. These results indicate that H76 genome lacks *dmsA2*-like genes.

5.2. General features of *S. algidipiscicola* H111 genome

The genome sequence reveals that H111 genome consists of 4.14 Mb and contains 3,678 potential protein coding genes (CDSs) without artifacts, 13 rRNAs and 90 tRNAs. The
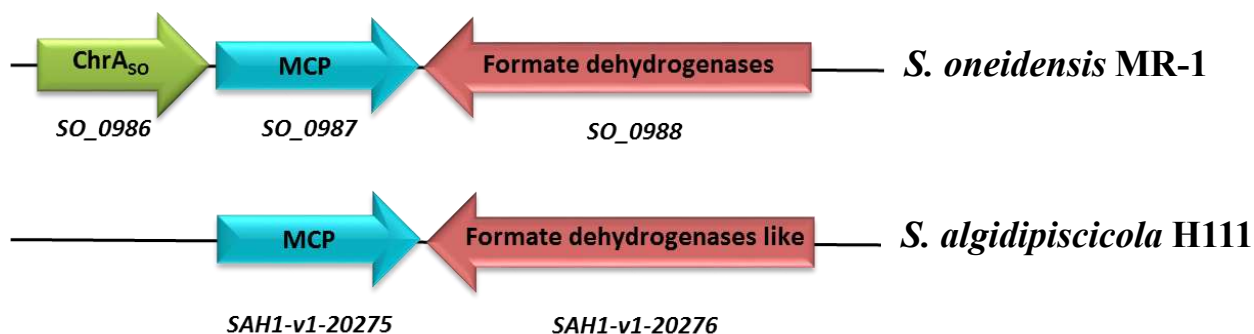
Figure 38. Schematic comparison of the organization of genes encoding Fdh-like proteins in strains MR1 and H111. The MCP and Fdh-like of H111 share 79.8 % and 66.01 % identity respectively with the corresponding proteins of MR1.

Table 1. Identity percentage of proteins present in MR-1, H76 and H111. NI: Not identified.

Proteins	ChrA		Fdh		DmsA1		DmsA2		ChrR (YieF)	
Strains	H76	H111	H76	H111	H76	H111	H76	H111	H76	H111
MR-1	61%	NI	69%	66%	63%	64%	33%	34%	60% + 69%	68%
H76	/	/	/	71%	/	79%	/	/	/	68% + 74%

mean G+C% value is 46.63. *S. algidipiscicola* H111 genome is one of the smallest so far sequenced in the *Shewanella* genus. Strikingly, the minimal gene set defined using the MaGe comprises the same 205 genes as *S. oneidensis* MR-1 (4.97 Mb) and H76 (4.874 Mb) suggesting that genome sequencing is nearly if not complete.

5.2.1. *chrA*- and *fdhA*-like genes

Proteins sharing homologies with the ChrAso and Fdh of MR-1 were searched in H111 genome using the BlastP. No *chrA$_{SO}$*-like gene could be detected on the chromosome. However, the gene *SAH1-v1-20276* coding a protein that displays 66 % of identity with Fdh of MR-1 strain is present. It is noticeable that the *fdh*-like gene of strain H111 is located close to a gene coding a putative MCP (*SAH1-v1-20275*) harboring 79.8% identity with the chromate-sensing MCP of MR-1 (*SO0987*) (Figure 38).

5.2.2. *chrR*-like and *nfsA*-like enzymes

We investigated the presence of homologous of ChrR-like azoreductase (*SO3585*) of MR-1 in H111 strain. Results of the BalstP search show the presence of a putative product of *SAH1_v1_10414* that shares 66.01% of identity with ChrR-like azoreductase of MR1. Moreover, a Synteny analysis shows that part of the genes surrounding *SO3585* is also conserved in H111.

No gene coding for NfsA-like, type II chromate reductase, is detected on the chromosome of strain H111.

5.2.3. *dmsA1*-like and *dmsA2*-like enzymes

We searched also the presence of proteins homologs to DmsA1 and DmsA2 of MR-1 using a BlastP search. Results demonstrate the presence of *SAH1_v1_50121* gene which product shares 64.41% of identity with DmsA1 from the strain MR-1. Moreover, the synteny analysis reveals that the entire region surrounding the *SAH1_v1_50121* gene is highly conserved. However, the research of DmsA2 homologs show only 33.66% of identity of *SAH1_v1_10414* gene product with DmsA2 from the strain MR-1, and there is no other putative protein of strain H111 presenting significant identity with it.

When blasted, the proteins Fdh-like, DmsA1-like and ChrR-like present in H76 show a high identity with their homologs in H111 strain. All results concerning this comparative analysis between strains MR-1, H76 and H111 are summarized in Table 1.

From the phenotypic study and the global genome analysis of both H76 and H111, we can notice the originality of *S. algidipiscicola* H111 strain. Despite the small size of the genome and the absence of several genes encoding enzymes known to play a role in chromate resistance and reduction, H111 strain is characterized by its strong ability to efficiently resist and reduce chromate at high concentrations. Therefore, we decided to publish its whole genome sequence (Article 2). To simplify, the *S. algidipiscicola* H111 strain is named in Article 2 as H1 strain.

Article 2.

Draft genome sequence of *Shewanella algidipiscicola* H1, a highly chromate-resistant strain isolated from Mediterranean marine sediments.

Hiba BAAZIZ, Olivier LEMAIRE, Cécile JOURLIN-CASTELLI, Chantal IOBBI-NIVOL, Vincent MEJEAN, Radia ALATOU, Michel FONS.

Draft genome sequence of *Shewanella algidipiscicola* H1, a highly chromate-resistant strain isolated from Mediterranean marine sediments.

Running title **(54 caractères + espaces / 54 max)**

Draft genome sequence of *Shewanella algidipiscicola* H1

Hiba BAAZIZ[ab], Olivier LEMAIRE[a], Cécile JOURLIN-CASTELLI[a], Chantal IOBBI-NIVOL[a], Vincent MEJEAN[a], Radia ALATOU[b], Michel FONS[a#].

[a]Unité de Bioénergétique et Ingénierie des Protéines, Institut de Microbiologie de la Méditerranée, Aix-Marseille Université, Centre National de la Recherche Scientifique, Marseille, France.

[b]Laboratoire de Biologie Moléculaire et Cellulaire, Université des Frères Mentouri Constantine 1, Constantine, Algeria.

[#]Corresponding author

Footnote

Address correspondence to Michel Fons, mfons@imm.cnrs.fr.

Abstract **(49 mots / 50 max)**

The ability of different *Shewanella sp.* to convert heavy metals and toxic substances into less toxic products by using them as electron acceptors has led to their use in environmental clean-up strategies. We present here the draft genome of *Shewanella algidipiscicola* H1, a strain resistant to high chromate concentration.

Text **(480 mots / 500 max)**

Most of *Shewanella sp.* were isolated from marine environments. The hallmark of many strains is their ability to utilize an extended array of final electron acceptors in addition to

dioxygen. This property, coupled to other adaptive features, allows their survival in various habitats and consequently their wide distribution in nature (1, 2, 3).

Chromate (Cr(VI)), the hexavalent form of chromium, is a powerful oxidant found in oxyanions form which are highly soluble in water. For bacteria, it is highly toxic because of its rapid entry into the cytoplasm where it can generate a whole spectrum of reactive oxygen species (ROS), which exert deleterious effects on cells (4).

Bacteria can repair the damages induced by chromate. To overcome Cr(VI)-stress, they have also developed different resistance mechanisms that directly target Cr(VI). These mechanisms comprise regulation of chromate ions uptake, their efflux from the cell cytoplasm and the reduction of Cr(VI) into Cr(III) (5, 6).

In aerobic conditions, Cr(VI) reduction is usually achieved in the cytoplasm by type I (like ChrR of *Pseudomonas putida* or YieF of *Escherichia coli*) or type II (NfsA from *E. coli*) chromate reductases (7, 8).

The *S. algidipiscicola* strain H1 was isolated from the muddy sediment of the Stora harbor (in the city of Skikda, 36°54'06.9"N 6°52'45.4"E, on the Mediterranean Algerian coast) sampled from the uppermost 3 centimeters. The genome sequencing of *S. algidipiscicola* H1 was carried out at the "Molecular Research LP (MR DNA) Laboratory" (USA). The library was prepared using Nextera DNA Sample Preparation Kit (Illumina) following the manufacturer's user guide and sequenced using the HiSeq 2500 system (Illumina). The draft genome consists of 4.14 Mb and contains 3,857 predicted genes. Among them 3,678 are potential protein coding genes (CDSs) without artifacts, 13 rRNAs and 90 tRNAs. Until now, only one *Shewanella sp*. genome from the Mediterranean environment was available, that of *S. woodyi* ATCC51908. Besides *S. putrefasciens* HRCR-6 (3.631350 Mb) (https://img.jgi.doe.gov/cgibin/pub/main.cgi?section=TaxonDetail&page=taxonDetail&taxon_oid=2540341238#statistics), *S. algidipiscicola* H1 genome is one of the smallest so far

sequenced in the *Shewanella* genus. Strikingly, the minimal gene set defined using the MaGe (Magnifying Genomes, http://www.genoscope.cns.fr) web-based interface comprises the same 205 genes as *S. oneidensis* MR1 (4.97 Mb).

Preliminary results show that strain H1 is able to grow in the presence of 3 mM chromate under aerobic conditions, whereas the model strain, *S. oneidensis* MR1, stops growing when Cr(VI) concentration is above 0.5 mM.

Unexpectedly, the analysis of the strain H1 genome revealed neither a *nfsA*-like gene nor a *chrA_{SO}*-like gene that was recently shown to significantly improve chromate resistance and reduction in *S. oneidensis* MR1 (9). However, a ChrR-like protein could be produced by strain H1, but the role of this azoreductase in chromate resistance is probably not crucial as only the initial rate of chromate disappearance is affected in the Δ*chrR* (*so3585*) *S. oneidensis* MR1 mutant (10).

The draft genome sequence of *S. algidipiscicola* H1 could thus provide with novel information concerning chromate resistance and reduction systems.

Accession number(s). The results obtained from this whole-genome shotgun project have been deposited at the European Nucleotide Archive (ENA) database under accession number XXXXXX.

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PART III:

CHROMATE REDUCTION BY PELLICLES

S. fidelis H76   **S. algidipiscicola H111**

B.
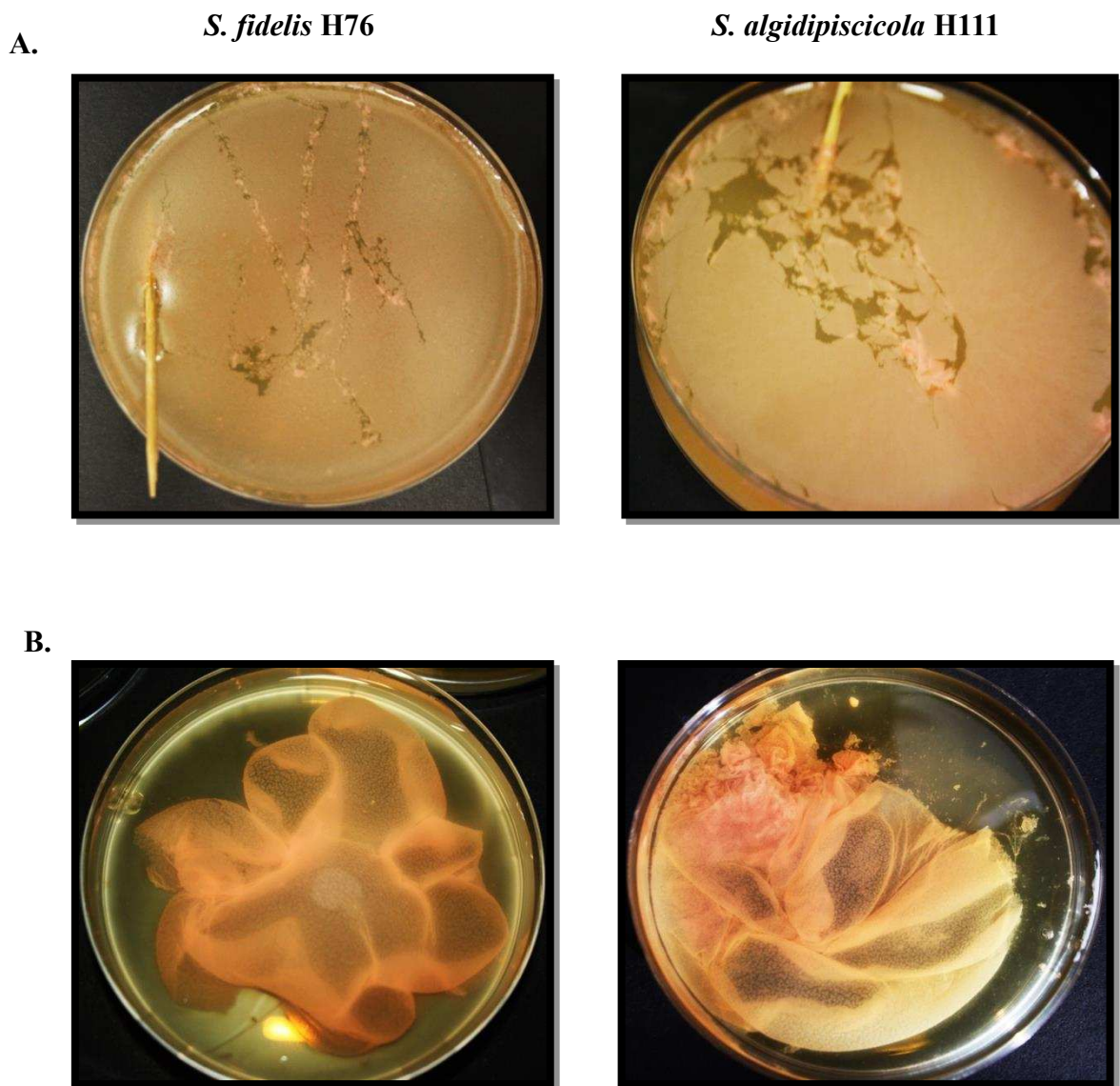
Figure 39: A. Pellicles of *S. fidelis* H76 and *S. algidipiscicola* H111 formed after 48h in LB medium supplemented with NaCl (15g/l). **B.** Pellicles of *S. fidelis* H76 and *S. algidipiscicola* H111 formed after 48h in LB medium supplemented with NaCl and enriched with traces metal solution. The cohesivity and thickness of pellicles was checked using a toothpicks.

1. Pellicle formation ability of *S. fidelis* H76 and *S. algidipiscicola* H111

The hallmark of bacterial biofilms is their remarkable resistance and tolerance to a wide range of toxic compounds. The capacity of *S. fidelis* H76 and *S. algidipiscicola* H111 to form biofilm at the air-liquid interface, named pellicle, was investigated under aerobic and static conditions. We searched culture parameters allowing *S. fidelis* H76 and *S. algidipiscicola* H111 to form a thick and cohesive pellicle. As a preliminary assay, we sought their ability to form pellicle under similar conditions needed for pellicle formation in *S. oneidensis* MR-1, with addition of NaCl (15g/l) to LB medium. After 48h of following pellicle formation, we observed a formation of a thin non-cohesive layer of pellicle for both strains using the toothpick test (Figure 39A).

As known, certain cations such as Mn^{2+}, Cu^{2+} or Zn^{2+} are required for the formation and maturation of pellicle in *S. oneidensis* MR-1 (Liang *et al*, 2010). We wondered if the enrichment of the culture medium would improve the cohesiveness of *S. fidelis* H76 and *S. algidipiscicola* H111 pellicles. Therefore, prior to incubation, we added traces metal solution, containing iron, molybdenum, cobalt, copper, manganese and zinc, to a culture suspension at an OD_{600nm} of 0.2. After 48h of incubation, we observed that *S. fidelis* H76 and *S. algidipiscicola* H111 form very thick and cohesive pellicles (Figure 39B).

Trace metals were also added separately to test the effect of each metal on pellicle formation capacity of *S. fidelis* H76 and *S. algidipiscicola* H111. After 48h of incubation, we noticed that the addition of each metal separately does not improve the cohesiveness of both strains pellicles.

2. Chromate reduction capacity of *S. fidelis* H76 and *S. algidipiscicola* H111 pellicles

Knowing that, usually, bacteria living in biofilm are more resistant to toxic compounds, including heavy metals, compared to planktonic bacteria cells when they are isolated as planktonic forms (free-swimming cells) studied before. We wondered whether the chromate reduction ability of *Shewanella* strains, MR-1, H76 and H111, could be improved when cells are in biofilm life form.
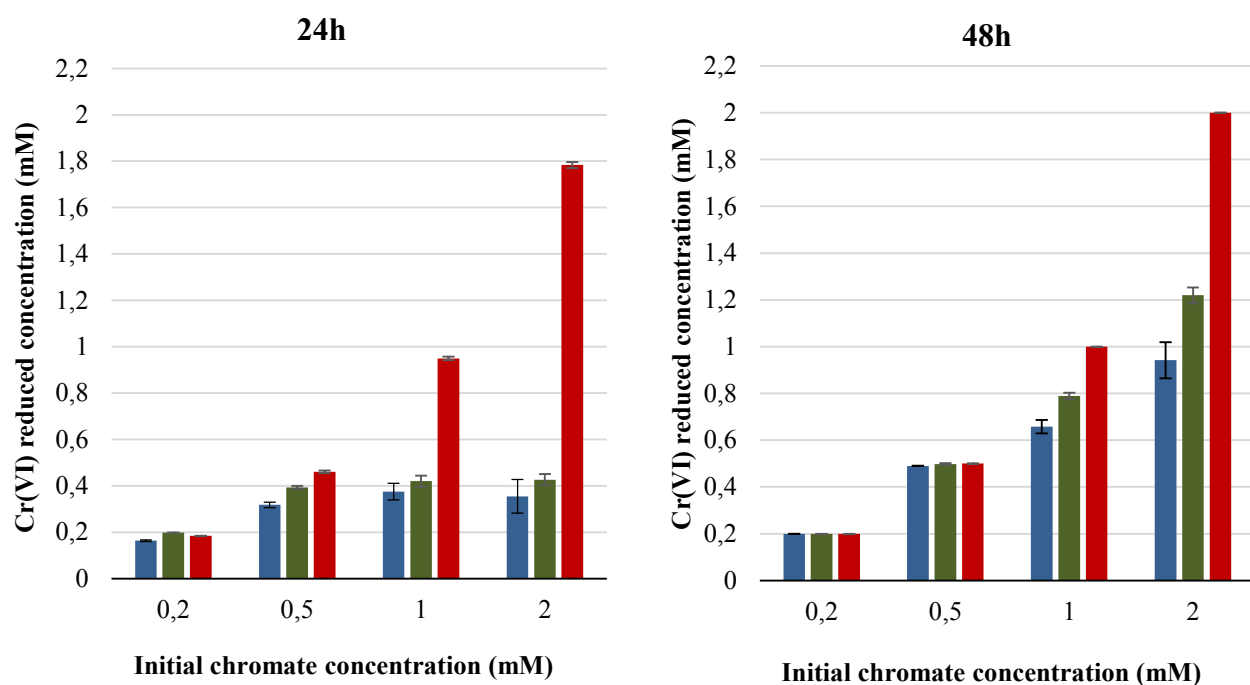
Figure 40. Chromate reduction by *S. oneidensis* MR-1 (Bleu bars), *S. fidelis* H76 (Green bars) and *S. algidipiscicola* H111 (Red bars) planktonic cells, after 24h and 48h of incubation at 28°C under semi-aerobic conditions, in the presence of various chromate concentrations: 0.2, 0.5, 1 and 2mM.
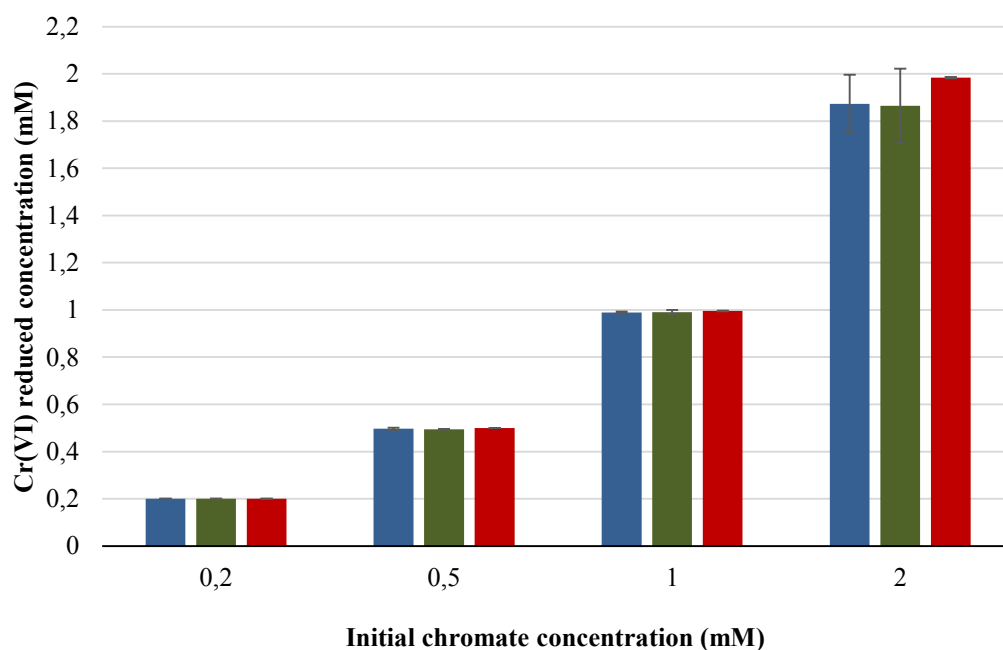
Figure 41. Chromate reduction by *S. oneidensis* MR-1 (Bleu bars), *S. fidelis* H76 (Green bars) and *S. algidipiscicola* H111 (Red bars) pellicles after 24h of incubation at 28°C under semi-aerobic conditions, in the presence of various chromate concentrations: 0.2, 0.5, 1 and 2mM.

In order to answer this question, we investigated the chromate reduction efficiency of pellicles and planktonic. To this end, mature pellicles formed by MR-1, H76 and H111 were collected and then incubated in fresh LB medium supplemented with various chromate concentrations (0.2, 0.5, 1 and 2mM) under static conditions (see Material and Methods) to maintain the three-dimensional architecture of pellicles. Simultaneously, strains cells grown overnight were gently centrifuged and resuspended in LB medium supplemented with chromate (0.2, 0.5, 1 and 2mM) to reach an initial OD_{600nm} of 2. The latter OD_{600nm} was chosen to approach pellicle assay conditions, where the final optical density of cells forming pellicle in culture medium was 2. After incubation of 24h and 48h in semi-aerobic and anaerobic conditions, the chromate reduction activity in both conditions, pellicles and planktonic cells, was determined by measuring residual Cr(VI) concentration using the DPC colorimetric assay and total chromate concentration using ICP-OES.

As shown in Figure 40, planktonic cells of H111 strains are able to reduce completely the initial chromate concentration of 1mM after 48h of incubation in semi-aerobiosis, while only 77.9 % and 65.7% are reduced by H76 and MR-1 cells, respectively. Furthermore, H111 is able to reduce completely the initial chromate concentration of 2mM within 48h of incubation under static conditions, contrary to H76 and MR-1 cells that are able to reduce only 59.7% and 47% of it, respectively. These results indicate that, in planktonic life form, the chromate reduction efficiency of H111 strain is higher than H76 and MR-1 under semi-aerobic conditions.

With respect to the chromate reduction capacity of pellicles, results shown in figure 41 indicate that in semi-aerobic conditions the initial chromate concentrations 0.2, 0.5 and 1mM are totally reduced within 24h. Moreover, the highest chromate concentration 2mM is totally reduced by H111 within 24h of incubation while it is totally reduced by H76 and MR-1 after 48h of incubation.

Overall, the chromate reduction rates of pellicles and planktonic cells of H76, H111 and MR-1 indicate that chromate reduction ability of strains in pellicle life form is more efficient than that of their planktonic cells.

Initial pellicules grown in the absence of chromate

After 48h of incubation in the presence of 1mM chromate

S. oneidensis MR-1
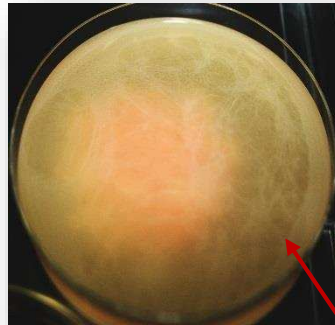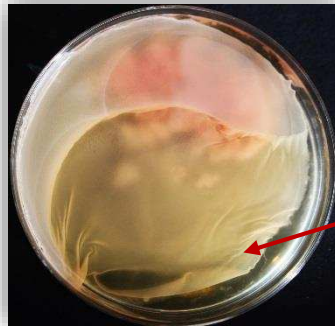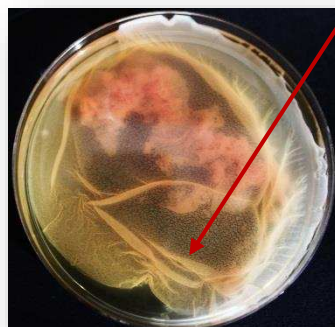
S. fidelis H76

New pellicules formed in the presence of chromate

S. algidipiscicola H111

Figure 42. Formation of pellicles of *S. oneidensis* MR-1, *S. fidelis* H76 and *S. algidipiscicola* H111 after 48h of incubation at 28°C under semi-aerobic conditions, in the presence of 1mM of chromate.

Under anoxic conditions, MR-1, H76 and H111, in both planktonic and pellicle life forms, show the ability to totally reduce all initial chromate concentrations tested (0.2, 0.5, 1 and 2mM) within only 24h of incubation (Results not shown), indicating that the chromate reduction ability of strains is more efficient in anaerobic conditions.

During our experiment, we noticed the capacity of *Shewanella* strains to reform a new pellicle when the initial pellicle is incubated 48h in the presence of chromate concentrations lower than 1mM. The pellicles formed after 48h of chromate incubation are slightly thinner than the initial ones. However, those formed by H76 and H111 are more cohesive that those of MR-1 (Figure 42).

3. Chromium accumulation capacity of pellicles

It is known that heavy metal ions in both soluble and insoluble forms can potentially be accumulated by bacterial cells (live or dead) and their byproducts such as the extracellular polymeric substances (EPSs) (Gupta & Diwan, 2017). We investigated the capacity of H76, H111 and MR-1 planktonic cells and pellicles to uptake and accumulate different chromate concentrations (0.2, 0.5, 1 and 2mM). To this end, cells grown overnight were gently centrifuged and then resuspended in LB medium supplemented with chromate to reach an initial OD_{600nm} of 2, while mature strains pellicles were gently collected and then moved into fresh LB medium supplemented with chromate. Afterward, planktonic cells and pellicles were incubated at 28°C in the presence of chromate for 24h and 48h under semi-aerobic conditions. After incubation, cultures were centrifuged to separate cells from supernatant. The total chromium concentration present in both cellular and supernatant fractions was measured using ICP-OES.

Results show that pellicles of all strains accumulate much better chromium compared to their planktonic cells. As an example, when exposed to initial chromate concentrations of 2mM for 24h, pellicles of MR-1, H76 and H111 show a chromium accumulation capacity 10-fold greater than that of their planktonic cells (Figure 43). Furthermore, H76 and H111 strains, in their pellicle life state, accumulate more efficiently chromium than the reference strain MR-1. As shown in figure 43, after 48h of incubation in the presence of the initial
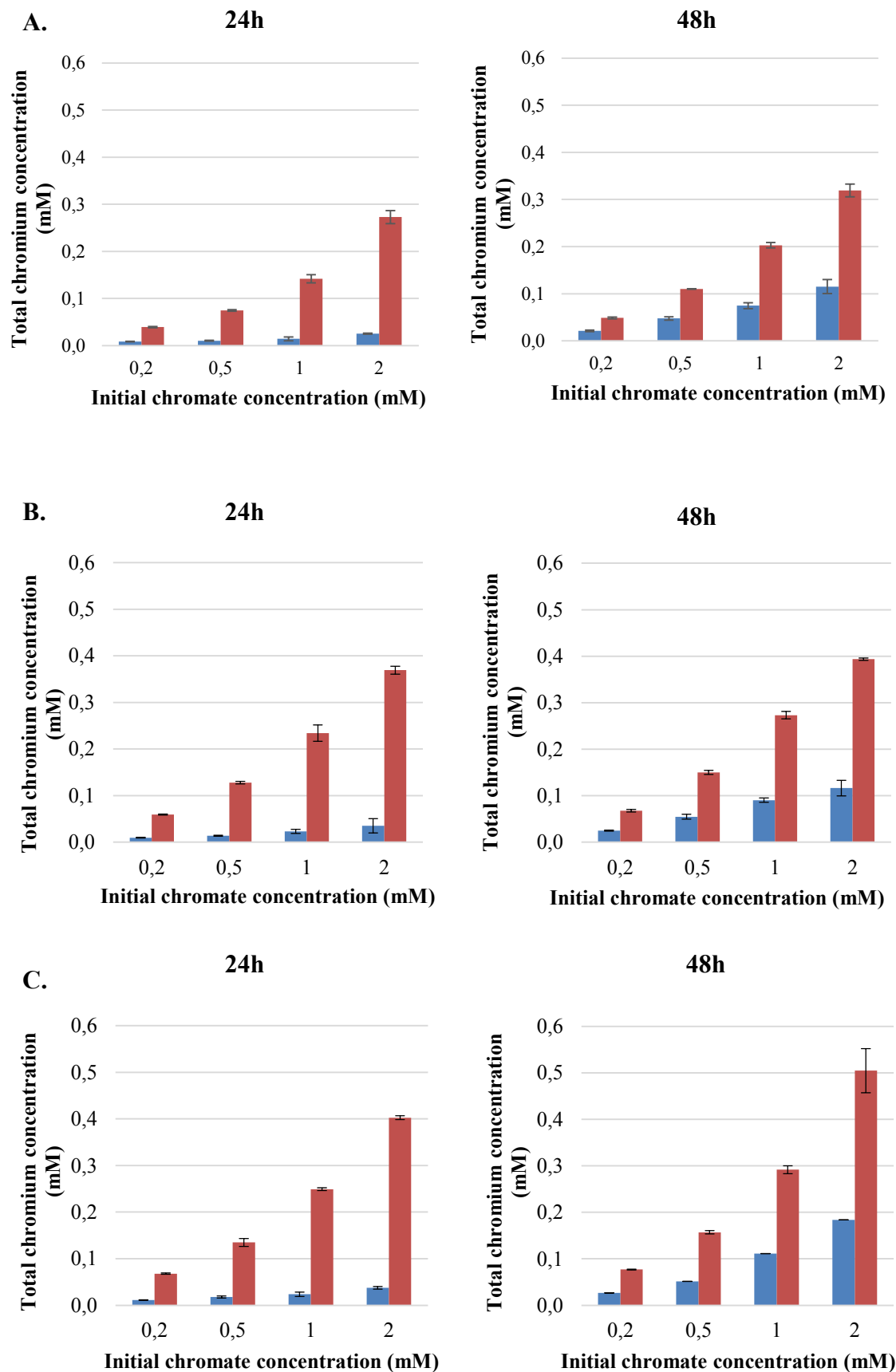
Figure 43. Total chromium accumulation by planktonic cells (bleu bars) and pellicles (Red bars) of *S. oneidensis* MR-1 (A), *S. fidelis* H76 (B) and *S. algidipiscicola* H111 (C) after 24h and 48h of incubation at 28°C under semi-aerobic conditions, in the presence of various chromate concentrations: 0,2, 0,5, 1 and 2mM.

chromate concentration 2mM, 19.68% and 25.23% of chromium are accumulated in H76 and H111 pellicles, respectively, while only 15.96% is accumulated by MR-1 strain in its pellicle life state. These results also show that the amount of total chromium accumulated in both planktonic cells and pellicles increases with increasing initial chromate concentrations.

To investigate the nature of chromium accumulated in planktonic cells and pellicles, we performed the specific DPC colorimetric assay in order to detect the presence of the hexavalent chromate Cr(VI) in cellular fractions of strains cultures. Results show clearly the absence of Cr(VI) in cellular fractions indicating that the chromium accumulated in planktonic cells and pellicles are the reduced forms of Cr(VI), most probably the stable form Cr(III) known to be less soluble than Cr(VI) (Data not shown).

DISCUSSION

AND PERSPECTIVES

Between the two stable chromium forms Cr(III) and Cr(VI), the latter is the highest toxic for bacterial cells due to its great solubility and rapid entry into the cytoplasm where it may exert its toxic effects. Among the various strategies that bacteria developed to resist chromate, efflux of chromate ions from the cytoplasm and reduction of Cr(VI) into Cr(III) are considered to be the main ones (Ramírez-Díaz *et al*, 2008). Although numerous studies have been conducted, the molecular mechanisms developed by *Shewanella* sp. to overcome chromate remain largely unknown. Therefore, the main aim of the present dissertation was to improve understanding about chromate resistance and reduction mechanisms of *Shewanella* sp. We studied first these mechanisms in the model strain *S. oneidensis* MR-1. The strain MR-1 is considered as a great candidate for chromate bioremediation investigations due to its metabolic diversity, available sequenced genome and easy growth in laboratory conditions.

We first confirmed the ability of *S. oneidensis* MR-1 to grow in the presence of chromate concentrations up to 0,5mM under semi-aerobiosis, which indicates that this bacterium is powered by mechanisms that allow its resistance to such high chromate concentration. We showed that chromate concentrations higher than 1mM alter cell growth while concentrations above 2mM inhibit the growth of MR-1 cells. These findings are in good agreement with previously published results which showed that the minimum inhibitory chromate concentration (MIC) for aerobically grown *S. oneidensis* MR-1 is 2mM (Brown *et al*, 2006). The cease of growth in the presence of high chromate concentrations results probably from the inhibition of some metabolic processes by chromate and mainly from the accumulation of Cr(III) precipitates in the cytoplasm, as physiological studies on the toxicity of chromate in *S. oneidensis* MR-1 previously reported (Viamajala *et al*, 2008; Parker *et al*, 2011).

Among the numerous studies on the chromate reduction by *S. oneidensis* MR-1, most were conducted under anaerobic conditions. Hence, we focused our investigation on chromate resistance/reduction under semi-aerobic conditions. We firstly confirmed the ability of *S. oneidensis* MR-1 to reduce, if not totally, a significant amount of chromate, even at high initial concentrations under semi-aerobic conditions. It is noteworthy to

mention that the strain MR-1 shows a higher chromate reduction efficiency in anoxic conditions, which may be explained by the absence of competition between oxygen and chromate as electron acceptors that usually results in a lower chromate reduction efficiency (Han *et al*, 2016) or by less ROS production. Aerobic and anaerobic chromate reduction by *S. oneidensis* MR-1 was previously reported (Lowe *et al.* 2003). However, there is no evidence until to now that MR-1 strain respires chromate by using it as a sole electron acceptor (Bencheikh-Latmani *et al*, 2005). *S. oneidensis* MR-1 may develop this capacity not to gain energy by using chromate as electron acceptor but to simply protect itself from toxicity under different specific conditions. This capacity could provide *S. oneidensis* MR-1 with a selective advantage over other sensitive bacteria, allowing its colonization in challenging environments (Trevors *et al*, 1985).

Knowing that chromate efflux pumps belonging to the superfamily of CHR can be involved in chromate resistance in other bacteria like *C. metallidurans* CH34 and *Pseudomonas aeruginosa*, we searched for the presence of this type of pump in *S. oneidensis* MR-1. Our bioinformatic analysis showed that chromosomal gene *SO0986* likely encodes a chromate efflux pump ChrA$_{SO}$ that belongs to long chain CHR subfamily.

We confirmed that ChrA$_{SO}$ is a chromate efflux pump, as demonstrated for some members of the CHR family (Pimentel *et al*, 2002; Aguilar-Barajas *et al*, 2008), by demonstrating, on the one hand, that its absence affects significantly the chromate reduction and resistance of *S. oneidensis* under semi-aerobic and anaerobic conditions. It is noticeable that the deletion of *chrA$_{SO}$* does not dramatically affect chromate reduction under anoxic conditions compared to semi-aerobiosis. This suggests that other transporters might be involved in this process under anaerobic conditions. Of interest, genes encoding the three components of a putative heavy metal efflux pump (*SO0518* to *SO0520* and CzcCBA) were observed to be up-regulated by chromate in anaerobic conditions (Bencheikh-Latmani *et al*, 2005). This suggests a role in chromate resistance, although this pump does not belong to the CHR family. It would be interesting to delete genes coding for this pump in order to investigate its involvement in chromate resistance.
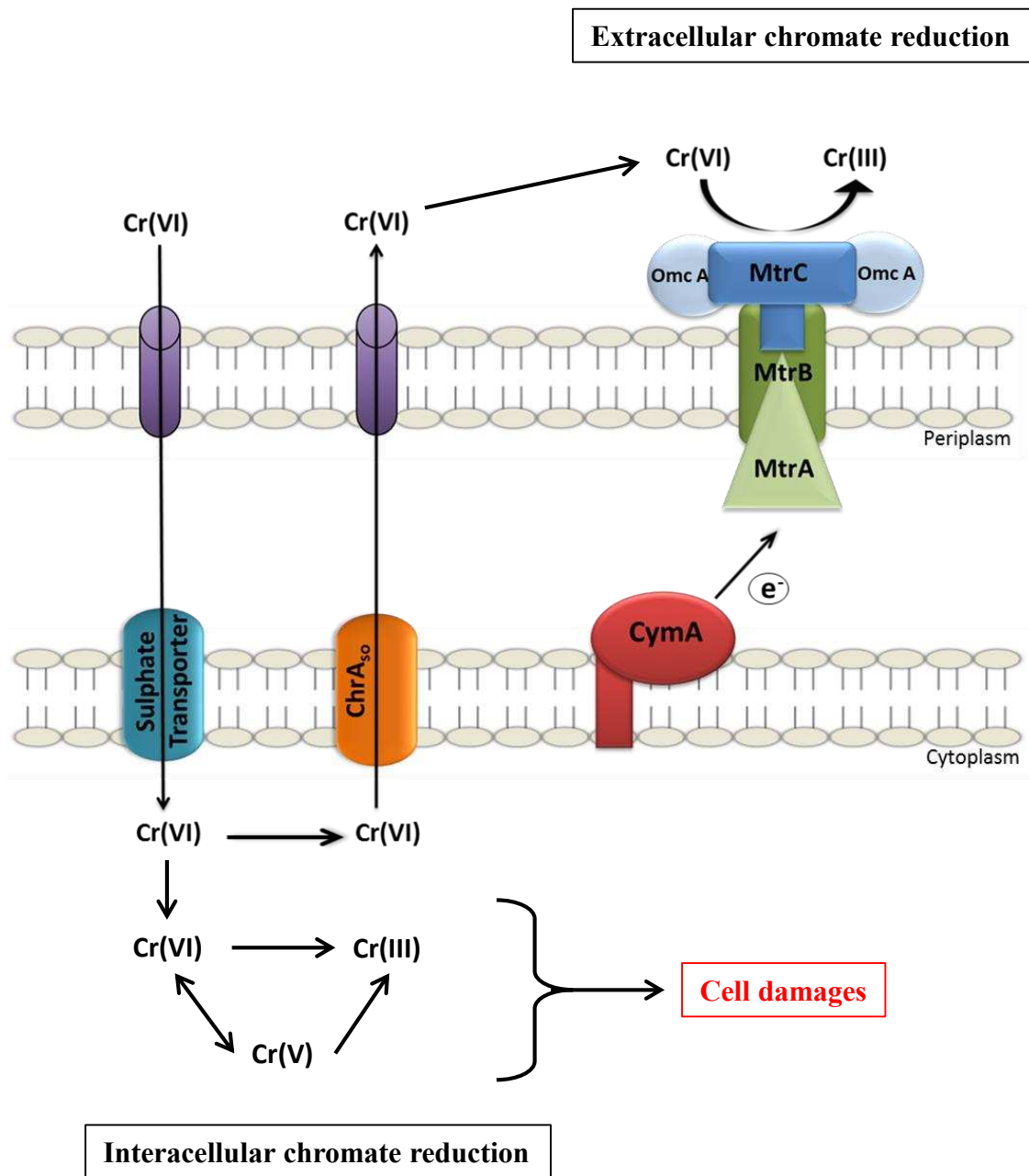
Figure 44. The role of ChrA$_{SO}$. Chromate enters cells via sulphate transporters, the role of ChrA$_{SO}$ is to decrease the rate of chromate inside the cells by extrude it from the cytoplasm to be later reduced extracellularly by outer membrane cytochromes MtrA and OmcA. Limiting the presence of chromate in the cytoplasm, protect the cell from damages resulting from its intracellular presence and reduction. Hence, cells grow better and therefore can detoxify theirs environement from chromate by reducing it, extracellularly, more efficiently.

In order to reaffirm the role of ChrA$_{SO}$, we demonstrated that the heterologous expression of *chrA$_{SO}$* in *E. coli* confers resistance to high chromate concentrations, which is also observed for the plasmid-encoded ChrA of *Shewanella* ANA-3 and for several ChrA of *Burkholderia xenovorans* LB400 (Aguilar-Barajas *et al*, 2008; Acosta-Navarrete *et al*, 2014). This heterologous expression strikingly improves the chromate reduction ability of *E. coli* cells. A direct role of ChrA$_{SO}$ in chromate reduction was ruled out since the specific activity of chromate reductase measured *in vitro* on crude extracts from both *S. oneidensis MR-1* and *E. coli* strains is similar in the absence or in the presence of ChrA$_{SO}$. Our hypothesis to explain the improvement of chromate reduction efficiency is that ChrA$_{SO}$ promotes chromate reduction by expulsing chromate outside the cells where it can then be reduced extracellularly by membrane bound reductases. In *S. oneidensis MR-1,* this scenario is worth considering as two outer membrane decaheme cytochromes, MtrC and OmcA, are known to be involved in extracellular chromate reduction (Belchik *et al*, 2011) (Figure 44). An additional effect could be that ChrA$_{SO}$ improves cell survival by lowering intracellular chromate concentration and consequently Cr-induced damages inside the cells (Joutey *et al*, 2015), which protects and allows the cells to grow and reduce chromate over an extended period of time (Figure 44). This could be true for both *S. oneidensis* MR-1 and *E. coli*.

We revealed that although *chrA$_{SO}$* is expressed in the absence of chromate, its level of expression is higher in the presence of chromate. Two previous studies performed on *S. oneidensis* using transcriptomic approaches missed this regulation, probably because the applied cut-offs were quite stringent (Bencheikh-Latmani *et al*, 2005; Brown *et al*, 2006). Indeed, these studies considered only the genes showing at least a 2-fold or a 3-fold change in expression, respectively. Moreover, the experimental conditions used in both studies were different from ours, which can also explain this discrepancy. The fact that *chrA$_{SO}$* expression is induced by chromate is reminiscent of what was previously observed for the expression of several *chr* genes in other bacteria. As an example, the transcription of the *chrA1* and *chrA2* genes of *C. metallidurans and B. xenovorans* LB400 were shown to be up-regulated by chromate (Juhnke *et al*, 2002; Acosta-Navarrete *et al*, 2014). The chromosomally located *chrBACF* operon of *Ochrobactrum tritici* 5bvl1 presents another

example since its expression is also induced by chromate (Branco *et al*, 2008). It was subsequently shown that the ChrB protein is a transcriptional regulator that binds to the *chrBACF* promoter region to regulate its expression (Branco & Morais, 2013). The fact that there is no ChrB homolog in *S. oneidensis MR-1*, suggests that the regulation of *chrA$_{SO}$* expression probably depends on a regulatory protein belonging to a family different from that of ChrB. It would be interesting to identify this protein, since no chromate-sensing regulator except from ChrB was previously described.

Besides extruding chromate outside the cytoplasm, the expression of chromate-reductases is an efficient strategy to resist to Cr(VI) under aerobic conditions. During our study, 3 loci of the strain MR-1 genome caught our attention. The first one, *SO0988*, codes for a putative formate dehydrogenase α subunit. It is located close to *chrA$_{SO}$* and to *SO0987* encoding an MCP known to detect chromate. The second one is *dmsAB-1* which codes for a DMSO reductase. Several studies demonstrated DmsAB-1 involvement in the respiration of DMSO despite the fact that *dmsAB-1* is not induced by DMSO (Bencheikh-Latmani *et al*, 2005). It have been shown that *dmsAB-1* cluster is upregulated under thiosulfate-reducing conditions as well as in the presence of chromate (Beliaev *et al.* 2005,Bencheikh-Latmani *et al.* 2005), which may be indicative of a broad substrate specificity of corresponding complex in the MR-1 strain. The third one is *dmsAB2* which is predicted to code for a second putative DMSO reductase. However, there is no evidence until now about the involvement of the second cluster *dmsAB-2* products in DMSO respiration. Previous studies revealed that the expression of *dmsAB*-2 cluster is not significantly induced by DMSO (Beliaev *et al*, 2005). However, it is interesting to note that the genomic context of *dmsA2* contains *SO4359* and *SO4360* genes whose products are not only redundant to cytochroms MtrB and MtrA known to be implicated in chromate reduction (Belchik *et al*, 2011), but they function as a terminal reductase localized at the cell surface (Schicklberger *et al*, 2013).

These data prompted us to wonder whether the 3 loci could play a role in chromate resistance and reduction in *S. oneidensis* MR-1, we therefore deleted *SO0988, dmsA1* and *dmsA*2 separately in order to investigate their involvement under semi-aerobic conditions.
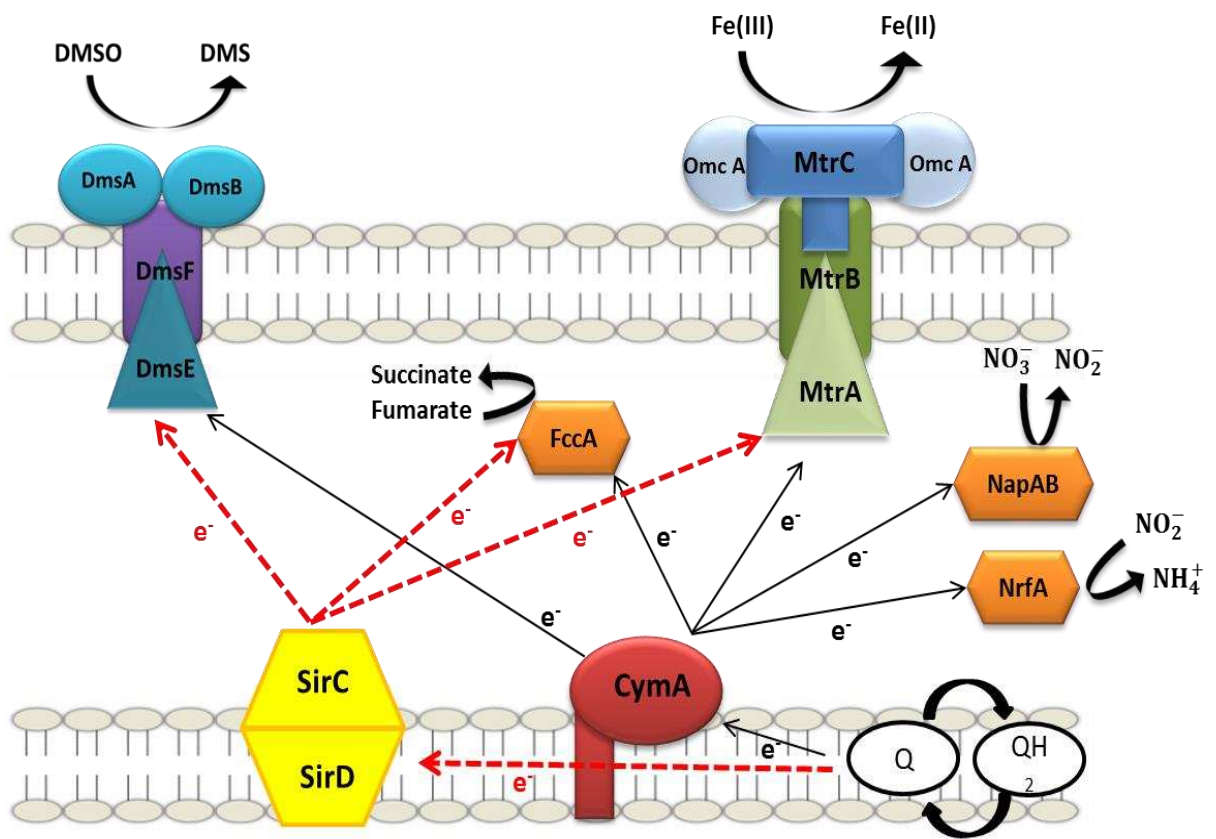
Figure 45. Partial functional replacement of CymA by SirCD in respiration of fumarate, DMSO and iron(III) (red arrows). Figure adapted from Cordova *et al.* 2011.

While the deletion of *dmsA1* affected slightly and transitionally the chromate reduction capacity of the strain in the first hours of incubation, the deletion of *SO0988* and *dmsA2* showed a constant drastic effect on the chromate reduction capacity of strains which reduced chromate 3-fold lower than the wild-type. These results indicate that the proteins encoded by these genes are involved either directly or indirectly in chromate reduction in semi-aerobiosis. The high impairment of chromate reduction in the absence of *SO0988* gene and *dmsA2* highlights the fact that efficient chromate reduction in semi-aerobiosis requires the presence of these important genes. Although, *SO0988* gene is annotated as a putative formate dehydrogenase α subunit, some authors predicted that the *SO0988* product is cytoplasmic because the gene does not encode a twin-arginine translocation pathway signal sequence contrary to other Fdh periplasmic α subunits known in *S. oneidensis* MR-1 (Kane *et al.* 2016). Moreover, the involvement of the *SO0988* product in formate oxidation has never been reported. Until now the function of *SO0988* product is unknown, therefore, further studies are needed to clarify the precise role of this cytoplasmic protein and its direct or indirect relation with chromate reduction under semi-aerobiosis. The collected data and the results obtained about DmsA2 strongly suggest that it plays a role as a chromate reductase. It would be interesting to investigate the chromate reductase activity *in vitro* of both DMSO reductases, especially that of DmsA2. The expression of both genes in the presence of different chromate concentrations under semi-aerobic and anaerobic conditions would be also interesting to investigate in order to complete our understanding about the expression regulation of these genes and likely the broad substrate specificity of their products.

Finally, we checked the involvement of the membrane bound c-type cytochrome in the efficiency of MR-1 strain to reduce Cr(VI). CymA is considered as a central element of multiple electron acceptor reduction pathways. It delivers electrons from the quinone pool to a number of terminal reductases located in the periplasm and in the outer membrane including the Mtr complex (Figure 45), whose coding genes (*mtrABC* and *omcA*) have been shown to be upregulated in the presence of chromate (Beliaev *et al*, 2005). Furthermore, it was reported that MtrC and OmcA complex are used by *S. oneidensis* MR-1 for extracellular chromate reduction (Belchik *et al*, 2011). Therefore, we sought the effect of

CymA deletion on chromate resistance and reduction in *S. oneidensis* MR-1 under both semi-aerobic and anaerobic conditions. On the one hand, we showed that CymA does not appear to play a key role in chromate resistance and reduction under anaerobic conditions, in agreement with results of previous studies (Bencheikh-Latmani *et al*, 2005). The first hypothesis to explain this phenomenon is that the Mtr complex could be supplied with electrons via other donors under anaerobic conditions. This scenario is worth considering as previous studies revealed that CymA could be partially substituted by the SirCD complex, which primarily function as quinol dehydrogenases in respiration of several electrons acceptors, such as fumarate, DMSO and iron(III) but has no effect on nitrate and nitrite reduction (Cordova *et al*, 2011) (Figure 45). A second hypothesis would be the presence of unknown chromate reductases CymA-independent, since even when the whole MtrBAC was deleted, the chromate reduction was not completely abolished (Bencheikh-Latmani *et al*, 2005). It would be interesting to investigate if the complex SirCD could fulfill the role of CymA in chromate reduction in anaerobiosis since it can transfer electrons to the Mtr complex to reduce Fe(III).

On the other hand, we demonstrated that CymA may play a partial role in chromate survival of *S. oneidensis* MR-1 under semi-aerobic conditions. Moreover, we showed that CymA is most likely involved in chromate reduction mechanisms of *S. oneidensis* MR-1 under semi-aerobiosis, since the chromate reduction ability of the *cymA*-mutant strain is strongly, but not completely, impaired. This impairment may explain the slight decrease of growth seen when *cymA*-mutant is challenged with chromate, as it is unable to reduce chromate effeciently and, consequently, chromate-induced damages result in a slight decline of cells viability. Overall, we demonstrated that under semi-aerobiosis, CymA may transfer electrons to terminal reductases that partially reduce chromate. The shortcoming of CymA as branching position for many pathways, is that bacteria can lose their ability to use various electron acceptors simultaneously, resulting in sever physiological damages, when CymA is mutated. Therefore, *S. oneidensis* MR1 evolved a strategy to circumvent the risk by developing functional substituent proteins for CymA. This may explain the residual chromate reduction activity of *cymA*-mutant under semi-aerobiosis. We suggest the presence of a protein that partially substitutes CymA under these conditions, which is not

surprising given that it has been reported that under nitrate/nitrite reduction and aerobic conditions the role of CymA could be partially fulfilled by the bc_1 complex that functions as quinol dehydrogenases to transport electrons to the cytochrome cbb_3 oxidase, the enzyme complex predominantly responsible for oxygen respiration and which expression is favored in aerobic conditions (Cordova *et al*, 2011; Fu *et al*, 2014). It will be interesting to investigate the possibility if *cb1* complex could substitute CymA under semi-aerobiosis by transferring electrons to chromate reductases.

Taken together, our findings highlight the fact that *S. oneidensis* MR-1 uses different strategies to resist and reduce chromate depending on the concentration of oxygen. The chromate reduction of this bacterium seems to overlap with its metabolic complex network. Therefore, further studies need to be pursued to decipher the enzymes implicated or related to chromate resistance and reduction in *S. oneidensis* MR-1 under different conditions.

Shewanella species are frequently isolated from marine and freshwater environments. More than 32 *Shewanella* genomes have been fully sequenced to date (https://www.ncbi.nlm.nih.gov/genome/browse/#!/overview/shewanella) due to their known energy and bioremediation capabilities, phylogenetic relatedness, and inhabitance of environments (Fredrickson *et al*, 2008). Among the marine species whose genome is available, only *Shewanella woodyi* ATCC 51908 was originally isolated from the Mediterranean Sea. During this thesis, we isolated and identified two novel Mediterranean *Shewanella* strains from sediment water of Stora harbor, located in the north east of Algeria and previously reported to be polluted by heavy metal (Gueddah & Djebar, 2014). Based on their 16SDNA and GyrB sequences, the strains H76 and H111 were identified as *Shewanella fidelis* and *Shewanella algidipiscicola*, respectively. The later were firstly isolated from the South China Sea in 2003 and the Baltic Sea of Denmark, respectively (Ivanova, 2003; Lee *et al*, 2016). The phylogenetic tree that we constructed using 16SDNA sequences of some of the nearest neighbors of H76 and H111, surprisingly showed that strains are present in different sub-groups of the first group of *Shewanella* that enclose piezotolerant and psychrotolerant species, contrary to MR-1 which belongs to the second group that contains pressure sensitive and mesophilic species. Phenotypic characterization

showed that the two strains can grow at temperatures ranging from 25°C to 35°C indicating that although both strain belong to the first group of *Shewanella* they cannot be psychrotolerants this may be due to the adaptation of species to Mediterranean environments conditions. It would be interesting to test the pressure sensitivity of our strains since no *S. fidelis* and *S. algidipiscicola* previously isolated strains have been reported as a piezotolerants.

Unlike MR-1, both strains require a relatively high salt concentration for optimal growth; consequently, they are able to grow over a range of NaCl concentrations from 15 to 30g/l, with an optimum growth at 15g/l of NaCl. These results are not surprising since H76 and H111 are originating from a marine environment whereas MR-1 was first isolated form freshwater. We demonstrated also the chemotaxis capacity of H76 and H111 towards few metals. The strains showed chemotactic behavior similar to that of MR-1 as they were repulsed from nickel and cobalt and were attracted to nitrate and chromate (Armitano *et al*, 2011). Two hypotheses can be suggested to explain the attraction toward chromate, either this attraction allows cells to localize favorable environments containing metals that can be used as electron acceptors (Baraquet *et al*, 2009) or confers selective advantage to cells by allowing their colonization in environments that are hostile for many other chromate-sensitive bacteria.

During this study, we revealed the capacity of both H76 and H111 strains to resist and reduce chromate more efficiently than the model strain MR-1 under semi-aerobiosis. This may indicate that both strains use an efficient strategy to cope with chromate toxicity. It is interesting to note that among the three studied strains, H111 showed striking chromate resistance and reduction. We demonstrated that its minimum inhibitory chromate (MIC) concentration would be above 3mM while the MIC of the reference strain MR-1 is below 2mM (Viti *et al*, 2014). These promising results intrigued and prompted us to set out a whole genome-sequencing project to analyze and decipher the genetic background of these particular chromate resistance and reduction efficiencies of both H76 and H111 strains.

Our genetic analysis was based on the investigation of the presence of *chrA$_{SO}$*-, *chrR*- and *nfsA*-like genes whose products were previously described to play a key role in Cr(VI)

resistance and reduction, as well as *fdh-*, *dmsA1-* and *dmsA2*-like genes for which we demonstrated earlier the involvement of their products in Cr(VI) reduction. Genome sequencing revealed that *S. fidelis* H76 genome size of 4.874 Mb is considered similar to that of the model strain MR1 (4.97 Mb), whereas *S. algidipiscicola* H111 genome is the one of the smallest so far sequenced in the *Shewanella* genus with 4.14 Mb. It contains the same 205 essential genes set as *S. oneidensis* MR1 and H76 suggesting that genome sequencing is almost complete.

We showed that the H76 strain can probably express ChrA-, Fdh-, ChrR- and DmsA1-like putative proteins that share a high homology with those present in the MR-1 strain. Interestingly, the strain H76 genome harbors a gene that code for a putative chromate reductase NfsA-like that presents a significant homology with that of *E. coli* (Ackerley *et al*, 2004). Despite the absence of a putative DmsA2 homologous to that of the MR-1 strain the chromate resistance and reduction of the H76 strain is more efficient than that of MR-1. One can ask whether the high chromate reduction observed in the H76 strain is due to the presence of *nfsA*-like gene. It would be interesting to confirm the role of these homologous proteins present in the H76 strain and to evaluate their involvement in chromate resistance and reduction mechanisms.

We showed that the H111 strain harbors Fdh-, ChrR- and DmsA1-like putative proteins sharing a significant homology with those present in the MR-1 strain. Unexpectedly, no putative proteins of the H111 strain presenting homology with the chromate reductase NfsA nor with the chromate efflux pump $ChrA_{SO}$ and DmsA2 were identified. In spite of the small size of the genome and the absence of these putative proteins known to play a key role in chromate resistance mechanisms in bacteria, H111 strain shows strong capacity to resist to high chromate concentrations. Unquestionably, these promising results reveal that H111 strain have original chromate resistance/reduction mechanisms. To decipher theses mechanisms, a new project was set up in our laboratory. A genomic library of *S. algidipiscicola* H111 using *E. coli* as surrogate is under construction. This library will help us targeting and identifying potential genes involved in chromate resistance by selecting clones presenting a chromate-resistance phenotype (Cr^R).

S. oneidensis MR-1 is able to form pellicle, and the machinery involved in the setting of this particular floating biofilm was previously described in detail (Armitano *et al*, 2013). By supplementing the culture medium with traces metals, we showed that the strains H76 and H111 are able to form a very cohesive and thick pellicle. This was not surprising as previous studies revealed that the traces metals that we add such as Mn^{2+}, Cu^{2+} or Zn^{2+} are required for the formation and maturation of pellicle in *S. oneidensis* MR-1 (Liang *et al*, 2010). We demonstrated afterwards that the chromate resistance and reduction efficiency of H76 and H111 was much higher when cells were in pellicle life form. For instance, within 24h, the pellicles of both strains were able to reduce entirely 1mM of chromate. Whereas, only 40 and 90% were reduced by H76 and H111 planktonic cells, respectively. This increase of chromate reduction might result from a better resistance of biofilm cells compared to free swimming cells, hence cell's metabolic functions, including those responsible for detoxifying chromate, are more protected resulting in an efficient chromate reduction. The differential between planktonic and surface biofilm resistance to heavy metal have been previously demonstrated in *Pseudomonas* (Teitzel & Parsek, 2003). A potential explanation for this, is that the extracellular polymeric substances (EPSs) encasing the pellicle cells may be responsible for protecting them from chromium toxicity by binding chromate ions and retarding their diffusion within the biofilm (Kaplan & Christaen, 1987; Nocelli *et al*, 2016; Gupta & Diwan, 2017), which allows bacteria to survive and reduce gradually chromate present in their environment. The contribution of EPSs of biofilm in immobilization of metals was previously shown in *Shewanella* sp.HRCR-1 (Cao *et al*, 2011).

We showed that the pellicles of H76, H111 as well as MR-1 strains are able to accumulate reduced-chromate forms better than their planktonic cells. This may be due to the accumulation of the reduced chromate as a precipitate not only in the cytoplasm but also outside the cells related to the large amount of matrix embedding pellicle cells (Belchik *et al*. 2011, Gupta and Diwan 2017). It is worth noting that H76 and H111 strains showed a better reduced-chromate accumulation than MR-1. This can be the result of their high chromate reduction capacity, hence their high generation of reduced-chromate forms. The nature of H76 strain and H111 pellicles may also play a role in this high accumulation, as both strain's pellicles were more cohesive than that of MR-1, which probably means that they can produce

more EPSs which can subsequently accumulate more chromium molecules. It would be interesting to quantify the contribution of EPSs, embedding pellicle cells, in immobilization of chromate and it reduced-forms.

Some *Shewanella* sp. were found to be potentially applicable for use in the remediation of toxic chromate and other metal contaminants (Aguilar-Barajas *et al*, 2008). The fact that both strains H76 and H111 are able to form cohesive pellicles, to tolerate high chromate concentrations, to reduce rapidly and efficiently chromate and to accumulate reduced chromate which facilitate its further physical removal, make them really interesting candidates for chromate bioremediation of polluted aquatic environments. The major advantages of the use of theses strains in bioremediation over conventional physicochemical methods include low cost, good efficiency, minimization of chemicals, and the possibility of recovering pollutant metals. Thus, further studies on chromate resistance and reduction of these strains are needed to better understand and control their capacities to cope with chromate for an optimal use in bioremediation.

Conclusion

During the present thesis, we studied the chromate resistance and reduction mechanisms of *Shewanella oneidensis* MR-1 under semi-aerobiosis. We showed that MR-1 is able to overcome chromate toxicity by using two main direct strategies. The first one is extruding chromate ions from the cytoplasm using the chromate efflux pump ChrA$_{SO}$ whose function has been confirmed during this thesis. The second strategy is to reduce chromate into a less toxic form. We demonstrated that the MR-1 strain has a complex network of proteins involved in chromate reduction and can use different pathway depending on the oxygen concentration present in its environment. We focused on the potential functions involved in chromate reduction under semi-aerobiosis. This investigation allowed us to reveal that, in contrast to anaerobic conditions CymA is crucial in chromate reduction under semi-aerobiosis as well as DmsA2 that we suggest to play the role of a chromate reductase. We also found that DmsA1 and Fdh are involved in chromate reduction under semi-aerobic conditions. We studied also the chromate resistance and reduction capacity of two *Shewanella* sp. strains, *S. fidelis* H76 and *S. algidipiscicola* H111 that we isolated from the Mediterranean Sea. We showed that, under planktonic and pellicle life forms, both strains resist and reduce higher chromate concentrations than MR-1, H111 strain shines with its exceptional chromate resistance and reduction capacities in spite of its small genome size and the absence of many putative proteins known to play a key role in chromate resistance mechanisms. Therefore, the powerful chromate resistance and reduction mechanisms of *S. algidipiscicola* H111 deserve further studies, which could lead to novel new possibilities in chromate bioremediation.

MATERIAL AND METHODS

Table 2. Strains used in this study

Strains	Characteristics	References
Shewanella oneidensis strains		
MR1-R	derivative of the Wild type, Rif[R]	(Bordi et al., 2003)
MR1 ΔchrAso	MR1 deleted from SO0986 gene (Chromate efflux pump)	This work
MR1 ΔchrAso pchrAso	MR1 deleted from SO0986 harboring pbad33 vector containing chrAso (complemented mutant)	This work
MR1 pchrAso	MR1 wild-type harboring pbad33 vector containing chrAso	This work
MR1 Δfdh	MR1 deleted from SO0988 gene	This work
MR1 Δfdh pfdh	MR1 deleted from SO0988 harboring pbad33 vector containing fdh (complemented mutant)	This work
MR1 ΔcymA	MR1 deleted from SO4591 gene	(Baraquet et al. 2009)
MR1 ΔcymA pcymA	MR1 deleted from SO4591 harboring pbad33 vector containing cymA (complemented mutant)	This work
MR1 pcymA	MR1 wild-type harboring pbad33 vector containing cymA	This work
MR1 ΔdmsA1	MR1 deleted from SO1429 gene	(Baraquet et al. 2009)
MR1 ΔdmsA2	MR1 deleted from SO4358 gene	(Baraquet et al. 2009)
MR1 ΔdmsA2 pdmsA2	MR1 deleted from SO4358 harboring pbad33 vector containing dmsA2 (complemented mutant)	This work
MR1 ΔdmsA1 ΔdmsA2	MR1 deleted from SO1429 and SO4358 genes	This work
MR1 ΔchrAso ΔdmsA2	MR1 deleted from SO986 and SO4358 genes	This work
Other Shewanella strains		
S. algidipisicola	Newly isolated from the Mediteranean sea, Algeria (H111)	This work
S. fidelis	Newly isolated from the Mediteranean sea, Algeria (H76)	This work
Escherichia coli strains		
CCC118λpir	Δ(ara-leu) araDE ΔlacX74 galE galK phoA20 thi-J rpsE rpoB argE (Am) recAl λpir lysogen	(Herrero et al., 1990)
1047/pRK2013	Mobilizing strain, Km[R]	(Herrero et al., 1990)
MC1061	Δ(ara-leu)7697 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 e14⁻ mcrA0 relA1 rpsL150(Str[R])	(Casadaban & Cohen, 1980)

1. Bacterial strains, plasmids and culture media

The bacterial strains and plasmids used in this study are described in Tables 2 and 3.The *E. coli* strains CC118 λpir and 1047/pRK2013 (Herrero *et al*, 1990) used for conjugation and MC1061 (Casadaban & Cohen, 1980) derivatives used for chromate resistance and reduction assays were routinely grown aerobically at 37°C or 30°C when specified, in Lysogeny Broth (LB) medium containing yeast extract (5g/L), bacto-tryptone (10g/L) and NaCl (5g/L) (Miller, 1972).

All *Shewanella* strains including *S. fidelis* H76 and *S. algidipiscicola* H111 were routinely grown at 28°C in classic LB medium for *S. oneidensis* MR-1 (Bordi *et al*, 2003) strains. However, addition of NaCl to a final concentration of 15g/L to LB medium was necessary for growth of H76 and H111 strains. Strains were either incubated under anaerobic (static) or aerobic (gentle agitation) conditions. In Anaerobic conditions, LB medium was supplemented with 20mM trimethylamine oxide (TMAO) as electron acceptor and buffered with 40mM of MOPS. When growth was performed in the presence of chromate, disposable tubes were used and incubated either statically or under agitation. The dissolved oxygen (DO) of the latter condition measured by a Clark electrode was 10%. This condition was then referred as semi-aerobic. Chromate challenge was carried out by supplementing LB medium at the required final concentration with a filter-sterilized stock solution of potassium chromate (K_2CrO_4, Sigma-Aldrich). If required, media were solidified by adding 17 g/L agar. When needed, antibiotics are added at the following concentration: chloramphenicol 25 μg/mL, ampicillin 50 μg/mL, kanamycin 10 μg/mL and streptomycin 100 μg/mL. Arabinose is added to induce the expression of a gene under the control of a pBAD promoter carried on a dedicated plasmid (pBAD33, Annexes, Figure S1). Growth was determined spectrophotometrically by monitoring changes in optical density at 600 nm compared to the same medium without bacterium (OD_{600nm}). The bacterial strains are kept in their culture medium supplemented with glycerol (20% final), then frozen at -80°C.

Table 3. Plasmids used in this study

Plasmids	Characteristics	References
pBAD33	Vector containing pBAD promoter with a p15 origin of replication, Cm^R	(Guzman *et al.*, 1995)
pchrA$_{so}$	*chrA$_{so}$* sequence cloned into pBAD33	This work
pdmsA2	*dmsA2* sequence cloned into pBAD33	(Baraquet *et al*. 2009)
pfdh	*fdh* sequence cloned into pBAD33	This work
pGE593	*lacZ* transcriptional reporter fusion cloning vector, Amp^R	(Eraso and Weinstock, 1992)
pACYC185	Cloning vector containing the p15A origin of replication, Cm^R	(Chang and Cohen, 1978)
pACYC184-lacZ	pACYC184 vector cotaining *lacZ* sequence from pGE593	This work
pmxd$_{450}$::lacZ	Promoter region upstream of mxdA cloned into pACYC184-lacZ	This work
pchrA$_{so}$::*lacZ*	Promotor region upstream of chrAso cloned into pACYC184-lacZ	This work
pRK2013	Broad-host-range helper vector; Tra$^+$,Kn^R	(Figurski and Helinski, 1979)
pKNG101	Marker exchange suicide vector; *sacB*, *mobRK2*, *oriR6K*, Sm^R	(Figurski and Helinski, 1979)

2. Molecular biology techniques

2.1. Isolation, purification and hydrolysis of DNA

The plasmid DNA was prepared using the «GenElute^{TM} Plasmid Miniprep» (Sigma Aldrich) and their concentration was estimated after agarose gel electrophoresis. Chromosomal DNA was obtained using the kit «GenElute^{TM} Bacterial Genomic DNA kit» (Sigma Aldrich). Restriction enzymes used come from New Biolabs. Conventional plasmid preparation techniques, restriction enzyme digestion and ligation digestion were performed under the conditions stipulated by the suppliers.

2.2. DNA amplification by PCR

In general, for a 50µL reaction, a mixture containing the template DNA (10 to 20 ng of chromosomal DNA or approximately 300µg of plasmid DNA), a pair of oligonucleotide primers (100 to 200 ng each primer), the four dXTPs (100µM) and a unit of Taq DNA polymerase « GoTaq® DNA Polymerase » (Promega) as well as 1X of its specific buffer, is subjected to a succession of cycles: 1-Initial denaturation: 2 min at 95°C; 2-Denaturation: 30 sec to 95°C; 3-Hybridization: 30 sec at 53°C; 4-Elongation: 1 min per kilobase at 72°C; 5-Final elongation: 3 min to 72°C.

Steps 2 to 4 are repeated between 20 and 30 times depending on the amount of DNA desired. The duration of step 4 varied according to the size of the DNA to be amplified, given that on average the elongation rate of the polymerase is greater than 1 kb per min. The temperature of step 3 may also vary depending on the Tm (melting temperature) of the primers used. If the enzyme used for PCR is the high-fidelity DNA polymerase « Q5 hot start High-Fidelity », with its specific buffer «Q5 Reaction Buffer» (Biolabs), we used the following program: 30sec of initial denaturation at 98°C followed by 24 amplification cycles including 10sec of denaturation at 98°C, 30sec of hybridization at 55°C and of 72°C for elongation, and finally 2 min at 72°C for the final elongation step.
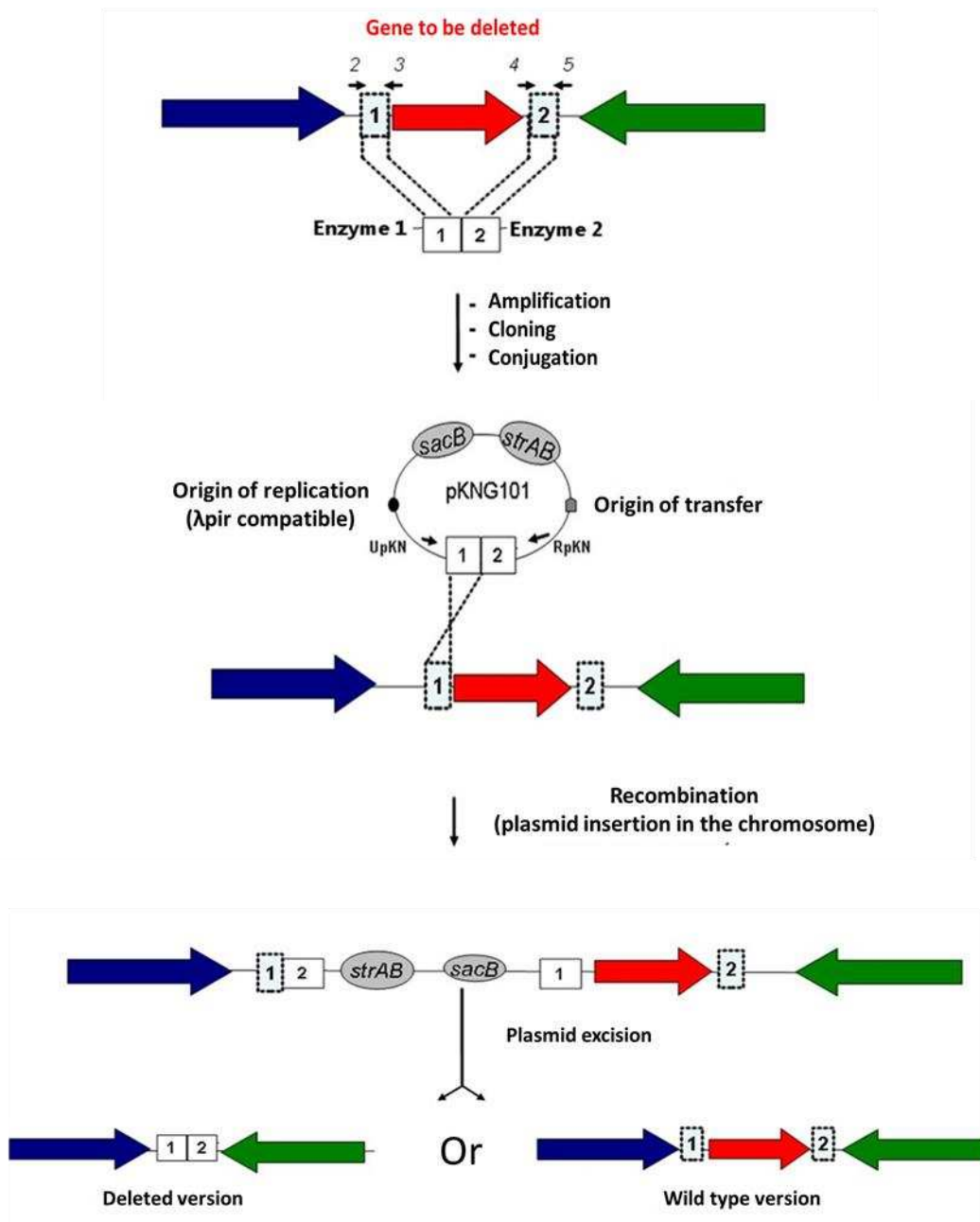
**Figure 46. Diagram of gene deletion by homologous recombination in *S. oneidensis.* The gene to be deleted is represented in red. The blue and green areas represent the flanking regions. Squares 1 and 2 are homologous recombination zones. Primers (2, 3, 4, 5, UpKN and RpKN) are represented as arrows. The plasmid pKNG101 is not replicative in *S. oneidensis*. It carries a gene coding for streptomycin resistance and a gene conferring sensitivity to sucrose. If both recombination events involve the same regions, the strain becomes wild again. If the homologous regions involved are different, then the region of interest is deleted.

2.3. Plasmid construction

The cloning vector pBAD33 (Guzman *et al*, 1995) carrying inducible promoter was used during this work (Annexes, Figure S1). In general, the genes to be cloned were amplified by PCR without their promoter, from chromosomal DNA of *S. oneidensis* MR1-R using « Q5 hot start High-Fidelity ». The primers allowed the generation of restriction sites compatible with those present in the multiple cloning sites of the expression vector pBAD33. The PCR products were then purified using « EZ-10 Spin Column PCR Products » kit (Bio Basic Inc) and digested and then cloned into the expression vector hydrolyzed by the same enzymes as the PCR product. Hybrid plasmids thus obtained were verified by DNA sequence.

The pACYC184-lacZ vector was constructed by cloning the β-galactosidase-encoding gene lacZ from pGE593 (Eraso & Weinstock, 1992) (Annexes, Figure S2) into the vector pACYC184 (Chang & Cohen, 1978) (Table 2, Annexes, Figure S3). The ligation product was transformed into *E. coli* strain MC1061. The resulting plasmid called p*chrA$_{SO}$*::lacZ was introduced into *S. oneidensis* MR1-R by conjugation. Plasmid construction was also checked by DNA sequencing. Another fusion used as control, named p*mxd450*::lacZ, was similarly constructed using a DNA fragment corresponding to the 450 bp upstream from the ATG of *mxdA*.

2.4. Construction of deletion mutants

The construction of deletion mutants in *S. oneidensis* was carried out by a four-step homologous recombination mutagenesis technique (Figure 46). The first step was to amplify the two 500-base pair (bp) fragments flanking the gene to be deleted by PCR using the high-fidelity DNA polymerase enzyme « Q5 hot start High-Fidelity » (Biolabs) and two pairs of primers that should be previously purified using the « GenElute^{TM} Bacterial genomic DNA » kit (Sigma) following the manufacturer's protocol. The first (primers 2 and 3) and the second pair (primers 4 and 5) used as template the chromosomal DNA of the wild strain of *S. oneidensis*. The PCR products obtained were then purified with the « EZ-10 Spin Column PCR Products » kit (Bio Basic Inc). Primers 3 and 4 share a

complementary sequence which allowed the realization of a second PCR with primers 2 and 5 using the products of the first PCR as DNA template. This second PCR resulted in the fusion of the two fragments flanking the gene of interest, thus obtaining a fragment of approximately 1kb. After purification, PCR products and the plasmid pKNG101 (Annexes, figure S2) were digested with the same restriction enzymes (Biolabs) for 1h30 at 37°C. After purification, the products resulting from the digestion were incubated for 16h at 16°C with DNA ligase of T4 phage (Biolabs) in order to fuse the gene of interest together with the plasmid.

The second step is the introduction of the obtained plasmid in *E. coli* CC118λpir strain by transformation. To this end, the *E. coli* strain CC118λpir should be treated by $CaCl_2$ to become competent. From an overnight preculture of *E. coli* CC118λpir strain, 50 ml of LB culture medium were inoculated with 1:100 of starter culture and then incubated at 37°C in a shaking incubator. Growth was stopped when OD_{600nm} reached values between 0.4 and 0.6, to be centrifuged at 4000 rpm for 10 min at 4°C. The pellets were then resuspended in 25 ml of 50mM $CaCl_2$ buffer (50mM $CaCl_2$ and 15% of glycerol) previously cooled to 4°C. After 20 minutes of incubation at 4°C, a second centrifugation was then carried out. The pellet was resuspended in 4 mL of the same buffer and left for another 20 min at 4°C before being divided into several aliquots and then stored at -80°C.

For the transformation, 100µL of competent cells were added to 7µL of ligation mixture for 20 min at 4°C. The cells were then subjected to a thermal shock for 2 min at 42°C, and immediately thereafter incubated for 2 min at 4°C. LB medium was then added, and the suspension was incubated at 37°C for 1h to allow expression of the antibiotic resistance genes. The vector pKNG101 (Figurski & Helinski, 1979) contains an λpir-dependent replication origin (R6K), a *sacB* cassette and a streptomycin resistance cassette (stp^R) (Annexes, Figure S4). The later allows the selection of colonies harboring the plasmid. Transformants were therefore selected on solid agar LB medium supplemented with Streptomycin at 37°C. The presence of the insert in the various transformants was confirmed by PCR using two primers, RpKN and UpKN, which hybridize on the plasmid on either side of the multiple cloning sites (Figure 46).

The third step was the introduction of the plasmid into *S. oneidensis* by conjugation. The mobilizing strain *E. coli* 1047 which harbors a pRK2013 plasmid (the helper) (Figurski & Helinski, 1979) possessing the genes encoding the proteins necessary for the synthesis of the conjugative bridge and the donor strain CC118λpir containing a non-replicative plasmid, were placed in contact on LB plates at 37°C for 2 h. This contact time allows the transfer of the plasmid "helper" into the donor strain CC118λpir. After that, the receptor strain *S. oneidensis* was added and the plates were then incubated at 28°C for 6h. This second contact time allows the transfer of the recombinant plasmid pKNG101 through the conjugation bridge to the receptor strain. Afterwards, the set of cells was resuspended in 1 ml of LB containing 4μl of colicin A, in order to eliminate the *E. coli* cells (Cascales *et al*, 2007), and incubated for 1h at 28°C. Since the plasmid pKNG101 is non-replicative in *S. oneidensis*, it must be inserted in the chromosome to be conserved and to confer resistance to streptomycin. The strain of *S. oneidensis* used in this study is naturally resistant to rifampicin, unlike *E. coli*. Thus, conjugants were selected on solid agar medium (LB agar) containing streptomycin (100μg/mL) and rifampicin (10μg/ml) at 28°C.

The fourth step consists of the excision of the plasmid from the chromosome using another recombination step. For this purpose, the streptomycin-resistant cells were plated on LB containing 6% sucrose, to select the second recombination event, and incubated for 2 days at 16°C, and then for 1 day at 28°C. In fact, the plasmid pKNG101 possesses the *sacB* cassette which makes the cells sensitive to sucrose. To survive, the cell must eliminate the plasmid sequence from its chromosome. Two possibilities of recombination were possible, either the strain becomes wild again or it loses the gene of interest. The colonies were then tested by PCR using the primers 1 and 5 which hybridize upstream and downstream of the gene of interest, to determine whether the strains obtained are wild or lacking the gene of interest.

3. Biochemical techniques

3.1. Measurement of β-galactosidase activities

The β-galactosidase activity was measured on the total extracts according to Miller's assay adapted for use with plate reader. The *S. oneidensis* MR1-R strain containing p*chrA*$_{SO}$::*lacZ* or p*mxd*450::*lacZ* was grown overnight on LB-agar plate. The overnight culture was diluted to an OD600nm = 0.1 in fresh liquid LB medium containing various concentrations of chromate (0, 0.05, 0.1 and 0.2 mM) and incubated in semi-aerobiosis at 28°C for 16 hours prior to β-galactosidase activity quantification. Thereafter, cells were transferred into a microtiter plate and OD_{600nm} was measured using a Tecan Spark 10M microplate reader. Cells were then lysed using 1µl of lysozyme at 10 mg/mL and 9µl of « PopCulture Reagent^{R} » (Novagen^{R}) prior to incubation with 100µl of Z buffer (62 mM Na_2HPO_4, 45 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) in each well. Forty microliters of O-nitrophenyl-ß-D-galactopyranoside (ONPG) at 4 mg/ml were added to the mix in each well, before kinetic quantifications of OD_{420nm} every second for 15 minutes. Slope of the obtained curves were calculated and β-galactosidase activity (arbitrary units) determined by the following equation:

$$(1000 \times slope)/(OD_{600nm} \times volume\ of\ reaction\ (\mu L)).$$

3.2. Measurement of chromate reduction

3.2.1. *In vivo* and *In vitro* Cr(VI)-reduction assay

In vivo Cr(VI) measurements were carried out on the supernatant of strain cultures exposed to several chromate concentrations during different time of incubation under anaerobic and semi-aerobic conditions. Cell cultures were centrifuged at 10 000 rpm for 5 min and residual Cr(VI) concentration present in the supernatant and cell pellets was determined using the S-diphenylcarbazide (DPC) method (Urone, 1955) slightly modified. Briefly, 10, 20, and 50 µL supernatant samples were added in a 2 mL tube containing 1 mL of H_3PO_4 0.5% and the volume of water necessary to obtain a 1960µL final volume. Forty microliters of DPC reagent (5 mg/mL in 95% acetone and stored in dark at room temperature) were added, gently mixed and kept at room temperature for 5−10 min.

Over night (O/N) culture

O/N culture diluted to OD_{600} of 0.2

Culture poured into Petri dish

Incubation at 28°C for 24h and/or 48h under static conditions

Pellicle formation on the air-liquid interface

Air

Pellicle

Liquid LB medium

Mature pellicle collection

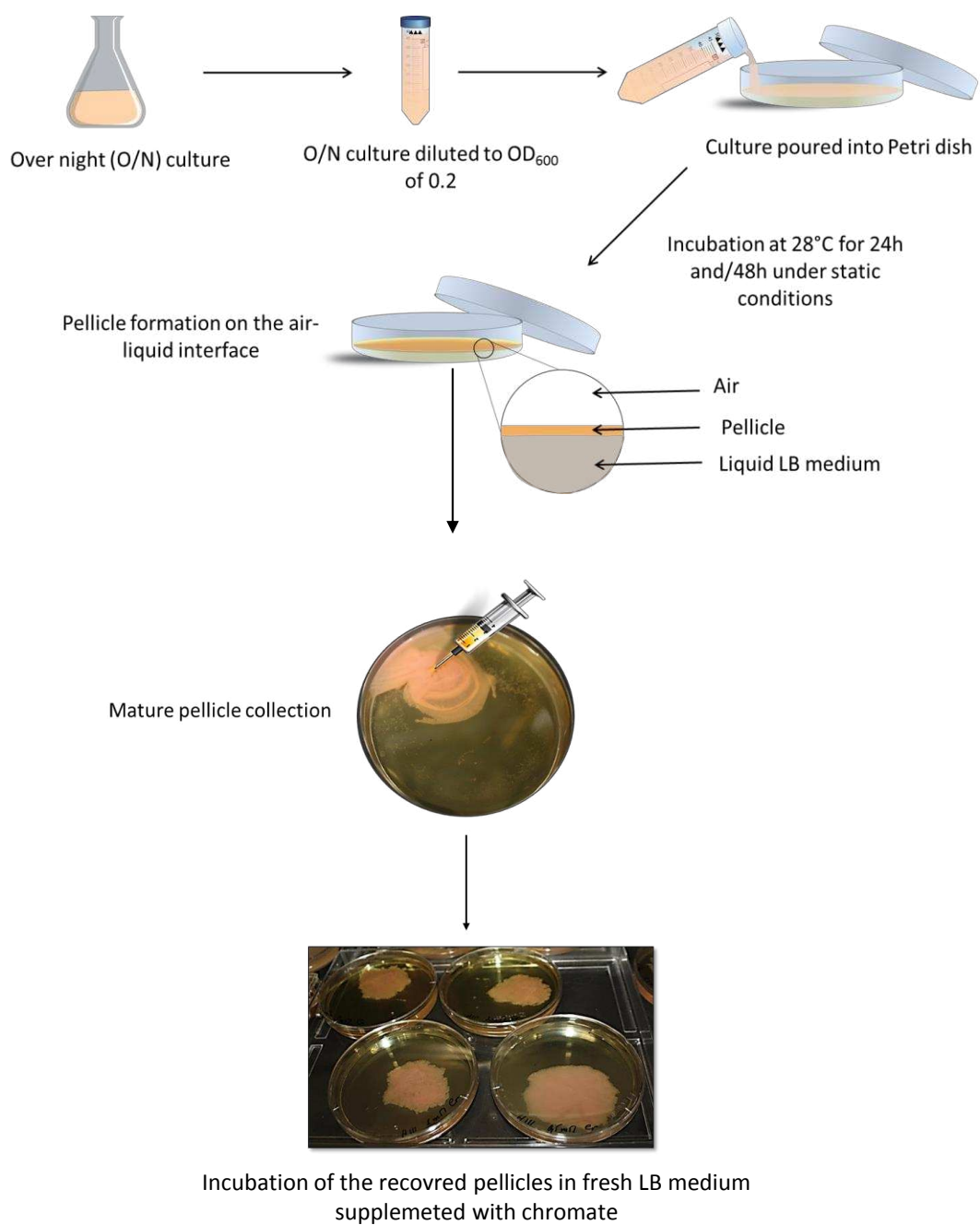Incubation of the recovred pellicles in fresh LB medium supplemeted with chromate

Figure 47. Schematic representation of the technique used prior to the measurement of Cr(VI) reduction of *Shewanella* strains pellicles.

Absorbance was measured at 540nm. Cr(VI) concentration in the samples was calculated from a standard curve.

In vitro measurements of chromate reductase activity of *S. oneidensis* strains (WT and the *chrA_{SO}* deleted mutant) and *E. coli* strains (containing either the pBAD33 vector or the plasmid p*chrA_{SO}*) were performed on crude extracts as described previously with slight modifications. Cells were grown aerobically overnight in LB medium at 28°C and 30°C respectively, and then harvested, washed with sodium phosphate buffer (pH7) and resuspended in the same buffer before disruption by French press. The cell lysates were then centrifuged and the supernatants (crude extracts) recovered. Protein concentrations were measured using Protein Assay Dye Reagent (Bio-Rad), and bovine serum albumin as standard. Reaction mixtures (1 mL) containing 100 μM of K_2CrO_4 [Cr(VI)] and 1.5 mg of proteins (crude extracts) were incubated at 30°C for 30 min. The residual Cr(VI) concentration in the reaction mixture was estimated using the DPC method as described above.

3.2.2. Cr(VI) reduction measurement of *Shewanella* pellicles

In order to measure pellicles chromate reduction rates, *Shewanella* mature pellicles were collected using a sterile syringe while avoiding the recovery of the LB medium that is underneath. Pellicles were then placed into an empty Petri dish. Thereafter LB medium supplemented with different chromate concentrations was poured gently on the recovered pellicles, which were then incubated for 24h and 48h at 28°C under static conditions. After incubation time, pellicles and LB medium were recovered and centrifuged at 10 000 rpm for 10-15 min prior to measurement of total chromium (As described below) and residual Cr(VI) in the cellular and supernatant fractions (Figure 47).

3.2.3. Total chromium measurement assay

Total chromium in both the resuspended pellet and the supernatant, resulting from centrifugation of cultures previously exposed to chromate, were measured by inductively coupled plasma optical emission spectrometry (ICP-OES). Prior to this measurement, 71.4μL samples (cellular or supernatant fractions) were resuspended in 428.6μL of LB.

Thereafter, 500µL of pure nitric acid (HNO_3) was added. The mixture of sample and HNO_3 was then incubated in 95°C water bath for 30 min, and 4 ml of 20% HNO_3 solution was then added to the treated samples.

4. Assessment of strains chromate resistance

4.1. Chromate resistance assays in *Shewanella* strains

Shewanella strains pre-cultures grown overnight on LB plates were suspended in LB medium and used to inoculate fresh LB medium to an initial OD_{600nm} of 0.2. Cells were then grown until an OD_{600nm} of 0.5 and submitted to two different assays. For the spot assay, 10-fold serial dilutions of cell cultures were spotted on LB plates supplemented or not with 0.5mM of chromate and incubated at 28°C. Plates were scanned after 4 days of incubation. For the viability assay, chromate was added to the cell cultures (at OD_{600nm} of 0.5) to a final concentration of 0.2mM. After 5 hours, cells were appropriately diluted in LB and spread onto LB agar, and incubated at 28°C. The total number of viable cells was estimated based on the number of colony-forming unit (CFU). Results were expressed as the percentage of viable counts measured in these conditions compared to that of the same culture grown in the absence of chromate.

4.2. Chromate resistance assay in *E. coli*

Pre-cultures of MC1061 derivatives containing either the plasmid p*chrA$_{SO}$* or the pBAD33 vector were used to inoculate fresh LB medium to an initial OD_{600nm} of 0.05. Cells were grown until an OD_{600nm} of 0.2 prior to the addition of chromate at various final concentrations (0, 0.2, 0.4, 0.8 and 1.2mM), then the cells were incubated at 30°C. The cultures were regularly sampled to measure the OD_{600nm} and to quantify the chromate using the DPC method (As described above).

Table 4. Trace metal solution composition

Component	Final concentration (g/l)
$CoCl_2$ $6H_2O$	0,01g/l
$CuSO_4$ $5H_2O$	0,01 g/l
$MnCl_2$ $4H_2O$	0,14 g/l
Na_2MoO_4 $2H_2O$	0,04 g/l
$ZnSo_4 7h_2O$	0,02 g/l
$FeCl_3$ $6H_2O$	2,15 g/l

5. Divers assays

5.1. Pellicle assay

Cells were grown overnight and then diluted in 50 ml of fresh LB medium, supplemented with NaCl in the case of *S. fidelis* H76 and *S. algidipiscicola* H111, to reach an OD_{600nm} of 0.2. This suspension was then transferred into a Petri dish and incubated at 28°C without being shaken for 24h for *S. oneidensis* MR-1 and 48h for H76 and H111 strains (Figure 47). For the two latter strains the LB medium was supplemented with different concentrations of trace elements either separately or combined in a solution a trace metal solution containing $CoCl_2$ $6H_2O$, $CuSO_4$ $5H_2O$, $MnCl_2$ $4H_2O$, Na_2MoO_4 $2H_2O$, $ZnSo_4$ $7H_2O$ and $FeCl_3$ $6H_2O$. The final concentration added of each component is indicated in Table 4.

5.2. Chemotaxis assay (µplugs microscopy technique)

The bacteria were grown aerobically in LB medium, supplemented with NaCl in the case of *S. fidelis* H76 and *S. algidipiscicola* H111 at 28°C to an optical density at 600nm of about 0.1-0.2. Afterwards strains cultures were centrifuged (10 min at 3500 rpm) and subsequently resuspended in buffer (LM supplemented with 15mM lactate, 0, 1% Tween 20 and 10 μg/ml tetracycline) in order to obtain an optical density at 600nm of 0.5. A bridge was constructed by placing two square coverslips (22mmx22mm) on each side of a plastic slide (76mmx26mm) (Figure 48). For this technique, since *S. oneidensis* MR-1 cells adhere to the glass, it is necessary to use plastic slides that made from PETG and manually cut from a plate Vivak (Bayer). A 5μL agarose drop prepared from 2% low-melting agarose containing either the test compound (nitrate, chromate, nickel and cobalt) or buffer was then placed on the PETG slide. The final concentrations of compounds tested were 50mM for nitrate and 20mM for chromate, nickel and cobalt. The whole was then covered with glass rectangular coverslip (24mmx60mm). This structure was held in place by cyanoacrylate glue. After 30 minutes, for the agarose and the adhesive to solidify, approximately 150μL of cells were introduced into the space between the slide and the coverslip. The microscopic images were taken after 30 minutes at the edges of the drops using a 10x objective (Figure 48).
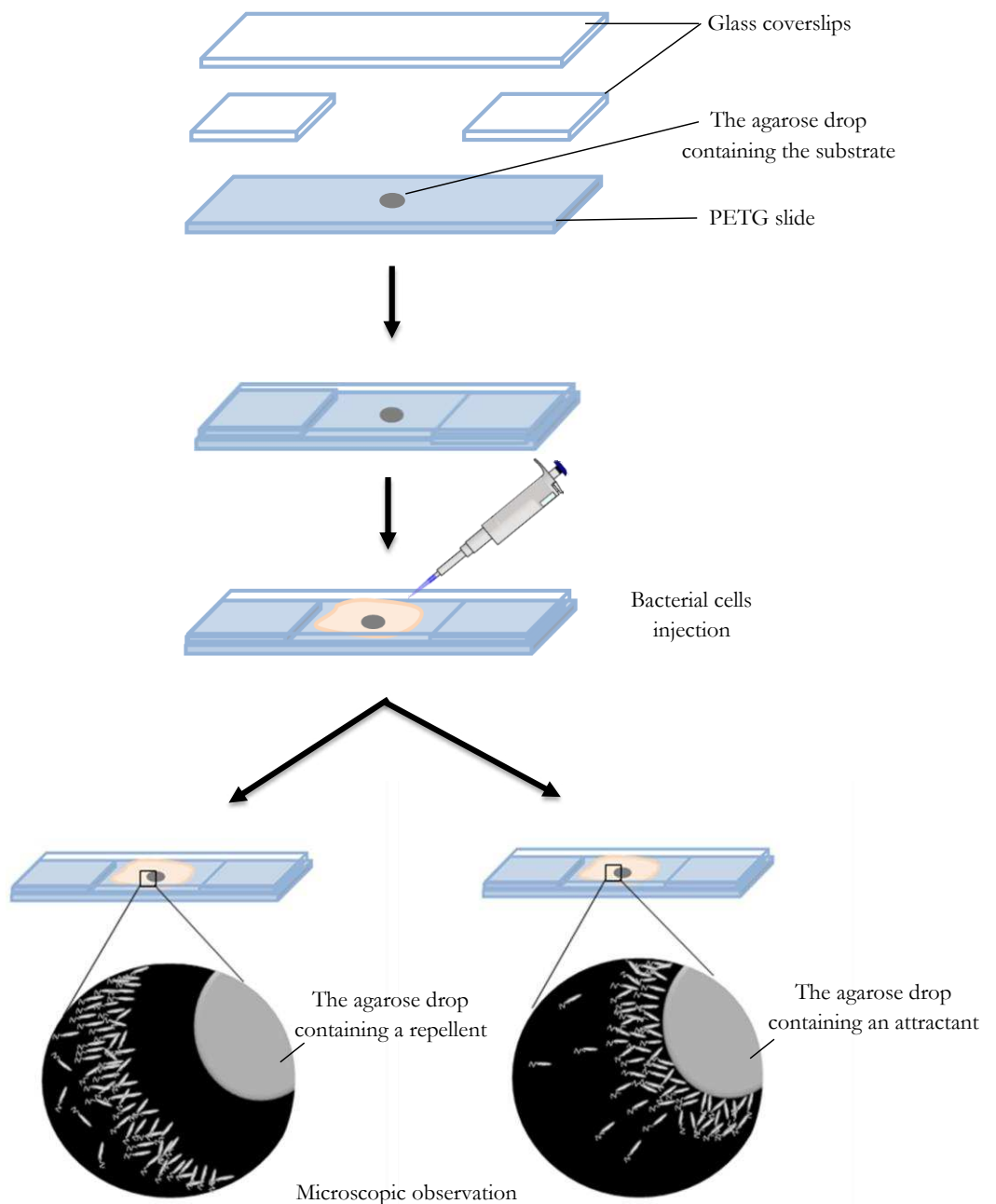
Figure 48. Representation of the µplugs microscopy technique The substrate to be tested is premixed with agarose and deposited on the PETG slide. The cells are placed between PETG slide and the coverslip.

6. Isolation of new *Shewanella* species method

6.1. Samples collection

To isolate new marine *Shewanella* sp. strains, more than ten samples of seawater and sediments coming from different Algerian harbors, beaches were collected. Aseptically using plastic bottles, seawater samples were taken from the uppermost 60 centimeters and sea-sediments samples were taken from about 10 to 15 meters deep. All samples were stored at room temperature until processed. The collected samples as well as the geographical coordinates of the sampling sites were summarized in Table 5

6.2. Isolation

To isolate *Shewanella* cells present in the samples, the latter were cultured in LB medium supplemented with NaCl (30g/l), TMAO (20mM) and MOPS (40mM) in aerobic conditions at 28°C to mimic marine environment conditions. After 24h of incubation, 10-fold serial dilutions were spread on LB plates containing TMAO and NaCl. Simultaneously, 10µl of the first culture was used to inoculate 15ml of fresh LB medium. Plates and liquid cultures were then incubated at 28°C. This procedure was repeated successively two more times in order to enhance the amount of *Shewanella* cells which facilitate their isolation. Colonies presenting similar phenotypic aspects of *Shewanella* species were selected and further subjected to PCR amplification using the primer set She211F and She1259R that specifically targets *Shewanella* 16SDNA.

7. Genome sequencing and bioinformatic analysis

The genome sequencing of H76 and H111 strains was carried out at the "Molecular Research LP (MR DNA) Laboratory" (USA). The library was prepared using Nextera DNA Sample Preparation Kit (Illumina) following the manufacturer's user guide and sequenced using the HiSeq 2500 system (Illumina).

The genome analysis was carried out using the programs of the Mage platform (http://www.genoscope.cns.fr/agc/microscope/home/index.php). The phylogenic tree representing the nearest neighbours of H76 and H111, based on 16S ribosomal DNA

Table 5. Sampling site, geographical coordinates and type of samples collected

Locations	Geographical coordinates	Type of samples
Annaba, Chapui beach	36°55'40.0"N 7°45'38.6"E	Seawater
Annaba, Sidi Salem beach	36°51'17.0"N 7°47'41.2"E	Seawater
Annaba, Maritime harbor	36°53'44.5"N 7°45'37.6"E	Seawater and sediments
Algiers, Moretti beach	36°45'38.4"N 2°51'38.8"E	Seawater
Algiers, Maritime harbor	36°45'50.3"N 3°03'44.4"E	Seawater and sediments
Béjaia, Les Aiguades beach	36°45'53.2"N 5°06'10.4"E	Seawater
Béjaia, Maritime harbor	36°44'39.6"N 5°05'14.2"E	Seawater and sediments
Sekikda, Ravin des lions-beach	36°54'52.5"N 6°52'59.8"E	Seawater
Sekikda, Stora harbor	36°54'06.9"N 6°52'45.4"E	Seawater and sediments
Sekikda, Maritime harbor	36°53'10.1"N 6°54'05.6"E	Seawater and sediments

sequences, was constructed using MOLE BLAST (http://blast.ncbi.nlm.nih.gov/*moleblast*/*moleblast*.cgi)

Proteins sharing homologies with ChrA$_{SO}$ were searched in the Bacteria kingdom using the BlastP software on the NCBI server (https://blast.ncbi.nlm.nih.gov). For the phylogenetic analysis, we used the "Phylogeny.fr" software in the "one-click" mode, i.e. with the default parameters optimized by the authors (http://www.phylogeny.fr/). To obtain the phylogenetic tree of a subset of ChrA proteins, we used the software "FigTree" version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) in which we entered the result in Netwick format obtained with "Phylogeny". The tree rooting was performed with the midpoint option. Prediction of transmembrane helices of ChrA$_{SO}$ was carried out using the TMHMM online software (http://www.cbs.dtu.dk/services/TMHMM-2.0/).
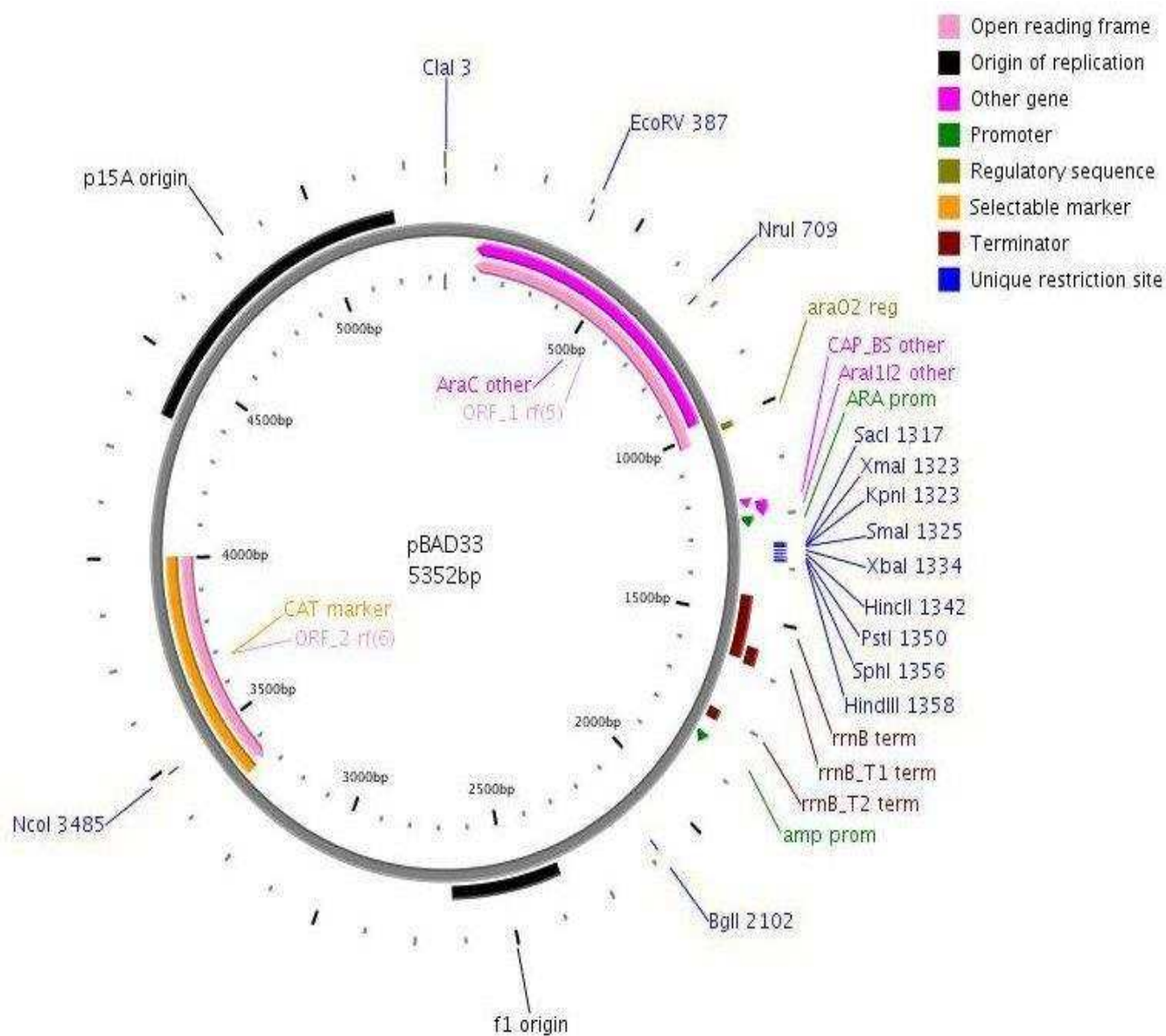
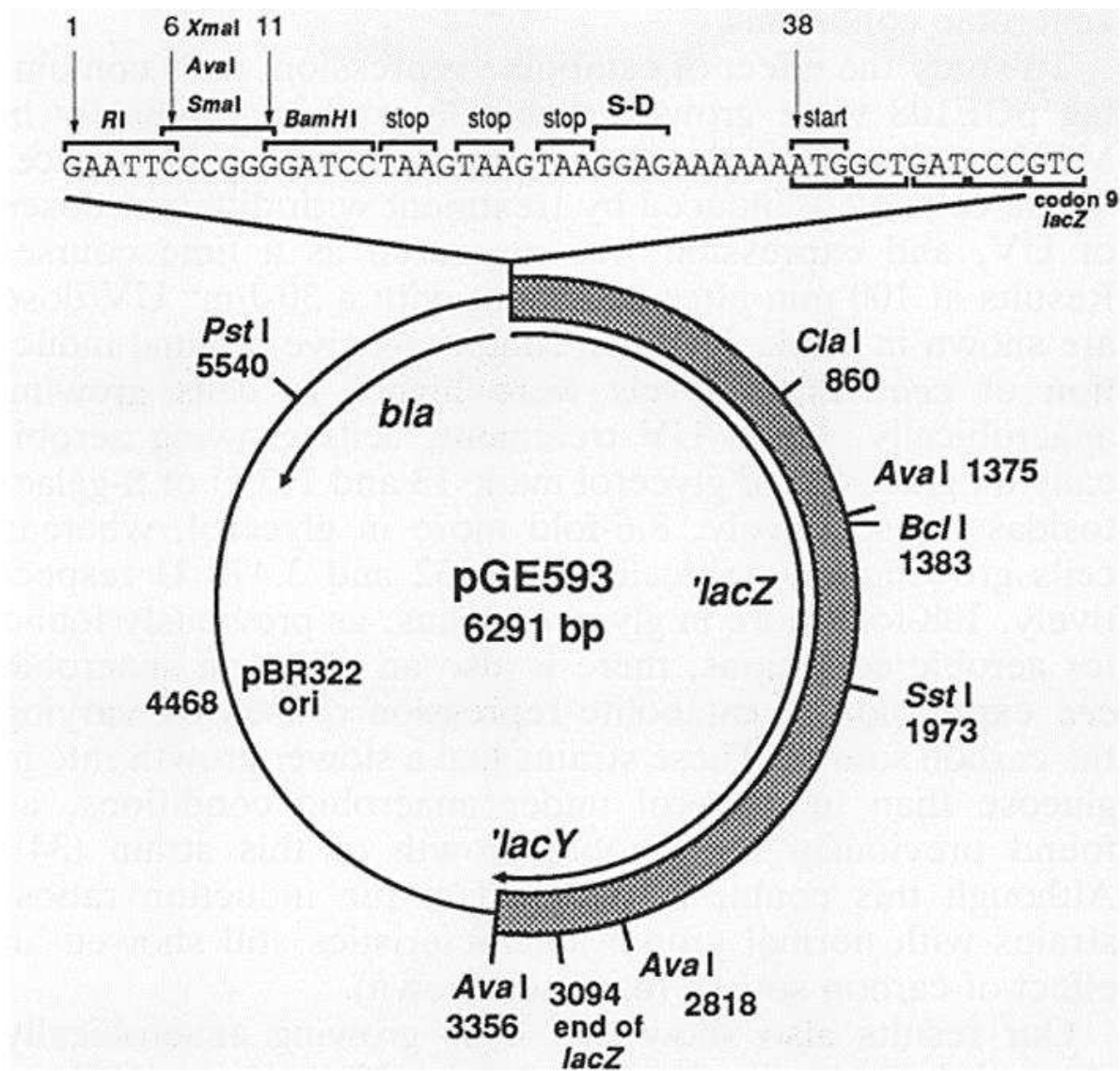# ANNEXES

Figure S1. pBad33 vector map

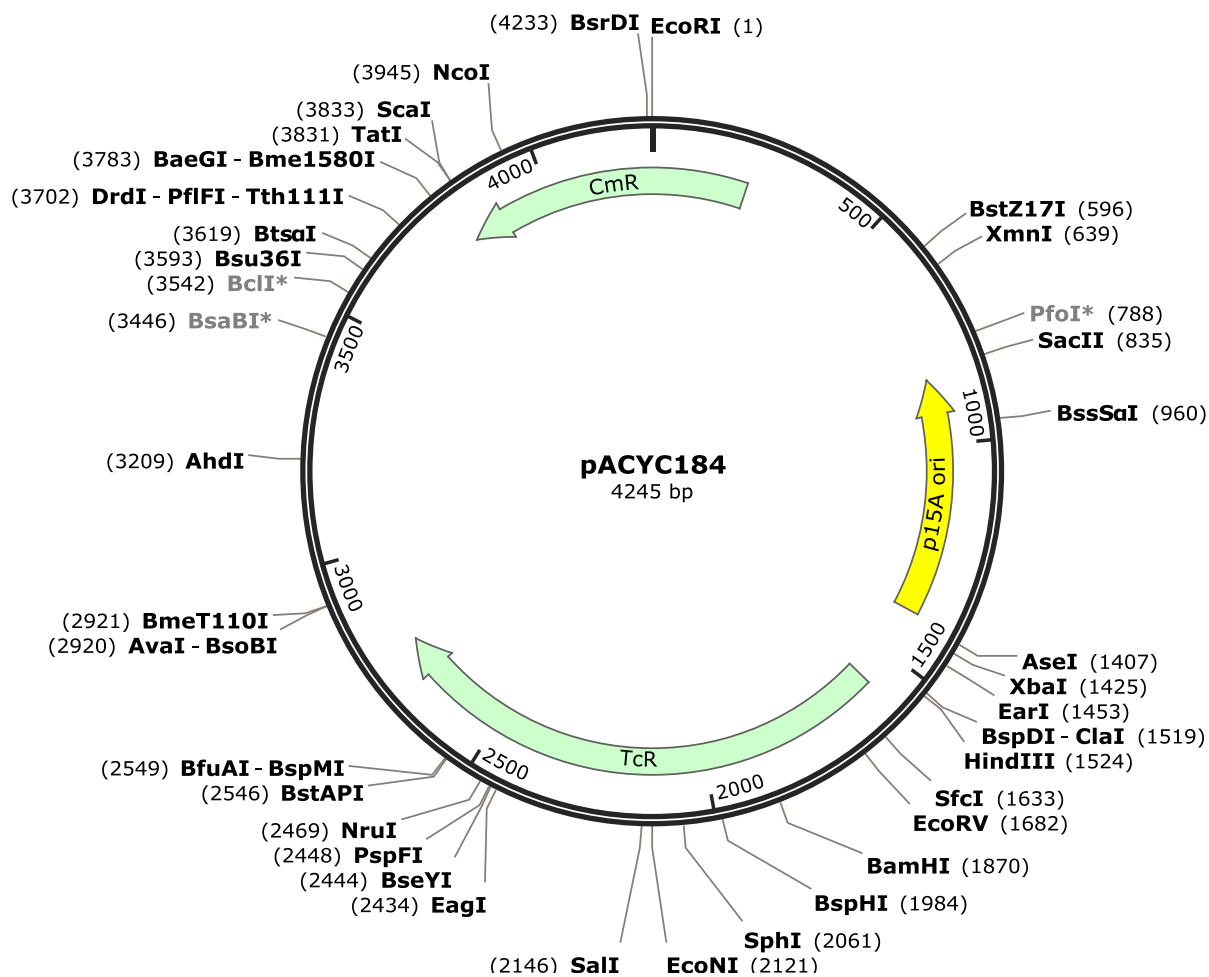**Figure S2. Operon fusion vector pGE593 map**
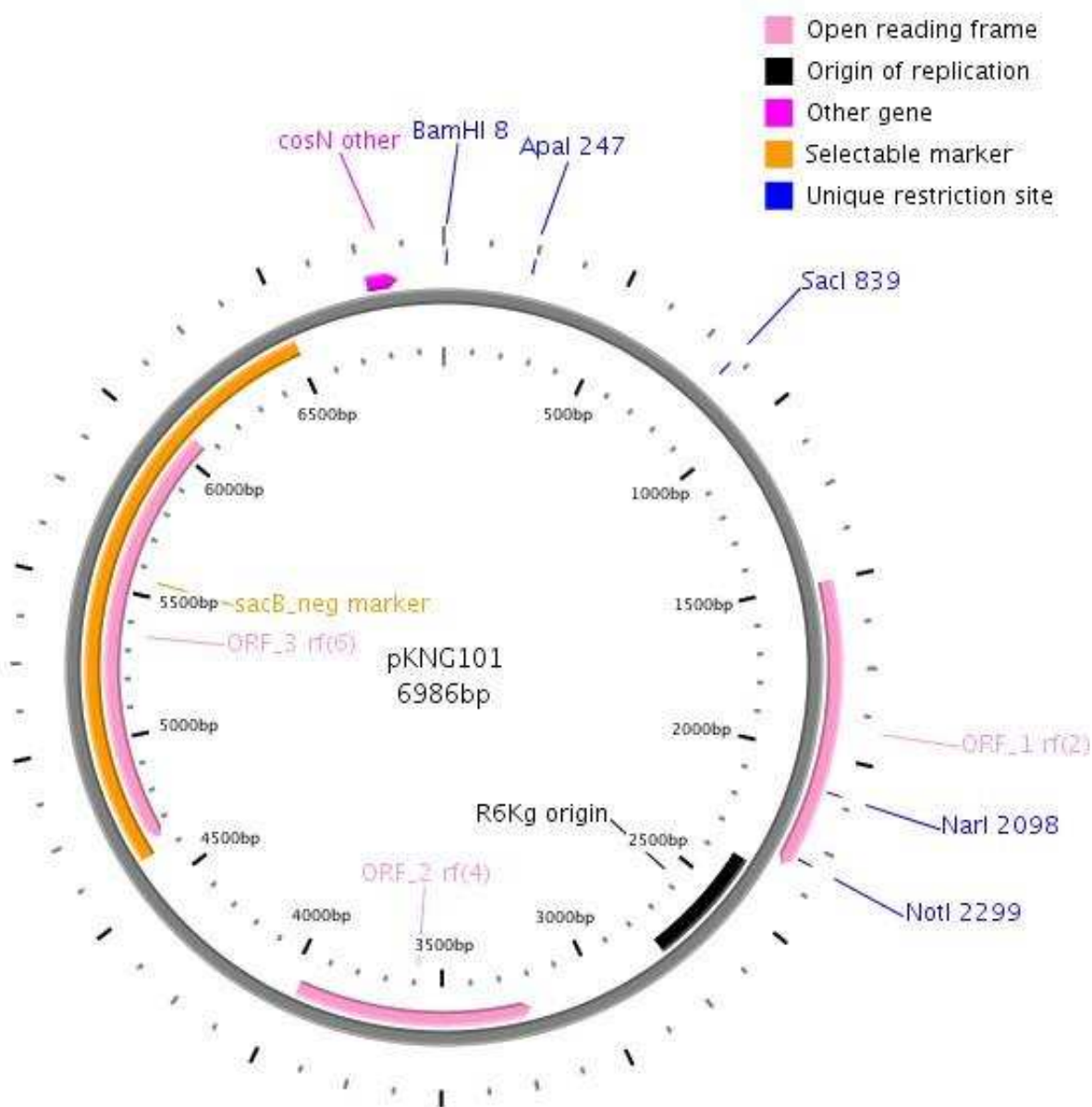
Figure S3. pACYC184 vector map

Figure S4. pKNG101 vector map used for gene deletion in *S. oneidensis*

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Abstract

The widespread use of the toxic heavy metal chromium (Cr) in industrial applications resulted in large quantities of Cr being discharged into the environment, causing severe contamination of global soil and water systems. Cr primarily exists in two stable forms, Cr(III) and Cr(VI). The latter is highly toxic due to its strong oxidizing nature and its high solubility. The model organism for bioremediation *Shewanella oneidensis* MR-1 has evolved diverse resistance mechanisms to cope with chromate toxicity.

The first aim of the present thesis was to study the chromate resistance and reduction mechanisms of this bacterium under semi-aerobic conditions. We showed that $chrA_{SO}$ gene is induced by chromate and its deletion impairs the chromate resistance and reduction capacity of MR-1 strain, we confirmed that its product functions as an efflux pump to extrude chromate ions from the cytoplasm protecting cells from chromate toxicity. With *cymA*-deletion mutants, we revealed the involvement of the c-type cytochrome CymA in chromate resistance and reduction. We also identified a potential chromate reductase DmsA2, as well as two other proteins Fdh and DmsA1 that are potentially involved in chromate resistance and reduction in MR-1 strain.

In the second part of this work, we isolated, identified and characterized two novel Mediterranean *Shewanella* sp. strains, *S. fidelis* H76 and *S. algidipiscicola* H111. Both strains are characterized by their great chromate resistance and their ability to reduce it efficiently even at high concentrations. Although the small size of its genome and the absence of several genes encoding enzymes known to play a role in chromate resistance and reduction, the H111 strain is the best chromate resistant strain. Interestingly, the air liquid interface biofilm (Pellicles) of both strains reduce more efficiently chromate than their free-swimming cells. Moreover, they can accumulate a significant amount of its reduced forms. These characteristics make those strains, in particular H111 strain, suitable candidates for chromate bioremediation.

Key words: *Shewanella* sp.,chromate, resistance, reduction, bioremediation.