Molecular and physiological analyses of hexavalent chromium biotransformation by marine bacteria

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in

Marine Sciences

by

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CERTIFICATE

This is to certify that the thesis entitled "Molecular and physiological analyses of hexavalent chromium biotransformation by marine bacteria", submitted by Mr. Elroy Joe Pereira, for the award of the degree of Doctor of Philosophy in Marine Sciences is based on original studies carried out by him under my supervision.

This thesis or any part thereof has not been previously submitted for any degree or diploma in any Universities or Institutions.

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DECLARATION

As required under the University Ordinance 0.19.8 (iv), I hereby declare that the present thesis entitled "Molecular and physiological analyses of hexavalent chromium biotransformation by marine bacteria" is my original work carried out in the National Institute of Oceanography, Dona Paula, Goa, and the same has not been submitted in part or in full elsewhere for any other degree or diploma.

The literature related to the problem analyzed and the investigated, has been appropriately cited. Due acknowledgements has been made wherever facilities and suggestions has been availed of.

Elroy Joe Pereira

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ABBREVIATIONS

ABBREVIATION	FULL NAME
°C	Degree Celsius
µL	Microliter
µM	Micromolar
Cd	Cadmium
CFU	Colony forming unit
ABC transporter	ATP binding cassette transporter
APDC	Ammonium pyrrolidine dithiocarbamate
As	Arsenic
AAS	Atomic absorption spectrophotometer
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BIS	Bureau of Indian Standards
BOD	Biochemical oxygen demand
bp	Basepair
clpc	ATP-dependent Clp protease ATP- binding subunit ClpC
Co	Cobalt
COD	Chemical oxygen demand
COG	Clusters of Orthologous groups
ComEA	Competence protein
Cr^{6+}	Chromate/hexavalent chromium
Cr^{3+}	Trivalent chromium
CRB	Chromate resistant bacteria
Cu	Copper
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DPC	Diphenylcarbazide
ECF	Energy-coupling factor
Fe	Iron
HF	Hydrofluoric acid
Hg	Mercury
ICP-OES	Inductively coupled plasma optical emission spectrometer
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS-QToF	Liquid chromatography mass spectrometry quadropole

Mg / Kg	Milligram / Kilogram
MIC	Minimum inhibitory concentration
ml	Milliliter
mM	Millimolar
Mn	Manganese
MPP	Mass Profiler Professional
NA	Nutrient agar
NADH	Nicotinamide adenine dinucleotide
NB	Nutrient broth
Ni	Nickel
NIOER	National Institute of Oceanography Elroy Ramaiah
NIOMR	National Institute of Oceanography Mandovi River
OsmC	Osmotically inducible protein C
OD	Optical density
PBS	Phosphate buffered baline
PCR	Polymerase chain reaction
pH	power of Hydrogen
PSU	Practical Salinity Scale
RecG	ATP-dependent DNA helicase RecG
RimM	Ribosome maturation factor
ROS	Reactive oxygen species
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SLP	S-layer/Surface layer protein
SOD	Superoxide dismutase
SWNA	Seawater nutrient agar
TDS	Total dissolved solids
TE	Tris-EDTA
TN	Total nitrogen
TP	Total phosphorous
TS/TSS	Total solids/Total suspended solids
TUF	Translation elongation factor
TVC	Total viable bacterial counts
UV	Ultraviolet
UvrABC	Subunit composition of UvrABC Nucleotide Excision Repair Complex= [UvrB] [UvrC] [UvrA]
Zn	Zinc

Chapter 1

Introduction

Introduction

"*If you don't like bacteria, you're on the wrong planet*." - **Stewart Brand**

Microbes. Numerously myriad microscopic affiliates of the Planet Earth. Invisible to our eyes, these tiny creatures- aplenty and diverse comprising of bacteria, fungi, protozoans and other microfauna- wear many coats. Inhabiting the Earth's terrestrial, aquatic and atmospheric (and beyond) realms, their basic functions of decomposition, nutrient derivation, assimilation, energy generation and growth for their self-replication and survival are the hallmarks of this teeming community of microbes. Notably, they also modulate metal transformation, again for their selfish reasons of survival. In turn, helping other comity of biota, if the transformation leads to detox of toxic metals/substances in the ecosystem.

Microbes are essential to all forms of life. There are countless types that serve as building blocks for all kinds of ecosystems. Most are beneficial carrying out functions necessary to sustain the food chain and to digest the food human's consume. Through their growth, reproduction and of photosynthesis (by micro-phytoplankton), respiration and degradation of complex organics into simpler molecules, they perform vital roles for normal ecological functioning. Essentially, environmental functioning as well as global biogeochemical cycling of elements is intrinsically managed by microbial activity in all of Earth's ecosystems including that of the oceanic ones. Any interference of these pathways and their habitat by external factors can drastically affect the ecosphere.

1.1 Importance of microbes in toxic metal biotransformation

Increase in industrialization over the past two centuries has caused an unimaginable increase in pollution. The indiscriminate use of non-renewable resources and the rapid growth of non-disposable waste have led to an accelerated decline of already sensitive habitats. Long as well as short term consequences include displacement of human settlements, economic loss, diseases, loss in biodiversity and health effects. These pollutants infiltrate natural environments hampering life processes. Due to metal persistence, it is therefore not surprising that microbes interact with them, developing means to either use them to their benefit, or defend against them when adverse. Their physiologic and genetic abilities allow them to transform metals, metalloids, hydrocarbons and organic chemicals to suit their needs. This adjustment can be termed collectively as tolerance or resistance.

While many components in natural communities have the potential for such adjustment, the focus of this thesis will be on bacterial resistance. With industrial pollution rampant, ability of bacteria to rapidly sense as well as detoxify/degrade them is of practical significance. Of the many metals anthropogenically released into the environment, release of chromium (**Cr**) metal has particular implications. Although chromium is not the most toxic among the priority metals of public health significance (others include arsenic, cadmium, lead and mercury), it is the most widely used (industrial applications) and distributed toxicant. Still, its high degree of toxicity coupled with its systemic presence has raised concerns over its potential effects. As such, the Agency for Toxic Substances and Disease Registry (ATSDR, 2005) listed chromium and its derivatives as dangerous pollutants affecting human health and the environment.

It is important to note that although these heavy metals naturally occur, most environmental contamination and human exposure is due to human activities. Chromium enters environmental matrices from release of industrial establishments. Therefore environments in close proximity to such exposure are at high-risk. It is estimated that more than a 100,000 people are affected each year by chromium in *India* alone. Numerous reports outline India as a major producer and user of chromium compounds/salts, categorizing extensive chromium pollution (Alka *et al*., 2017). It is thus imperative that efforts be made to find an indigenous solution.

Microbial interactions with metals are illuminating as only prokaryotes include organisms capable of oxidizing metal ions like Mn^{2+}, Fe^{2+}, Co^{2+}, AsO_2^-, Hg^{2+} etc., or reducing Fe^{3+}, Cr^{6+}, Co^{3+} on a large scale. Though studies of metal tolerance and detoxification, on bacteria from contaminated environments are extensive, these aspects are not explored in uncontaminated environments. Often, these bacteria (from uncontaminated environments) utilize different metabolic pathways making it likely that they may be better suited for environmental remediation and may be valuable.

The ecological distribution of chromium resistant bacteria (**CRB**) is dependent on (i) population and types of heterotrophic bacteria; and (ii) the degree of chromium and other metal pollution. A comparison of CRB abundance with respect to time and space will be useful in detection of heavy metal pollution, possibly being useful also in efforts directed at detecting coastal pollution. The potential of highly chromium resistant strains to detoxify chromium might be valuable in biotechnological applications by facilitating biological processes or bioremediation. Further, characterization of key chromium detoxifying isolates will provide a concurrent state of knowledge on metal-microbe

interaction and may bridge the research gap between terrestrial and marine microbial bioremediation. Keeping in view the large body of work on CRB worldwide as well as the apparent lack of the same from Indian coasts, this research was planned to pursue the following major objectives.

1.2 Objectives

- **Ecological analyses in relation to the occurrence and abundance of chromium resistant bacteria (CRB) vis-à-vis general bacterial populations from one each of mangrove, effluent outfall, coastal and offshore locations**

  The rationale behind this objective was to study the prevalence and abundance of marine chromium tolerant/resistant populations, and compare their occurrence and distribution across mangrove, coastal and offshore locations from the coastline of Goa in comparison to those of an effluent outfall. This was done to (i) understand the extent of pollution levels, (ii) compare and evaluate the contributions of physicochemical parameters, and (iii) taxonomically study prevalent chromium resistant native phylotypes. Since there is a lack of studies detailing chromium tolerant or resistant bacteria from these coastal regions, this objective was pursued.

- **Evaluation of chromate tolerance limits and chromate biotransformation potential of a select set of CRB representative strains under varying physiological settings**

  Native bacteria posses ability to sense and remove toxic substances from their vicinity in order to promote their survival. Detailed study on chromium tolerance

limits, biochemical identification, their ability to tolerate other metals as well antibiotics and physiological characterization was carried out for this purpose. In addition, detoxification of chromate by select isolates was also evaluated to understand physiological aspects of CRB from the study locations.

- **Functional genomics and analyses of expressional proteomics from amongst the select set of CRB evaluated for chromate tolerance and biotransformation**

  Bacteria inherently possess capability to detoxify chromium. The genes/proteins responsible or that play a role in survival and/or detoxification may help identify what mechanism of action marine isolates use, helping to differentiate them from their terrestrial counterparts. Proteomic studies will supplement this with information on what processes are induced, repressed or suppressed under chromate stress. Keeping the absence of a proteomic outlook or reports of protein information regarding marine bacteria in view, this major objective was pursued.

Chapter 2

Review of literature

2.1 Introduction

The quality of life is linked inseparably to the quality of the surrounding environment. Although industrialization has long been considered the hallmark of civilization, the fact remains that its emergence has a negative effect on the environment. The environmental reduction of chromate ($\mathbf{Cr^{6+}}$) to trivalent chromium ($\mathbf{Cr^{3+}}$) greatly benefits the ecosystem due to its highly toxic nature and mobility in aquatic systems, while Cr^{3+} is less mobile, readily forms insoluble precipitates and is 10^3 times less toxic than the former. With rise in industrialization, there is an increased deposition of wastes and metals into the environmental sink, which inhibit biological remediation processes. Even still, biological Cr^{6+} reduction is limited, to organisms that can actively reduce it and its toxicity to such organisms. In view of the environment, there have been a large number of reports that outline the role of bacteria that can perform this metal detoxification function. Since metals are valuable resources for various applications, their recovery and recycle is also important.

2.2 Chromium

Nicholas-Louis Vaquelin first discovered chromium in 1797 and observed the brittleness and infusibility of the new mineral. Less than 150 years later, the metal had become indispensible by industrialized nations and is now essential in the formation of alloys for modern cast iron, steel and non-ferrous metallurgy. Basically, without chromium, the development of most of today's structures like high-speed trains, automobiles, satellites, associated space industry, machine based industry, construction based industry etc. may have not been possible (Nieboer and Jusys, 1988).

2.2.1 Occurrence in nature

Chromium is the seventh most abundant element on earth with most of it residing in the earth's core and mantle. It ranks as the 21st most abundant element in the earth's crust and its crustal distribution shows a particular preference for concentration in ulrabasic rocks and basic rocks. Chromium content in soils depends on parent materials and concentrations range from 10-150mg kg^{-1} (Bertine and Goldberg, 1971). Ranking fourth among the 29 elements of biological importance, there are 40 known chromium containing minerals, with chromite being the only one with economic importance. In nature this mineral has highly variable chemical composition containing around 46% chromium. These chromium containing ores are classified based on their composition and industrial use into high-chromium (46-55% Cr_2O_3) for metallurgical uses; high-iron (40-46% Cr_2O_3) for chemical and metallurgical uses, and high-aluminum (33-38% Cr_2O_3) used widely as refractory material. Major chromium deposits are known to occur in three principal geologic settings: (1) Stratiform-type deposits, (2) Podiform or Alpine-type, and (3) Lateritic (placer) deposits, with most of the worlds's chromite reserve residing in Southern Africa (Yassi and Nieboer, 1988). In freshwater, total chromium concentrations range from 0.1-6.0µg mL^{-1}, while seawater values range from 0.2-50µg mL^{-1} (Bowen, 1979). Chromium largely exists as immobile Cr^{3+} in natural water. Atmospheric Cr is mostly due to windblown dust, forest fires, meteoric showers, sea spray or volcanic eruptions ranging from 0.015-0.03µg m^{-3} (Nieboer and Jusys, 1988).

2.2.2 Chromium cycling and dynamics

The major links in the cycling of chromium in soils and natural waters lie between the trivalent and hexavalent form, dependent on redox conditions and on solubility. Three reactions: oxidation (redox), sorption-desorption and precipitation-dissolution govern the distribution of chromium naturally. Trivalent chromium is the most stable form in neutral conditions of water and soil and adsorbs or retains on soil particles, hence its low mobility and bioavailability. It binds with adjacent molecules of the same species to form polynuclear complexes precipitating as $Cr(OH)_3$ (Salem *et al*., 1989). Chromate is a strong oxidant and is stable under high redox potential albeit in the absence of reductants (Adriano, 2001). The pH of soils also plays a crucial role affecting mobility as well as oxidation states. Soils and sediments in partial equilibrium with atmospheric oxygen house both oxidized manganese and reduced carbon (Bartlett, 1991). As a result, the oxidation of 3+ to 6+, or reduction from 6+ to 3+ can occur simultaneously, since they are both thermodynamically spontaneous reactions. Manganese-oxide containing minerals such as birnessite or lithiophorite are effective oxidizers of Cr^{3+} where as in water, Cr^{3+} gets oxidized by chlorinated products (Clifford and Chau, 1988). Trivalent chromium is thus likely to be complexed by organic functional groups implicit in its oxidation first reported by Bartlett and James in 1979 (Bartlett and James, 1979). They also reported that manganese oxides present, in reactive forms generally in fresh, moist, nonacid field soils, served as an electron link between Cr^{3+} and atmospheric oxygen, with the amount of Cr^{3+} oxidized proportional to the manganese reduced. Rate of oxidation is also determined by chromium speciation and mobility; and by the presence of immobile manganese-oxide surfaces. Chromate can be reduced in the presence of electron donors

such as Fe^{2+}, reduced sulfur or organic matter like humic and fulvic acids either by abiotic or biotic processes (Kotas *et al*., 2000). The natural biogeochemical cycling of chromium is largely affected by anthropogenic inputs, as is in the case of other metals like Aluminum, Iron and Zinc

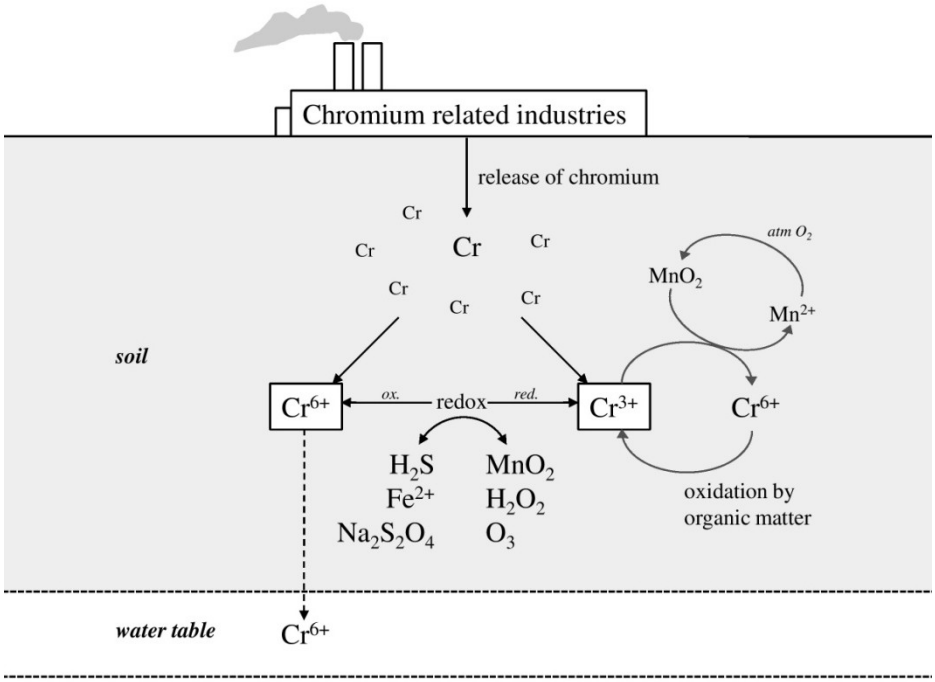


Figure 2.1 Chromium cycling in the environment

2.2.3 Uses

Different industrial applications demand for different forms of chromium such as chromite, ferrochromium, chromite refractory bricks or foundry sands, chromium metal or other such chromium compounds. The 10 million tons of chromite used globally annually is distributed among three main industrial applications; ~80% for metallurgical, ~10% for refractory and ~10% for chemical applications (DeYoung *et al*., 1984). The pattern of use differs across countries and economic communities. Stainless steel with 15-30% chromium is widely used in architecture, automotive and rail-road industries.

Refractories use chrome ore due to its high melting point (2040°C), and its chemically inert nature making it resistant to acids, bases and high temperature, in the formation of refractory bricks, mortars, castables, ramming gunning mixes, in furnaces or for repairs.

Table 2.1 Uses of chromium in industrial sectors. *Modified from* Nieboer and Jusys (1988)

Uses in industrial sectors	
Metallurgical	*Chemicals*
Wrought stainless and heat resisting steels	Pigments
Tool steels	Metal finishing
Wrought-alloy steels	Leather tanning
Cast-allow steels	Drilling muds
Alloy castirons	Wood preservatives
Non-ferrous alloys	Chemical manufacture
Electroplating	Textile preservatives
	Catalysts
Refractories	Mordants
Chrome and chromite magnesite	Pyrotechnics
Magnesite-chrome brick	Photosensitization
Granular chrome bearing	Metal primers
Granular chromite	Antifouling agents

Over 70 chromium compounds are used commercially as chemicals, with some such as sodium chromate, potassium dichromate, chromic acid and basic chromic sulfate produced in large quantities, primarily for leather tanning applications. In addition to being used in electroplating applications as metal surface treatments to prevent corrosion and improve product durability, chromium compounds also have numerous uses as anti-freeze agents, anti-corrosive agents, in printing processes, oxidizing agents in fireworks, tattoo coloring agents, in petrochemical industry, in the synthesis of other chemicals or as chemicals for research purposes.

2.2.4 Release into the environment

Chromium is found throughout the environment, including air, water, soil and all biota and although large amounts of it exist naturally, most is primarily locked in sea-beds, sediments and in crustal rocks.

Table 2.2 List of chemicals and uses of chromium compounds. *Modified from* Nieboer and Jusys (1988)

Name	**Use**
Oxidation state 6+	
Aluminum Chromate	in ceramics
Barium chromate	printing and photosensitization
Cadmium chromate	catalyst, pigment
Lithium chromate	corrosion inhibitor
Pyridine-chromic acid	research oxidant
Silver chromate	in catalysts
Oxidation state 3+	
Chromic acetate	printing and dyeing textiles
Chromic acetylacetonate	preparation of Cr complexes in catalysts
Chromic chloride	chromizing
Chromic fluoride	mordant in catalysts

The main source of Cr into the environment especially into water bodies and soils is therefore that based on anthropogenic inputs, relative to natural background levels. Like every contaminant, chromium metal has a unique set of characteristics and issues that lead to its release into the environment. There are several sources such as direct infiltration of leachate from landfills and mines, sewage sludge, seepage from industrial lagoons, leaks from industrial metal processing or other industrial activities (Choppala *et al*., 2013). Because of its toxic nature, chromium creates multiple environmental problems in the form of waste products, mine wastes and post-manufacturing slag piles

(Hawley *et al*., 2004). The high volumes of Cr consumed in electroplating and manufacturing industries result in large amounts of Cr related wastes such as chromic acid, Cr^{6+} cleaners, Cr^{6+} containing compounds, Cr^{3+} wastes and Cr as a metal. Management of wastes from industries is always a problematic one usually associated with cost-cutting and political affiliations. As a result, Cr is commonly released into the environment as a contaminant in association with other wastes affecting tens of thousands each year.

2.2.5 Toxicity

Trivalent chromium is an essential dietary nutrient in trace amounts, required to potentiate insulin for normal glucose metabolism (Cohen *et al*., 1993). Chromium toxicity is mainly attributed to the Cr^{6+} form, and all related compounds are considered as potential carcinogens. Exposure to Cr can be through non-occupational sources like air (combustion and metal industries), water (tanning, electroplating etc industries dispose wastes into water bodies; or through leaching into groundwater), soils (communities affected by slags) or as occupational hazards (chrome, leather tanning, welding industries, textile manufacture). Entry routes of Cr into the human body are dependent on whether it is ingested inhaled or exposed to skin (dermal absorption). Once inside, Cr^{6+} is metabolized to Cr^{3+} by gastric juices or by the epithelial lining in the lungs to form reactive intermediates. Depending on the dosage, route of entry and exposure duration, effects range from respiratory effects (on inhalation) like asthama, chronic bronchitis/pharyngitis, ulceration; to skin (dryness, erythema, swelling), renal, hepatic, gastrointestinal, cardiovascular, carcinogenic, genotoxic and mutagenic effects. The

mechanism of Cr-induced damage is not fully understood but is based on the damage caused by reactive intermediates and Cr itself targeting and damaging DNA, proteins and other cellular molecules, interfering with their function. Cr^{3+} in comparison is not readily adsorbed due to its insolubility in water, increased gastrointestinal transit time and with organic Cr more readily absorbed than inorganic chromium (ATSDR, 2008).

2.2.6 Conventional treatment

Chromium treatment strategies depend on whether it contaminates soils or water bodies. Treatment technologies can be further classified as:

(a) *Reduction of toxicity*

Carcinogenic effects of chromium on a receptor species is a product of exposure and inherent toxicity of the amount consumed. A method to minimizing damage is to reduce chromium toxicity by converting it to a chemical form that is biologically unavailable or inert when ingested. This can occur naturally by biological or geological processes (Haq *et al*., 1998). In the case of pollution, chemical (abiotic) reduction of toxicity by addition of electron donors like hydrogen sulphide, sodium dithionite, calcium dithionate, Iron(II); or biotic reduction by phytoremediation (phyto-accumulation/stabilization/extraction is done.

(b) *Removal*

Removal strategies in soils include excavation, off-site disposal of or separation of chromium from soil by soil washing, soil flushing, solvent extraction or concentration of Cr into smaller volumes (by phytoextraction or electrokinesis).

Removal of chromium from water bodies include excavation and dumping of pumped groundwater or, separation by membrane technology (ultra/nanofiltration, reverse osmosis), ion exchange or granular activated carbon.

(c) *Containment*

Containment technologies focus on minimizing spread to larger areas. This can be made possible by stabilization, biostabilization, solidification, precipitation, encapsulation, vitrification or phytostabilization in the case of contaminated soils. Ground waters can be contained by use of slurry walls or other physical barriers including chemical and hydraulic barriers (Hawley *et al*., 2004).

Although conventional Cr contamination treatment has been largely successful especially by the use of a cheap alkali like lime (calcium hydroxide), high capital, high operational, chemical and labor costs, risk of water fouling, risk of secondary pollution, precipitation of large quantities of solid sludge for disposal, high energy consumption, low efficiency and production of waste water produced that is unusable make other treatments like bioremediation more viable, attractive and environmentally friendly (Owlad *et al*., 2009; O'Connell *et al*., 2008).

2.3 Bacteria and Chromium

To exert toxicity or to partake in physiological reactions, Cr metal ions first have to traverse cellular membranes (Nies, 1999). Chromate in the form of oxyanions, avoids binding to anionic components of bacterial membranes, whereas cationic Cr^{3+} ions bind tightly to them. Hexavalent chromium derivatives (chromate and dichromate, CrO_4^{2-},

$Cr_2O_7^{2-}$) share structural similarity to sulfate and phosphate anions (SO_4^{2-}/ PO_4^{2-}) and as a result can be easily transported across membranes by sulfate transporters (Collins *et* al., 2010.

2.3.1 Chromium toxicity

Chromium is essential for microbial growth in trace quantities but is highly toxic at elevated levels. The Cr^{6+} is more toxic (than Cr^{3+}) due to its strong oxidizing potential that invariably damages cells (Kotas et al., 2000). Being mutagenic (Puzon et al., 2005), carcinogenic (Codd et al., 2003) and teratogenic (Qureshi et al., 1998) Cr^{6+} enters cells easily via sulfate transporter pathway. Once inside, it gets reduced to Cr^{3+} by various metabolic processes, producing short-lived intermediates. These short lived intermediates (Cr^{4+}/Cr^{5+}) exert deleterious effects by principally bringing about Cr mediated carcinogenesis and apoptosis, (Ye et al., 1999) in addition to binding directly with phosphate groups on DNA. The reactive oxygen species (**ROS**) generated as part of the redox cycle causes considerable oxidative stress on cells by interacting with proteins, nucleic acids and cellular components (Cheung, et al., 2007). In contrast, Cr^{3+} is less toxic due to its tendency to form insoluble oxides/hydroxides reducing its bioavailability (He *et al*., 2009). Intracellularly, it reacts with carboxyl and thiol groups of enzymes inactivating them (Cervantes *et al*., 2001).

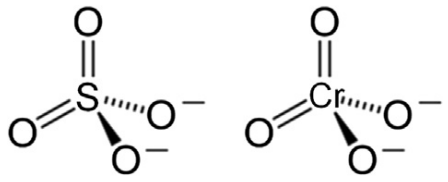


Figure 2.2 Structural similarity of chromate and sulfate ions
Adapted from Thatoi *et al*. (2014).

2.3.2 Bacterial resistance to Chromium

Resistance in bacteria makes use of strategies like un/specific C^{6+} reduction, removal of free radicals, DNA damage repair (Morais, *et al*., 2011), DNA methylation, metal efflux, adsorption uptake, and/or processes associated with sulpur, sulfate or iron homeostasis (Ramirez-Diaz *et al*., 2008). Some utilize metal biotransformation either by direct enzyme action or through formation of complexes with metabolites (Camargo *et al*., 2005)

(a) *Reduced uptake of chromate*

An effective mechanism of minimizing toxic effects is the reduced uptake of Cr^{6+} through the sulfate pathway or through Sulfur or Iron homeostasis. Chromate ion can easily pass through cell membranes via sulfate transport pathway aided by non-specific anion (sulfate or phosphate) carriers (Wenbo *et al.*, 2000). Its entry is impeded in bacteria that have chromosomally encoded uptake pathways that are altered or mutated. Bacteria that reside in contaminated environments rapidly develop mutations or incorporate genetic information that result in reduced uptake (Kümmerer *et* al., 2004)

(b) *Extracellular (indirect/non-enzymatic) chromate reduction*

Chromate can be reduced extracellularly by binding to functional groups that reside on the bacterial surface. Peptidoglycan components in cell walls have been reported to be dynamic binders of Cr^{3+} (Ngwenya *et al*., 2011). Reactive functional groups like carboxyl, amines, phosphate and hydroxyl groups have also been reported to play a role (Parmar *et al*., 2000). Trivalent chromium binds

to these electro-negatively charged groups which serve as nucleation sites for precipitation, or undergoes complexation with the outer envelope and capsular exopolymer preventing it from re-entry (McLean and Beveridge, 2001). In addition to indiscriminate binding to cell wall components, some bacteria are also capable of Cr^{6+} reduction by biotic-abiotic coupling, where oxidation of organic compounds and H_2 is coupled with the reduction of Fe^{3+} and SO_4^{2-} to Fe^{2+} and H_2S, under oxidative stress (Nevin and Lovley, 2002). Chromate reduction is brought about by reaction with H_2S, which diffuses out into the anaerobic medium (like soil-environments) followed by Cr^{3+} precipitation (Kamaludeen, *et al*., 2003).

(c) *ROS detoxification/scavenging*

During Cr^{6+} reduction to Cr^{3+}, Cr^{4+} and Cr^{5+} short-lived highly reactive radical intermediates are formed, a. Cr^{5+} is oxidized back to Cr^{6+} giving up its electron to dioxygen in a Fenton-like reaction, generating ROS in the process. This generation of ROS results in oxidative stress. Resistant bacteria possess inherent ability to nullify this stress by detoxifying enzymes such as gluthathione transferase, superoxide dismutase (**SOD**), catalase etc. (Ackerley *et al*., 2004) which mop up free radicals minimizing their damage.

(d) *DNA repair*

DNA that has been damaged by free radicals can have drastic effects on the life cycle of a bacterial cell. ROS generated stress damages DNA by forming base modifications, single stranded breaks, double-stranded breaks, Cr-DNA adducts,

chromosomal breaks and mutations (Zhitkovich, 2011). These damages can be repaired by dedicated DNA repair mechanisms like the SOS response enzymes (RecA, RecG) or by recombinational DNA repair systems (Hu *et al*., 2005).

(e) *Efflux of chromate from bacterial cells*

Efflux of Cr^{6+} is an active mechanism mediated by transporters encoded by specific plasmid-borne genes that prevents accumulation of Cr in the cell as observed by (Ramirez-Diaz *et al*., 2008). The best understood efflux system is that of *P. aeruginosa* ChrA protein, a hydrophobic membrane spanning protein from the chromate ion transporter CHR superfamily. ChrA has been studied to confer resistance by Cr^{6+} efflux mechanism by acting as a chemiosmotic pump, exporting intracellular Cr extracelluarly. Proton motive forces have been assumed to power the process (Alvarez *et al*., 1999).

(f) *Biosorption and bioaccumulation*

Biosorption is a physico-chemical process that selectively sequesters metal ions. It is metabolically independent, passive, and governed solely by components on the bacterial cell surface that act as biosorbents. This extracellular binding involves surface molecules like S-layer protein (**SLP**) (Gerbino *et al*., 2015) involving one or a combination of: physical adsorption, van der Waals forces, ion exchange, complexation or inorganic microprecipitation (Srinath *et al*., 2002). Bioaccumulation is a two-step process, with the first one identical to the process of biosorption. The second step involves the ATP-driven active transport of bound ions into the cytosol in a slow, metabolically dependent process. Ions

from extracellular binding sites are thus internalized and bound to intracellular binding sites (Chojnacka, 2010) localized within specific organelles. Metal ions are complexed with ligands or proteins to prevent reaction with other biomolecules. Some bacteria eventually export bound ions by efflux, but the main function in both sorption and accumulation is to restrict ions from interfering with normal function by binding them (Martin-Gonzalez *et al*., 2006).

(g) *Bacterial chromate reduction*

It has been reported that both Cr^{6+} resistant as well as non-resistant strains are able to reduce Cr, but growth of non-resistant ones are inhibited at higher Cr concentrations (Bopp and Ehrlich, 1988).. Although Cr resistance is possible through Cr reduction which decreases intracellular Cr^{6+} concentration, they are two independent processes (Nies *et al*., 1989).

2.3.3 Bacterial chromate reduction

In addition to indirect extracellular Cr^{6+} reduction (Section **2.3.2** (b)); three underlying mechanisms of Cr^{6+} reduction have been reported:

(a) *Non-enzymatic anaerobic chromate reduction*

Under anaerobic conditions, cellular protoplasm components like amino acids, nucleotides, carbohydrates, vitamins, organic acids, glutathione, flavoproteins and hemeproteins can act as electron donors to Cr^{6+}, a terminal electron acceptor reducing it (Cheung *et al*., 2007).

Table 2.3 Bacterial resistance mechanisms

Enzyme/system	Bacterial species	Function	Reference
Reduced uptake of chromate			
Cys operon products	*Shewanella oneidensis*	Sulphate transport	Brown et al. (2006)
TonB receptor	*S. oneidensis*	Iron tranport	
Cys operon products		Sulphur metabolism	
Ferritin	*S. oneidensis*	Sulphur metabolism	
Adenylyl sulphate kinase		Iron biniding	
Extracellular reduction			
Lipopolysaccharide	*Pseudomonas aeruginosa*		Langley *et al*., (1999)
Peptidogycan	*Escherichia coli, Bacillus subtillis*		Hoyle *et al*., (1984)
Polypeptide	*B. subtillis, Klebsiella pneumoniae*	Metal binding functional groups on bacterial surface component	Beveridge *et al*., (1980)
Metallothioneins	*Ralstonia eutropha*		Valls *et al*., (2000)
Phospholipids	Bacteria		Beveridge *et al*., (1989)
ROS detoxification			
SOD, catalase	*E. coli*	mops up oxidative stress	Ackerly *et al*., (2004)
Outer membrance proteins	*Caculobacter crescentus*	general stress response	Hu *et al*., (2005)
DNA repair			
RuvB	*Ochrobacterium tritici 5bvl1*	DNA damage repair	Morais *et al*., (2011)
RecG, DNA helicases	*P. aeruginosa*	DNA damage repair	Miranda *et al*., (2005)
SO368, UvrD and HrpA helicases	*S. oneidensis*	DNA damage repair	Chourey *et al*., (2006)
Efflux/transport			
ChrA transporter	*P. aeruginosa*	Efflux of cytoplasmic Cr6+	Alvarez *et al*., (1999)
ChrA transporter	*A. eutrophus*	Efflux of cytoplasmic Cr6+	Nies *et al*.,1990
chrBACF operon	*Ochrobactrum tritici* strain 5bvl1	Efflux of cytoplasmic Cr6+	
Reduction			
chrB, chrC	*O. tritici* 5bvl1	Resistance to superoxide generating agents	Branco *et al*., (2008)

(b) *Aerobic chromate reduction*

Aerobic Cr^{6+} reduction is mostly associated with soluble proteins that require NAD(P)H as electron donors and are localized in the cytoplasm (Shen and Wang, 1993). NAD(P)H dependent soluble reductases have been reported in aerobes by Puzon et al. (2002) and Shen and Wang, (1993) which were able to reduce Cr^{6+}. They perform this function with the help of functional groups present on the cell surface. Ishibashi *et al.*, (1990), Suzuki *et al.*, (1992) and Chen and Hao (1998) have all reported reductases inside and outside the cell that, utilizes diverse electron donors. Processes regulated by these enzymes are energy taxing, co-metabolic and highly regulated. As a result, they are inducible and independent from transport mechanisms.

(c) *Anaerobic chromate reduction*

Anaerobic Cr^{6+} reduction is generally associated with membrane bound reductases. Examples include flavin reductases, cytochromes and hydrogenases that form part of the electron transport system (Wang, 2000) and in the absence of oxygen, use Cr^{6+} as the terminal electron acceptor in a dissimilatory reaction. These reductases operating under anaerobic conditions often require H_2 or glucose as electron donors (Ibrahim *et al*., 2012). There have been reports of bacteria where Cr^{6+} reduction has been inhibited in the presence of oxygen as observed by Wang et al. (1989) in *E. cloacae* HO1. It was also found that some sulfate reducing bacteria (growing in anaerobic conditions) that use nitrate and sulfate as terminal electron acceptors in the respiratory chain, replaced them with chromate (Chardin *et al*., 2003).

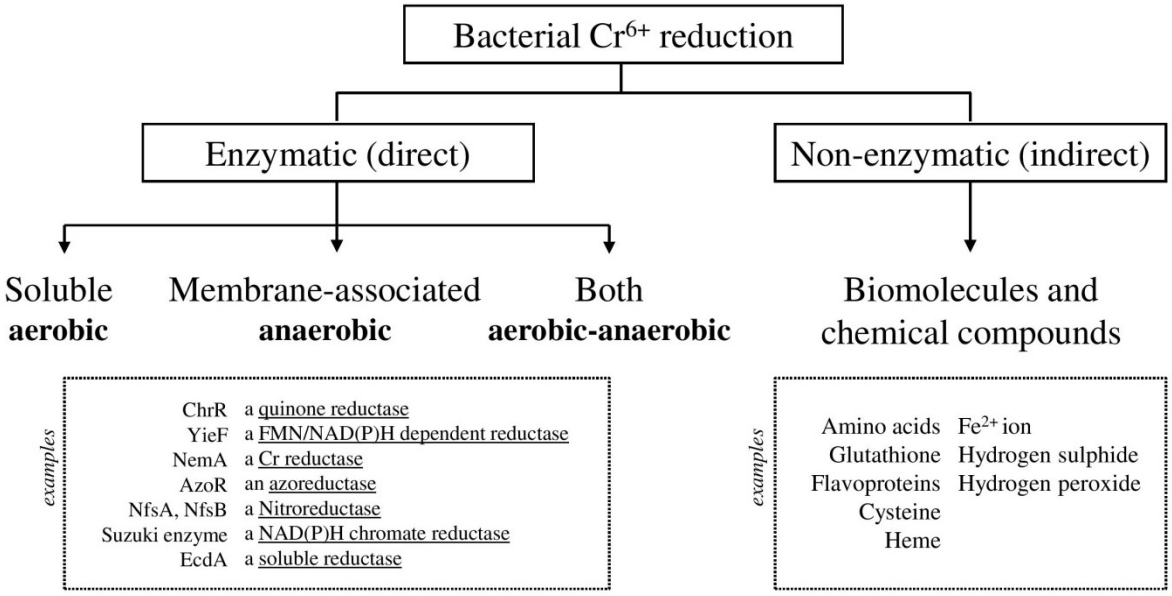


Figure 2.3 Schematic diagram of direct and indirect bacterial chromate reduction

As outlined, enzymatic Cr^{6+} reduction can be aerobic, anaerobic or in some bacteria like *P. fluorescens* LB300 both aerobic-anaerobic (Bopp and Ehrlich, 1988). Enzymes that function in each condition can be intracellulary or extracellularly active. Cheung and Gu (2007) reported Cr reductases such as flavin reductases, nitrate reductases and ferrireductases acting extracellularly by electron-shuttling coupled to membrane directed reduction. In contrast, intracellular reductases play an intermediate role between associated biological electron donors like NADH and NADPH used by enzymes as reductants, to reduce Cr^{6+} (Puzon *et al*., 2005).

2.3.4 Genetic mechanism of chromate resistance and tolerance

According to Gadd (1992), *resistance* is "the ability of a microorganism to survive toxic effects of metal exposure by means of detoxification mechanisms as a direct response to the metal species concerned", whereas *tolerance* is defined as "the ability to survive

metal toxicity by means of intrinsic properties and/or environmental modification of toxicity". Ability to resist abnormally high concentrations of Cr is generally suggestive of resistance, encoded either chromosomally or extra-chromosomally (Li and Krumholz, 2007). Several genes have been reported to confer resistance namely *chrR*, *chrB*, *chrC* and *chrF* all located chromosomally (Aguilar-Barajas, *et al*., 2008). Other genes have been reported to moonlight providing indirect Cr resistance such as *RuvB* (Morais, *et al*., 2011). Plasmid genes that confer resistance are generally devoted to offering protection from oxidative stress generated on exposure or, encode membrane transporters which mediate Cr^{6+} efflux. These elements in some cases, are transmittable across species as observed by Branco et al. (2008) in the case of *chrBAF* operon, or the plasmid pLHB1 from *P. fluorescens* LB300 (Shen and Wang, 1993). Chromate reduction is a complicated process and is regulated by an operon structure as observed in *Bacillus cereus* SJ1 or in *B. thuringiensis* strain 97-27 (He *et al.*, 2010). Here genes seem to be regulated by certain promoters and were inducible.

2.3.5 Chromate resistant bacteria in bioremediation

Since the isolation of the first Cr^{6+} resistant and reducing strain, *Psueudomonas* sp. by Romanenko and Koren'kov in 1977, several Cr resistant bacteria have been reported including *Psuedomonas*, *Bacillus*, *Enterobacter*, *Deinococcus*, *Shewanella*, *Agrobacterium* etc. (Ohtake *et al*., 1987). Although many indigenous bacteria are sensitive to Cr^{6+} few exceptions aside, bacteria isolated from Cr^{6+} contaminated sites are reported to be highly resistant. Camargo et al (2005) reported that Cr^{6+} contamination promotes natural selection and biodiversity among Cr resistant bacteria. Among bacteria

that have been studied, Gram positive bacteria have shown significant resistance to Cr toxicity at high concentrations, Gram negative bacteria on the other hand have been described to be more sensitive to toxic effects of Cr (Coleman, 1988).

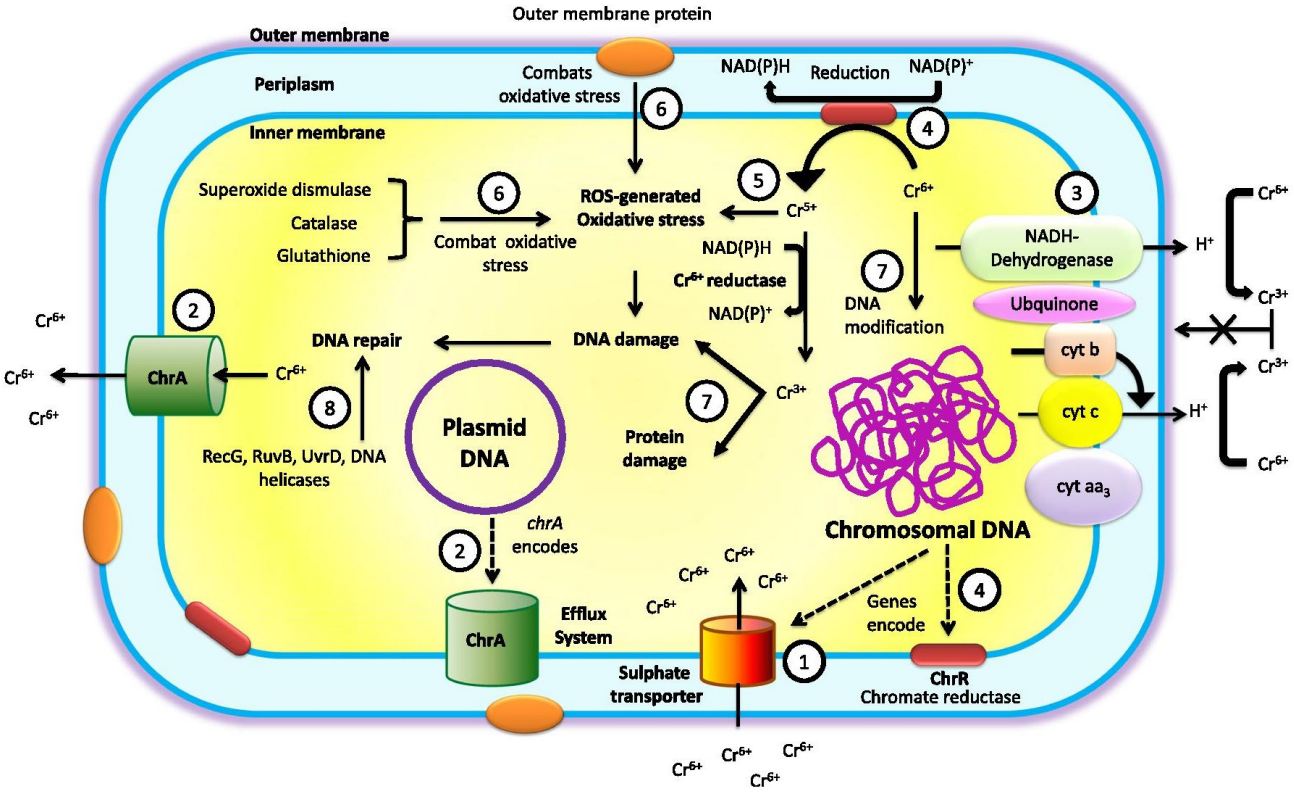


Figure 2.4 Schematic depiction of chromium resistance and toxicology in a bacterial cell.

Bacterial cell membranes are impermeable to Cr^{3+} due to the insolubility of Cr^{3+} derivatives (**1**) Cr^{6+} enters bacterial cells via sulfate transporters; (**2**) plasmid derived efflux systems expel intracellular Cr^{6+} extracellularly; (**3**) soluble reductases bring about aerobic Cr^{6+} reduction to Cr^{3+} with NAD(P)H as an electron donor, anaerobic Cr^{6+} reduction occurs in the electron transport pathway with cytochrome b (cyt b) or cytochrome c (cyt c) along the respiratory chains in the inner membrane; (**4**) Membrane-associated chromate reductase reduces Cr^{6+} anaerobically in the presence of electron donors; (**5**) Cr^{5+} intermediated formed during the redox cycle of Cr^{6+} produces oxidative stress by generation of reactive oxygen species (ROS); (**6**) ROS-generated oxidative stress, is nullified by protective metabolic enzymes in the cytosol or by some outer membrane proteins; (7) Cr^{6+} and generated Cr^{3+} binds to nucleic acids and proteins damaging them; (8) damaged DNA is repaired by DNA repair systems. *Adapted from* Ahemad (2014)

2.3.6 Reports of chromate resistant bacteria in India

There are a number of reports of outlining the isolation of bacteria from the Indian sub-continent. Shakoori *et al.*, (1999, 2000) isolated multiple bacteria from industrial effluents. Hasnain *et al.*, (2005, 2006) have isolated *Bacillus*, *Micrococcus* and *Ochrobactrum* sp. from contaminated soils. Shukla *et al.*, (2007), Dhal *et al.*, (2010), Bhide *et al.*, (1996), Mistry *et al.*, (2010), Poormima *et al.*, (2010), Kathiravan *et al.*, (2007), Rajkumar *et al.*, (2005), Desai *et al.*, (2008) and Singh *et al.*, (2010) have all isolate various *Pseudomonas* and *Bacillus* and *Staphylococcus* sp. from tannery effluent, sewage, chromite mine soils, Cr^{6+} landfills and other contaminated sites.

2.3.7 Reports of marine chromate resistant bacteria

Cycling of chromium is limited to terrestrial bodies due to the lack of reductants in aqueous bodies. Compared to their terrestrial counterparts, marine bacteria have many additional requirements mainly maintenance of osmotic balance by pumping of potassium and sodium ions (McLeod and Onofrey, 1957). Other exclusive features include facultative psychrophilicity (Bedford, 1933), high tolerance to pressure (Zobell and Morita, 1957) and ability to fine-tune and rapidly respond to constantly changing environmental conditions (Lauro *et al.,* 2009). This ability to adapt to varying sea surface temperature, pH, UV and light patterns, terrestrial inputs, pressure and salt concentrations (Dash *et al*., 2013) may be useful in bioremediative efforts. Few studies have been reported with respect to marine Cr resistant bacteria. Subramanian *et al.*, (2012) isolated a halotolerant Cr^{6+}reducing *Planococcus maritimus* VITP21 from marine water. On investigation they found Cr^{6+} reducing function associated with the soluble cytosolic

fraction of the cell. Cheung *et al.*, (2003, 2006, 2007) isolated *Bacillus megaterium* TKW3 from marine metal-contaminated sediments off the shore of Hong Kong. It was capable of rapidly reducing Cr^{6+} (ca. 170µg mL^{-1} in 7days) under anaerobic conditions, as part of a sulfate-reducing bacterial consortia which relied on sulfur metabolism to reduce Cr^{6+} in marine environments. Das *et al.* (2009, 2006, 2007) outlined the biosorption ability of *Bacillus*, *Streptococcus* and *Pseudomonas* strains isolated from the Uppanar estuary (Tamil Nadu). Additionally they also suggested the selection of these bacteria and their use as bioindicators of metal pollution and environmental status. There are also reports of marine bacteria tolerant to other metals (De *et al*., (2008); Nagvenkar *et al*., 2010) that have been investigated for remediation. De *et al*. (2006, 2008) investigated the ability of mercury resistant bacterial strains in the detoxification of mercury, as well as cadmium and lead. Nagvenkar *et al*. (2010) evaluated the arsenic detoxification ability of arsenic resistant strains isolated from estuarine environments.

Screening and examination of literature allows clear insight on how limited reports are of marine related metal resistant/tolerant bacteria. An overview on the current knowledge of chromium metal, its cycling, transport, toxicity, and detoxification is important. The literature here, focused mainly on chromium resistant bacteria, their ability to detoxify Cr^{6+} and their potential in Cr^{6+} bioremediation. Detailed literature pertinent to each chapter is added.

Chapter 3

Abundance and distribution of chromate tolerant bacteria

3.1 Introduction

The omnipresence of microbes impacts entire ecosystems. In order to understand their role and function in regulating biogeochemical systems, their ecology must be evaluated. Investigations on microbial populations that specialize in a certain ecological functions would provide further insight on the importance of these communities and how they perform transformation of certain substrates. Studies that enumerate the prevalence and abundance of certain bacteria are important in indicating levels of pollution. Reports of Ramaiah *et* al. (2002, 2003 & 2004), Nagvenkar and Ramaiah (2009) Rodrigues *et al*. (2011). Gardade and Lidita (2017) and Noroozi *et al*. (2017) describe pollution levels and indicator bacteria. As such, ecological processes in coastal and offshore environs are greatly affected by anthropogenic activities.

In order to determine to what extent presence of external factors influences abundance and ecology of bacterial populations, water quality measurements are important. Environmental bacteria are subject to multiple factors and parameters both physical and chemical. Measurements of each would help in differentiating populations spatially and temporally, as well as relate environmental characteristics and microbial load/diversity.

In recent years, there have been concerns over the release of chromium-related compounds into the environment with a wide range of toxic effects to native organisms as well as humans. Studies evaluating metal-tolerating/resistant native microflora are thus important, as they can help realize the extent of metal contamination and may be valuable in metal detoxification efforts. Past studies have shown that chronic Cr^{6+} stress alters microbial community structure, resulting in decreased biomass, activity and diversity

(Francisco *et al.*, 2002). Despite this, bacteria that can tolerate toxicity or more rapidly decompose pollutants are more likely to persist. As a result, successful microbial-based Cr^{6+} remediation technologies require a better understanding of microbial abundance and the population response to Cr^{6+} stress. Isolation and characterization of the culturable microbial community of chromium tolerant bacteria, and evaluation of water quality (from which they were isolated) would help evaluate Cr^{6+} resistance and remediating ability of autochthonous bacterial populations. This would help determine whether characteristics pertaining resistance are exclusive to one microbial group, or are shared abilities.

3.2 Methods

3.2.1 Sampling locations

Three sampling locations off Goa were selected for enumeration and isolating of chromium tolerant bacteria. These are off Dias Beach (**DB**), in the Divar Mangroves (**DM**), and in the offshore site (**G5**) off Candolim, which has been sampled routinely for many years (**Fig. 3.1**)

Dias (**DB**; 15° 27' 12.5496" N, 73° 48' 4.9284" E) *Beach* is a secluded pocket beach on the south side promontory where there is confluence of both Mandovi and Zuari estuaries. Adjacent to the Dona Paula headland, it is situated opposite the Mormugao harbor (an important port for maritime trade) and is exposed to contributions from shipping-related, tourist-related and hotelier activities. This site is described geographically, geologically (Chandramohan *et al*., 1998) and edaphically (Ansari and Gauns, 1996).

Divar (**DM**; 15° 30' 31.4604" N, 73° 54' 43.8876" E) *island Mangroves* are separated from the main land by the river Mandovi. In addition to being exposed to an influx of metal effluents from iron ore mining activities, (Krishnan*et al*., 2007) this site is also exposed to indiscriminate outfall of urban waste. This site has been described geographically and edaphically by Attri and Kerkar (2011) and Divya *et al*. (2009).

Offshore site G5 (**G5**; 15°30'31 N, 73°40'27 E) is a station along the Candolim time series (CaTS) transect in the offshore waters off the coast of Candolim (9.5 km) with depth ~28m. This ecosystem has been described geographically by Bhaskar *et al.* (2011).

Pallavaram Tannery (**T**; 12°57'44"N 80°8'8"E) is in an industrial town situated in the suburbs of Pallavaram, located in Chennai, Tamil Nadu, India, where around 150 tanneries are concentrated to form the CEMCOT- Pallavaram Tanners Industrial Effluent Treatment Co. (PTIETC) which treats ca. 3000m^3 of tannery effluent per day (Krishnan *et al*., 2016).

3.2.2 Sampling

Surface water and sediment samples were collected at low-tide during pre-monsoon (March 2015), monsoon (July-August 2015) and post-monsoon (November 2015-January 2016) seasons from Dias beach and Divar mangrove locations. Samples were collected in sterile polypropylene tubes, and held on ice till further analyses in the laboratory.

Sediment samples were collected (0-2cm depth) by penetrating an undisturbed area with a 5cm diameter core. At Divar mangrove, some samples were collected from low-lying stagnant pools.

Offshore location– surface water samples were collected along CaTS on a fishing trawler (as part of CaTS) during pre-monsoon (May 2015), monsoon (September 2015) and post-monsoon (November 2015) seasons and stored on ice till analyses. Water samples were collected from a Niskin bottle sampler.

From Pallavaram – raw tannery wastewater samples were collected with the permission of authorities from the collection tank of the Common Effluent Treatment Plant (CETP, Pallavaram). They were transported in 10L cans. A small volume was held and transported on ice for bacteriological analyses. Samples were collected during pre-monsoon (July 2014) and monsoon (September 2015) seasons.

3.2.3 Measurement of physico-chemical parameters

3.2.3.1 *Water samples*

Temperature at each location was measured on-site using a hand-held mercury thermometer. Salinity and pH of collected water was measured using a hand-held refractometer (ATAGO, ATC-S/Mill-E) and a bench top Accumet Basic AB15 – pH meter (Fisherbrand, USA) calibrated with standard buffers. Samples were analyzed for dissolved oxygen (DO) following Winkler's procedure (Parsons *et al.*, 1984) (**Appendix D**). Nutrients such as nitrate (NO_3-N), nitrite (NO_2-N) and phosphates (PO_4-P) were analyzed according to Parsons et al. (1984) (**Appendix D**). At site **G5**, surface profile of

temperature and salinity were taken using a portable Conductivity Temperature Depth (CTD) system (Sea Logger, Sea Bird, SBE 25).

3.2.3.2 *Sediment samples*

Temperature, salinity and pH of the overlying water were measured as outlined earlier.

3.2.3.3 *Tannery effluent samples*

All physical and chemical parameters analyzed in triplicate, followed standard methods outlined in American Public Health Association (APHA, 2005). Biochemical oxygen demand (BOD) was determined using the 5-day test based on difference in sample initial DO and fifth day DO. Chemical oxygen demand (COD) was determined by open reflux oxidation method. Total nitrogen (TN) was calculated using Kjeldahl's method and total phosphorous (TP) by ascorbic method. Total dissolved solids (TDS), total suspended solids (TSS) and total solids (TS) were estimated by gravimetric methods. For measurement of TDS and TSS, fixed sample volumes were filtered through pre-weighed 0.7µm glass microfiber filter papers. The residual solids on filter paper and in the filtrate were dried at 180°C, cooled, weighed and calculated as TSS and TDS. TS was measured by weighing residual solids present in a known volume of sample, after drying in pre-weighed vessels (**Appendix D**).

3.2.3.4 *Metal analysis*

Water- concentrations of dissolved heavy metals (solvent-extracted) were estimated in samples using an inductively coupled plasma optical emission spectrometer (ICP-OES)

(Optima 7300DV, PerkinElmer, Singapore), following protocols outlined in APHA (2005). Fixed volume (400ml) of water samples were prepared for ICP-OES by acidifying with concentrated nitric acid. Dissolved metals were then pre-concentrated by chelation to ammonium pyrrolidine dithiocarbamate (APDC, 2%), followed by double extraction into chloroform and back-extraction with nitric acid (returned to aqueous layer); finally made up to 20mL with sterile de-ionized water (Anas *et al*., 2015). Processed samples were stored at 4°C till ICP-OES analysis.

Sediment- samples were digested as per guidelines in Balaram *et al.*, (1995). Sub-samples for metal analysis were dried at 60°C for 48h before disaggregating in a dry agate mortar (washed multiple times prior use with 5% nitric acid and then de-ionized water). A known quantity (~0.5g) of sample was digested in teflon vessels, with 10ml acid mixture of 7:3:1 concentrated hydrofluoric acid (HF), nitric acid (HNO_3) and perchloric acid ($HClO_4$). The teflon vessels were then heated on a hot plate in a fume hood, step-wise up to 240°C, until dryness. This step was repeated with 5ml of acid mixture. To the residue, 4ml of HNO_3 was added. The mixture was then warmed, allowed to cool and carefully filtered (Whatman No. 1 filter paper) into an acid-washed, dry standard flask. Final volume was made up to 25ml with double distilled water. Concentration of chromium was measured using a flame atomic absorption spectrophotometer (Thermo Electron Corp., S-Series, SOLAR S AAS) with an air–acetylene flame for Chromium element. Sub-volumes of the sample are fed to the instrument. Blank and standard solutions were run similarly for calibration and prior to analysis. Certified reference material MAG-1 (USGS) was used as a standard with a comparable matrix where handling, analytical and instrumental accuracy was assessed

that yielded readings between the prescribed reference value range (Flanagan, 1967; 1976). It was digested independently along with the sediment samples.

Tannery wastewater samples were tested for chromium content following protocol outlined in APHA (2005) by Diphenylcarbazide (DPC) method (described in detail in **Section 6.2.1**). Prior to colorimetry, samples were nitric acid digested, filtered and oxidized to hexavalent chromium form. The digest was filtered using 0.45µm filters before oxidation, as per test's requirements.

3.2.4 Microbiological analyses

3.2.4.1 *Total viable counts*

For enumerating total viable bacterial counts (**TVC**), aliquots of 0.1-0.3ml sample were spread onto seawater nutrient agar (SWNA, with composition [L^{-1}]: peptic digest of animal tissue 5.0 g, sodium chloride 5.0 g, beef extract 1.5 g, yeast extract 1.5g, agar 15g, distilled water 1000mL, pH 7.4 ± 0.2) as well as SWNA with 25-150µg mL^{-1} Cr^{6+} (prepared by mixing appropriate amounts of sterile potassium dichromate [$K_2Cr_2O_7$, A.R grade from Merck] stock solution for final cationic concentrations of µg mL^{-1}, **Appendix F**) prepared with 25 or 50% aged seawater, in triplicate. Sediments and effluent samples were processed accordingly, by serial dilution in sterile phosphate buffer (pH 7.2). Plates were incubated at 28°C for 24-48h and bacterial counts were taken as colony forming units (CFU) mL^{-1}. (Pereira *et al*., 2017). Initial weight of the sediment used in dilutions was determined by drying the filtered sediment at 60°C. Effluent samples were spread onto NA prepared without seawater. The pH of Cr^{6+}stock solutions were adjusted to 7.0

with conc. HNO_3 and/or 1N NaOH, and filter sterilized through 0.22µm filter membranes.

3.2.4.2 *Isolation of chromate tolerant bacteria*

For isolation of chromate tolerant bacteria, discrete colonies were picked at random from Cr^{6+} amended plates (**Section 4.2.1**).

3.2.5 Statistical analysis

Basic descriptive statistics were applied on raw data sets with mean ± standard deviation of marine waters, sediment and tannery wastewater parameters determined separately for each sampling location. Further statistical analysis was only performed on water parameters, with Pearson's correlation test used to estimate the significance of association between the various physico-chemical parameters spatially.

3.3 Results

The state of Goa has a tropical climate with generally humid conditions. Atmospheric temperatures ranged from 22.1 to 36.8 °C during the study period. Three seasons were chosen (Goa- pre-monsoon (March - May), monsoon (June - September) and post-monsoon (October - February) and for Chennai- pre-monsoon (March – May), monsoon (June – September) and post-monsoon (October - December) demarcated by the presence or absence of rainfall. Depth range was minimal as only surface waters were considered, even though the offshore site had a depth up to 28m. Total heterotrophic bacteria were reported from all sites for all seasons with the exception of the tannery wastewater site. In this chapter the seasonal physico-chemical parameters that may affect the numbers of

bacteria are reported.

3.3.1 Measurement of physico-chemical parameters

3.3.1.1 *Water*

Physico-chemical analysis of water samples showed variation in temperatures ranging from 26.1 to 32.1°C, a pH range of 6.88 to 8.13 and salinity from 3.9 to 36.3PSU in water sampled at different sites (**Table 3.1**). Waters collected were slightly alkaline with water from offshore site consistently displaying higher pHs for all seasons. It was also observed that pH was lowest during the monsoon season with salinity closely mirroring changes in pH. Divar Mangrove was observed as having the largest salinity range (3.9 to 33.5PSU). The nitrite profile of the water samples generally varied from 0.00 to 0.73$\mu molL^{-1}$ throughout the study period (max. observed at Dias beach). Nitrate values ranged from 0.27 to 5.37$\mu molL^{-1}$, with phosphate levels ranging from 0.15 to 1.53$\mu molL^{-1}$. Dissolved oxygen profile obtained in this study varied significantly ($P< 0.05$) and ranged from 1.14 to 4.89mgL^{-1}during pre-monsoon, from 3.63 to 5.71mgL^{-1} during monsoon and; from 1.47 to 4.79mgL^{-1} during post-monsoon. **Figure 3.2** shows normalized seasonal parametric values.

3.3.1.2 *Sediment*

Sediment parameters were observed similarly to water parameters and it was found that overlying waters displayed the same pH and salinity of their water counterparts. Sediment temperatures varied instead being generally warmer in comparison.

3.3.1.3 *Tannery effluent*

Nine parameters were analyzed by standard APHA protocol (**Table 3.2**) in addition to temperature, pH and chromium content. The raw effluent was greenish-brown in colour, had a mild but offensive odor and was found to be turbid possibly due to the high concentrations of carbonate, chloride and sodium commonly used in the tanning process. It had a pH range similar to seawater with much lower salinity. pH was recorded as 7.63 and 7.76 which was well within the tolerance limits of 5.5-9.0 prescribed by the Bureau of Indian Standards (BIS, 2009). Levels of total solids, total suspended solids and total dissolved solids found in the effluent (TS: 6761-9500; TSS: 1487-1595 and TDS: 5166-8166mgL^{-1}) were found to be extremely high and were much greater than permissible limits. COD was tested to determine degree of pollution and was found to be 12-14 times higher that permissible limits. BOD underlined the amount of organic matter in wastewater and was observed to be much higher (1520-1096mgL^{-1}) than the prescribed BIS limits (30$mgL^{-`1}$). The high COD, BOD, total nitrogen, total phosphates and phenols observed were consistent with those in untreated effluents (Krishnan *et al*., 2016).

3.3.1.4 *Metal analysis*

All samples were tested for their chromium content by various techniques. In water and sediment samples, although there are reports of chromium being found in this region (Kerkar and Ranjan, 2017), no chromium was observed (**Table 3.1**, **Supplementary table 1**). Tannery effluent contained chromium and its total content ranged from 9.57 to 12.31mgL^{-1} (**Table 3.2**).

3.3.2 Abundance of bacterial populations

Bacteria were recorded from all samples from coastal (Dias Beach), mangrove (Divar) and offshore locations. It was observed that bacteria were more abundant in sediments and tannery effluent than in water samples.

3.3.2.1 *Total viable counts*

Total viable counts (TVC) are one of the most important indexes in evaluating water quality. The TVC in water samples ranged from 13.5 to 401.7 x 10^2 colony forming units (CFU) ml^{-1}, with the highest TVC observed in Divar during pre-monsoon season, and the lowest at offshore site G5 also during pre-monsoon season. The TVC in water samples from both Divar mangrove and Dias beach were higher (exception being Dias during Pre-monsoon) than those from offshore location in all three seasons. Sediment and tannery samples showed a much higher microbial load in terms of order with the high TVC in tannery effluent directly correlating to poor quality. Dias beach site showed highest counts during the monsoon season, whereas Divar mangrove site showed highest microbial load during the pre-monsoon season (**Table 3.1**). Total viable counts for media amended with Cr^{6+} showed an obvious trend with decrease in counts as chromium concentration increased (**Table 3.3**). In general, the percentage of TVC in water samples grown in media amended with 25µg mL^{-1} Cr^{6+} ranged from 17-65% of the total observed in the control. This percentage reduction varied seasonally and from location to location. Dias beach showed 26, 40 and 65% of the control during pre, monsoon and post-monsoon seasons. Similarly, Divar demonstrated 40, 46 and 32%; and offshore site 65, 32 and 17% respectively, during pre, monsoon and post-monsoon season. Samples from

tannery effluent showed a similar reduction in TVC, with a range of 42-43% of the control.

3.3.2.2 *Chromate tolerant bacteria*

Based on TVC (**Table 3.3**), a sizable amount of the cultivable population (**% TVC**, TVC at 100µg mL^{-1}/ total CFU) showed tolerance to chromium ranging from 0.95 to 12.59% of the total. A larger percent of bacteria was found to tolerate chromium during the monsoon season (4.58-12.47%) (**Table 3.1**). The proportion of tolerant bacteria was lower during the other seasons at Dias beach and Divar. Offshore site had highest percentage of Cr^{6+} tolerant bacteria during pre-monsoon and followed a different trend compared to the other two sites. Each site showed a wide range in % of tolerant bacteria seasonally. In contrast, the range was within narrow limits in tannery samples with 5.01-5.81% of the total cultivable population demonstrating Cr^{6+} tolerance (**Table 3.2**). Similarly, seasonal variation also affected percents of Cr^{6+} tolerant bacteria from sediments collected at Dias beach, but not sediments from Divar mangrove.

3.3.3 Statistical analysis

Pearson correlation applied revealed certain relationships among and between the physico-chemical indicators (**Table 3.4**, **3.5** and **3.6**). At all three sampling sites, nutrients showed a negative relationship with salinity and pH. Both Dias beach and offshore site G5 also showed a similar negative relation with temperature. At Divar and Dias, DO shared a positive relationship with nutrients. Offshore site G5 on the other head showed a negative relationship with the same. Total viable counts showed an inconsistent relationship with the parameters measured at the various sites.

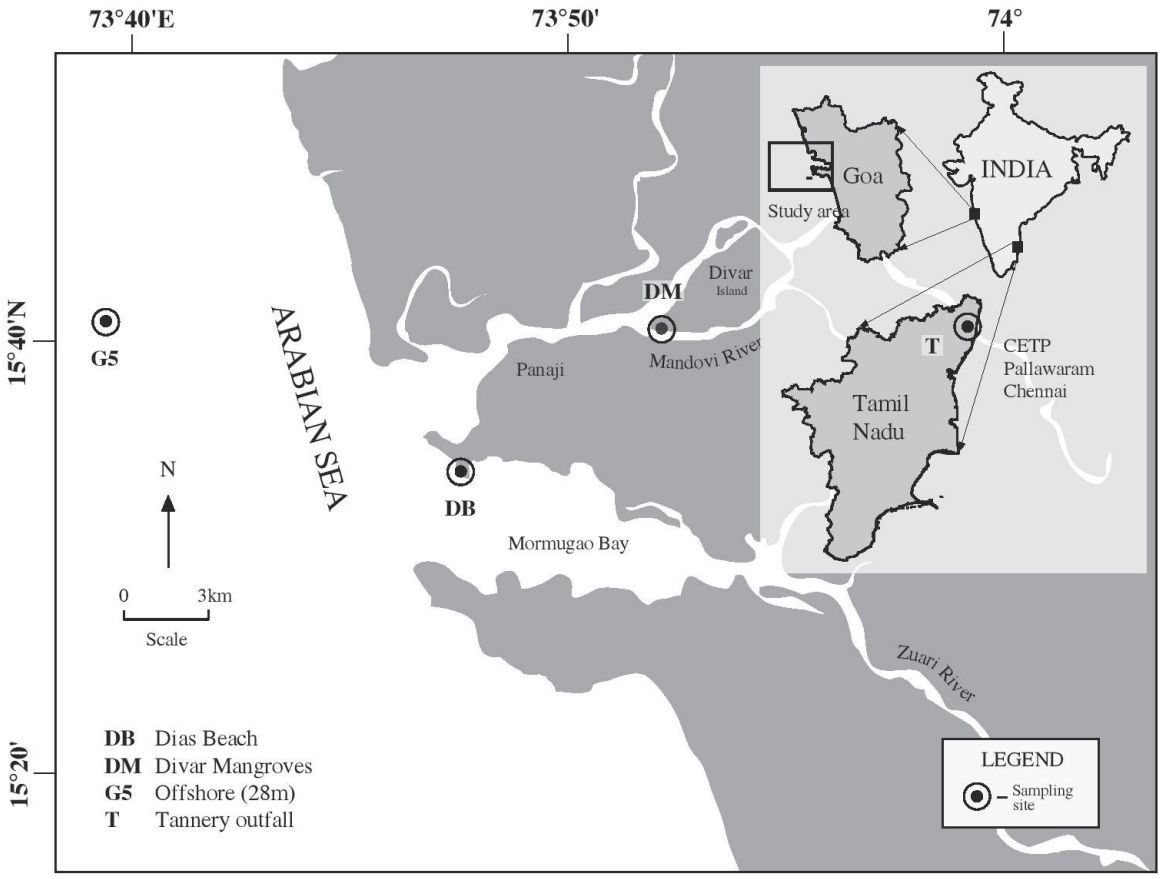


Figure 3.1 Map showing sampling locations (**DB**) Dias beach in Zuari estuary, (DM) Divar Mangrove site in Mandovi estuary, (G5) Offshore site off Candolim along the Goa coast and; Tannery outfall site (**T**, CETP, Pallavaram, Chennai) in Tamil Nadu

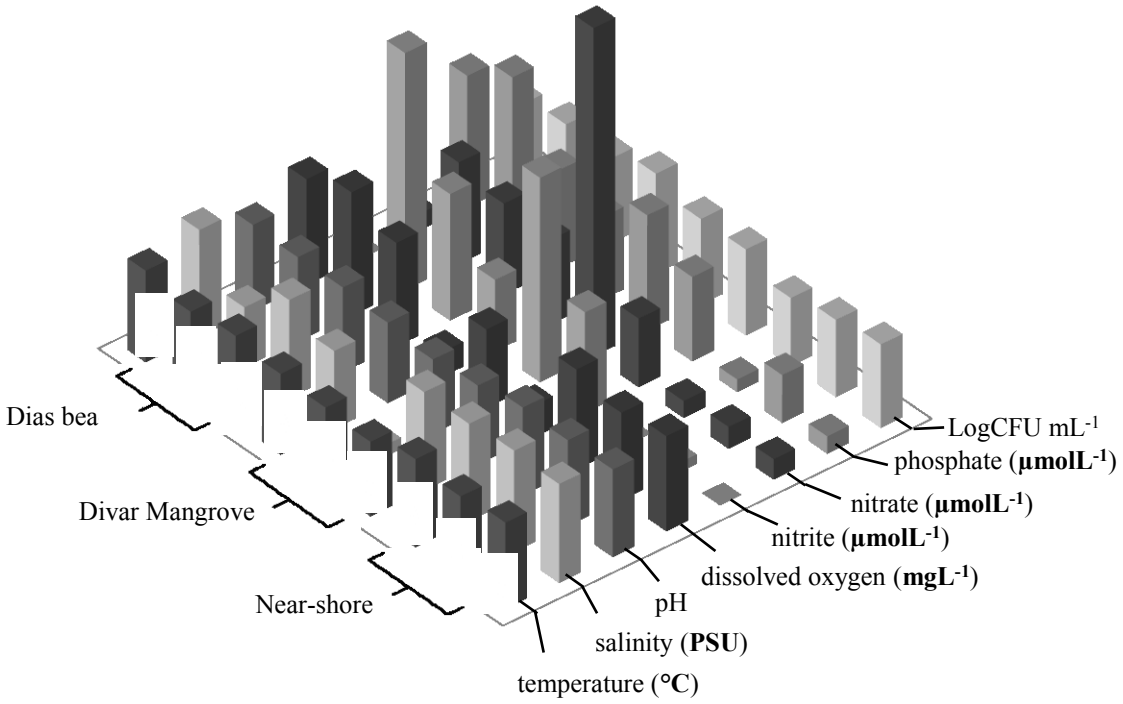


Figure 3.2 Physicochemical parameters normalized up to a score of 100 with their averages, from Dias beach, Divar Mangrove and Offshore site during the pre-monsoon (**A**), monsoon (**B**) and post-monsoon (**C**) seasons

Table 3.1 Physico-chemical characteristics of water samples

Parameter	Dias Beach			Divar Mangrove			Offshore G5		
	Pre- monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon
Temperature (°C)	32.07 ± 0.06	28.47± 0.06	30.77 ± 0.06	28.33 ± 0.06	27.30 ± 0.00	26.07 ± 0.12	30.76 ± 0.00	28.54 ± 0.00	30.16 ± 0.00
Salinity (PSU)	36.30 ± 0.00	20.43± 0.06	33.43 ± 0.06	26.47 ± 0.06	3.93 ± 0.15	33.53 ± 0.06	33.22 ± 0.00	34.54 ± 0.00	33.69 ± 0.00
pH	7.93 ± 0.07	7.79± 0.09	7.85 ± 0.04	7.44± 0.00	6.88 ± 0.00	7.31 ± 0.00	8.13 ± 0.00	7.96 ± 0.00	8.10 ± 0.00
Dissolved oxygen (mgL^{-1})	4.89 ± 0.02	5.71± 0.02	4.79 ± 0.01	1.14 ± 0.02	3.63 ± 0.01	1.47 ± 0.01	4.63 ± 0.00	4.06 ± 0.00	4.40 ± 0.00
Nitrite ($\mu molL^{-1}$)	0.00 ± 0.00	0.73± 0.00	0.39 ± 0.01	0.22 ± 0.01	0.63 ± 0.01	0.31 ± 0.01	0.00 ± 0.00	0.03 ± 0.00	0.00 ± 0.00
Nitrate ($\mu molL^{-1}$)	0.27 ± 0.00	1.66± 0.00	1.49 ± 0.00	1.39 ± 0.01	5.37 ± 0.01	1.12 ± 0.02	0.28 ± 0.00	0.36 ± 0.00	0.33 ± 0.00
Phosphate ($\mu molL^{-1}$)	1.53± 0.02	1.87± 0.03	1.14 ± 0.03	0.96 ± 0.02	1.34 ± 0.02	0.98± 0.03	0.15 ± 0.00	0.56 ± 0.00	0.2 ± 0.00
Cr ($\mu g\ mL^{-1}$)	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
CFU ($no.$ x 10^2 mL^{-1})	34.20 ±10.18	109.90 ± 9.67	76.50 ± 12.72	401.70 ± 78.00	86.60 ± 37.18	108.80 ± 8.20	13.50 ± 5.46	34.90 ± 9.40	67.70 ± 3.80
Cr^{6+} tolerant/total CFU(%) $no.\ ml^{-1}$	**2.63**	**12.47**	**3.92**	**0.95**	**11.20**	**2.93**	**12.59**	**4.58**	**1.92**
Sediment parameters									
Temperature(°C)	33.70	32.30	32.50	29.10	27.90	26.50	-	-	-
Cr ($\mu g\ mL^{-1}$)	BDL	BDL	BDL	BDL	BDL	BDL	-	-	-
CFU ($no.$ x 10^4 mL^{-1})	17.94 ± 1.79	7.60 ± 1.41	45.5 ± 10.60	463.8 ± 155.33	292.96 ± 71.45	446.5 ± 37.47	-	-	-
Cr^{6+} tolerant/total CFU (%) $no.\ ml^{-1}$	**1.68**	**8.82**	**0.66**	**9.62**	**9.93**	**9.97**	-	-	-

BDL below detection limit

Table 3.2 Physico-chemical characteristics of raw tannery effluent (**T**)

Parameter	**Pre-monsoon**	**Monsoon**	**Maximum permissible limits** (BIS, 2009)
Temperature (°C)	36.4	34.8	-
pH	7.63 ± 0.00	7.76 ± 0.00	5.5–9.0
Salinity (PSU)	11.30 ± 0.45	15 ± 0.30	-
Total solids (mgL^{-1})	9500.00 ± 67.30	6761 ± 50.20	2200
Total suspended solids (mgL^{-1})	1487.00 ± 27.60	1595.00 ± 30.40	100
Total dissolved solids ($mg\ L^{-1}$)	8166.67 ± 301.30	5166.67 ± 288.70	2100
BOD (mgL^{-1})	1096.00 ± 39.30	1520.00 ± 42.50	30
COD ($mgL^{-1}L$)	3520.00 ± 47.80	3070.00 ± 51.20	250
Total Nitrogen (mgL^{-1})	834.00 ± 52.70	822.00 ± 51.20	100
Total Phosphates (mgL^{-1})	0.79 ± 0.1	1.89 ± 0.05	1.0
Phenols (mgL^{-1})	10.1 ± 2.75		5 - 50
Total Cr (mgL^{-1})	12.31 ± 0.36	9.57 ± 0.28	2.0
CFU mL^{-1} (*no.* x 10^4 mL^{-1})	2.06 ± 0.05	1.94 ± 0.06	-
Cr^{6+} tolerant/total CFU (%) *no.* ml^{-1}	**5.01**	**5.84**	-

Standard deviation, **n=3**

Published in Das *et al.*, (2017)

Table 3.3 Colony forming units (CFU) of microbial populations from sampling sites in chromium amended and unamended media, determined by spread plate viable count method, 48h after incubation

	Concentration of Cr^{6+} ($\mu g\ mL^{-1}$)					
	0	**25**	**50**	**75**	**100**	**150**
CFU in **water** (*no.* x mL^{-1})						
Pre-monsoon						
Dias	3420	910	470	180	90	50
Divar	40170	16320	3350	1270	380	250
G5	1350	880	710	350	170	320
Tannery effluent	205600	88700	39100	29100	10300	7500
Monsoon						
Dias	10990	6830	3950	3250	1300	1530
Divar	8660	4020	2470	1930	970	310
G5	3490	1130	760	250	160	160
Tannery effluent	193600	82800	35300	27600	11300	5400
Post-monsoon						
Dias	7650	1830	650	250	300	50
Divar	10880	3530	1010	670	260	110
G5	6771	1200	530	390	130	40
Tannery effluent	-	-	-	-	-	-
CFU in **sediment** (*no.* x 10^4 g^{-1} *dry wt.*)						
Pre-monsoon						
Dias	17.94	1.20	0.50	0.50	0.30	0.10
Divar	463.87	114	77.38	62	44.63	32.63
G5	-	-	-	-	-	-
Monsoon						
Dias	7.60	4.16	2.06	1.53	0.67	0.32
Divar	292.96	128	55.83	37.42	29.08	18.5
G5	-	-	-	-	-	-
Post-monsoon						
Dias	45.50	16.00	3.50	0.50	0.30	0.10
Divar	446.50	301.5	162.5	91.25	44.5	10.5
G5	-	-	-	-	-	-

Table 3.4 Correlation matrix of physico-chemical parameters of water from Dias beach during pre-monsoon, monsoon and post-monsoon

	Temperature	Salinity	pH	DO	NO_2	NO_3	PO_4	TVC
Temperature	1							
Salinity	**0.982**[c]	1						
pH	**0.971**[c]	**0.907**[b]	1					
DO	-0.894[b]	-0.963[c]	-0.762[a]	1				
NO_2	-0.980[c]	-0.925[b]	-0.999[c]	**0.789**[a]	1			
NO_3	-0.842[b]	-0.723[a]	-0.946[c]		**0.932**[c]	1		
PO_4		-0.742[a]		**0.894**[b]			1	
TVC	-0.974[c]	-0.913[b]	-1.000[c]	0.771[a]	1.000[c]	**0.942**[c]		1

Table 3.5 Correlation matrix of physico-chemical parameters of water from Divar Mangroves during pre-monsoon, monsoon and post-monsoon

	Temperature	Salinity	pH	DO	NO_2	NO_3	PO_4	TVC
Temperature	1							
Salinity		1						
pH		**0.904**[b]	1					
DO		-0.939[c]	-0.996[c]	1				
NO_2		-0.905[b]	-1.000[c]	**0.996**[c]	1			
NO_3		-0.985[c]	-0.964[c]	**0.984**[c]	**0.965**[c]	1		
PO_4		-0.962[c]	-0.986[c]	**0.997**[c]	**0.987**[c]	**0.995**[c]	1	
TVC	**0.803**[c]		**0.717**[a]		-0.715[a]			1

Table 3.6 Correlation matrix of physico-chemical parameters of water from Offshore site G5 during pre-monsoon, monsoon and post-monsoon

	Temperature	Salinity	pH	DO	NO2	NO3	PO4	TVC
Temperature	1							
Salinity	-0.996[c]	1						
pH	**0.991**[c]	-0.975[c]	1					
DO	**0.988**[c]	-0.998[c]	**0.959**[c]	1				
NO2	-0.965[c]	0.936[c]	-0.991[c]	-0.914[b]	1			
NO3	-0.890[b]	**0.929**[c]	-0.823[a]	-0.950[c]	**0.741**[a]	1		
PO4	-0.990[c]	**0.973**[c]	-1.000[c]	-0.957[c]	**0.992**[c]	**0.818**[b]	1	
TVC								1

[a] Correlation is significant at the *0.05* level
[b] Correlation is significant at the *0.01* level
[c] Correlation is significant at the *0.001* level

3.4 Discussion

Marine habitats are complex ecosystems that are strongly influenced by physical, chemical and biological processes. Physical parameters such as temperature, salinity, pH, turbulence, waves etc. therefore have a considerable impact (Lenihan, 1999) in the dynamics of marine ecosystems (Varadharajan and Soundarapandian, 2014). Subtle changes in physical conditions can have profound effects on water quality of the study system, which may in turn affect the spatial and temporal distribution of nutrients and/or biological communities. In addition to structuring water masses and providing environmental conditions that support marine life, physical parameters of seawater can be altered by human activity, especially in coastal areas. Since physical conditions, nutrient concentrations and primary productivity are intrinsically linked, biological monitoring is important to describe community constituents. Additionally, they may be used to assess variability in seasonal patterns as well environmental stressors, which are relative to pollution levels (Twomey *et al.*, 1980).

Physico-chemical interpretation

Chromium is a priority metal pollutant, and this study was focused on the dissolved fraction (dissolved + sediment bound fraction). Surface water temperatures primarily rely on intensity of solar radiation, evaporation and mixing up of tidal waters. Low water temperatures are generally due to overcast skies and rainfall. The prevalence of rainfall (along with lower temperatures) leads to freshwater influx in sinks, and dilution of seawater. As a result, salinity tends to drop and this phenomenon has been reported by James and Najmuddin (1986). In this study, lower salinity values and pH were observed

during monsoon when compared to non-monsoon seasons. James and Najmuddin also reported heavy land runoff during monsoons, which contribute to organic material decomposition and accumulation of organic acids. This is accompanied by a decrease in pH (acidic condition) which explains decreased pH at both Divar Mangrove and Dias Beach during this season. This is corroborated by Pearson correlation matrix (significance level of $P<0.05$) which highlighted a negative relationship between (nutrients) vs. (temperature, pH or salinity). In contrast, pH did not change as much in Offshore site G5 (in monsoons) as freshwater influx/inputs may be variable or reduced in comparison.

Dissolved oxygen (DO) was observed to be higher during monsoons. This may be due to (i) enhanced solubility at lower temperatures and mixing up, due to influx of fresh water (Anilakumary and Azis, 1992), and/or (ii) a decrease in salinity (Menzel, 1970). Menzel (1970) also reported solubility of oxygen in seawater to be inversely related to salinity. In this study, similarly, DO was found to form an inverse relationship with salinity, with strong negative correlation. Additionally, DO levels from Divar ranked as lowest. This highlighted that water from Divar had low oxygen tensions and was of poor quality. The hypoxic nature is possibly due to high amounts of decomposing organic material in the vicinity and a lack of turbulence (Shenai-Tirodkar *et al*., 2016).

In this study, nitrites and nitrates were higher in monsoons. This was also observed by (Nair *et al*, 2015) who reported similar readings. Low oxygen tension due to saturation of water during non-monsoon seasons leads to rapid conversion of nitrate to nitrite-dentirification (Dawson and Murphy, 1972). In monsoon condition, nitrite is expected to accumulate more than nitrate due to higher concentrations of dissolved

oxygen, this was observed to be true at Dias beach and Divar Mangroves. The conversion of nitrite to nitrate is also favoured by elevated temperatures (Dawson and Murphy, 1972). From the results obtained, the negative correlation between nitrites and temperature, as well as the positive correlation between nitrites and DO confirm these processes. Comparatively, the levels of nitrites and nitrates were found to be higher at Divar. This again indicates a septic system (poor water quality) and may be related to oxidation of decaying matter, reported earlier by Chanda *et al*., (1996) in Mandovi estuary. Higher levels of phosphate during the monsoon could be attributed to high adsorption rate as a result of low pH (Rajagopal and Reddy, 1984), or by leaching from sediments into overlying water. It followed the same trend as nitrogen derivatives measured spatially.

Bacterial abundance interpretation

Notwithstanding the deficiencies of bacterial enumeration using plate counts (Stanley and Konopka, 1985), culturable bacteria are useful to obtain insights on prevalence of certain bacteria as well as the isolation of potential remediators. Analyses for bacterial TVC in water systems therefore help assess water quality and their relationships with relevant environmental factors. The Mandovi and Zuari estuaries have long been associated with raw sewage disposal (Ramaiah *et al*., 2007) and there have been multiple reports (Ramaiah *et al*., 2001, 2002) which examined and affirmed the presence of allochthonous microflora. An understanding of bacterial incidence is therefore important to coastal pollution surveillance. Although these aspects from nearby tropical estuaries have been investigated (Nagverkar and Ramaiah, 2009), there are no reports outlining Cr^{6+} resistant

bacteria in autochthonous populations. In general, higher abundances were observed spatially at, Dias and Divar. With rainfall, large amounts of land material drain in to the Mandovi river (Shankar *et al.*,2004) affecting related physical parameters at both sites. The greater runoff explains the higher bacterial abundances. Between the two sites, Divar had higher bacterial abundances. In addition to high runoff, it is also subject to urban sewage outfall as well as barge-related wastes (from iron-ore mining) (Sawkar *et al*., 2003). This is evident from the low oxygen tension, and with nutrient data, points to low water quality in comparison to the other two sites. Attri and Kerkar (2011), and Alagarsamy (2006) have also reported similar results.

Temporal trends at each location were different. Abundance at Dias was most during monsoon as a result of freshwater influx and runoff. Abundances at Divar and offshore site did not follow this trend. Nitrates showed a negative correlation ($P< 0.05$) with TVC at Divar, this can be attributed to its active utilization (Khandeparker *et al.*, 2017). It is known that Nitrogen (N) is a major limiting nutrient in marine ecosystems (Elser *et al.,* 2007). Surge in levels of nitrates in monsoons (Divar) as a result of loss of N due to denitrification correlates well with decrease in abundance and may be responsible for what I have observed. Although nutrient levels varied little at offshore site, nitrates were found to accumulate in monsoon and post-monsoon seasons, which coincide with higher counts. Other studies (Anand *et al.*, 2014a, 2014b; Khandeparker *et al.*, 2017) have also shown notable intra-seasonal co-variation of nutrients and bacterial abundance. These point out bacterial populations are heavily influenced by physical processes. As such, each of the sites had unique nutrient trends and formed micro-environments.

Raw tannery effluent samples as expected were of extremely low quality with high viable counts, similarly observed as Krishnan *et al*., (2016) from the same site. From measurements, the rich nutrient content (as a microbial energy source) resulted in high bacterial numbers (Calheiros *et al.*, 2008). Abundance was high in sediments as expected. Sediments from Divar showed exceptionally high abundance throughout, with the least abundance observed during monsoon, as in its water samples from monsoon.

Chromate tolerating bacteria

Kerkar and Das (2017) reported 0.021µg mL^{-1} and 8.9µg g^{-1} Cr^{6+} in dissolved water and sediments collected from Ribander (Goa) salt pans. Attri and Kerkar, (2011) observed 27.6, 17.5 and 10.3µg g^{-1} of Cr^{6+} during pre-monsoon, monsoon and post-monsoon seasons in sediments collected from Divar mangroves. Although these reports outline presence of Cr^{6+} in said environments, no chromium was detected in marine samples, in this study. It is likely that chromium was present below detection limits. Around 9-12µg $mL^{-1}Cr^{6+}$ was measured in tannery effluent. Concentrations of chromium in natural waters that have not been affected by waste disposal are commonly less than 10µg mL^{-1} (Hem, 1989).

A large fraction of the cultivable population was tolerant to Cr^{6+} (2.6-12.6% of the total). This suggests that most bacteria are physiologically equipped to deal with chromium metal and/or other pollutants. Importantly, presence of Cr^{6+} would most likely result in incidence of higher number/percentages of Cr-tolerant bacteria, but this is not the case as the ratio of Cr-tolerant to total CFU is much higher in natural sources in comparison to ratios for effluent. This means that (i) Cr^{6+} contamination increases the

likelihood of the presence Cr^{6+} tolerant/resistant strains, but is not necessary for resistance and/or resistant isolates and; (ii) it is likely that local environmental influences/stresses mainly micro-climate, vegetation and anthropogenic inputs play a role.

The comparative assessment of abundance and the presence of Cr^{6+} tolerant isolates are important to infer that the Mandovi estuary experiences impacts of urban outfalls. This is further supported by parametric data and high abundances that indicate some level of pollution at Dias, but not on the same level as that observed at Divar in this study as well as reports outlined here. Tannery effluent from Pallavaram, was of poor quality, having high nutrient and low Cr^{6+} content. High bacterial abundances are indicative of this. It is also evident from the substantial fractions of native bacteria growing in medium with Cr^{6+} that marine environments are home to a diverse set of high potential strains. These strains appear to be metabolically equipped to grow in elevated Cr^{6+}. As such, their abundance is intrinsically linked to physical factors which are limiting in nature.

Chapter 4

Phylogenetics of chromate tolerant bacteria

4.1 Introduction

Ecological, physiological and molecular biological studies from a location can provide insights into many processes that operate in that ecosystem (Pace, 1997). Widespread use of chromium compounds in industries, and indiscriminate dumping of its wastes has led to release of this metal and its compounds into the environment (Francisco *et al*., 2002). Field studies have shown the adverse and deleterious effects of Cr^{6+} in environments surrounding related industries (Turpeinen *et al.*, 2006). This has ultimately led to an increase in potential chromium resistance in the microbial community. Although there are reports of environmental responses to exposure within bacterial functional groups (Giller *et al.*, 1998), it is unknown how these compounds affect microbial community composition or the prevalence and form of metal resistance (Sheik *et al*., 2012).

Camargo *et al*. (2005), Sheik *et al*. (2012), Roane and Kellogg (1996) and Nakatsu *et al*. (2005) all report of bacterial communities from chromium-contaminated environments. There are also a plethora of reports (Rooney-Varga *et al*., 2005; Bouvier and Giorgio *et al*., 2006) outlining bacterial diversity from marine environments, some from the coastline of Goa (Singh *et al.*, 2010) (Khandeparker *et al*., 2011) but none so far providing information on a dynamic marine environment (Cr^{6+} contamination free) where microorganisms have considerable potential for Cr^{+6} detoxification.

Diversity can be studied (Muyzer *et al.*, 1993; Smit *et* al., 1997) by cultivation dependant or independent techniques. Cultivation independent methods are much more in vogue these days as more comprehensive assessment (DeLong, 2002) of native microflora biomass type is possible rather quickly. Although in retrospect, cultivation dependant studies cover only a small fraction of the total diversity in complex

environmental samples, they are valuable (Jain et al., 2014). Considering that chromate (Cr^{6+}) resistant bacteria (CRB) have a prospect in bioremediation applications, traditional culturing and 16S rRNA genes as molecular markers in the assessment of CRB are useful for isolating novel organisms and exploring their properties.

Although the role of CRB is recognized to be ecological vital, detailed information on their populations and its relation to sub-surface mobility of Cr^{6+} in marine ecosystems is lacking. It is important to emphasize that this study aims to describe representative subset of bacteria, but primarily the subset that demonstrates potential for high Cr^{6+} resistance. In that regard, chromate tolerant bacteria isolated from Dias Beach (DB), Divar Mangroves (DM), offshore site (G5) and Pallavaram tannery effluent samples were identified based on 16S rDNA sequencing. This sequencing information was used to establish evolutionary relationships of chromate tolerant bacteria.

4.2 Methods

4.2.1 Isolation of chromate tolerant bacteria

For isolation of chromate tolerant bacteria, discrete colonies were picked at random from plates with 100-150µg mL^{-1} Cr containing 30-200 colonies. These were purified (by quadrant streak subculture), and subsequently maintained on NA slants containing 50µg mL^{-1} Cr, at 4°C. For extraction of DNA, pure isolates were grown overnight in Nutrient Broth (NB, containing [L^{-1}] 5.0 g peptone and 3.0g yeast extract), at 120rpm and 28°C.

4.2.2 Extraction of genomic DNA

DNA was extracted from as many as 278 chromate tolerant bacterial isolates from different sampling locations using GenElute™ Bacterial Genomic Kit (Sigma-Aldrich,

USA), according to the manufacturer instructions. Around 1.5mL of bacterial culture, grown in NB was pelleted by centrifugation and the culture medium discarded. Pellets were re-suspended in 200µL lysozyme (~35mg/mL) and incubated for 30min at 37°C. This was followed by 2min incubation at room temperature after the addition of 20µL of RNase A solution. To this suspension, 20µL of Proteinase K solution and 200µL of Lysis Solution C were added, vortexed and incubated at 55°C for 10min. To the following lysate, 200µL of ethanol added was homogenized; and entirely transferred to an activated Miniprep Binding Column assembled in a collection tube. After centrifuging at 6500 x g for 1min, the column was washed with wash solutions provided with the collection tube changed at each step. In a fresh collection tube, the bound DNA was eluted by addition of Elution Solution. The eluate was then tested for DNA quality and integrity using Nanodrop and agarose gel electrophoresis, respectively.

4.2.3 PCR amplification and purification

16S rRNA gene was amplified from DNA extracted using two general 16S rRNA primers, 27F (5'-AGAGTTTGATCACTGGCTCAG-3') (Weisberg *et* al., 1991) and 1492R (5'-CTACGGCTACCTTGTTACGA-3') (Reysenbach *et al*., 1992). Each PCR reaction tube contained 2µL of template (30-50µg DNA μl^{-1}), 1µL each of forward and reverse primer (10pmol), 12µL of PCR Master Mix (Thermo, USA) and 9µL of Nuclease free water (Thermo, USA) (final reaction volume- 25µL). PCR was conducted through a thermal cycler (Veriti, Applied Biosystems, USA) with parameters:

94°C initial denaturation	4min	
94°C denaturation	1min	┐
55°C annealing	1min	├ x *35 cycles*
72°C extension	1min	┘
72°C final extension	7min	

The resulting PCR amplicons were electrophoresed through 1% agarose gel alongside 1kb molecular ladder ranging from 300bp to 10000bp to confirm amplification. Prior to sequencing, the amplicons were purified using UltraClean® PCR Clean-Up Kit (MoBio) according to the manufacturer's instructions (30µL final volume). Products were stored at -20°C.

4.2.4 Sequencing

Sequencing was performed with 15-50ng of the PCR amplicon (volume calculated based on Nanodrop readings) and 1pmol each of forward and reverse primer (27F and 1492R) using an ABI 3130 genetic Analyzer, based on dideoxy chain termination. Resulting sequences obtained were compared with known sequences on National Center for Biotechnology Information (NCBI) database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for their closest match. Sequences submitted to NCBI-GenBank database were given the accession numbers **MG205785** to **MG206062**.

4.2.5 Phylogenetic analysis

Phylogenetic trees were constructed using obtained sequences, and sequences of reference/type strains retrieved from EzTaxon database **(**http://eztaxon-e.ezbiocloud.net**)**

(Kim *et al.*, 2012). Reference strains were selected based on closest match with 16S rRNA sequences. All sequences (in FASTA format) entered into MEGA6 were aligned and their molecular evolutionary distances were assessed following the Maximum Composite model (Tamura *et al.* 2011). Phylogenetic trees were constructed using neighbor joining (NJ) algorithm (Tamura *et al*., 2004), and bootstrap analysis was performed to evaluate stability of relationships by performing 1000 replications for tree topology (Felsenstein, 2004).

4.3 Results

4.3.1 Bacterial isolates

A total of 278 Cr^{6+} tolerant isolates (193 from water, 61 from sediment and 24 from tannery effluent) were picked out randomly from SWNA with 100µg mL^{-1} Cr^{6+}. Genomic DNA from all isolates was extracted and purity ensured based on A_{260nm}/A_{280nm} ratio (within 1.7-2.0).

4.3.2 16S rDNA sequence analysis

Extracted DNA was used as template for amplification of 16S rRNA gene and the products were confirmed by electrophoresis on 1% agarose gel. Amplified ribosomal DNA in all samples was around ~1500bp in size. The sequences obtained (**MG205785** to **MG206062**) represented phylotypes that were compared with known sequences (>97% similarity) in the database. The bacteria isolated grouped into Actinobacteria, Firmicutes and Proteobacteria phyla, and were further divided based on their classes from water, sediment and effluent for each location sampled (**Fig. 4.1**). In all locations, bacilli

dominated representation (62-90%) with Actinobacteria (class), Betaproteobacteria and Gammaproteobacteria forming minor groups. Actinobacteria formed ~16% of the isolates from water sampled from the offshore site (**G5**) and were also observed in water from Dias beach (**DB**). Gamaproteobacteria formed ~22% of the isolates at **G5** with the species in this group (*Halomonas* sp.) found only in this locale. Betaproteobacteria were only observed in samples from tannery. The 278 isolates were found to represent as many as 15 genera belonging to *Arthrobacter*, *Brevibacterium*, *Curtobacterium*, *Janibacter*, *Kocuria*, *Microbacterium* and *Rothia* (Actinobacteria); *Bacillus*, *Exiguobacterium* and *Staphylococcus* (Bacilli); *Vibrio*, *Psychrobacter*, *Halmonas* and *Serratia* (Gammaproteobacteria); and *Alcaligenes* (Betaproteobacteria)

4.3.3 Phylogenetic analysis

Phylogenetic trees (**Fig.2**) imply high homology between isolates and show the diverse chromate tolerant bacteria from water, sediment and effluent. Each tree indicates the distribution of tolerant isolates into bacterial classes namely Bacilli, Actinobacteria, Gammaproteobacteria and Betaproteobacteria with all isolates showing a high degree of similarity to related isolates and related type strains. **Figure 2** shows phyolgenetic trees based on 16S rDNA sequences and isolates cluster into clades based on similarity or difference in rDNA sequence composition. The scale bar in each tree indicates fixed mutation per nucleotide position to denote evolutionary distances.

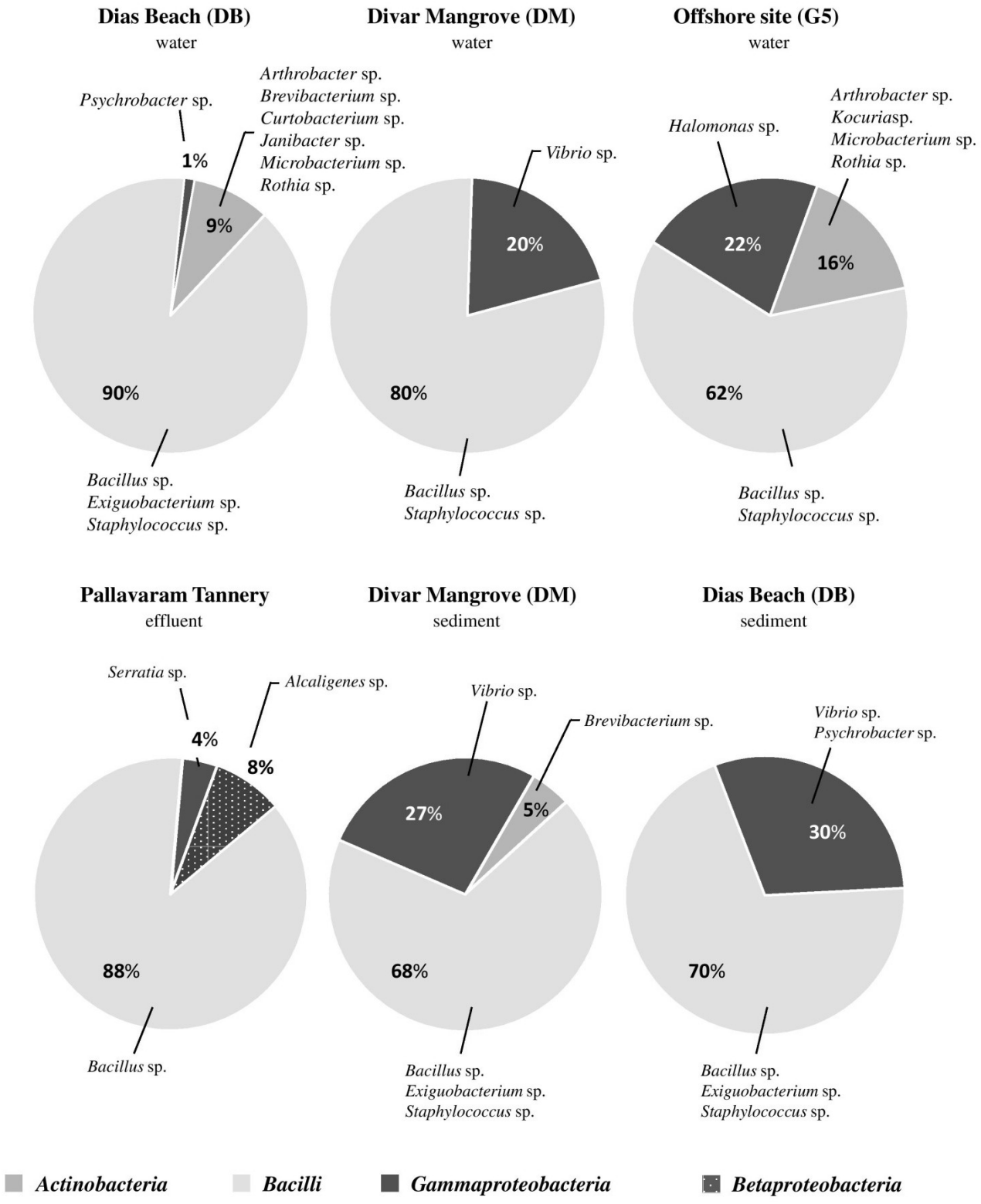
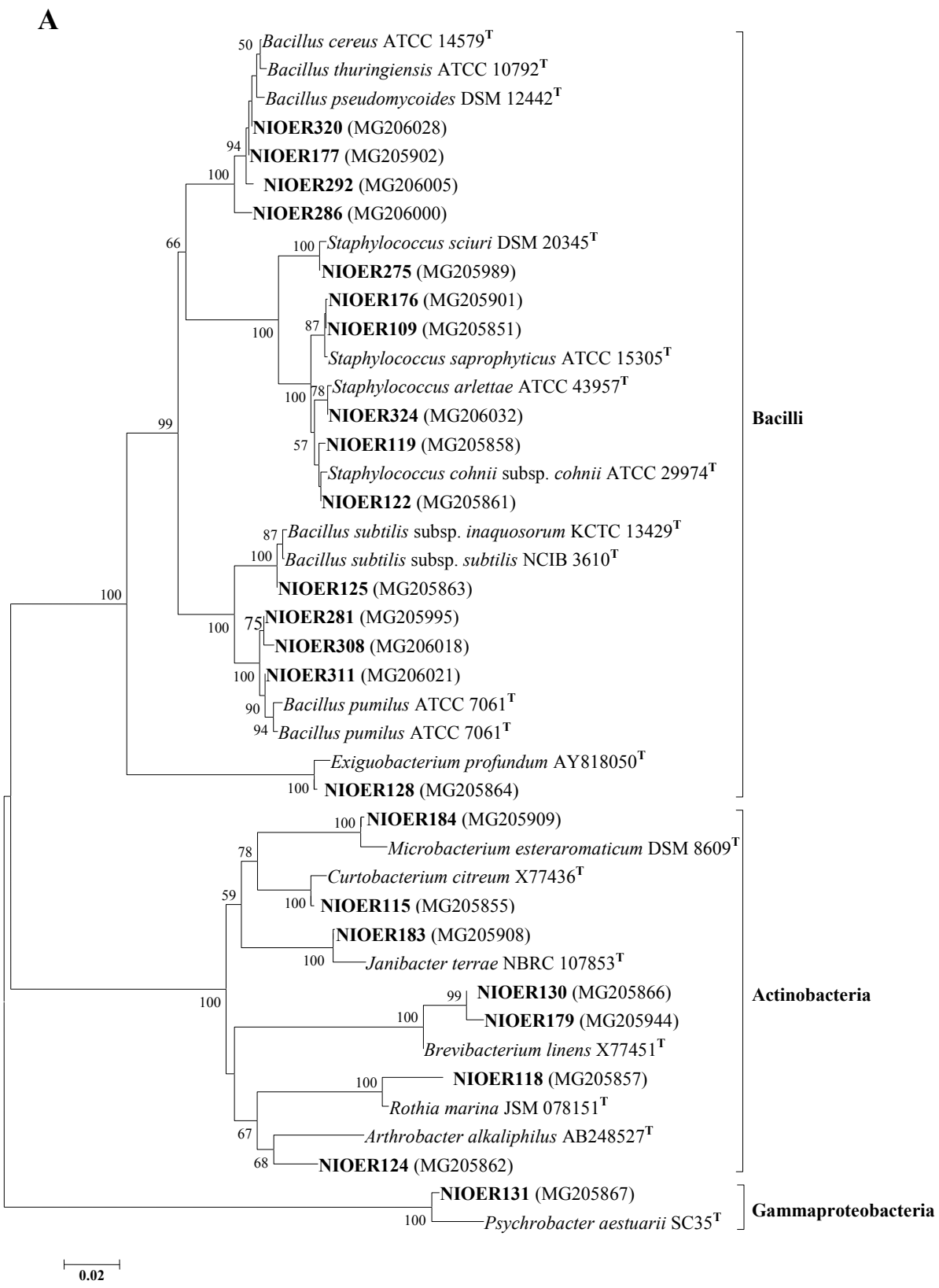
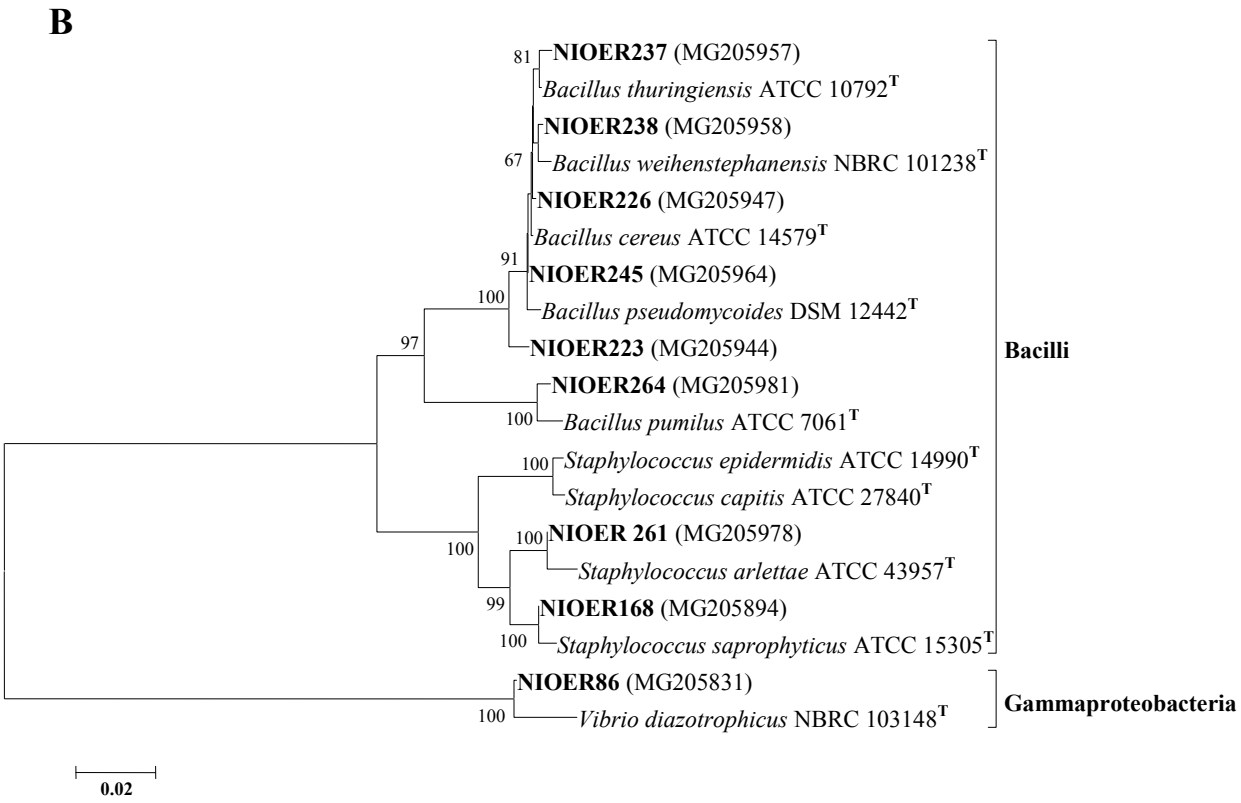
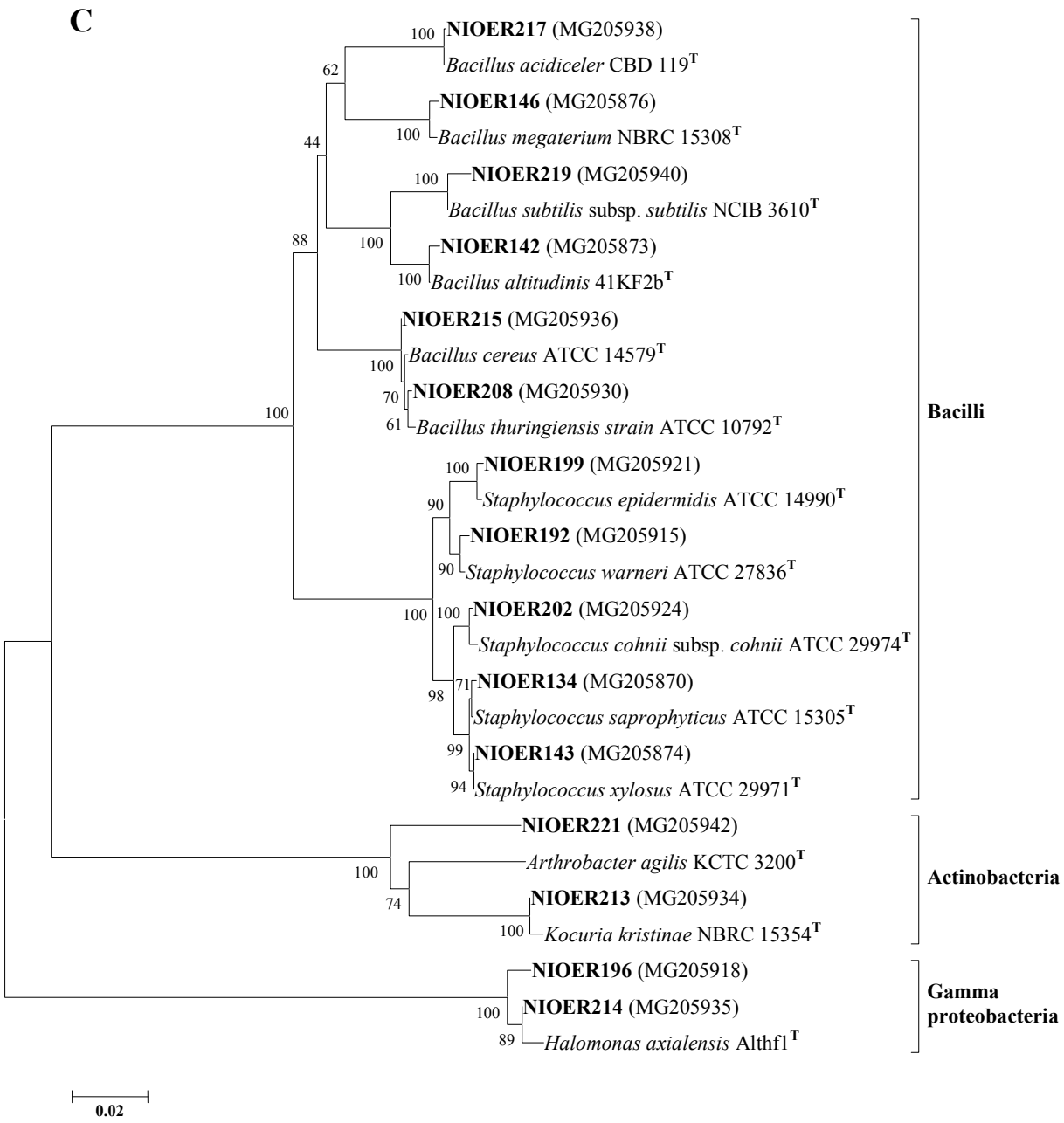
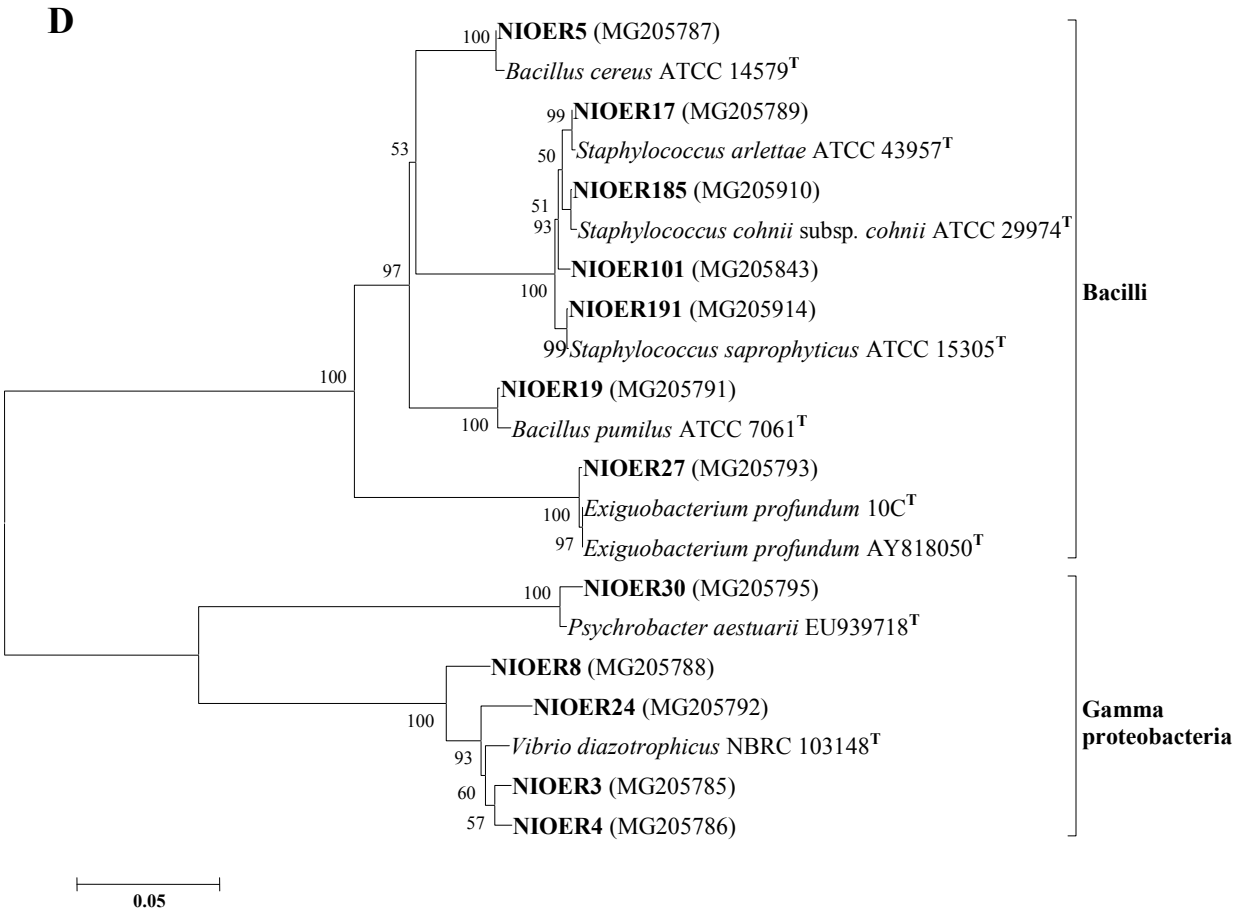


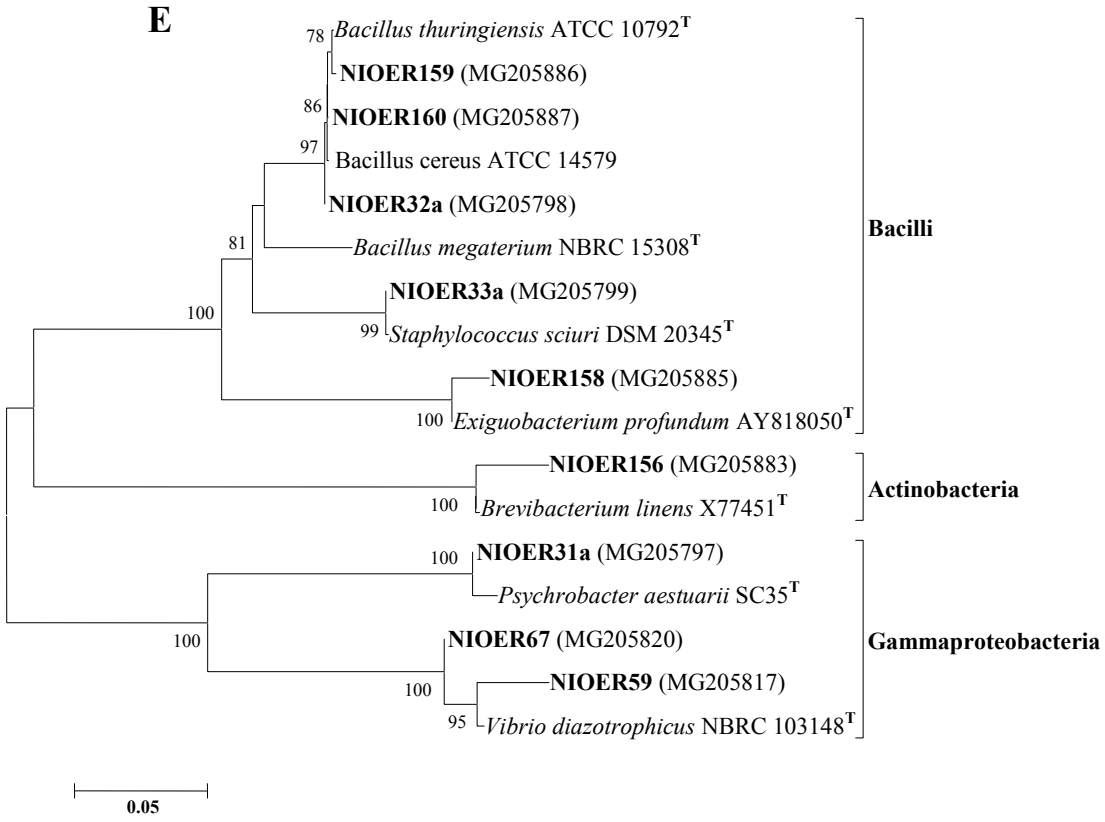
Figure 4.1 Pie-chart representation of the distribution of chromate tolerant bacteria from samples taken from Dias beach, Divar Mangrove, offshore site G5 and Pallavaram tannery effluent


A
Bacillus cereus ATCC 14579T
Bacillus thuringiensis ATCC 10792T
Bacillus pseudomycoides DSM 12442T
NIOER320 (MG206028)
NIOER177 (MG205902)
NIOER292 (MG206005)
NIOER286 (MG206000)
Staphylococcus sciuri DSM 20345T
NIOER275 (MG205989)
NIOER176 (MG205901)
NIOER109 (MG205851)
Staphylococcus saprophyticus ATCC 15305T
Staphylococcus arlettae ATCC 43957T
NIOER324 (MG206032)
NIOER119 (MG205858)
Staphylococcus cohnii subsp. cohnii ATCC 29974T
NIOER122 (MG205861)
Bacillus subtilis subsp. inaquosorum KCTC 13429T
Bacillus subtilis subsp. subtilis NCIB 3610T
NIOER125 (MG205863)
NIOER281 (MG205995)
NIOER308 (MG206018)
NIOER311 (MG206021)
Bacillus pumilus ATCC 7061T
Bacillus pumilus ATCC 7061T
Exiguobacterium profundum AY818050T
NIOER128 (MG205864)
Bacilli
NIOER184 (MG205909)
Microbacterium esteraromaticum DSM 8609T
Curtobacterium citreum X77436T
NIOER115 (MG205855)
NIOER183 (MG205908)
Janibacter terrae NBRC 107853T
NIOER130 (MG205866)
NIOER179 (MG205944)
Brevibacterium linens X77451T
NIOER118 (MG205857)
Rothia marina JSM 078151T
Arthrobacter alkaliphilus AB248527T
NIOER124 (MG205862)
Actinobacteria
NIOER131 (MG205867)
Psychrobacter aestuarii SC35T
Gammaproteobacteria
0.02


B
81
NIOER237 (MG205957)
Bacillus thuringiensis ATCC 10792T
NIOER238 (MG205958)
67
Bacillus weihenstephanensis NBRC 101238T
NIOER226 (MG205947)
Bacillus cereus ATCC 14579T
91
NIOER245 (MG205964)
100
Bacillus pseudomycoides DSM 12442T
97
NIOER223 (MG205944)
Bacilli
NIOER264 (MG205981)
100
Bacillus pumilus ATCC 7061T
100
Staphylococcus epidermidis ATCC 14990T
Staphylococcus capitis ATCC 27840T
100
NIOER 261 (MG205978)
100
Staphylococcus arlettae ATCC 43957T
99
NIOER168 (MG205894)
100
Staphylococcus saprophyticus ATCC 15305T
NIOER86 (MG205831)
100
Vibrio diazotrophicus NBRC 103148T
Gammaproteobacteria
0.02


C
NIOER217 (MG205938)
Bacillus acidiceler CBD 119T
NIOER146 (MG205876)
Bacillus megaterium NBRC 15308T
NIOER219 (MG205940)
Bacillus subtilis subsp. subtilis NCIB 3610T
NIOER142 (MG205873)
Bacillus altitudinis 41KF2bT
NIOER215 (MG205936)
Bacillus cereus ATCC 14579T
NIOER208 (MG205930)
Bacillus thuringiensis strain ATCC 10792T
NIOER199 (MG205921)
Staphylococcus epidermidis ATCC 14990T
NIOER192 (MG205915)
Staphylococcus warneri ATCC 27836T
NIOER202 (MG205924)
Staphylococcus cohnii subsp. cohnii ATCC 29974T
NIOER134 (MG205870)
Staphylococcus saprophyticus ATCC 15305T
NIOER143 (MG205874)
Staphylococcus xylosus ATCC 29971T
NIOER221 (MG205942)
Arthrobacter agilis KCTC 3200T
NIOER213 (MG205934)
Kocuria kristinae NBRC 15354T
NIOER196 (MG205918)
NIOER214 (MG205935)
Halomonas axialensis Althf1T
Bacilli
Actinobacteria
Gamma proteobacteria
0.02


D
100
NIOER5 (MG205787)
Bacillus cereus ATCC 14579T
99
NIOER17 (MG205789)
53
50
Staphylococcus arlettae ATCC 43957T
NIOER185 (MG205910)
51
93
Staphylococcus cohnii subsp. cohnii ATCC 29974T
97
NIOER101 (MG205843)
100
NIOER191 (MG205914)
99
Staphylococcus saprophyticus ATCC 15305T
100
NIOER19 (MG205791)
100
Bacillus pumilus ATCC 7061T
NIOER27 (MG205793)
100
Exiguobacterium profundum 10CT
97
Exiguobacterium profundum AY818050T
Bacilli
100
NIOER30 (MG205795)
Psychrobacter aestuarii EU939718T
NIOER8 (MG205788)
NIOER24 (MG205792)
100
93
Vibrio diazotrophicus NBRC 103148T
60
NIOER3 (MG205785)
57
NIOER4 (MG205786)
Gamma proteobacteria
0.05


E
78
Bacillus thuringiensis ATCC 10792T
NIOER159 (MG205886)
86
NIOER160 (MG205887)
97
Bacillus cereus ATCC 14579
NIOER32a (MG205798)
81
Bacillus megaterium NBRC 15308T
NIOER33a (MG205799)
100
99
Staphylococcus sciuri DSM 20345T
NIOER158 (MG205885)
100
Exiguobacterium profundum AY818050T
Bacilli
NIOER156 (MG205883)
100
Brevibacterium linens X77451T
Actinobacteria
100
NIOER31a (MG205797)
Psychrobacter aestuarii SC35T
100
NIOER67 (MG205820)
100
NIOER59 (MG205817)
95
Vibrio diazotrophicus NBRC 103148T
Gammaproteobacteria
0.05

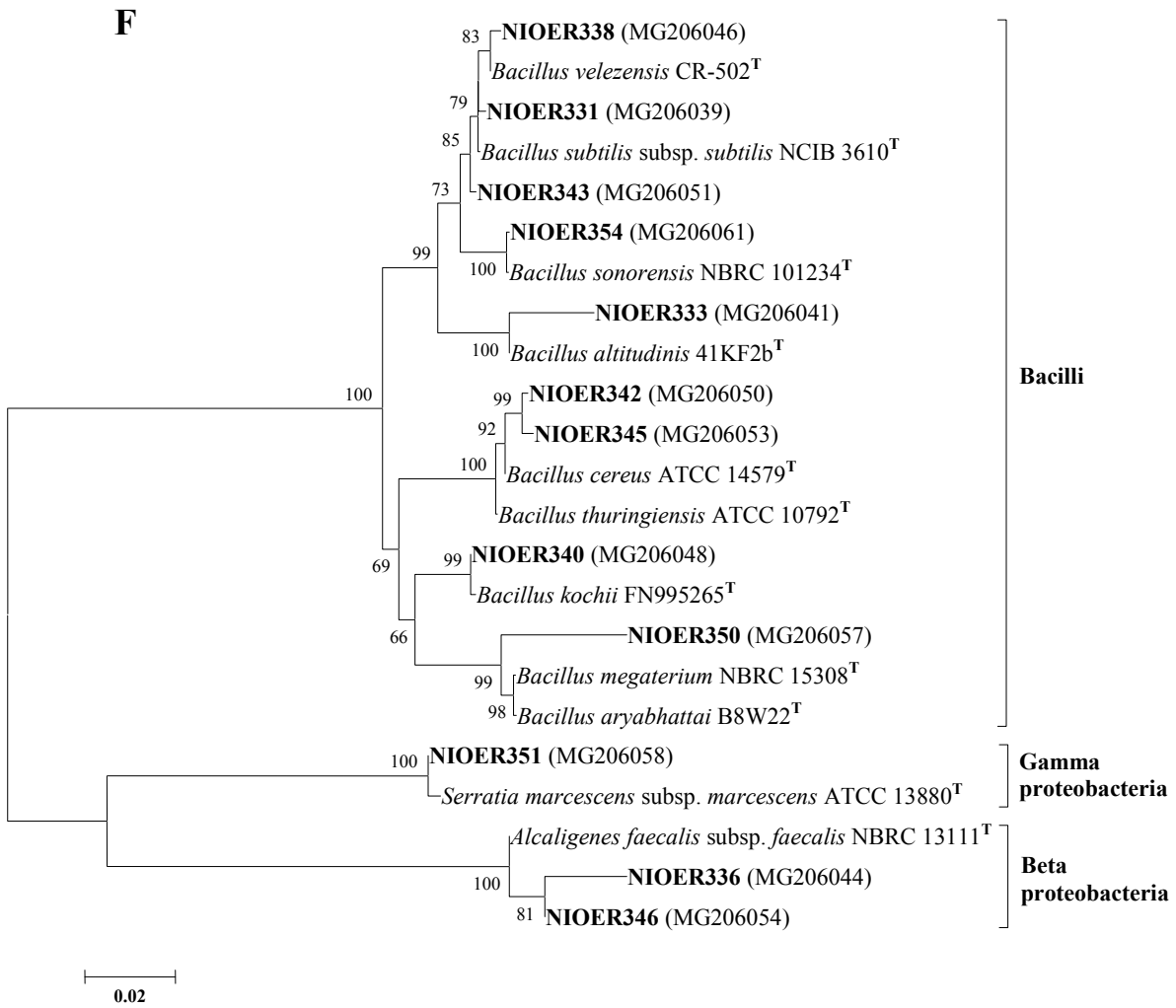


Figure 4.2 Phylogenetic trees showing the relationships of chromate tolerant cultures from (A) Dias beach waters (B) Divar mangrove waters (C) offshore waters (D) Dias beach sediments (E) Divar mangrove sediments and (F) Pallavaram tannery effluent. The trees were inferred from 16S rRNA sequence data by the neighbor-joining method. Molecular sequences for reference strains were obtained from EzTaxon database. Boldface type indicates isolate code of strains from this study, with the accession numbers given in brackets. The scale bar indicates fixed mutation per nucleotide position. Numbers refer to bootstrap values for each node out of a total 1000 replicate resamplings (values below 50 are not shown)

Table 4.1 16S rDNA sequence based identification of chromate tolerant bacteria bearing ≥ 97% similarity with NCBI accession numbers. CRB isolates included to represent the genus/species in the phylogenetic trees are underlined and in **bold**

S. No	Genus/group	No. of isolates	Accession No. (% similarity ≥97%)
1	*Alcaligenes faecalis*	2	**MG206044**[T], **MG206054**[T]
2	*Arthrobacter nicotianae*	1	**MG205862**[DBW]
3	*Arthrobacter protophormiae*	2	MG205856[DBW], **MG205942**[G5]
4	*Bacillus acidiceler*	2	**MG205938**[G5], MG205939[G5]
5	*Bacillus aerius*	1	MG205979[DMW]
6	*Bacillus aerophilus*	2	MG205967[DMW], MG205967[DBW]
7	*Bacillus altitudinis*	9	**MG206041**[T], MG206055[T], **MG205873**[G5], MG205965[DMW], MG205975[DMW], MG205993[DBW], **MG205995**[DBW], MG206014[DBW], MG205962[DMW]
8	*Bacillus baekryungensis*	1	MG205898[DMW]
9	*Bacillus cereus*	64	MG206045[T], MG206062[T], MG205823[DMsed], MG205824[DMsed], MG205850[DBW], **MG205887**[DMsed], MG205888[DMsed], **MG205902**[DBW], **MG205936**[G5], **MG205947**[DMW], MG205948[DMW], MG205950[DMW], MG205953[DMW], MG205959[DMW], MG205960[DMW], **MG205964**[DMW], MG205966[DMW], MG205970[DMW], MG205971[DMW], MG205973[DMW], MG205977[DMW], MG205980[DMW], MG205982[DMW], MG205983[DMW], MG205985[DMW], MG205987[DBW], MG205991[DBW], MG205994[DBW], MG205996[DBW], MG205998[DBW], MG205998[DBW], MG205999[DBW], MG206002[DBW], MG206003[DBW], MG206009[DBW], MG206010[DBW], MG206011[DBW], MG206013[DBW], MG206016[DBW], MG206020[DBW], MG206026[DBW], MG206026[DBW], **MG206028**[DBW], MG206030[DBW], MG206033[DBW], MG206034[DBW], MG206037[DBW], MG206038[DBW], **MG205944**[DMW], MG206040[T], MG206043[T], MG206047[T], **MG206050**[T], MG205800[DMsed], MG205802[DMsed], MG205803[DMsed], MG205806[DMsed], MG205808[DMsed], MG205822[DMsed], MG205880[DMsed], MG205903[DBW], MG205943[DMW], MG205945[DMW], MG205955[DMW], MG205963[DMW], MG206004[DBW], MG206060[T], **MG205787**[DBsed], MG205813[DMsed]
10	*Bacillus kochii*	2	MG206052[T], **MG206048**[T]
11	*Bacillus megaterium*	3	**MG205876**[G5], MG205798[DMsed], **MG206057**[T]
12	*Bacillus pseudomycoides*	11	MG205951[DMW], MG205952[DMW], MG205972[DMW], MG205974[DMW], **MG206000**[DBW], MG206001[DBW], MG206006[DBW], MG206012[DBW], MG206015[DBW], **MG205944**[DMW], MG206007[DBW]
13	*Bacillus pumilus*	7	**MG205981**[DMW], **MG206018**[DBW], MG206019[DBW], **MG206021**[DBW], MG205869[G5], MG205968[DMW], MG205990[DBW]
14	*Bacillus sonorensis*	1	**MG206061**[T]
15	*Bacillus subtilis*	9	MG206056[T], MG205929[G5], MG206042[T], **MG205863**[DBW], MG205868[G5], MG205875[G5], **MG205940**[G5], **MG206039**[T], MG206051[T]
16	*Bacillus thuringiensis*	15	MG205854[DBW], **MG205886**[DMsed], **MG205957**[DMW], MG206008[DBW], MG206017[DBW], MG206036[DBW], **MG206053**[T], MG205810[DMsed], MG205815[DMsed], MG205946[DMW], MG205954[DMW], MG205997[DBW], MG206005[DBW], MG206035[DBW], **MG205930**[G5]
17	*Bacillus velezensis*	1	**MG206046**[T]
18	*Bacillus weihenstephanensis*	1	**MG205958**[DMW]

19	*Bacillus sp.*	28	MG206022^{DBW}, MG205969^{DMW}, MG205976^{DMW}, MG205807^{DMsed}, MG205949^{DMW}, MG205992^{DBW}, MG205911^{DBsed}, MG206027^{DBW}, MG206023^{DBW}, MG206059^{T}, MG205916^{G5}, MG205796^{DBsed}, MG205881^{DMsed}, MG206049^{T}, MG205913^{DBsed}, MG205986^{DBW}, MG205895^{DMW}, MG205988^{DBW}, MG205812^{DMsed}, MG205877^{DMsed}, MG205878^{DMsed}, MG205884^{DMsed}, MG205896^{DMW}, MG205905^{DBW}, MG205923^{G5}, MG205984^{DMW}, MG206024^{DBW}, MG205879^{DMsed}
20	*Brevibacterium casei*	1	MG205904^{DBW}
21	*Brevibacterium iodinum*	1	MG205882^{DMsed}
22	*Brevibacterium linens*	2	**MG205883**^{DMsed}, **MG205866**^{DBW}
23	*Curtobacterium citreum*	1	**MG205855**^{DBW}
24	*Exiguobacterium aestuarii*	4	MG205794^{DBsed}, MG205825^{DMsed}, **MG205864**^{DBW}, MG205907^{DBW},
25	*Exiguobacterium profundum*	4	MG205811^{DMsed}, MG205821^{DMsed}, **MG205885**^{DMsed}, **MG205793**^{DBsed}
26	*Halomonas aquamarina*	2	MG205917^{G5}, **MG205918**^{G5}
27	*Halomonas axialensis*	2	MG205922^{G5}, MG205926^{G5}
28	*Halomonas meridian*	5	MG205941^{G5}, MG205941^{G5}, MG205920^{G5}, MG205927^{G5}, **MG205935**^{G5}
29	*Janibacter terrae*	1	**MG205908** ^{DBW}
30	*Kocuria kristinae*	5	MG205937^{G5}, MG205931^{G5}, MG205932^{G5}, MG205933^{G5}, **MG205934**^{G5}
31	*Microbacterium esteraromaticum*	1	**MG205909**^{DBW}
32	*Psychrobacter aestuarii*	3	**MG205867**^{DBW}, **MG205795**^{DBsed}, **MG205797**^{DMsed}
33	*Rothia marina*	1	**MG205857**^{DBW}
34	*Serratia marcescens*	1	**MG206058**^{T}
35	*Staphylococcus arlettae*	8	MG205912^{DBsed}, **MG205978**^{DMW}, **MG205789**^{DBsed}, MG205842^{DBsed}, **MG205843**^{DBsed}, MG205853^{DBW}, MG206025^{DBW}, **MG206032**^{DBW}
36	*Staphylococcus cohnii*	8	MG205836^{DMW}, **MG205924**^{G5}, **MG205858**^{DBW}, **MG205861**^{DBW}, MG205865^{DBW}, MG205906^{DBW}, **MG205910**^{DBsed}, MG205925^{G5}
37	*Staphylococcus saprophyticus*	27	**MG205921**^{G5}, MG205837^{DMW}, MG205897^{DMW}, MG205919^{G5}, MG205844^{DBsed}, MG205859^{DBW}, MG205928^{G5}, **MG205851**^{DBW}, MG205889^{DMW}, MG205890^{DMW}, MG205891^{DMW}, MG205893^{DMW}, MG205899^{DBW}, MG205900^{DBW}, MG205845^{DBW}, MG205847^{DBW}, MG205848^{DBW}, MG205849^{DBW}, MG205860^{DBW}, **MG205870**^{G5}, MG205892^{DMW}, **MG205894**^{DMW}, **MG205901**^{DBW}, **MG205914**^{DBsed}, MG205956^{DMW}, MG205871^{G5}, MG205872^{G5},
38	*Staphylococcus sciuri*	5	MG205852^{DBW}, MG205961^{DMW}, **MG205989**^{DBW}, MG206031^{DBW}, **MG205799**^{DMsed}
39	*Staphylococcus warneri*	1	**MG205915**^{G5}
40	*Staphylococcus xylosus*	3	MG206029^{DBW}, **MG205874**^{G5}, MG205846^{DBW}

41	*Vibrio diazotrophicus*	27	**MG205785**[DBsed], **MG205786**[DBsed], MG205790[DBsed], **MG205792**[DBsed], MG205804[DMsed], MG205805[DMsed], MG205809[DMsed], MG205816[DMsed], **MG205817**[DMsed], MG205818[DMsed], MG205819[DMsed], **MG205820**[DMsed], MG205826[DMW], MG205827[DMW], MG205828[DMW], MG205829[DMW], MG205830[DMW], **MG205831**[DMW], MG205832[DMW], MG205833[DMW], MG205834[DMW], MG205835[DMW], MG205838[DMW], MG205839[DMW], MG205840[DMW], MG205841[DMW], MG205814[DMsed]
42	*Vibrio hispanicus*	1	MG205801[DMsed]
43	*Vibrio tubiashii strain*	1	**MG205788**[DBsed]

Key: DBW, Dias Beach water; **DMW**, Divar Mangrove water; **G5**, water from offshore site; **DEBsed**, Dias Beach sediment; **DMsed**, Divar Mangrove sediment; **T**, Tannery effluent sample

4.4 Discussion

Since Cr^{6+} remediation by bacteria is a subject of intense investigation, isolation and identification of these bacteria has taken precedent (Turic and Apel, 1997; Valls and De Lorenzo, 2002; Gadd and White, 1993; Webb *et al.*, 1998; White and Gadd, 1998; Neis, 2000 Barkay and

Schaefer, 2001 and Ghosh *et al*., 2003). Results from this study show the presence of different bacterial genera in coastal waters off Goa despite Cr concentrations reported to be very low (Kerkar and Das, 2017). High concentration of Cr^{6+} contamination exerts positive selective pressure (Sheik *et al*., 2012). In its absence, a generally low diversity with respect to resistant isolates *in situ* is expected. However, the sampling locations in this study are exposed to discharges from mining areas (Nair *et al.,* 2003) inducing selection of certain species with metabolic capability to tolerate and grow in medium with high Cr^{6+}. It is known that increase in metal tolerance in a community is attributed to an immediate effect due to the death of sensitive species and the following competitive abilities and adaptation of surviving bacteria (Di´az-Ravin˜a and Bååth, 1996). Keeping in view the lack of Cr^{6+} stress, but the presence of Cr^{6+} resistant bacteria, it can therefore be proposed that resistance mechanism in autochthonous bacteria is independent of Cr levels.

In this study, phylogenetic exploration of CRB was investigated by culture-dependant approach. The 16S rRNA gene sequencing revealed a significant bacterial diversity. The sequenced 278 bacterial strains divided into four major classes; Actinobacteria, Beta and Gammaproteobacteria, as well as Bacilli. The dominant group was Bacilli having ~79% bacterial isolates, 12% were of Gammaproteobacteria, only ~8% isolates belonged to Actinobacteria, and less than 1% were Betaproteobacteria. Bacilli were dominant consistently across locations whereas the minor groups varied.

Chromate resistant bacilli in <u>water and sediment</u>

There are multiple reports exploring diversity of Cr^{6+} resistant/reducing bacteria finding the prevalence of Bacilli (Jain *et al.*, 2014; Camargo *et al.*, 2005). Bacilli were highly represented in waters and sediments collected in this study, forming 90, 88, 80, 70, 68 and 62% of the total isolates respectively from: water from Dias Beach, effluent from Pallavaram, water from Divar

Mangrove, sediment from Dias, sediment from Divar and water sampled from offshore site G5. The percent of *Bacillus* sp. constituent of Bacilli, varied from 35 (sediment from Dias) up to 100% (effluent from tannery) with an average of 69%, of the total bacilli class. These findings are similar to results of previous works that report of the dominance of *Bacillus* sp. in contaminated environments (Ross *et al.*, 1981; Konopka and Zakarova, 1999; Kamala-Kannan *et al*., 2007; Rehman *et al*., 2008 and Verma *et al*., 2009). These observations suggest that in contaminated environs, the presence of Cr^{6+} has little to no effect on viability of *Bacillus* species. The probable presence of metal ion transporters, and/or their absence in other species, their cell physiology, ability to form endospores (Rowan *et al.*, 2001) and/or processes unique to their survival and environmental adaptation may be why. This may explain their predisposition and abundance in the group of Cr^{6+} resistant isolates in this study.

Assemblage of other chromate resistant bacteria in water and sediment

The percent of other groups ranged from 10-38% with isolates from Actinobacteria and sub-groups of Proteobacteria. Sheik *et al.* (2012) and Branco *et al.* (2005) have both reported the presence of Proteobacteria, Actinobacteria and Acidobacteria(in addition to Firmicutes) in Cr^{6+} contaminated soils/sediments. There are also multiple reports of *Gammaproteobcateria* isolated from surface waters and sediments (Cho and Giovannoni, 2004; Nikrad *et al*. 2014; Feng *et al*. 2009). In this study, *Gammaproteobacteria* were observed at all sites although their levels varied. Represented by *Vibrio*, *Psychrobacter*, and *Halomonas* sp., these genera were specific to a location. *Halomonas* sp. isolated only from offshore site is commonly observed in saline conditions (Vreeland *et* al., 1980; Mata *et al*., 2002). *Vibrio* and *Psychrobacter* isolated from the other marine sites are generally associated with urban contamination as Dumontet *et al*. (2000) have reported in fecal contaminated seawater. The presence of such coliform/bacterial indicators suggests that waters from these two locations are contaminated. This validates physcio-chemical

parameters reported in this study that describe these waters to be of poor quality. Duran *et al*., (2015), Bull and Stach, (2007) Subramani and Aalbersberg, (2012) and Ward and Bora, (2006) report of marine *Actinobacteria*. *Actinobacteria* were isolated from Dias beach (W), Divar mangroves (S) and largely in offshore site G5. This suggests that they may be present/prevalent in all the marine environments sampled even though representatives were not observed at all sites (Divar Water, Dias Sediment)

Assemblage of other chromate resistant bacteria in Tannery effluent from Pallavaram

Krishnan *et al*. (2016) studied physcio-chemical parametric and microbial shifts in different treatment stages in CETP (Pallavaram). They reported, with decrease in pollution concentration at each stage, a shift in community structure. Primarily, Actinobacteria, Proteobacteria, Bacteroidetes and Firmicutes were in high abundance in all stages, acting as indicator populations. In raw effluent, Cyanobacteria as well as Spirochaetes were observed in low levels. In this study, bacilli were again dominant in effluent samples with *Serratia* sp. (Gammaproteobacteria) unique to this sampling. In addition, Betaproteobacteria were also uniquely observed. Other studies (Pruesse *et al*., 2007; Xia *et al*., 2010; Snaidr *et al*., 1997) have reported the dominance of Proteobacteria in tannery sludge. It is likely that Cr^{6+} resistance selection favored under-representation of Proteobacteria.

These findings signify the shift of the community structure towards Cr^{6+} resistant bacteria which become prevalent in comparison to other groups. Reports of marine bacterial diversity summarize many representatives of *Firmicutes*, *Actinobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* (Bull and Stach, 2007; Gilbert *et al*., 2010) *Acidobacteria* and *Bacteroidetes* (Roane and Kellogg, 1996; Nakatsu*et al*., 2005) by culturing. However, in this study, some of these groups could not be brought into culture. This could be

due to a lack of diversity as reported in some Cr^{6+} contaminated sites (Nemergut *et al*., 2004; Brofft *et al.*, 2002 and Roling *et al.*, 2002) or, a reduction in diversity due to use of metals ions (Cr^{6+} in this study) as part of selective media to obtain resistant strains. Examples include (i) Nagvenkar and Ramaiah (2010) who used arsenic amended seawater NA to isolate marine arsenic resistant bacteria; and reported (Nagvenkar, 2014) isolates distributed across *Actinobacteria*, *Proteobacteria* and *Firmicutes*; (ii) De and Ramaiah (2008) who isolated mercury resistant bacteria from water and sediment samples using SWNA amended with mercury and reported (De, 2004) isolates from *Actinobacteria*, *Firmicutes* and *Proteobacteria*; and (iii) Naik (2012) who isolated lead resistant *Proteobacteria* from surface waters (using minimal media).

To summarize, as many as 15 different genera were observed in Cr^{6+} resistant isolates that were readily cultivable. Although they cannot be construed to reflect the functional and phylogenetic diversity present, they indicated the dominant classes of bacteria that were present and Cr^{6+} resistant. The main advantage of culturing was therefore to establish pure cultures of representatives, a prerequisite of marine biodiscovery, so that they may be available for experimentation (Joint *et al*., 2010). Results from this study imply that these new reports of CRB could be useful in providing insight on distribution of CRB in different environmental settings and that these naturally occurring CRB can be potential candidates for Cr^{6+} detoxification.

Chapter 5

Physiological and biochemical characteristics of chromate transforming bacteria

5.1 Introduction

Wide spread heavy metal pollution has led to an increase in appearance of metal resistant/tolerant microbes. To date, many chromate resistant bacteria (CRB) have been evaluated from different ecological settings (Ramírez-Díaz *et al.* 2008). Some of these Cr^{6+} resistant bacteria are reported to reduce/remove Cr^{6+} (Chirwa and Molokwane, 2011) which is of great ecological significance. Most, are isolated from environments that are contaminated with Cr^{6+} (Ahemad, 2014), but there are a few reports of Cr^{6+} reducing bacteria from uncontaminated environments. The abundance and preponderance of CRB populations provided in chapter 3 indicate that many bacteria possess the potential for Cr^{6+} resistance and detoxification. Observations also imply that a large percent of abundance capable of high Cr^{6+} tolerance may be experiencing physiological or genetic modification to withstand and adapt to dynamic ecological settings.

In the marine biosphere, the appearance of heavy metal resistant phylotypes (Nagevenkar and Ramaiah, 2010; De and Ramaiah, 2008; Subramanian *et al*., 2012; Cheung *et al*., 2006) is accompanied by adaptations to counter many other toxicants. These adaptations may be genetic and/or morphological to promote positive selection. The potential of marine bacteria is severely limited by a lack of understanding of these adaptations. In addition, establishment of laboratory culture may destroy cell-to-cell communication in natural marine environment and/or single out strains from bacterial consortia (reliance on other bacteria for nutrients and substrates). It is thus worth considering the principles that allow for Cr^{6+} resistance and detoxification. Description of select CRB was carried out to evaluate some of their biochemical properties and

antibiotic sensitivity. Molecular identities of these five isolates as well as the all the other isolates was established through 16S rDNA sequencing.

5.2 Methods

5.2.1 Isolation of tolerant bacteria and screening for chromate resistant bacteria

Chromate tolerant bacteria from water and sediment samples were isolated across different sampling locations during pre-monsoon, monsoon and post-monsoon seasons. All samples were spread on NA plates containing up to 100µg ml^{-1} Cr^{6+}. As many as 278 cultures of bacteria were isolated from plates with 100µg ml^{-1} Cr^{6+}. Each culture was spot inoculated and tested for their potential to grow in media amended with Cr^{6+} ranging from 200 to 600µg mL^{-1} Cr^{6+}. Twenty five isolates (chromium resistant bacteria) were able to grow well at 600µg mL^{-1} Cr^{6+} and were tested for their ability to detoxify Cr^{6+} using DPC method as described in chapter 6. Five isolates that were able to reduce Cr^{6+} were further characterized.

5.2.2 Morphological and biochemical characterization

16S rRNA sequence analysis of chromate resistant bacteria (CRB) was performed and the results are discussed in chapter 4. The CRB (maintained on NA with 50µg ml^{-1} Cr^{6+}) were also tested for their biochemical characteristics according to Holt *et al.* (2000). Gram staining (**Appendix B.1**) was done on 24h old cultures and observed microscopically. Shape was observed microscopically while evaluating Gram character. Production or elaboration of various enzymes (**Table 5.1**) by these isolates was carried out according to Holt *et al.* (2000). Production of methyl-red, Voges-Proskauer reaction

and hydrogen sulphide, reduction of nitrate and oxidation-fermentation reactions were tested following Holt *et al.* (2000) (**Appendix C**).

5.2.3 Minimum inhibitory concentration of chromate

Minimum inhibitory concentration (**MIC**) at which no growth (turbidity) occurred was determined by broth dilution method (Pei *et al*., 2009). Test-tubes containing NB supplemented with different concentrations of $K_2Cr_2O_7$ were inoculated (0.1% (v/v) of primary inoculum ~1 x 10^8 cells mL^{-1}) aseptically with a culture of CRB in early-exponential growth phase. The tubes, in triplicate were incubated at 28°C, at 200rpm for 24-48h. The minimum concentration of Cr^{6+} at which no turbidity (negative growth) was observed indicated MIC. Medium with only culture served as control and medium with only Cr^{6+} as blank.

5.2.4 Effect of temperature, pH and chromate on growth

Optimum growth conditions of CRB cultures were checked under different conditions of temperature, pH and Cr^{6+} concentrations for the best growing parameters

5.2.4.1 *Temperature*

To determine optimum temperature, triplicate sets of volumetric flasks (250mL capacity) containing 50ml NB, with or without 100µg mL^{-1} Cr^{6+} (set at pH 6), were inoculated with fresh inoculums and incubated at different temperatures. Each set was incubated at 20, 24, 28, 32, 37 or 42 °C, in a temperature controlled orbital shaker, set at 120rpm.

Growth was monitored by measuring optical density (OD_{600}) every 6h (Faisal *et al.*, 2004).

5.2.4.2 *pH*

Growth at different pH ranging from 4, 5, 6, 7, 8, 9 and10 was tested to determine optimum pH in NB with or without 100µg mL^{-1} Cr^{6+}. The pH of each set (250mL volumetric flasks containing 50mL NB, in triplicate) was adjusted as predetermined using conc. HNO_3 or 1N NaOH before inoculation, and grown at 28°C at 120 rpm. Optical density was measured every 6h.

5.2.4.3 *Growth kinetics with and without chromate*

All five CRB isolates were examined for their growth in NB as well as in NB amended with 100µg mL^{-1} Cr^{6+} (pH 6). Both sets were grown in 500mL volumetric flasks on a shaker set at 120rpm at 28°C. Aliquots of culture (1mL) were taken at hourly intervals and optical densities (OD_{600}) measured with non-inoculated control used as blank.

In all the parametric tests, 0.1% (v/v) of primary inocula grown overnight was used as inoculum.

5.2.5 Antibiotic susceptibility test

Twenty –four-hour old broth cultures of CRB were spread on NA plates to attain lawn cultures. Antibiotic discs were placed and incubated at room temperature for 24h. Inhibition zones (Bauer *et al*., 1966) around the discs were measured and sensitivity of the isolates to a particular antibiotic was evaluated as per chart provided by the manufacturer ((Himedia, India, CLSI, 2015).

5.2.6 Resistance to other heavy metals

The resistance of CRB to other toxic heavy metals was evaluated by broth dilution method in NB amended with appropriate stock solutions of metals salts (mercury chloride, cadmium chloride, copper sulphate, zinc sulphate, arsenic trioxide, nickel chloride and chromium trichloride) (**Appendix F**).

5.2.7 Morphological characterization of cultures grown in media with chromate

Samples (5ml) of broth cultures from test flasks with Cr^{6+} were centrifuged at (~1000 x g) for 5min. The cells were washed twice with 0.1M physiological buffered saline (PBS, **Appendix B**), pre-fixed in 2.5% glutaraldehyde with 75mM phosphate buffer (PB) (**Appendix B**), pH 7 for 2h, and fixed overnight in 3% glutaraldehyde (prepared in 0.1M PBS) at 4°C. The cells were washed with PBS and distilled water before dehydration in a graded ethanol series in increasing order, i.e. 10, 30, 50, 80, 90 and absolute for 20min each and air-dried. Samples were placed on brass stubs, gold sputter coated and examined by scanning electron microscopy (JEOL JSM- 5800LV, USA) to study morphological changes.

5.3 Results

5.3.1 Bacterial isolates

Five CRB, NIOER168 (*Staphylococcus saprophyticus*168, NIOER176 (*Staphylococcus saprophyticus*176), NIOER208 (*Bacillus thuringiensis*208), NIOER273 (*Bacillus cereus*273) and NIOER324 (*Staphylococcus arlettae*324) were examined for their physiology.

5.3.2 Morphological and biochemical characterization

The five CRB strains were identified after 16S rDNA sequencing by an array of biochemical and morphological tests. All five were Gram-positive. Both *Bacillus thuringiensis*208 and *Bacillus cereus*273 were observed to be short rods (<2µm), whereas the remaining *Staphylococcus saprophyticus*168, *Staphylococcus saprophyticus*208 and *Staphylococcus arlettae*324 were cocci in shape. All five were negative for coliform group specific tests (M, VP and IT). Both *Bacillus thuringiensis*208 and *Bacillus cereus*273 were positive for citrate utilization. Nitrate was only reduced by *Staphylococcus saprophyticus*168 and the presence of various enzymes can be discerned from the responses listed in **Table 5.1**. All five produced catalase, while only some produced gelatinase, amylase and casease. SEM analysis of *Bacillus cereus*273 showed alteration of its cell morphology when grown in Cr^{6+} (**Fig. 5.1**). Without Cr^{6+} stress, the shape of the cells was short and rod like. In the presence of 100µg mL^{-1} Cr^{6+}, cells became filamentous and slender in nature appearing as long connected chains.

5.3.3 Effect of temperature, pH and chromate on growth

Growth responses of the five CRB were tested at different temperatures and pH (**Fig. 5.2** & **5.3**). Cultures of all five isolates grew well at most temperatures and pHs showing optimal growth between a range of 28-37°C and pH 7-9. In the presence of chromate, maximal growth was observed at pH 9.

From growth kinetics based on optical densities (OD_{600}), it was evident that the lag phase was prolonged and generation times were longer in presence of Cr^{6+}. In Cr^{6+}-free media, rapid growth (log phase) was observed between 6-8h with growth maxima

(stationary phase) within 24h. All isolates had reduced growth maximas when grown in chromate with two isolates, *Bacillus thuringiensis*208 and *Bacillus cereus*273 displaying diauxic growth under Cr^{6+} metal stress.

5.3.4 Minimum inhibitory concentration of chromate and resistance to other metals

The minimum inhibitory concentration of all five isolates to Cr^{6+} ranged from 250-350µg mL^{-1} (**Table 5.2**). The tolerance limits to other metals was also tested (**Table 5.2**). It was found that metals such as Hg, Cd and Zn were more toxic comparatively and that the isolates were less sensitive to the effects of Cu and Ni. The general order of resistance to metals for all isolates was As>Cr>Ni>Cu>Zn>Cd>Hg in media. As was the least toxic of all the metal ions tested. The trivalent form of chromium was much less toxic than the Hexavalent form.

5.3.5 Antibiotic susceptibility test

In addition to the five Cr^{6+} removing isolates, representative isolates from the set of 25 CRB were tested for sensitivity to antibiotics (**Table 5.3**). Multiple broad and narrow-spectrum antibiotics were used from all classes and a variety of responses for each were recorded. All isolates were sensitive to amikacin, chloramphenicol, ciprofloxacin , clindamycin, gentamycin, levofloxacin, netillin (netilmicin sulphate), ofloxacin, tetracycline and tobramycin. All isolates were resistant to ampicillin, amoxyclav, aztreonam, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, oxacillin and piperacillin.

Table 5.1 Morphological and biochemical characteristics of a select set of bacterial isolates capable of chromate detoxification

Characteristics	**A**	**B**	**C**	**D**	**E**
colony shape	Round	Round	Round	Round	Round
colony colour	cream	cream	cream-white	cream	cream
shape	cocci	cocci	rod	rod	cocci
GS	+	+	+	+	+
CT	+	+	+	+	+
NRT	+	-	-	-	-
CUT	-	-	+	+	-
GHT	-	-	-	+	+
SHT	-	-	+	+	-
CHT	-	-	-	+	-
IT	-	-	-	-	-
MR	-	-	-	-	-
VP	-	-	-	-	-
TSI	AB&S	AB&S	AB	AB	AB

Key: A *S. saprophyticus*168; **B** *S. saprophyticus*176; **C** *B. thuringiensis*208; **D** *B. cereus*273; **E** *S. arlettae*324. **GS**, Gram Staining; **CT** Catalase; **NRT** Nitrate Reduction; **CUT** Citrate Utilisation; **GHT** Gelatin Hydrolysis; **SHT** Starch Hydrolysis; **CHT** Casein Hydrolysis; **IT** Indole; **MR** Methyl Red; **VP** Voges Proskauer; **TSI** Triple Sugar Iron; **C** cocci; **SR** straight rod; **+** positive; **-** negative; **AB** acidic butt (glucose fermenter); **AB & S** acidic butt and slant (lactose and sucrose fermenter)

Table 5.2 Resistance profiles of a select set of bacterial isolates capable of chromate detoxification to different heavy metals

	Toxic metals ($\mu g\ mL^{-1}$)[a]							
	Cr^{6+}	Hg	Cd	Cu	Zn	As	Ni	Cr^{3+}
*S. saprophyticus*168	300	20	40	140	60	800	300	850
*S. saprophyticus*176	300	15	30	120	40	750	300	900
*B. thuringiensis*208	250	10	30	130	50	850	300	900
*B. cereus*273	350	35	45	160	70	1100	300	950
*S. arlettae*324	300	20	40	140	50	500	300	850

[a] Salts of different metals dissolved to attain above mentioned concentrations of toxic metals. Data shown here represents the tolerance of isolates to different toxic metals

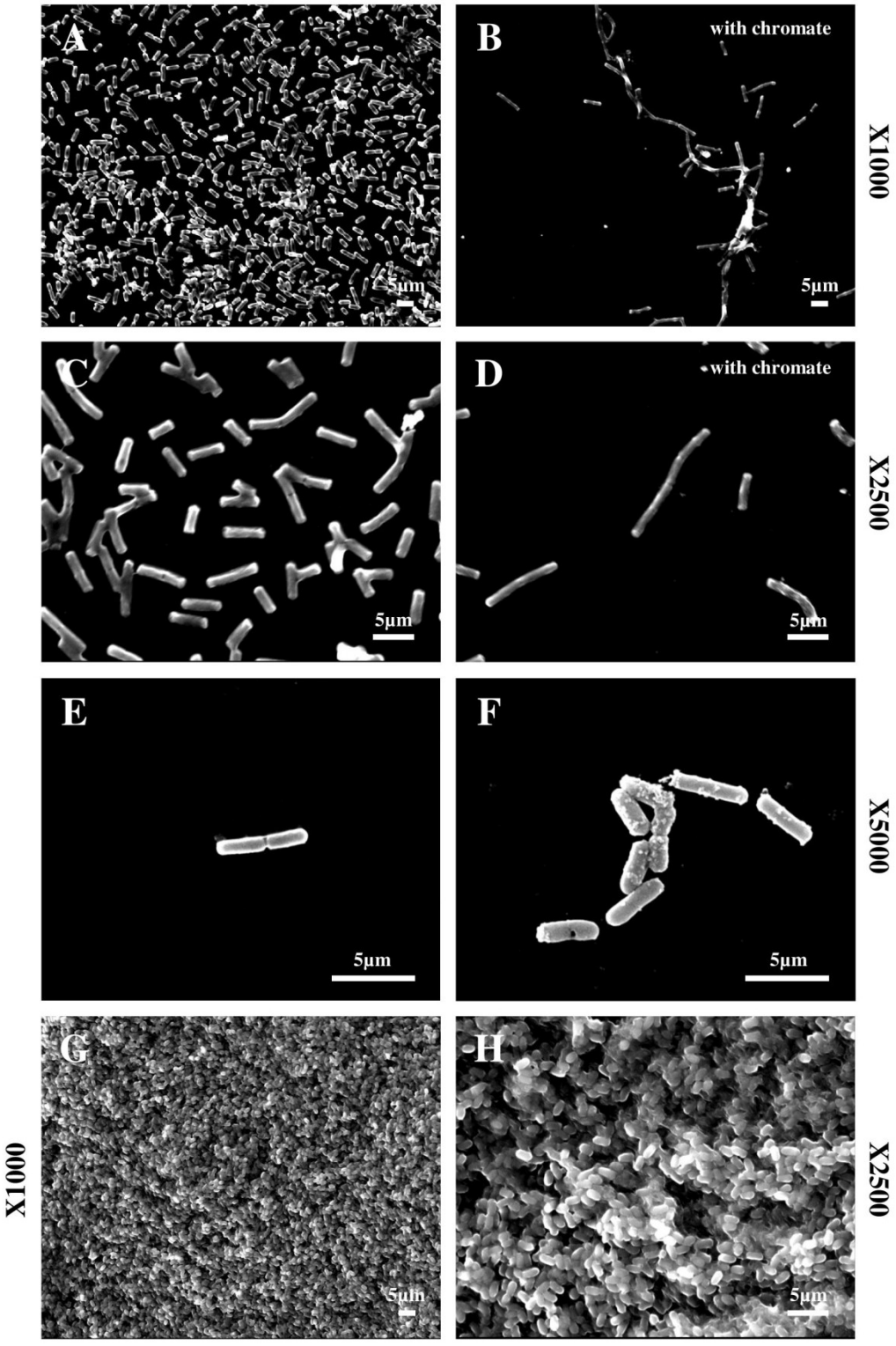


Figure 5.1 SEM-photomicrograph of chromate resistant isolate *B. cereus*273 grown with (100µg mL^{-1} Cr^{6+}) and without Cr^{6+}. (A, C, E, F and G) control cells (without Cr^{6+}) and (B and D) cells grown with 100µg mL^{-1} Cr^{6+} are shown at different magnifications; scale bar (white) in each photograph represents 5µm

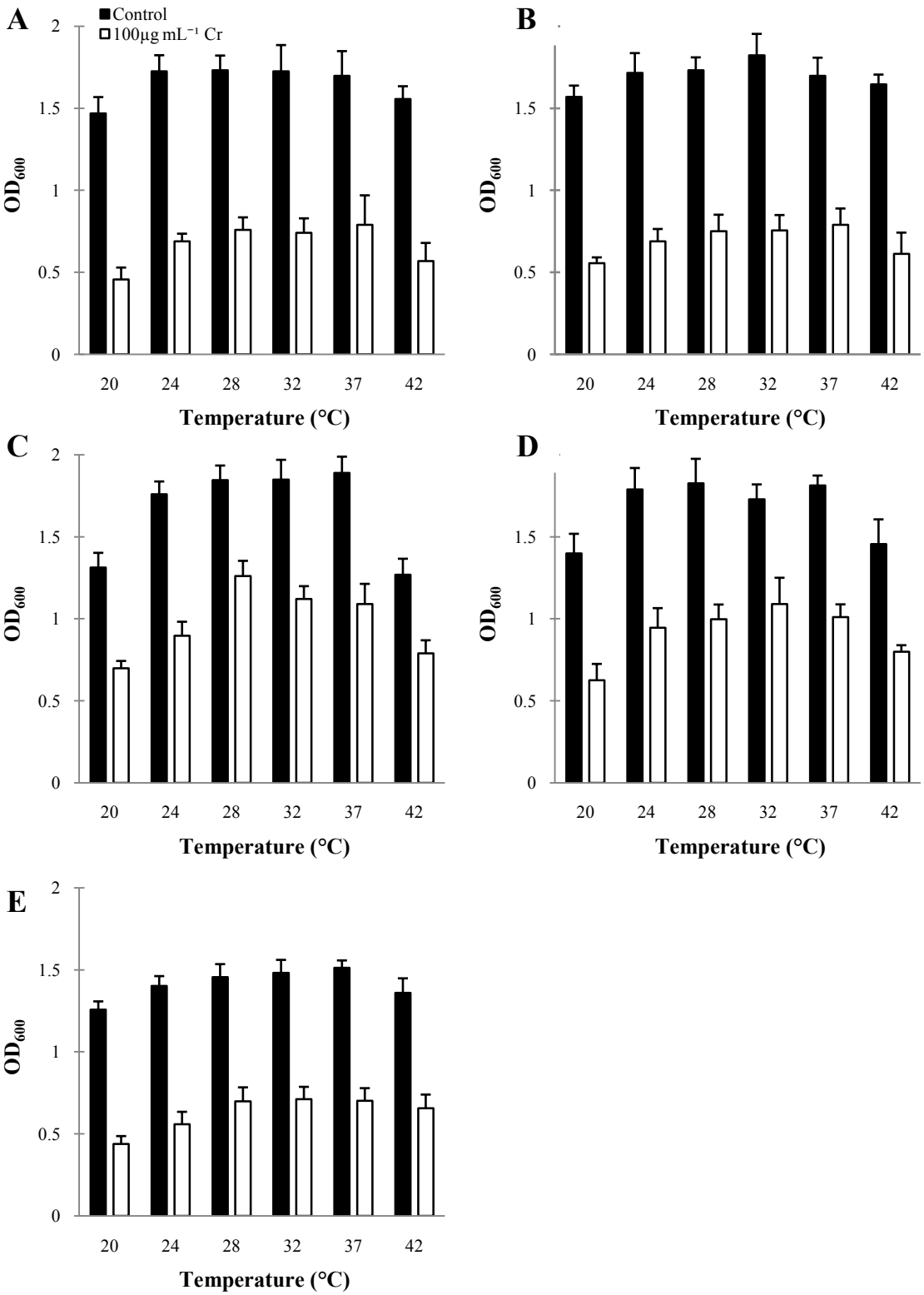


Figure 5.2 Growth (OD_{600}) responses of bacterial strains (A) *S. saprophyticus*168, (B) *S. saprophyticus*176, (C) *B. thuringiensis*208, (D) *B. cereus*273 and (E) *S. arlettae*324 in chromate supplemented (□; 100µg mL^{-1}) and chromate free (■) nutrient broth at different temperatures, *n=3*

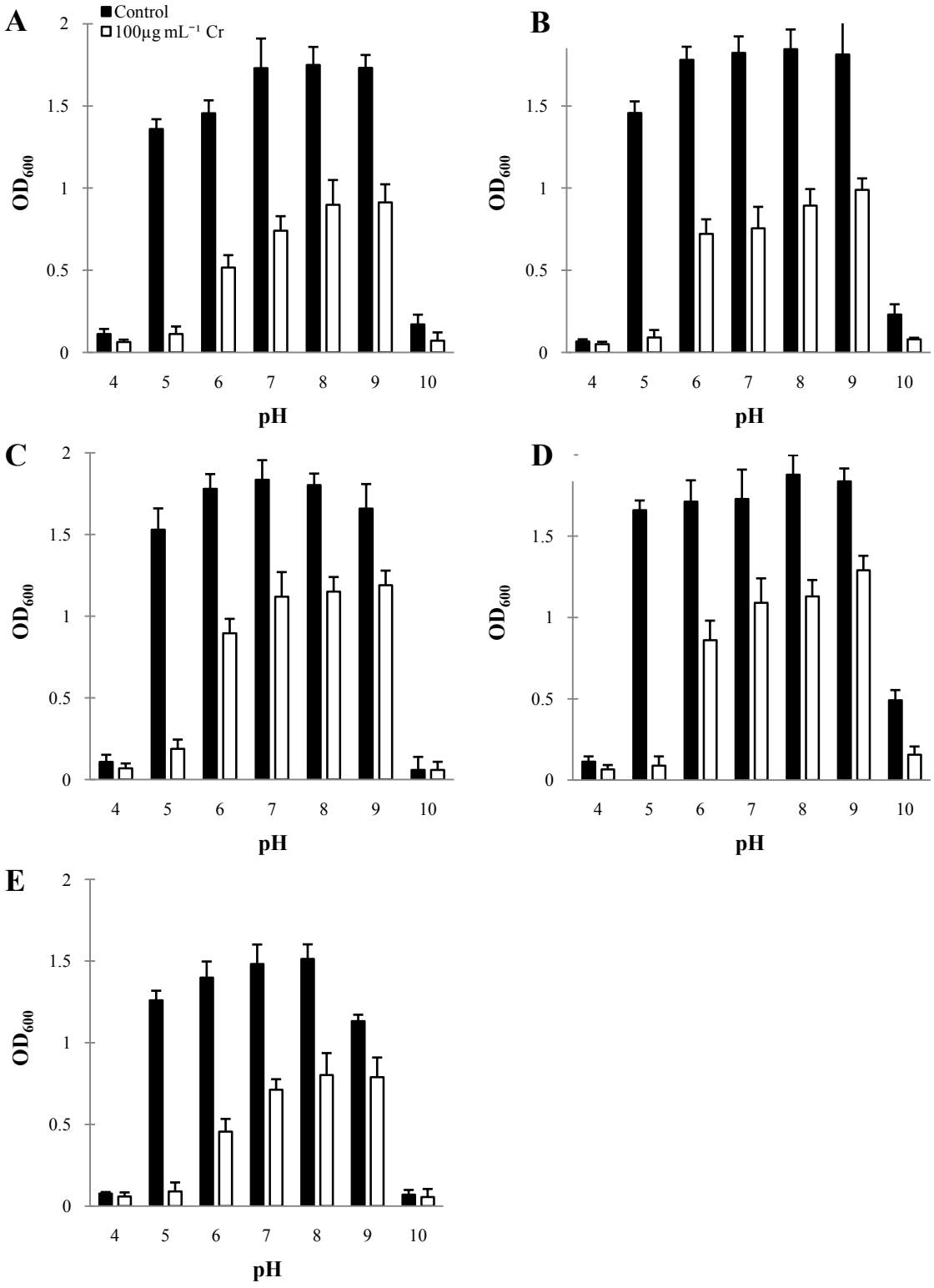


Figure 5.3 Growth (OD_{600}) responses of bacterial strains (A) *S. saprophyticus*168, (B) *S. saprophyticus*176, (C) *B. thuringiensis*208, (D) *B. cereus*273 and (E) *S. arlettae*324 in chromate supplemented (□; 100µg mL^{-1}) and chromate free (■) nutrient broth at different pH, *n=3*

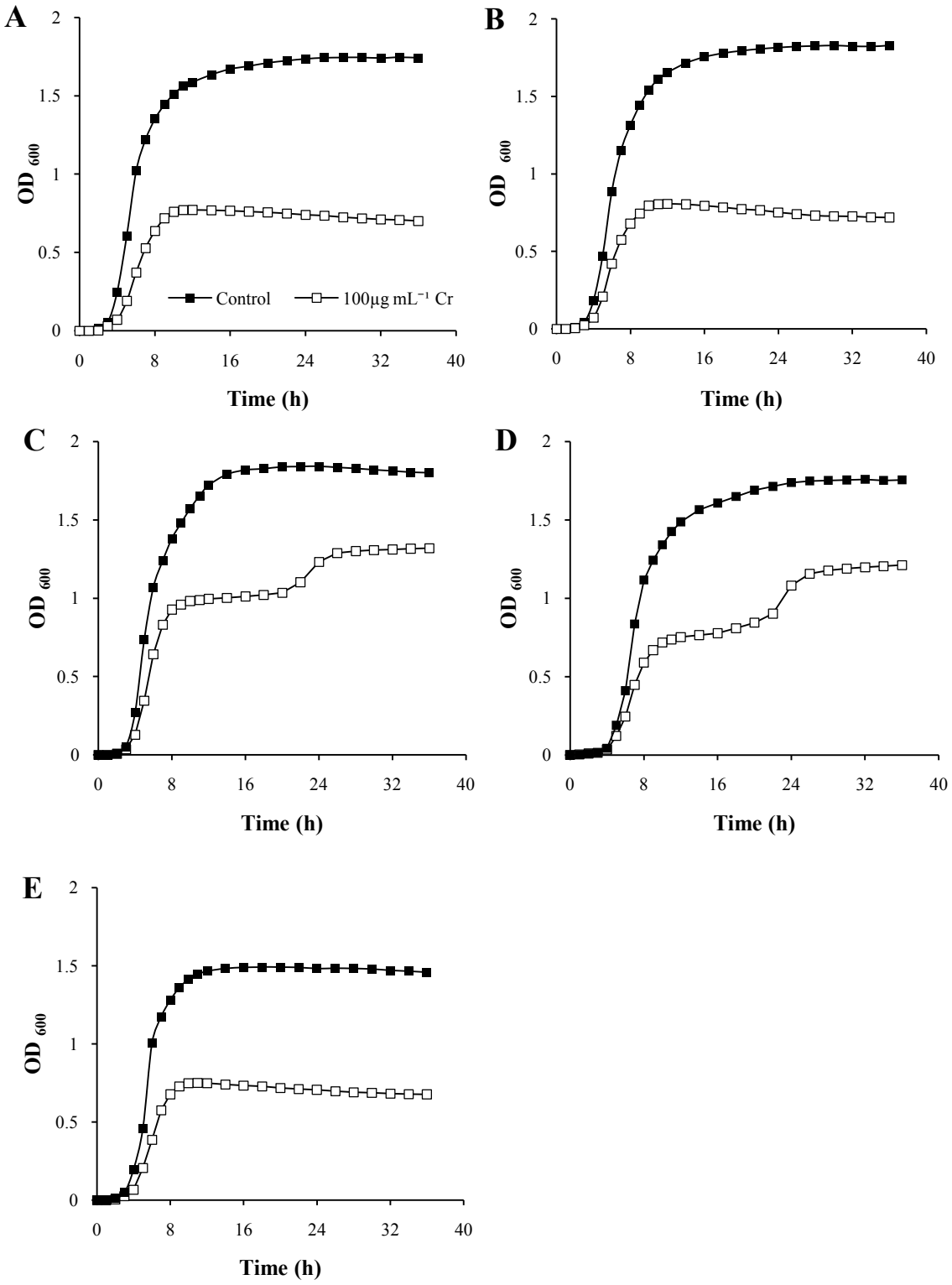


Figure 5.4 Growth curves (OD_{600}) of bacterial strains (A) *S. saprophyticus*168, (B) *S. saprophyticus*176, (C) *B. thuringiensis*208, (D) *B. cereus*273 and (E) *S. arlettae*324 in chromate supplemented (□; 100µg mL^{-1}) and chromate free (■) nutrient broth

Table 5.3 Antibiotic susceptibility of chromate resistant bacteria (CRB) (**bold** and underlined, Cr^{6+} detoxifying bacteria)

Antibiotic used	**Tested conc.** (µg/disc)	**Sensitivity limit** (mm)	Isolates of chromate resistant bacteria (CRB), *zone of inhibition* (mm)									
			A	**B**	**C**	D	E	F	**G**	**H**	I	**J**
AK	30	17-14	S (28)	S (29)	S (30)	S (25)	S (24)	S (27)	S (29)	S (27)	S (22)	S (29)
AMP	10	29-28	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)
AMC	30	20-19	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)
A/S	10/10	15-11	I (12)	R (0)	R (0)	R (0)	I (13)	S (22)	R (0)	R (0)	S (22)	R (0)
AZM	15	18-13	S (19)	S (24)	S (20)	S (20)	R (12)	R (0)	S (22)	S (21)	I (14)	S (19)
AT	30	21-17	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)
CTX	30	23-14	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)
CX	30	22-21	R (0)	R (0)	R (0)	R (0)	R (12)	R (11)	R (9)	R (8)	S (25)	R (0)
CAZ	30	18-14	R (5)	R (0)	R (0)	R (4)	R (6)	R (5)	R (5)	R (5)	I(15)	R (4)
CTR	30	21-13	I (15)	I (16)	R (0)	I (16)	I (18)	I (14)	R (9)	I (17)	S (21)	R (0)
CXM	30	18-14	R (13)	R (12)	R (11)	R (9)	R (9)	R (9)	R (10)	R (12)	R (9)	R (12)
CEP	30	18-14	S (23)	R (7)	R (7)	S (18)	S (25)	S (18)	R (5)	R(5)	S (34)	R (9)

C	30	18-12	S (25)	S (23)	S (27)	S (25)	S (23)	S (20)	S (23)	S (21)	S (30)	S (20)
CIP	5	21-15	S (27)	S (29)	S (30)	S (36)	R (13)	S (40)	S (30)	S (29)	S (35)	S (29)
CD	2	21-14	S (31)	S (35)	S (35	S (32)	S (30)	S (36)	S (42)	S (40)	S (30)	S (26)
COT	25	16-10	S (20)	R (7)	S(20)	S (29)	S (26)	S (31)	S (22)	S (20)	S (28)	S (23)
DO	30	16-12	S (32)	I (14)	R (11)	S (27)	S (24)	S (40)	S (25)	S (22)	S (33)	I (14)
E	15	23-13	R (9)	S (34)	S (37)	R (7)	S (23)	R(10)	S (31)	I (18)	S (32)	S (34)
GAT	5	23-19	I (22)	S (29)	S (25)	S (36)	S (36)	S 25)	S(23)	S (24)	S (30)	S (28)
GEN	10	15-12	S (20)	S (24)	S (21)	S (23)	S (16)	S (26)	S(27)	S (26)	S (24)	S(24)
LE	5	19-15	S (32)	S (30)	S (29)	S (27)	S (30)	S (28)	S (29)	S (30)	S (39)	S 32)
LZ	30	21-20	S (24)	S (26)	S (28)	S (25)	R (19)	S (30)	S (29)	S (30)	S (31)	S (30)
NA	30	19-13	S (25)	S (23)	S (28)	S (24)	S (22)	S (33)	S (32)	S (22)	S (24)	S (20)
NET	30	15-12	S (21)	S (25)	S (30)	S (31)	S (27)	S (25)	S (26)	S (25)	S (21)	S (20)
NIT	300	17-14	R	R	I	S(1	R	R	R	R	R(1	R(1

			(13)	(14)	(16)	8)	(10)	(9)	(11)	(10)	4)	4)
OF	5	18-14	S (27)	S (30)	S (39)	S (27)	S (23)	S (44)	S (35)	S (30)	S (34)	S (31)
OX	1	20	R (5)	R (7)	R (5)	R (7)	R (7)	R (5)	R (7)	R (5)	R (5)	R (5)
P	10U	29-28	R (18)	R (8)	R (6)	R(22)	R (17)	S (30)	R (9)	R (11)	R (27)	R (12)
PI	100	21-17	R (10)	R (12)	R (11)	R (13)	R (12)	S (25)	R (9)	R (15)	R (14)	R (9)
SPX	5	19-15	S (27)	S (24)	I (18)	S (22)	S (20)	S (25)	S (21)	S (21)	S (30)	S (20)
TEI	30	14-10	S (15)	S 16)	S (18)	S (15)	I (13)	S (21)	S (22)	S (17)	S(23)	S (20)
TE	30	19-14	S (25)	S (21)	S (26)	S (27)	S (27)	S (32)	S (25)	S (22)	S (40)	S (28)
TOB	10	15-12	S (21)	S (25)	S (17)	S (18)	S (21)	S (18)	S (22)	S (24)	S (21)	S (19)
VA	30	17-14	R (8)	R (8)	I (16)	R (10)	R (6)	R (8)	I (16)	R (5)	S (18)	R (7)

R resistant, **S** sensitive, **I** intermediate, 0mm- no zone of inhibition

A *S. xylosus*165; **B** *S. saprophyticus*168; **C** *S. saprophyticus*176; **D** *Exiguobacterium aestuarii*182; **E** *S.arlettae*188; **F** S. *cohnii*202; **G** *B. thuringiensis*208; **H** *B. cereus*273; **I** *B. pseudomycoides* 303; **J** *S. arlettae*324.

Zone size interpretative criteria as per CLSI standard (CLSI, Jan, 2013)

AK Amikacin; **AMP** ampicillin; **AMC** amoxyclav; **A/S** ampicillin/sulbactam; **AZM** azithromycin; **AT** aztreonam; **CTX** cefotaxime; **CX** cefoxitin; **CAZ** ceftazidime; **CTR** ceftriaxone; **CXM** cefuroxime; **CEP** cephalothin; **C** chloramphenicol; **CIP** ciprofloxacin; **CD** clindamycin; **COT** co-trimoxazole; **DO** doxycycline hydrochloride; **E** erythromycin; **GAT** gatifloxacin; **GEN** gentamycin; **LE** levofloxacin; **LZ** linezolid; **NA** nalidixic acid; **NET** netillin; **NIT** nitrofurantoin; **OF** ofloxacin; **OX** oxacillin; **P** penicillin G; **PI** piperacillin; **SPX** sparfloxacin; **TEI** teicoplanin; **TE** tetracycline; **TOB** tobramycin and **VA** vancomycin

Table 5.4 Well characterized examples of shared structural and functional characteristics of bacterial metal and antibiotic resistance systems. Modified from Baker-Austin *et al*. (2006)

Resistance mechanism	**Metal Ions**	**Antibiotics**	**Reference**
Reduction in permeability	As; Cu Zn, Mn, Co, Ag	Cip, Tet, Chlor, ß-lactams	Silver, 1996
Drug/ metal alteration	As, Hg	ß-lactams, Chlor	Mukhopadhyay, 2002
Drug/metal efflux	Cu, Co, Zn, Cd, Ni, As	Tet, Chlor, ß-lactams	Nies, 2003; Levy, 2002
Alteration of celllar target(s)	Hg, Zn, Cu	Cip, ß-lactams, Rif	Barkay, *et al.* 2003
Drug and metal sequestration	Zn, Cd, Cu	CouA	Roberts, 2005

Chlor chloramphenicol; **Cip** ciprofloxacin; **CouA** coumermycin A; **Rif** rifampicin and **Tet** tetracycline

As arsenic; **Cu** copper; **Zn** zinc; **Mn** manganese; **Co** cobalt; **Ag** silver; **Hg** mercury; **Cd** cadmium and **Ni**, nickel

5.4 Discussion

Of the 278 chromate tolerant isolates (193 from water; 61 from sediment samples, 24 tannery samples) collected from the coastline of Goa (and tannery effluent), twenty-five isolates were exceptionally resistant to Cr^{6+}. Results in chapter 6 discuss the reduction abilities of each and five were found to reduce/remove/transform Cr^{6+} adequately. These five isolates were characterized for their morphological, physiological and nutritional properties following Holt *et al*., (2000).

Biochemical and Morphological characterization

Differences were observed in their responses to characterization. Biochemical and morphological tests supported molecular identification with only isolates belonging to *Bacillus* genera producing amylase. Isolates displayed mesophily common to marine bacteria and their growth range was similar to the temperature and pH range of samples collected. Prokaryotes from the marine environments are ecologically resilient since they are continually exposed to varying pH, salinity, sea surface temperature, water currents, precipitation, wind speed, etc. Some undergo physical adaptation but many have been reported to possess adequate genetic potential to sense, undergo transduction and integrate external stimuli essential for their ability to rapidly respond to dynamic environmental conditions (Lauro *et al*., (2009). This results in effective regulation of nutrients including toxic metal influx or excretion. Based on this, many marine bacteria have been reported to transform, degrade or accumulate toxic substances (Hirak *et al*., (2013). When grown in Cr^{6+} and different pH, isolates showed a particular decrease in growth at lower pHs. A trend was observed as pH was lowered from 7 downwards. Although this may be due to the inability of the bacterial culture to grow at low pH, it is more likely that Cr^{6+} increases in bioavailability. The relative solubility of Cr^{6+} is dependent on conditions such as redox potential, temperature and pH (Marsh and McInerney, 2001). In acidic conditions, the fate and mobility of

$Cr^{6+}$is less affected by the presence of other organic and inorganic molecules (Katz, 1999). As a result, it is more bioavailable and to impair culture growth.

Growth parameters

In this study, all five isolates showed high resistance to chromate both in liquid and solid media. Being facultative oligotrophs, isolates behaved like copiotrophs (Schut *et al*., 1997) growing rapidly in nutrient chromate containing medium. Both *B. thuringiensis*208 and *B. cereus*273 emulated diauxic growth in medium amended with Cr^{6+}. This implies an adaptation in primary metabolism followed by a diauxic delay allowing reorientation of metabolism and a second round of growth (Novotna *et al*., 2003). Lag in growth was also observed in the other isolates when grown in Cr^{6+} implying their adaptation to toxic milieu, as has also been observed by De and Ramaiah (2007) for MRB and by Yin et al. (Yin *et* al., 2016). As Egler *et al.* (2005) suggested, such a lag may be for switching on relevant metabolic genes.

Antibiotic and metal resistance

The antibiotics tested were selected to represent seven groups: penicllins, aminoglycosides, tetracyclines, macrolides, cephalosporins, fluoroquinolones and sulfonamides. All isolates tested were sensitive to aminoglycosides (gentamycin, netillin, tobramycin) and resistant to cephalosporins (cefotaxime, cefoxitin, ceftazidime, ceftriaxone, cefuroxime). Very few were sensitive to ß-lactams (ampicillin/sulbactam, aztreonam) with variable responses to other groups. Resistance to multiple toxic metals also showed that tolerance was not confined to a single metal. This may be due to reduced membrane permeability, rapid metal efflux or change in cellular targets (Baker-Austin *et al*., 2006). Research has also documented the association of metal contamination and specific patterns of antibiotic resistance (Sabry *et al.*1997; Sinegani and Younessi, 2017; Onuoha *et al.*, 2018). Several mechanisms underlie this co-selection such as (i)

the close proximity of genes encoding resistance for either, on the same plasmid and/or transposon (Summers, *et* al., 1993), (ii) same genetic determinants responsible for resistance to both or (iii) indirect but shared responses such as biofilm formation or efflux pumps that target both toxicants (Hayashi *et al*., 2000; Baker-Austin *et al*., 2006). Baker-Austin *et al*. (2006) has elaborated on examples of shared structural and functional characteristics of bacteria antibiotic and metal resistance systems.

Examples include reduction of membrane permeability, drug/metal alteration, drug/metal efflux, alteration of cellular targets and drug/metal sequestration (**Table 5.4**). Isolates tested in this study showed high resistance to both ß-lactams and cephalosporins. This is of importance as they share similar modes of action disrupting the synthesis of the peptidoglycan layer in bacterial cell walls (Shearer *et al* 1988). Resistance to both indicates reduction in permeability, ability for drug/metal alteration and drug/metal efflux resistance as described in **Table 5.4** (modified from Baker-Austin *et al*. 2006).

SEM analysis

SEM analysis of *B. cereus*273 was done to evaluate morphological changes when grown in Cr^{6+}. In the presence of Cr^{6+}, cells became filamentous and slender. Transformation (increased cell size) in cell morphology has been reported to reduce the relative surface for toxic metal to attach (Neumann *et al.*, 2005; Justice *et al*., 2008; Shamim *et al*., (2013); Sharma *et al*, 2017). This relative reduction of cell surface area represented an adaptive mechanism of Cr^{6+} resistant *B.cereus*273 to minimize Cr^{6+} induced stress. It was also observed that *B. cereus*273 produced some form of exo-secretion. It is possible that this may be bio-film remnants as they have been known to form an effective mechanism of protection to both toxic metals and antibiotics (Costerton *et al*., 2003).

In summary, results in this study describe the characterization of Cr^{6+} detoxifying CRB. The growth of all isolates was inhibited when grown with Cr^{6+}, and showed fewer differences in growth rate with increase in Cr^{6+} concentration as observed by Viti *et al.* (2003). It is also important to note that all Cr^{6+} or antibiotic resistant organisms do not possess the ability to detoxify Cr^{6+}, and both phenomena are independent of each other (Viti *et al.*, 2003). Both temperature and pH govern the bioavailability of Cr^{6+}. It was observed that between the two, pH was more important in controlling the bioavailability of Cr^{6+} as cultures were less immune to the toxic effects of Cr^{6+} at acidic pH, as is expected. Antibiotic resistance profiles highlight possible mechanisms that strains in this study may utilize for survival. Additionally they also point out that resistance paths are shared between different metals, and in the case of isolates in this study, may show high resistance to manganese, cobalt and silver as well which were not tested for resistance in this study. The physiological and morphological characterization of potentially important strains in this study is important in outlining the dominant mechanisms of co-selection for metal/antibiotic resistance in addition to the ease of horizontally transferrable resistance determinants. It also highlights the prevalence of antibiotic-resistance mechanisms in environments that lack anthropogenic sources of antibiotics, which may be novel.

Chapter 6

Biotransformation and bioremediation of chromate

6.1 Introduction

Increase in industrialization has lead to unprecedented use of metal minerals. The threat of heavy metal pollution has thus lead to an increased interest in developing systems whether biological, physical or chemical that can remove and/or neutralize its deleterious effects in soils, waters and sediments (Kamaludeen *et al*., 2003). Biological systems offer unique advantages and approaches that are valuable in the removal of chromium metal. Chromate (Cr^{6+}) one of the two stable states of chromium, is highly toxic to humans, plants, animals and microbes alike (Thompson *et al.*, 2007). Interestingly, many species of microbes have developed mechanisms of Cr^{6+} resistance as result of selection. In addition to strategies such as exclusion of Cr^{6+} compounds through bacterial membranes, active efflux of Cr^{6+}, biosorption or bioaccumulation, up-regulation of genes and proteins active in oxidative stress response and DNA repair, bacteria have also been known to reduce Cr^{6+} as a survival mechanism (Ahemad, 2014). Bacteria that catalyze Cr^{6+} reduction are patently ubiquitous in Cr^{6+}/metal contaminated environments (Cheung and Gu, 2007), but have also been isolated from uncontaminated water (Ibrahim *et al*., 2012; Subramanian and Jayaraman, 2012), soils (Pal *et al.*, 2005; Jayalakshmi and Rao, 2012) and sediments (Beller *et al*., 2013). Bacterial Cr^{6+} reduction involves a myriad of conditions but can be classified under (i) cellular components that reduce Cr^{6+} under anaerobic condition (Ramírez-Díaz *et al*. 2008) (ii) enzymes that reduce Cr^{6+} under aerobic condition (Shen and Wang, 1993; Lovely and Philips, 1994) and, (iii) enzymes that reduce Cr^{6+} under anaerobic conditions (Wang *et al.*, 2000; Cheung and Gu, 2007). Reduction of Cr^{6+} to trivalent chromium (Cr^{3+}) (the other stable but innocuous state), can be brought about by Cr^{6+} reducing aerobes that utilize NADH and/or endogenous cell

reserves, by Cr^{6+} reducing anaerobes that rely on electron shuttling and cytochromes for dissimilatory Cr^{6+} reduction, or by enzymes that participate in other metabolic functions like iron reductases, nitroreductases etc. (Ahemad, 2014). Additionally, other mechanisms of Cr^{6+} remediation include bioaccumulation and biosorption. Reports of Nourbakhsh *et al*., (19994), Srinath *et al*., (2002), Khanafari *et al*., (2008), Pun *et al*., (2013), Pandian *et al*., (2014) etc. outline how binding of Cr^{6+} ions on cellular surfaces, within the cell walls, on to intracellular metal binding proteins or reaction with extracellular bacterial polymers to precipitate metal make them promising methods for Cr^{6+} removal.

From both ecological and economic perspectives, mechanisms of bacterial Cr^{6+} reduction, biosorption and/or accumulation are of importance. These processes reduce and immobilize chromium derivatives decreasing their bioavailability. In view of the many known potential of bacteria from marine environs, the Cr^{6+} detoxification/removal ability of select CRB isolated from waters and sediments were tested. In addition, this ability is also tested in the remediation of Cr^{6+} spiked soil to check if it can be made fit for the growth of (*Vigna radiata*) plantlings.

Biotransformation

6.2 Methods

6.2.1 Screening chromate resistant bacteria for their ability to remove chromate

The CRB were tested by diphenylcarbazide (DPC) method (Fulladosa *et al.*, 2006) to estimate their ability to remove/reduce Cr^{6+} when grown in liquid medium containing either 20 or 50µg mL^{-1} Cr^{6+}. Static cultures conditions were maintained in 250mL flasks containing 50mL of medium with initial pH set at 6 and temperature maintained at 28°C.

Aliquots (1mL) from cultures, taken at regular intervals were spun down at 14,000rpm (6500 x g) for 5 min and used to estimate Cr^{6+} remaining in the medium. From this, 100µL of cell free supernatant was transferred to 10mL glass-distilled water in an acid-washed test-tube. This was followed by addition of 1mL DPC solution (prepared by dissolving 0.25g DPC in 100mL acetone) and 2 drops of phosphoric acid. The mixture was kept at room temperature for 10min to develop colour, and the absorbance read at OD_{540}. Readings were monitored (up to 5 days in 20µg mL^{-1} and up to 12 days in 50µg mL^{-1}) and recorded. A series of standard potassium dichromate solutions were prepared from stock (diluted with double distilled water) ranging from 0-100µg mL^{-1} to generate a calibration curve. Accuracy of preparation (of the stock and subsequent dilutions) was also checked by flame atomic absorption spectroscopy (Thermo Electron Corp., S-Series, SOLAR S AAS) to 1ppm. All DPC tests were conducted in duplicate with water and non-inoculated controls.

6.2.2 Chromate removal potential under static and shake culture conditions

Bacterial cultures were tested in NB amended with 20µg mL^{-1} of Cr^{6}, in triplicate at room temperature (28 ± 2.5°C), pH 6, in both shake culture and static culture conditions for ability to remove Cr^{6+}. In addition to static culture, two agitation speeds were used (50 and 120rpm). Culture aliquots **(**1mL**)** were taken every 6h and residual Cr^{6+} in solution estimated following DPC test until no Cr remained in solution.

6.2.3 Isolation of chromate resistant bacteria for bioremediation experiment

Water samples were retrieved at low tide from a location along the Mandovi River (Lat-15 ° 30′ 19.206″ and Lon-73 ° 50′ 9.5748″) and 5mL was filtered through 0.22µm pore size membrane filters. These membranes were placed on SWNA plates amended with 100µg mL^{-1} Cr^{6+}. After overnight incubation at room temperature, isolates were picked up and purified by quadrant streaking. Isolates were designated as Cr^{6+} resistant after they were able to grow in NA amended with150µg mL^{-1} Cr^{6+}. These CRB were evaluated for their Minimum Inhibitory Concentration (MIC) of Cr^{6+} by broth dilution method (as described in **Section 5.2.3**). Minimum concentration of Cr^{6+} at which no growth was observed was considered as MIC. Two CRB isolates NIOMR3 and NIOMR8 were highly resistant, resisting up to 400µg mL^{-1} Cr^{6+}. By 16S rDNA sequencing, these were identified to be *Staphylococcus xylosus*3 and *Staphylococcus gallinaram*8.

6.2.4 Growth of isolates NIOMR3 and NIOMR8 in chromate

Cultures of *Staphylococcus xylosus*3 and *Staphylococcus gallinaram*8 were grown in NB and NB amended with 100µg mL^{-1} and incubated at room temperature in a shaker set at 110rpm. For optimum temperature, sets of flasks in triplicate were incubated at 20, 24, 28, 32, 37 and 42°C in shaking condition. For optimum pH, NB, in sets of flasks in triplicate was adjusted with conc. nitric acid or sodium hydroxide to achieve starting pHs of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, in each set. These were incubated at a fixed temperature in shaking condition. Culture aliquots were taken and optical densities measured to monitor growth.

6.2.5 Removal of chromate by isolates *S. xylosus*3 and *S. gallinaram*8

Diphenylcarbazide methods (Fulladosa *et al.*, 2006) outlined in **Section 6.3.1** was adhered to evaluate the ability of *S. xylosus*3 and *S. gallinaram*8 to remove/reduce Cr^{6+} when grown in liquid medium containing 20µg mL^{-1} Cr^{6+}.

6.2.6 Clean-up of garden soil by isolates *S. xylosus*3 and *S. gallinaram*8

Remediation ability of *S. xylosus*3 and *S. gallinaram*8 was evaluated. This was done by spiking locally obtained garden soil with Cr^{6+} to achieve final concentrations of 50 or 100µg mL^{-1} Cr^{6+} and treating them *S. xylosus*3 or *S. gallinaram*8. Soils were mixed in batches to ensure uniform Cr^{6+} strength (separately for 50 and 100 µg mL^{-1} Cr^{6+}) and dispensed (50g) into sets of petri-dishes. Each was treated with cultures of *S. xylosus*3 or *S. gallinaram*8 as outlined

Group I, bacterial set with **50**µg mL^{-1} Cr^{6+}

(i) *S. xylosus*3 + V. radiata + 50µg mL^{-1} Cr^{6+}

(ii) *S. gallinaram8* + V. radiata + 50µg mL^{-1} Cr^{6+}

Group II, bacterial set with **100**µg mL^{-1} Cr^{6+}

(i) *S. xylosus*3 + V. radiata + 100µg mL^{-1} Cr^{6+}

(ii) *S. gallinaram8* + V. radiata + 100µg mL^{-1} Cr^{6+}

Group III, bacterial **free** set of:

(i) V. radiata + no Cr^{6+}

(ii) V. radiata + 50µg mL^{-1} Cr^{6+}

(iii) V. radiata + 100µg mL^{-1} Cr^{6+}

After soils were inoculated with cultures (day 0), bacterial growth was supported further for a period of five days by the addition of sterile 0.2% yeast extract (w/v). On day 5, each plate was sown with ~10 surface sterilized mung seeds (*Vigna radiata*) which were allowed to germinate and grow in uniform light conditions set in laboratory. Dampened with water every 48h, shoot lengths of all the sprouts was recorded daily.

6.3 Results

Biotransformation

6.3.1 Removal of chromate

Twenty-five CRB isolates were tested for their ability to remove/reduce 20µg mL^{-1} Cr^{6+} for up to 10 days (**Table 6.1**). Five isolates namely *Staphylococcus saprophyticus*168, *Staphylococcus saprophyticus*176, *Bacillus thuringiensis*208, *Bacillus cereus*273 and *Staphylococcus arlettae*324 were able to reduce Cr^{6+} concentrations below detectable limits fewer than 4 days. These five isolates were also evaluated for their removal of 50µg mL^{-1} Cr^{6+} (**Fig. 6.1**) taking up to 10 days for completion. Rates of Cr^{6+} removal varied between 0.231 - 0.274µg mL^{-1} h^{-1}, with *S. saprophyticus*176 the most efficient.

6.3.2 Effect of static and shaking conditions

All five isolates reduced/removed Cr^{6+} with high performance. The capacity for reduction or removal of Cr^{6+} was evaluated under varying conditions of aeration (**Fig. 6.2**). Agitation speeds were used to regulate levels of aeration, with static cultures most poorly aerated and cultures at maximum rpm (100rpm) to be well-aerated. Growth increased linearly with increase in rpm, but the Cr^{6+} removal/reduction rate decreased inversely. This trend was observed for all isolates except *S.saprophyticus*176, whose levels or Cr^{6+} decrement remained fairly consistent throughout.

Bioremediation

Due to the experimental set-up, the following cultures were tested for bioremediation

6.3.3 Identification and characterization of *S. xylosus*3 and *S. gallinaram*8 for bioremediation experiment

NIOMR3 and NIOMR8 were identified as *Staphylococcus xylosus*3 and *Staphylococcus gallinaram*8 by 16S rDNA sequencing. Both tested negative for coliform group tests (M, VP and IT, discussed in **Section 5.2.2**). With Cr^{6+}MIC of 300 and 400µg mL^{-1} respectively, they were also found to be resistant to Hg, Cd, Cu, Zn, As, Ni and Cr^{3+} metal.

6.3.4 Growth of *S. xylosus*3 and *S. gallinaram*8 in chromate

Both isolates were impaired when grown in the presence of Cr^{6+} (**Fig. 6.3**) and were able to grow in a wide range of temperatures from 24 - 37°C, 24°C being the optimal temperature for growth in Cr^{6+}-free media. Alklaine pH was preferred by both (8-9).

6.3.5 Removal of chromate by isolates *S. xylosus*3 and *S. gallinaram*8

Both isolates were observed to remove Cr^{6+} only in static condition and were able to remove Cr^{6+} completely in medium in around 10 days. *S. xylosus*3 had a removal rate of 0.09µg mL^{-1} h^{-1} and *S. gallinaram*8 a rate of 0.08µg mL^{-1} h^{-1}.

6.3.6 Clean-up of garden soil by isolates *S. xylosus*3 and *S. gallinaram*8

Mung seeds that germinated after overnight incubation in garden soil spiked with Cr^{6+} were noted. Shoots that formed were measured for their lengths and were found to be

consistently longer in Cr^{6+} spiked soil remediated with either *S. xylosus*3 or *S. gallinaram*8, in comparison to soils not bacterially treated (**Table 6.3**). Except for Group III (ii and iii, both grown with Cr^{6+} but without bacteria), leaves were observed in all conditions. Unsurprisingly, shoots were observed to be stunted, thin, dry and yellowish in Groups III(ii) and III(iii) (**Fig. 6.4**).

Table 6.1 List of isolates tested for chromate removal and the ones selected for further experiments (in **bold**)

Bacterial isolate	**Bacteria identified by 16S rRNA gene**	**Source**	**Accession No.**	**Removal of Cr^{6+}** (20µg mL^{-1} in days)	**Cr^{6+} removal rate** (µg mL^{-1} h^{-1})
NIOER164	*Staphylococcus saprophyticus*	$Divar^{W}$	MG205890	did not	-
NIOER165	*Staphylococcus xylosus*	$Divar^{W}$	MG205891	did not	-
NIOER168	***Staphylococcus saprophyticus***	$Divar^{W}$	MG205894	**~3**	**0.270**
NIOER176	***Staphylococcus saprophyticus***	$Dias^{W}$	MG205901	**4**	**0.260**
NIOER182	*Exiguobacterium aestuarii*	$Dias^{W}$	MG205907	did not	-
NIOER185	*Staphylococcus cohnii*	Dias S	MG205910	did not	-
NIOER188	*Staphylococcus arlettae*	Dias S	MG205912	8	0.104
NIOER191	*Staphylococcus saprophyticus*	Dias S	MG205914	5	0.167
NIOER202	*Staphylococcus cohnii*	$G5^{W}$	MG205924	5	0.169
NIOER203	*Staphylococcus cohnii*	$G5^{W}$	MG205925	did not	-
NIOER208	***Bacillus thuringiensis***	$G5^{W}$	MG205930	**4**	**0.231**
NIOER236	*Staphylococcus saprophyticus*	$Divar^{W}$	MG205956	did not	-
NIOER261	*Staphylococcus arlettae*	$Divar^{W}$	MG205978	8	0.105
NIOER273	***Bacillus cereus***	$Dias^{W}$	MG205987	**4**	**0.253**
NIOER285	*Bacillus cereus*	$Dias^{W}$	MG205999	did not	-
NIOER286	*Bacillus pseudomycoides*	$Dias^{W}$	MG206000	9	0.093
NIOER289	*Bacillus cereus*	$Dias^{W}$	MG206002	8	0.104
NIOER292	*Bacillus thuringiensis*	$Dias^{W}$	MG206005	5	0.168
NIOER299	*Bacillus pseudomycoides*	$Dias^{W}$	MG206012	5	0.167
NIOER303	*Bacillus pseudomycoides*	$Dias^{W}$	MG206015	did not	-
NIOER307	*Bacillus thuringiensis*	$Dias^{W}$	MG206017	5	0.165
NIOER320	*Bacillus cereus*	$Dias^{W}$	MG206028	did not	-
NIOER321	*Staphylococcus xylosus*	$Dias^{W}$	MG206029	9	-
NIOER324	***Staphylococcus arlettae***	$Dias^{W}$	MG206032	**4**	**0.260**
NIOER325	*Bacillus cereus*	$Dias^{W}$	MG206033	5	0.167

Key: W water, **S** sediment

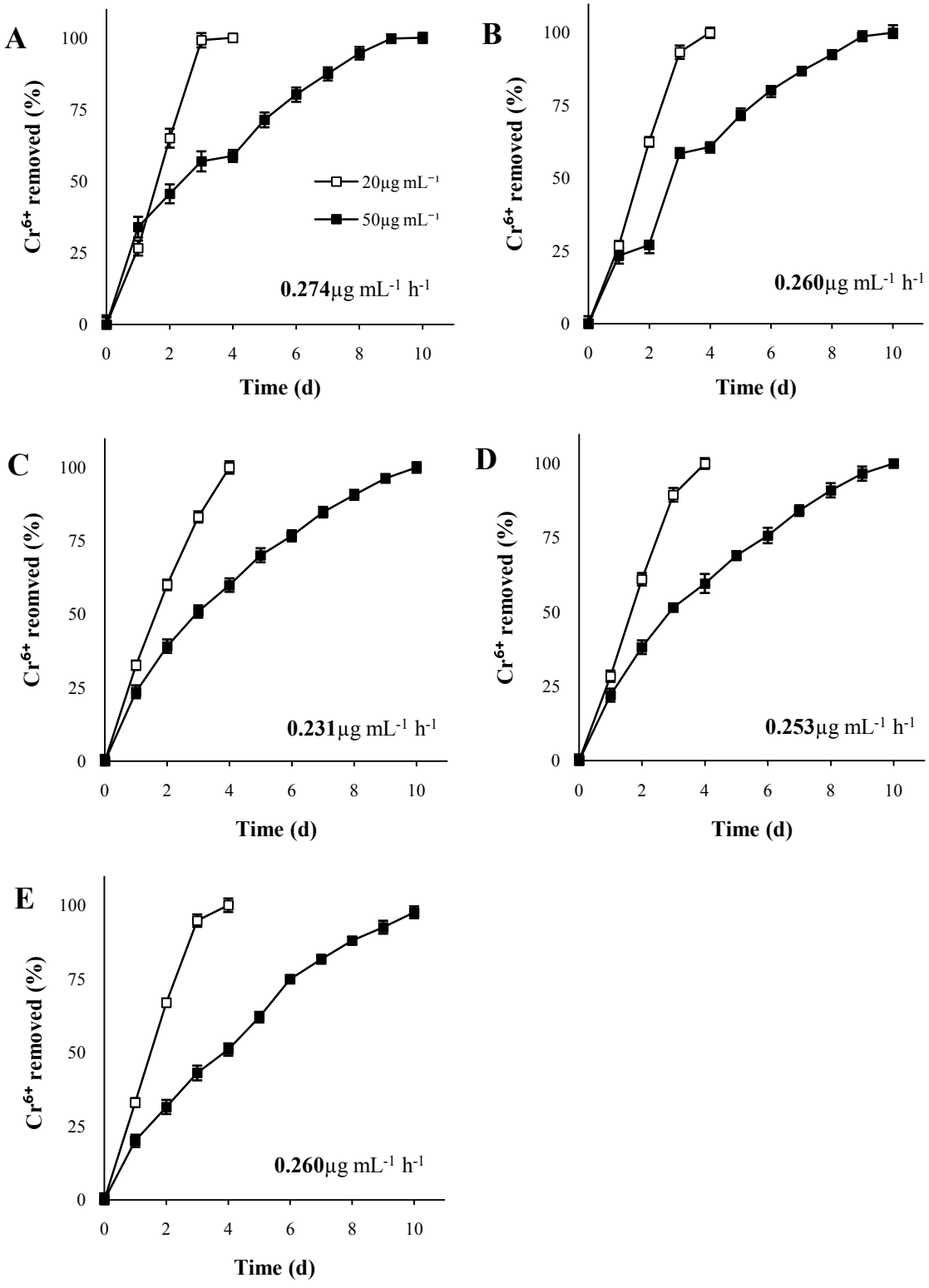


Figure 6.1 Chromate detoxification by (A) *S. saprophyticus*168, (B) *S. saprophyticus*176, (C) *B. thuringiensis*208, (D) *B. cereus*273 and (E) *S. arlettae*324 from medium containing (■) 20 and (□) 50µg mL^{-1} chromate. Estimations were done routinely every 24h up to 10 days of incubation; *n=3*. Value in the legend indicates rate of removal of Cr^{6+}.

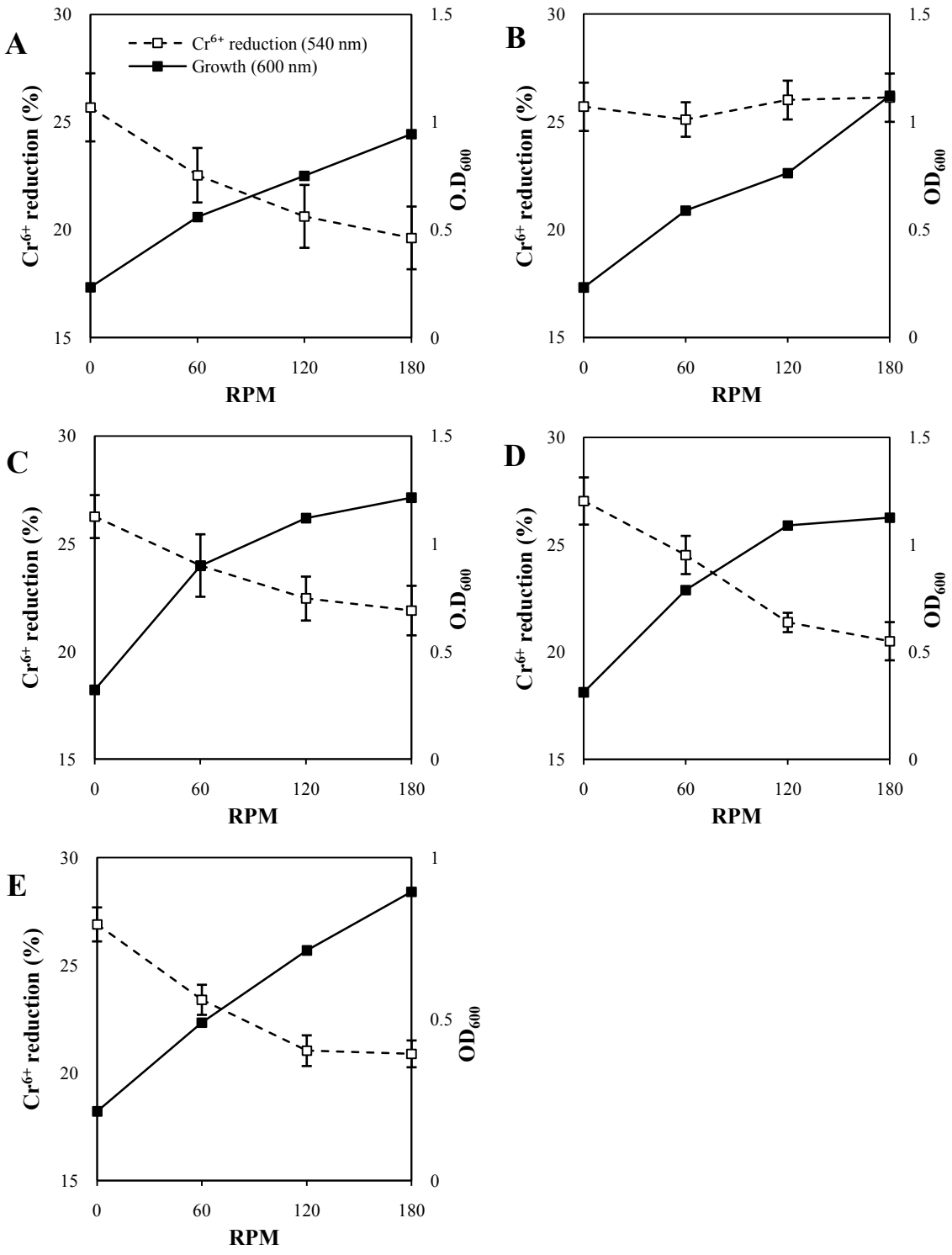


Figure 6.2 Effect of agitation on (■) growth and (□) chromate detoxification, by (A) *S. saprophyticus*168, (B) *S. saprophyticus*176, (C) *B. thuringiensis*208, (D) *B. cereus*273 and (E) *S. arlettae*324 in medium containing 20µg mL^{-1} chromate; *n=3*

Table 6.2 16S rRNA gene identity of *S. xylosus*3 and *S. gallinaram*8 with their MIC (µg mL^{-1}) of heavy metals, biochemical characterization and Cr^{6+} removal rate

	Isolate	**NIOMR3**	**NIOMR8**
	Bacteria identified by	*S. xylosus*	*S. gallinarum*
	Accession. No.	KY672997	KY673000
heavy metals (µg mL^{-1})[a]	Cr^{6+}	300	400
	Hg	20	20
	Cd	40	40
	Cu	120	140
	Zn	50	60
	As	700	750
	Ni	300	300
	Cr^{3+}	800	850
biochemical characteristics	Colony shape	Round	Round
	Colony elevation	convex	flat
	Colony colour	dull yellow	yellow
	GS	+	+
	CT	+	+
	NRT	-	+
	CUT	-	-
	GHT	+	+
	SHT	-	-
	CHT	-	+
	IT	-	-
	MR	-	-
	VP	-	-
	TSIT	AB&S	AB&S
Cr^{6+} removal rate (µg mL^{-1} h^{-1})		**0.08**	**0.09**

Key: GS, Gram Staining; **CT** Catalase; **NRT** Nitrate Reduction; **CUT** Citrate Utilisation; **GHT** Gelatin Hydrolysis; **SHT** Starch Hydrolysis; **CHT** Casein Hydrolysis; **IT** Indole; **MR** Methyl Red; **VP** Voges Proskauer; **TSI** Triple Sugar Iron; **C** cocci; **SR** straight rod; + positive; **-** negative; **AB** acidic butt (glucose fermenter); **AB & S** acidic butt and slant (lactose and sucrose fermenter)

[a] Salts of different metals dissolved to attain above mentioned concentrations of toxic metals

Figure 6.3 Growth (OD_{600}) of bacterial strains (A) *S. xylosus*3 and (B *S. gallinaram*8 in chromate supplemented (□; 100µg mL^{-1}) and chromate free (■) nutrient broth

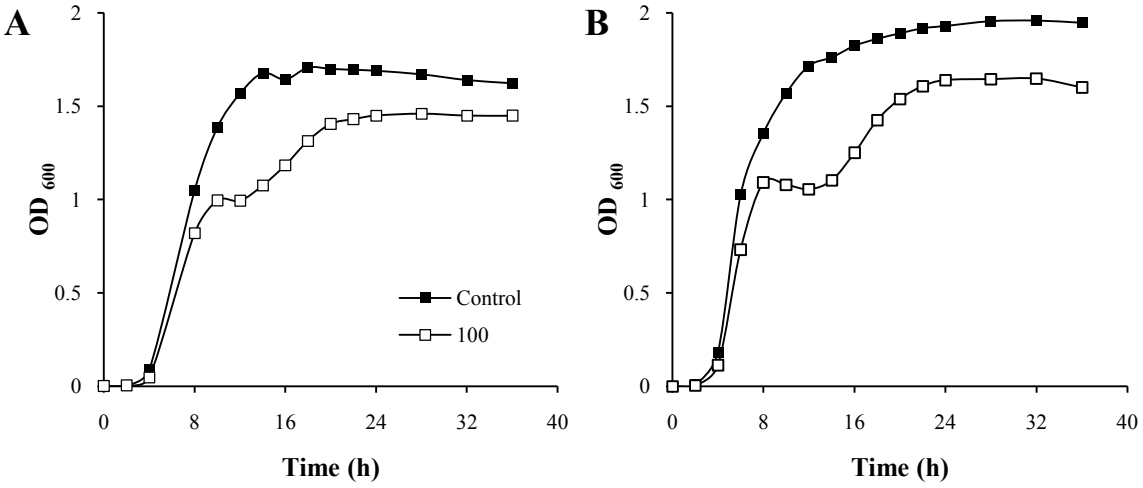


Table 6.3 Shoot lengths of *Vigna radiata* grown in 50 and 100µg mL^{-1} Cr^{6+} contaminated garden soils, in presence of 7 treatments

	Day 1		**Day 3**		**Day 5**		**Day 7**		**Day 10**	
µg mL^{-1} Cr^{6+}	50	100	50	100	50	100	50	100	50	100
Control	0	0	0.100± 0.01	0	0.27± 0.04	0.12± 0.03	0.45± 0.09	0.26± 0.07	0.46± 0.04	0.26± 0.30
*S. xylosus*3	0.400 ± 0.01	0.380 ± 0.01	1.146 ± 0.20	1.271 ± 0.03	2.870 ± 0.49	2.669 ± 0.31	4.592 ± 0.43	3.828 ± 0.5	5.192 ± 0.69	4.228 ± 0.54
S. gallinaram 8	0.470 ± 0.05	0.310 ± 0.03	1.370 ± 0.43	1.372 ± 0.20	3.528± 0.73	2.869± 0.53	4.668 ± 0.91	3.584 ± 0.35	5.198± 0.55	4.080 ± 0.30
Control (w/o Cr^{6+})	0.90±0.1		2.47±0.23		4.79±0.35		6.89±0.59		9.97±0.46	

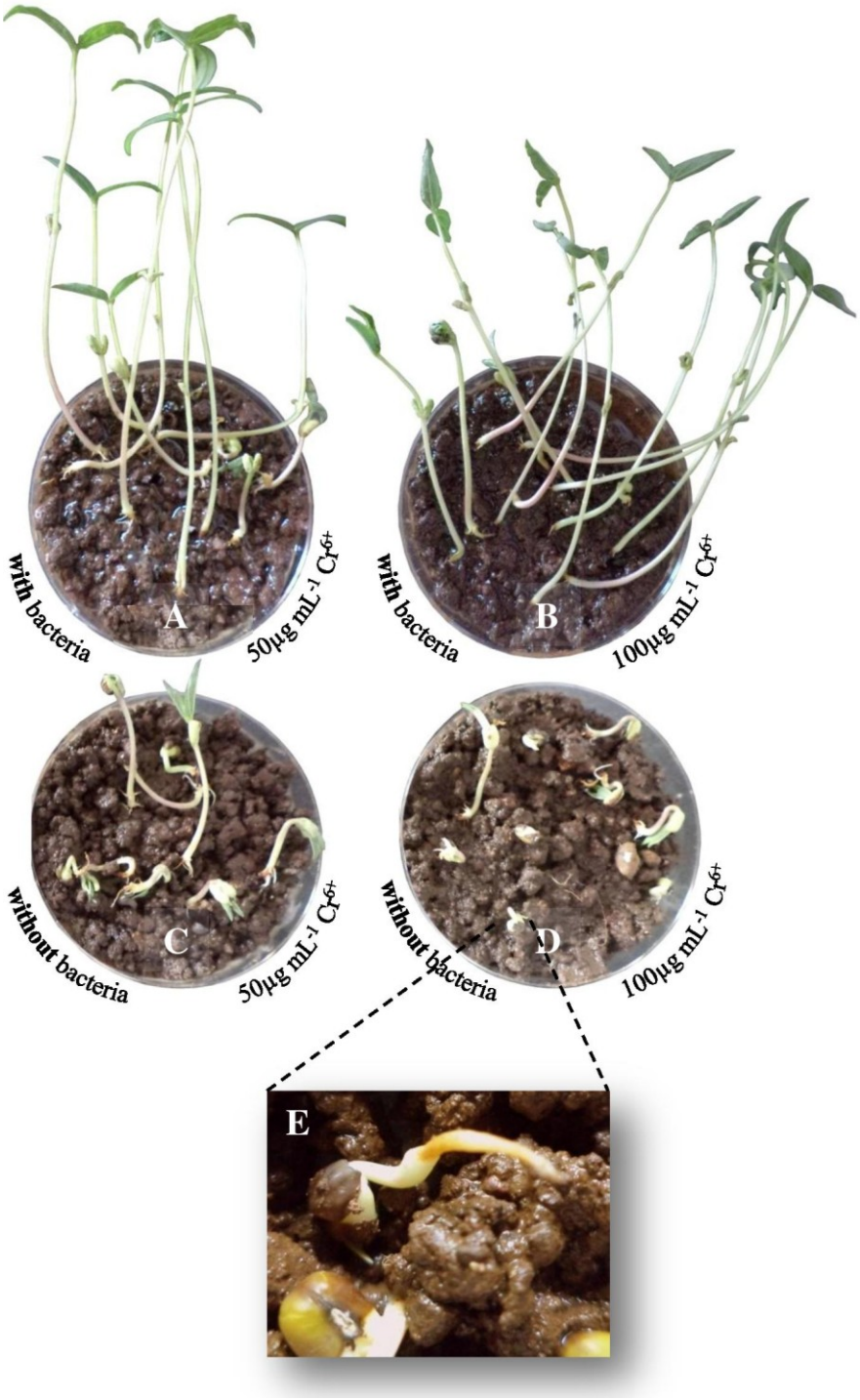


Figure 6.4 In situ approach for chromate detoxification of soil using marine bacteria (*S. xylosus*3 and *S. gallinaram*8). *Vigna radiata* was allowed to germinate and grow in Cr^{6+} (either 50 or 100µg mL^{-1}) contaminated soil pre-treated with bacteria (A and B) for 5 days. *V. radiata* grown in control without bacterial pre-treatment (C and D) were stunted with yellowing of roots (E).

6.4 Discussion

Biotransformation

Chromate resistant bacteria indigenous to various environments are reported by several researchers (Brown *et al.*, 2006; Cheung and Gu, 2007; Congeevaram *et al*., 2007; Alam and Ahmad, 2013). Some of these bacterial strains characterized for their ability to remediate chromate were from bacterial genera like *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Staphylococcus*, *Ochrobactrum*, *Thermus* , *Shewanella* (Aguilar-Barajas *et al*., 2008), *Vibrio* (Zenno *et al*., 1998), *Escherichia*, *Micrococcus* sp. (Sultan and Hasnain, 2005; Faisal and Hasnain, 2004), *Serratia (*Deng *et al.*, 2015), *Rhodobacter* (Nepple *et al*., 2000), *Geobacillus (*Chatterjee *et al*., 2010), *Desulfomicrobium* (Michel *et al*., 2003) *Desulfovibrio* (Chardin *et al*., 2002) and *Alcaligenes* (Peitzsch *et al*., 1998). Most of these reports outline strains originating from contaminated niches like tannery waste water or tannery effluent irrigated soil. Although it is evident that strains capable of Cr^{6+} detoxification are ubiquitous in Cr^{6+} contaminated environments, there is a need for the biodiscovery of potential Cr^{6+} reducers from other environs, especially marine niches. This is due to the possibility of a new mechanism (and/or the combination of mechanisms) of action as well as the unique properties marine environments confer on such bacteria. In this study, isolates capable of Cr^{6+} detoxification were from two genera, *Staphylococcus* and *Bacillus*.

All five isolates evaluated were able to remove Cr^{6+} (with Cr^{6+} removal rates ~ 0.2µg mL^{-1} h^{-1}) rapidly in comparison to NIOMR3 and NIOMR8 (*S. xylosus*3 and *S. gallinaram*8) reported earlier (Pereira *et al*., 2017a) with reported rates ~0.09µg mL^{-1} h^{-1}. Comparison with strains from other investigations revealed that these removal/reduction rates were on

par with those reported (Liu *et al*., 2004; Chirwa and Wang, 2000; Wang and Xiao, 1995). Song *et al*., (2009) reported the use of *Pseudomonas aeruginosa* CCTCC AB91095 to reduce Cr^{6+} over a concentration range of about 2 – 40µg mL^{-1}. Rates of reduction reportedly varied from 0.16 to 0.24µg mL^{-1} h^{-1} with average rates declining as Cr^{6+} concentrations were raised above 20µg mL^{-1}. This was also demonstrated by isolates in this study (rates reduced when tested for in 50µg mL^{-1}) revealing the existence of a finite Cr^{6+} removal ability which diminishes due to toxic effects on biological activity. Contrastingly, Chirwa and Wang (2000) and Wang and Xiao (1995) tested for Cr^{6+} reduction capacity and found a proportional increase in rates with increase in Cr^{6+} concentrations. They also reported similar rates of Cr^{6+} removal ranging from 0.3-0.4 µg mL^{-1} h^{-1}. Cordoba *et al.* (2008) tested the reduction rate of *Arthrobacteri* CR47 with a first order kinetic parameter of 0.071µg mL^{-1} h^{-1}. They improved this rate in a recirculating reactor fed with either laboratory or industrial model solutions to achieve removal rates of 0.79 and 0.49µg mL^{-1} h^{-1}. It is therefore possible, that in a similar setup for continuous culture instead of batch mode used in this study albeit under laboratory conditions, removal rates may be increased.

The ability to perform Cr^{6+} removal under different shaking conditions was evaluated and found to be most efficient under static conditions. Similar results were observed by Bachate *et al.* (2013), Ibrahim *et al.* (2012) and Wang *et al.* (1989) where isolates removed Cr^{6+} only under anaerobic conditions although growing and resisting the same under aerobic conditions. This lack of detoxification under shaking conditions could be due to decreased uptake of Cr^{6+} under static conditions as identified by Ohtake *et al.* (1987) or by the increased use of chromate as a terminal electron acceptor under

static conditions (Bachate *et al*., 2013). However, since the cells were not grown in strict anaerobic conditions, this possibility will have to be further tested.

In summary, results in this study describe the Cr^{6+} removal ability of bacterial strains isolated from marine environments that can tolerate multiple metals and antibiotics. Knowledge of parameters that may control growth whether negative or positive may be useful in optimizing conditions for Cr^{6+} removal, particularly in the case of isolates in this study which have high potential for remediation. In addition to being sturdy and resilient, these isolates are amendable to further testing and it is likely that they will perform to better standards in industrial settings, although this has to be evaluated. The rates of removal in this study are relatively high and comparable with those of augmented strains or strains isolated from contaminated environments. As such these isolates are valuable for a bioremediation aspect as they can be adapted to specific contamination problems.

Bioremediation

Marine bacteria are subject to varying physical, chemical and ecological change. As a result, like other copiotrophs they possess the genetic tools to sense and integrate external stimuli important for their ability to respond to and cope with dynamic environmental conditions (Pereira *et al*., 2017a). As a consequence, nutrient and toxic metabolite influx and excretion are tightly regulated making them ecologically versatile. This versatility and ability to transduce toxicants such as hydrocarbons, metals etc., has been evaluated by quite a few investigators and are summarized by Hirak, (2013). Exploiting this remediative potential thus, may provide an environmentally-safe way to clean-up pollution. In water samples collected from downstream the Mandovi River, two CRB

isolates, *S. xylosus*3 and *S. gallinaram*8 were found to be resistant to Cr^{6+}. Both *staphylococci* exhibited diauxic delay indicative of reorientation of metabolism and a second round of growth (Novotna *et al*., 2003) which may be to switch on relevant metabolic genes. Cr^{6+} removal rates of both indicated that although they were capable of gross Cr^{6+} removal, they were not as efficient as *S. saprophyticus*168, *S. saprophyticus*176, *B. thuringiensis*208, *B. cereus*273 and *Staphylococcus arlettae*324, all which exhibited rates above 0.2µg mL^{-1} h^{-1}. This difference in the rate of Cr^{6+} removal among the different isolates in the present investigation could be attributed to different resistance mechanisms and/or a difference in regulatory sequences. The resulting altered expression of enzymatic or metabolic activity may suggest differences in these rates.

Testing *S. saprophyticus*168, *S. saprophyticus*176, *B. thuringiensis*208, *B. cereus*273 and *S. arlettae*324 for remediation was not possible due to the experimental set-up, which was set prior to isolation of these strains. Instead, bioremediative potential of *S. xylosus*3 and *S. gallinaram*8 was evaluated to assess if they were capable of removing Cr^{6+} from a soil matrix, enough so, that mung seeds were able to proliferate. When grown in Cr^{6+} without bacterial presence, the shoot leaves were faded and leaves and roots were shrunken. This reduced growth, damaged root cells and chlorosis in leaves is due to reduced pigment content, altered enzymatic profile and ultra-structural changes in cell membranes as reported by Panda and Choudhury (2005), indicated Cr^{6+} uptake.

Growth and reduction of Cr^{6+} by *S. xylsous*3 or *S. gallinaram*8 enabled mung plants to grow substantially in comparison to the stunted and poor/no growth observed when mung seeds were sown in Cr^{6+} spiked soil without the addition of marine bacterial isolates. It is evident that there might be a reduction of toxic Cr^{6+} by metal

immobilization, sequestration or a direct reduction to innocuous Cr^{3+}. It is also likely that these bacteria may promote plant growth by speeding up germination and growth as reported by Ramı´rez-Dı´az *et al*. (2008).

This study highlights the potential of CRB isolates in bioremediation and their application in *in situ* strategies. These are advantageous as they avoid the costs and secondary dispersal problems associated with physical transportation of contaminated soils or waters (Valls *et al*., 2002). Although the usefulness of such an approach is unpredictable, there have been reports of the use of bacteria in metal immobilization in polluted soils (Diels *et al*.,2002; Achour *et al*., 2007; Achal *et al*., 2011; Ahmady-Asbchin *et al*., 2015; Banerjee *et al*., 2015). Valls *et al*. (1998, 2000) reported the introduction of a metal-tolerant bacterium into contaminated peat soil, which was able to immobilize cadmium, protecting plants from toxic effects. Similarly in this study, it is likely that Cr^{6+} underwent some degree of precipitation as a result of bacterial action, corresponding to decreased bioavailability. This is particularly useful in the remediation of moderately polluted agricultural fields where bacteria evaluated in this study could be employed to immobilize Cr^{6+}, allowing the use of these fields. As such, the knowledge obtained regarding microbial processes especially that, that help bacteria better adapt to actual conditions in which treatments are performed (like temperature, pH and nutritional requirements) may be applied. Thus this study has added new marine bacterial isolates capable of rapid reduction of Cr^{6+} which can ameliorate bioremediative technologies.

Chapter 7

Chromate induced protein expression

7.1 Introduction

There is a burgeoning growth of knowledge on capabilities of heavy metal tolerance transformation and turnover by marine organisms. The functional aspects of their abilities to carry out transformation or tolerance are not investigated enough to provide species specific deviations in generally well-known metal microbe interactions and/or end products. Application of proteomics can help determine the proteins and pathways involved in mechanisms that confer resistance and biotransformation in a given species/strain. Being both structural and functional entities of a cell, proteins are pivotal to the living world (Viti *et al*., 2014). As detailed by Chourey *et al*. (2006); Bar *et al*. (2007); Kilic *et al*. (2010); Zaka *et al*., 2013 and Zhai *et al*. (2017) their expressional patterns describe the intra-cellular environment in terms of metabolic pathways affected. In the presence of Cr stress, as may be the case with other toxic metals (Yung *et al*., 2014; Siripornadulsil *et al*., 2014; Ray *et al*., 2013), proteins are either up or down-regulated. Identifying these proteins and their fold-changes will allow for understanding intracellular adjustment and how these proteins may play a role in conferring resistance or Cr^{6+} transformation ability.

This study is divided into two parts: (i) the proteomic responses of NIOER168, NIOER176, NIOER208, NIOER273, and NIOER324 to chromate challenge; and (ii): the proteomic response of NIOMR3 to chromate challenge. NIOMR3 (*Staphylococcus xylosus*) was tested for its ability to remediate Cr^{6+} contaminated soil. In this regard, differential protein expression studies were conducted to ascertain its stress response to Cr^{6+}.

7.2 Methods

7.2.1 Bacterial strains, growth conditions and chromate metal induction

NIOER168, NIOER176, NIOER208, NIOER 273 and NIOER324 isolated (identified as *Staphylococcus saprophyticus*168, *S. saprophyticus*176, *Bacillus thuringiensis*208, *B.cereus*273 and *S. arlettae*324) from water samples collected were studied for their protein expression. For analysis, NB supplemented with potassium dichromate (50, 100, 150 and 200µg mL^{-1}) and freshly prepared primary inoculum (0.1% (v/v)) was grown until mid-exponential phase ($O.D_{600}$~ 0.4; time taken ca. 7h) and the cells harvested, by centrifugation at 14,000rpm at 4°C for 10min. Cell pellets were washed twice with PBS to ensure removal of media containing metal. Inoculated unamended NB was processed similarly and used as control.

7.2.2 Strain NIOMR3, growth conditions chromate induction

Bacterial strain *S. xylosus*3 (NIOMR3, Acc No.: KY672997) identified by 16S rDNA sequencing was isolated as discussed in **Section 6.2.3** from water sample collected at the Betim fishing point (Mandovi River). The culture was grown on in SWNA amended with Cr^{6+}. Liquid cultures grown in NB were used for proteomic analysis. Overnight culture was transferred into fresh broth amended with 50, 100, 150 or 200µg mL^{-1} Cr^{6+}, and allowed to grow for 6-7h- mid-exponential phase before being harvested by centrifugation. Culture grown in plain NB was used as control. Cell pellets were washed twice with 1X PBS to remove media and Cr^{6+}.

7.2.3 Cellular protein extraction

For *S. saprophyticus*168, *S. saprophyticus*176, *B. thuringiensis*208, *B. cereus*273 and *S. arlettae*324, resulting cell pellets were dissolved in 500µL urea thiourea (UT) buffer (**Appendix B**) in bead-bashing tubes (zirconia beads) and the cell slurry homogenized twice at 6.5m/s for 40s (FastPrep, MP Biomedicals, USA). The tubes were held in ice for rest duration of 5min between each homogenization. The bacterial cells and debris were then centrifuged at 14,000rpm for 20min at 4°C, and supernatant transferred to a fresh microcentrifuge tube. For extraction of residual proteins, another 500µL urea thiourea buffer was added to cell debris in the bead bashing tube, and re-homogenized. The supernatants were pooled and proteins in suspension were maintained in extraction buffer. A volume of 50µL was used for protein estimation and based on calculations, volumes were dispensed and made ready for precipitation. The remaining volume of protein sample was stored at -20°C for further use.

Similarly, for *S. xylosus*3, cell pellets were resuspended in 500µL of UT buffer and processed as described above. In the resultant supernatant, protein concentrations were estimated and eluted based on calculations. The stock mixture was stored at -20°C, and eluted sub-samples were prepared for LC/MS-QToF analysis

7.2.4 Protein estimation and methanol precipitation

To 50µL protein sample (in extraction buffer), 1:9 (v/v) 100% methanol (Fluka MS grade) was added and incubated at 4°C for a minimum of 1h. Tubes were then centrifuged at 14,000rpm for 20 min at 4°C and supernatant discarded before addition of 90% methanol (Friedman, 2007). After incubation and centrifugation, pellets were

vacuum dried for 20min at 30°C (care was taken not to over-dry) and re-suspended in de-ionized water. Standard procedure was followed for protein estimation (Lowry *et al.*, 1951). To 1mL of sample, 5ml of A+B (**Appendix D**) added was incubated at room temperature for 10min before addition of 0.5mL of Folin and Ciocalteu's Phenol reagent. After a dark incubation for 30min, absorbance was read at $O.D_{660}$ with protein extraction buffer used as blank and Bovine serum albumin as standard for calibration. Readings were used to estimate protein content and based on calculations, volumes were dispensed and methanol precipitated containing a fixed concentration of protein (for each sample). This was to ensure equal amount of protein was represented in each sample in subsequent tests.

7.2.5 Gel electrophoresis

Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve bacterial proteins in a Mini-Protean electrophoresis unit (Bio-Rad Laboratories), according to Laemmli (1970. Precipitated total protein samples (100µg μL^{-1} each) re-suspended in sample solubilizing buffer (15µL) (**Appendix E**) were heated in a dry bath at 95°C for 5min before being loaded on to a 6% stacking gel overlying a 12% resolving gel (**Appendix E**). Medium range marker (97.4-14.3KDa) (Perfect protein marker, GeNei) was prepared similarly and all tubes were spun shortly to make sure whole volume is loaded. Electrophoresis was carried out in 1X running buffer (**Appendix E**) at 90V till the dye-front reached the bottom. Gels were treated with fixative (**Appendix E**) for 1-2h, stained with silver nitrate following Simpson's protocol (2007) (**Appendix E**) and documented using a G-Box F3 Gel Documentation System (Syngene, UK).

7.2.6 In-gel digestion

Bands of interest (*B. cereus*273) from visualized SDS gels were further analyzed by preparation for Mass spectrometry as per Shevchenko (2007). The entire gel slab was rinsed in water for 2-3h before the band was excised using a clean razor blade on a glass plate. The cut band was chopped into cubes (ca. 1 x 1mm), transferred to a tube and spun down. In-gel reduction and alkylation were performed (**Appendix B**) followed by de-staining. All solutions were removed, and the gel pieces were completely covered with 20ng mL^{-1} trypsin (porcine, proteomics grade, Sigma) for 30-90min (**Appendix B**). After complete trypsin saturation, tubes with gel pieces were incubated overnight (ca. 14-16h) at 37°C for digestion. Peptide digestion products were extracted by addition of extraction buffer (**Appendix B**) and incubation at 37°C for 15min while shaking. The supernatant was withdrawn carefully, avoiding gel pieces, transferred to a fresh tube and adjusted to pH 3-4 by addition of formic acid.

7.2.7 In-solution digestion

In-solution trypsin digestion (Promega Sequencing Grade Modified Trypsin Product Information Sheet; Kinter and Sherman, 2000) was performed to prepare whole-cell protein samples of (NIOER isolates: *S. saprophyticus*168, *B. thuringiensis*208 and NIOMR isolate: *S. xylosus*3) for LC-MS QToF analysis. Precipitated sample protein pellets were reconstituted and dissolved in 6M urea (**Appendix B**), following which the mixture is reduced and alkylated in dark conditions at room temperature. To this, 500µL of 50mM Tris-HCl was added to dilute urea concentration before addition of trypsin solution. Tubes were then incubated overnight at 37°C and finally acidified using formic acid.

7.2.8 LC/MS-QToF Analyses

Aliquots (50-60µL) of acidified tryptic digests were transferred to auto-sampler MS vials, sealed and analyzed on a 6538 UHD Accurate Mass QToF LC/MS (Agilent Technologies, USA). Sub-volumes (8µL) were injected into the auto-sampler and LC separations were carried out over a gradient elution using water and Acetonitrile (ACN) on a Prot ID Chip 150 II 300A C18 150 mm column. Peptides were eluted using a standardized 100min protocol. MS/MS for separated ions were carried over a range from 50 to 2000 m/z in positive mode at 2 ppm accuracy with nitrogen gas inside the collision cell. Spectral data was acquired using Mass Hunter Software ver. 5.0 (Agilent Technologies, USA) (Pereira *et al.*, 2017b).

7.2.8.1 *Identification of Proteins*

Spectra collected through Mass Hunter were analyzed following manufacturer's instructions on Spectrum Mill MS Proteomics Workbench ver. B.04.01.141 (Agilent Technologies, USA). MS/MS search was done against a specific database sub-set (specific to each species) created using SwissProt database at an accuracy at 50 ppm (for MS), and 100 ppm (for MS/MS). All MS searches were auto validated using a false discovery rate of 1.2%, followed by the generation of peptide and protein summary files. Cutoff scores having hits >1, and % SPI >10 were considered for further analysis.

7.2.8.2 *Statistical Analyses*

Mass Profiler Professional ver. 13 (MPP, Agilent Technologies, USA) was used to analyze data generated in Spectrum Mill. Technical replicates of samples were grouped

with respect to chromate concentrations (0, 20, 50 and 100µg mL^{-1}) where each entity in the sample was filtered based on frequency of occurrence among the samples in at least one condition. The differential significance of samples was assessed with a p-value cut-off of 0.2 and a fold-change cut-off of 2.0 when control samples (no chromate) and chromate amended samples were compared. Proteins satisfying fold-change cut-off were used to generate Venn diagrams to check whether they were up or down-regulated at different chromate concentrations and whether they were common to all conditions. Proteins thus identified were used to create a functional based classification where the gene's corresponding functions were grouped, based on the Kyoto Encyclopedia of Genes and Genomes (**KEGG**, Ver. 37) pathways and Clusters of Orthologous Groups (**COG**s) of the proteins, cross-referenced with their UniProtK Accession numbers.

7.3 Results

7.3.1 Gel electrophoresis

Proteins that were differentially regulated in the presence of Cr^{6+} in a select few CRB isolates were examined in this study. Protein profiles of bacterial isolates differed (**Fig**. **7**.**1**) when grown in absence and presence of Cr^{6+} where synthesis of many proteins was altered (being decreased or increased). Proteins with molecular weight ranging from 97-15kDa were suppressed as Cr^{6+} concentration increased. SDS-PAGE profile of *S. saprophyticus*168 (**Fig. 7**.**1**A) revealed that fragments corresponding to 99, 84, 65, 55, 49, 34, 20 and 15kDa were apparently suppressed as intensity of each decreased as Cr^{6+} concentration was increased. SDS-PAGE profile of *B. thuringiensis*208 revealed a different set of fragments that were suppressed (**Fig**. **7**.**1**C). Comparing strain specific

patterns illustrated the presence of reproducible strain-specific distortions. SDS-PAGE profiles of *S. saprophyticus*168, *S.saprophyticus*173 and *S. arlettae*324 were visually very similar revealing similar sets of fragments that are suppressed or induced, the exception being the induction of a fragment corresponding to 24kDa in *S. saprophyticus*176 and the suppression of a fragment corresponding to 120kDa in *S. arlettae*324. Both *B. thuringiensis*208 and *B. cereus*273 shared strain-specific distortions and were visually similar. *B. cereus*273 showed an intense band which was induced with Cr^{6+}. This band was processed for in-gel digestion before being analyzed by MS/MS to identify the fragment. SDS-PAGE profiles (**Fig**. **7.2**) also indicate expression of proteins in Cr^{6+} concentrations up to 200µg mL^{-1}. The protein profile of *S. xylosus*3 (**Fig. 7.3**) was similar to protein profiles of *S. saprophyticus*168, *S.saprophyticus*173 and *S. arlettae*324,

7.3.2 In-solution digestion

7.3.2.1 *Protein identification*

*S. saprophyticus*168 and *B. thuringiensis*208

Three conditions of increasing Cr^{6+} concentrations *viz*., 20, 50 and 100µg mL^{-1} were examined to observe dose-dependent variation in protein expression in *S. saprophyticus*168 and *B. thuringiensis*208. As many as 762 proteins were identified from *S. saprophyticus*168 by mass spectroscopic measurements across all samples with 611, 453, and 474 proteins identified when grown in 0, 20, 50 and 100µg Cr^{6+} mL^{-1}. As many as 687 proteins were identified across all samples in *B. thuringiensis*208. Among these, 308, 334, and 292 proteins were expressed in this isolate grown respectively in 0, 20, 50 and 100µg Cr^{6+} mL^{-1}. More than 150 proteins were differentially expressed in both

isolates under each condition including proteins that were up and down regulated. Of the 762 (*S. saprophyticus*168) and 687 (*B. thuringiensis*208) proteins, nearly 20% (156 and 148 proteins) were without known function.

*S. xylosus*3

Four Cr^{6+} conditions of 50,100, 100, 150 and 200µg mL^{-1} were compared with control containing no Cr^{6+}. As many as 613 proteins were identified by MS measurements across all samples with 83, 83, 366, 94 and 150 proteins enumerated respectively when grown in increasing Cr^{6+} from 50 to 200µg mL^{-1}. Nearly 45% (293 proteins) were unidentified with annotated function as per UniProt database (www.unitprot. org).

7.3.2.2 *Comparison of protein profiles in cultures with and without exposure to chromate*

*S. saprophyticus*168 and *B. thuringiensis*208

Quantitative analysis revealed significant changes in protein expression. In *S. saprophyticus*168 the numbers of up-regulated proteins (**Fig. 7.4.1**A) were 346, 140 and 168 grown in medium with Cr^{6+} metal exposure. As many as 27 (~4%) and 144 proteins (~19%) were commonly up and down-regulated respectively for all conditions compared (**Fig. 7.4.1**B). In *B. thuringiensis*208 (**Fig. 7.4.2**A), 180, 209 and 219 proteins were up-regulated in *B. thuringiensis*208 grown with 20, 50 and 100µg mL^{-1}. As many as, 28 proteins (~4%) were commonly up-regulated and126 proteins were commonly down-regulated (**Fig. 7.4.2**B). Each Cr^{6+} condition/concentration revealed a set of proteins unique to each, with the most number (249 unique proteins) observed when *S. saprophyticus*168 was grown in 20µg mL^{-1}. Substantial down-regulation of proteins was

also observed. In *B. thuringiensis*208, number of proteins unique to each Cr^{6+} concentration was fairly similar.

*S. xylosus*3

Quantitative analyses of proteomic data showed significant expressional changes on metal exposure. As many as 59, 364, 300 and 86 proteins were uniquely up-regulated when *S. xylosus*3 was grown in medium with 50, 100, 150 or 200µg mL^{-1} Cr^{6+} (**Fig**. **7**.**4.3**A). Down-regulation of proteins were also observed, 554, 294, 313 and 527 proteins were down-regulated when grown in medium with 50, 100, 150 or 200µg mL^{-1} Cr^{6+} (**Fig**. **7.4.3**B)

7.3.2.3 *Chromate induced proteins*

*S. saprophyticus*168 and *B. thuringiensis*208

Chromate induced proteins were expressed differentially under varying Cr^{6+} concentrations in both *S. saprophyticus*168 and *B. thuringiensis*208 (**Table** 7.**1**). When grown in medium with Cr^{6+}, stress indicator proteins like alkaline shock proteins, and damage inducible proteins were observed. Additionally, multiple proteins related to protein folding, sorting and degradation like molecular chaperones and preprotein translocase subunits, were up-regulated. Similarly, stress response proteins (thioredoxin, L-lactate dehydrogenase, peroxiredoxin), DNA repair proteins (Rad50, ABC-ATPase UvrA), energy metabolism related (cytochrome P450, ECF transporter S component, F0F1 ATP synthase subunit gamma), metal related (ArsR family transcriptional regulator, arsenate reductase (thioredoxin), cadmium transporter), Iron and sulphur metabolism related (Ferrichrome transport system, sulfite reductase flavoprotein subunit)

and biofilm/sporulation related proteins (putative biofilm-associated protein, stage V sporulation protein B) were also up-regulated.

*S. xylosus*3

Under different Cr^{6+} dosage conditions, different proteins were expressed. When grow in medium with 50μg mL^{-1} Cr^{6+}, one stress protein (OsmC-like protein) and one DNA repair protein (UvrABC system protein A) were up-regulated. When grow in medium with 100μg mL^{-1} Cr^{6+}, six stress proteins (Universal stress family protein, Thioredoxin reductase, oxidoreductase, Alkyl hydroperoxide reductases subunit C, ATP-dependent Clp protease, and SOD), five DNA repair proteins (DNA repair protein radA, DNA 3-methyladenine glysosylase, DNA repair protein RecN, UvrABC system protein A and ImpB family protein), and five peptide folding and degradation related proteins (Bifunctional preprotein translocase, oligopeptide transport ATP-binding protein OppD, Chaperone protein DnaK, 60 kDa chaperonin and ABC transporter) were up-regulated. When grow in medium with 150μg mL^{-1} Cr^{6+}, four stress proteins (SOD, ATP-dependent Clp protease, Alkyl hydroperoxide reductases subunit C and Alkyl hydroperoxide reductase subunit F), six DNA repair proteins (ImpB family protein, UvrABC system protein A, UvrABC system protein B, DNA repair protein and ATP-dependent helicase), and five peptide folding and degradation related proteins (oligopeptide ABC transporter, oligopeptide transport system oppB, ABC transporter, Chaperone protein DnaK and 60 kDa chaperonin) were up-regulated. When grow in medium with 200μg mL^{-1} Cr^{6+}, one DNA repair protein (Competence protein comEA), and three peptide folding and degradation related proteins (ribosome maturation factor RimM, Chaperone protein

DnaK and 60 kDa chaperonin) were up-regulated. No stress proteins were up-regulated when grown in medium with 200µg mL^{-1} Cr^{6+}.

7.3.2.4 *Comparison between isolates*

Proteins in *S. saprophyticus*168 and *B. thuringiensis*208 that were differentially regulated in varying Cr^{6+} concentrations (**Table 7.1**) outlines the classification of proteins expressed by both isolates in response to Cr^{6+} conditions. It was observed that many stress indicator proteins were up-regulated in *S. saprophyticus*168 when grown in 20µg mL^{-1}, in comparison to all the other sub-sets. The stress response was more pronounced in *B. thuringiensis*208 with a greater number of response proteins observed. Proteins involved in DNA repair was more numerous in *S. saprophyticus*168 and unique to each isolate with Ku protein only observed in *B. thuringiensis*208. Proteins involved in protein folding and degradation were similar in both isolates. Energy metabolism was highly up-regulated in *B. thuringiensis*208 whereas metal-related proteins were, in *S. saprophyticus*168. Proteins involved in iron metabolism were greatly expressed in both isolates with maximum number in *S. saprophyticus*168 at 20µg mL^{-1}. Both isolates demonstrated biofilm forming proteins with *B. thuringiensis*208 expressing spore-formation related proteins in large numbers.

7.3.2.5 *Functional characterization of differentially expressed proteins*

All differentially expressed proteins in response to Cr^{6+} conditions were segregated based on Clusters of Orthologous groups (COG) categories (**Fig**. **7.5**). Percentage of proteins in each group varied under different Cr^{6+} conditions. A fraction of proteins (*S.*

*saprophyticus*168: 156 & *B. thuringiensis*208: 148) did not have matches in the UniProt database and were grouped under proteins with "unknown functions". COG proteins from *S. saprophyticus*168 with annotated functions in lipid metabolism, glycan metabolism, cofactor and vitamin metabolism, terpenoid metabolism and secondary metabolite biosynthesis grouped under "other metabolic processes" formed the largest group accounting for ~12% of the total differentially expressed proteins. Following this, the largest groups of proteins were involved in carbohydrate metabolism (~9%), translation (~8%) and membrane transport (~7%), followed by enzymatic functions (~5%) and amino acid metabolism (~5%) (**Fig. 7.5**A). COG proteins from *B. thuringiensis*208 followed a similar pattern with translation (8%), carbohydrate (6%), and amino acid (6%) metabolism accounting for similar fractions (**Fig 7.5**B). In contrast, a greater number of proteins responding to stress (2.1% compared to *S. saprophyticus*168's 0.7%) and energy metabolism (4.3% compared to *S. saprophyticus*168's 2.3%) were observed in *B. thuringiensis*208. For *S. xylosus*3, proteins that were uncategorized or without annotated function were grouped as "Uncharacterized". The largest functionally annotated groups of differentially expressed proteins were involved in enzymatic role (11%), translation (10%), and membrane transport (5%) (**Fig. 7.5**C).

7.3.2.6 *Common proteins under various chromate concentrations*

The 27 commonly up-regulated proteins among the three Cr^{6+} conditions (**Table 7.2**) had a variety of predicted constitutive functions in replication, bio-molecule metabolism, translation, energy metabolism and other cellular processes. Each was observed in all Cr^{6+} conditions but were absent in the control. Fold-changes for each protein (**Table 7.2**)

indicate their levels of expression, for each Cr^{6+} induced stress condition. Similarly for *S. xylosus*3, ten proteins were commonly up-regulated in all the Cr^{6+} conditions with a variety of functions in translation, replication, and energy metabolism. Protein's functions are annotated in **Table 7.3**. As many as 36 proteins were expressed exclusively in the control and were not observed in any Cr^{6+} dosage. These commonly down-regulated proteins were part of transcription, replication and metabolic functions such as organic acid metabolism, terpenoid metabolism and other enzymatic functions.

7.3.3 In-gel digestion

SDS-PAGE of *B. cereus*273 grown in Cr^{6+} revealed a protein band that was intensely expressed (**Fig**. **7**.**1**D). Its intensity increased proportionally with increase in Cr^{6+} concentration. Bands from *B. cereus*273 grown in 50 as well as 100µg mL^{-1} was cut (**Fig. 7.6**A), processed for MS/MS based peptide fragmentation fingerprinting and proteins within were identified. Data searches of both bands (band 1 & 2) revealed the protein to be S-layer protein, based on its high number of distinct peptides (25* and 34* respectively) (**Fig**. **7**.**6**B, *orange and yellow*), high peptide coverage (29.3% and 34.7% respectively) (of the whole protein) and reproducibility.

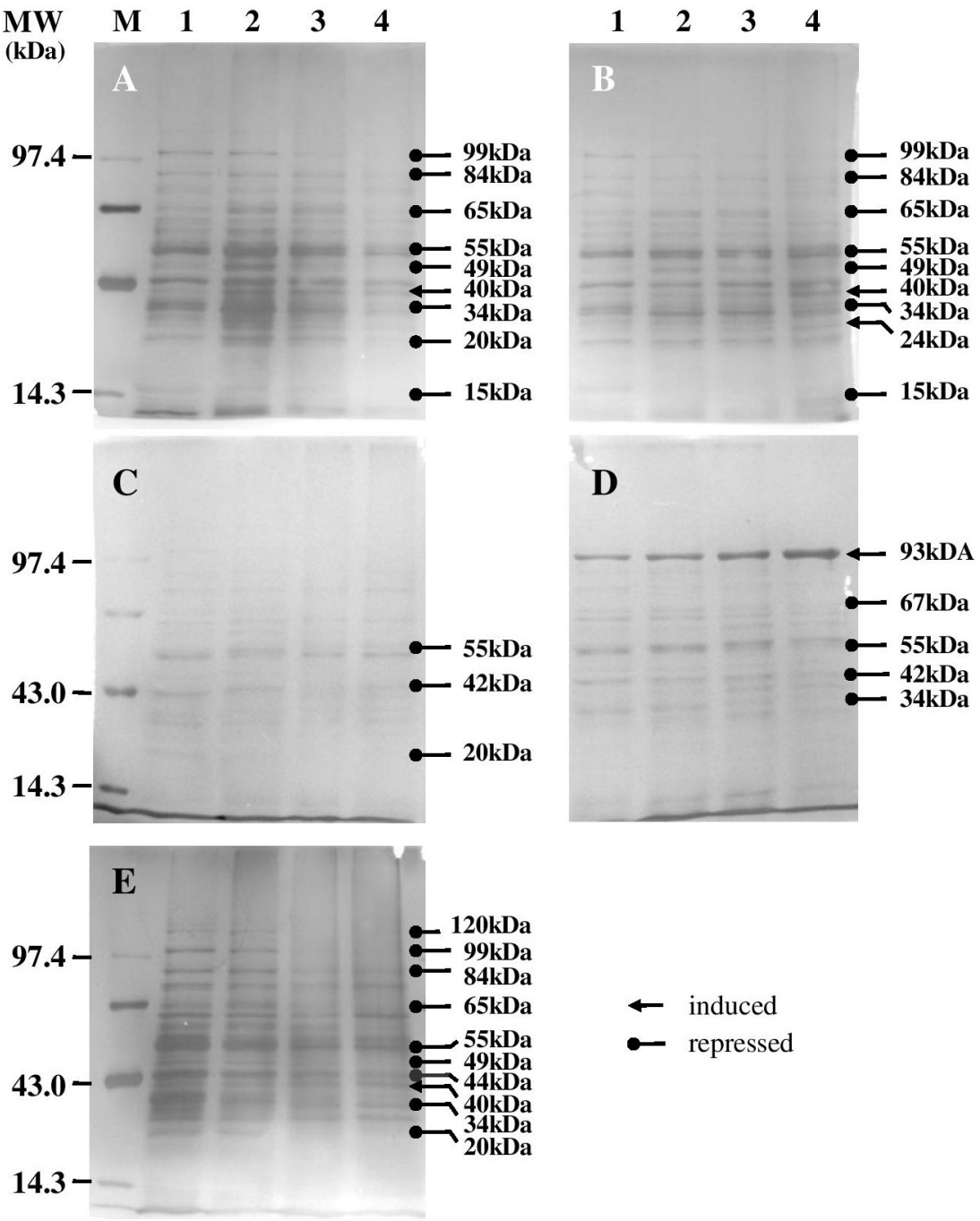


Figure 7.1 SDS-PAGE protein profiles for (A) *S. saprophyticus*168, (B) *S. saprophyticus*176, (C) *B. thuringiensis*208, (D) *B. cereus*273 and (E) *S. arlettae*324 grown in the absence and presence of chromium. *Lanes* **M**, Medium range protein molecular marker (14.3-97.4kDa); **1**, isolate grown in absence of Cr^{6+}; **2**, isolate grown in 20µg mL^{-1} Cr^{6+}; **3**, isolate grown in 50µg mL^{-1} Cr^{6+}; **4**, isolate grown in 100µg mL^{-1} Cr^{6+}. Arrows indicate major proteins that are induced or repressed. Molecular weight of protein standards in kDa are shown on the left

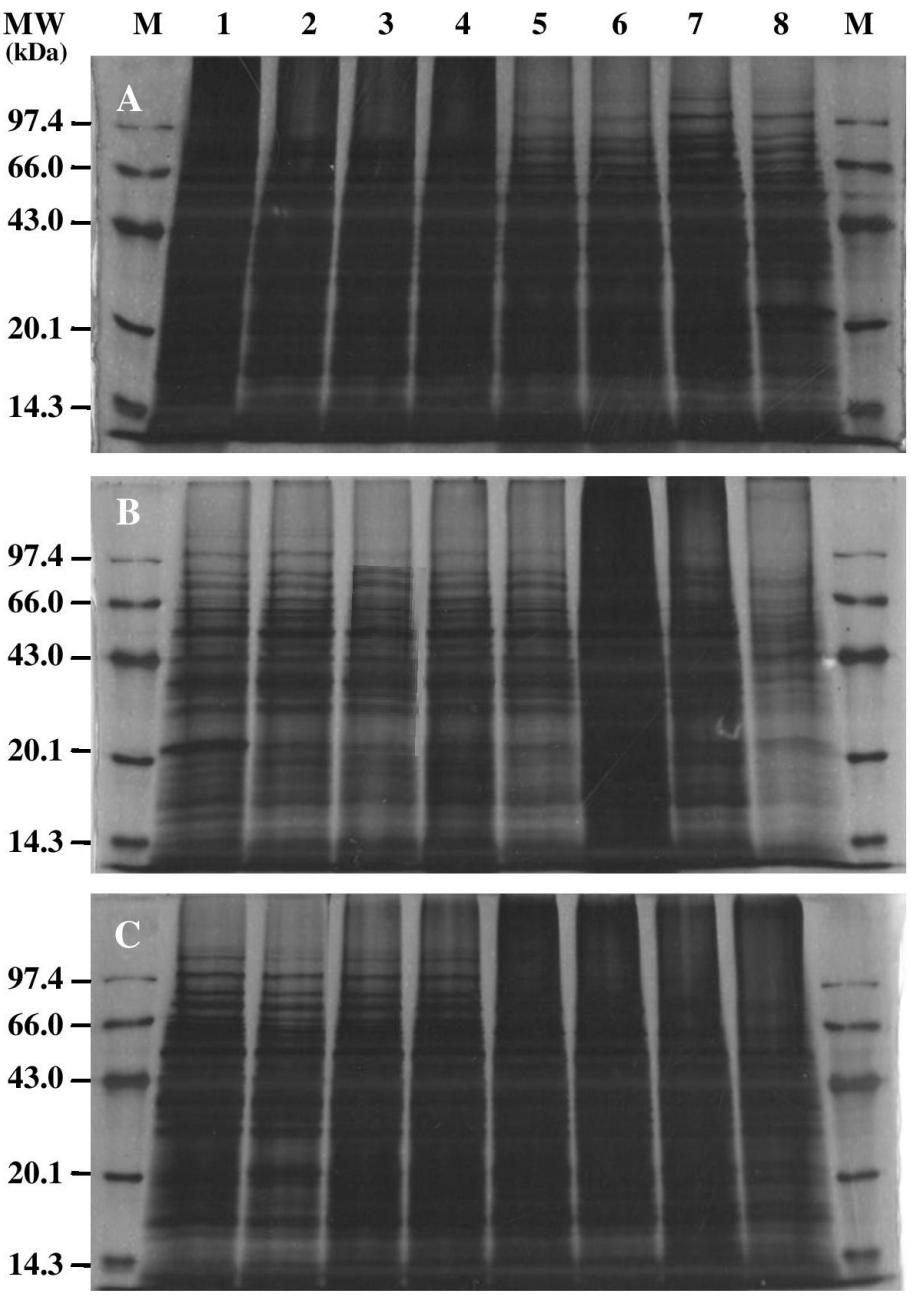


Figure 7.2.1 SDS-PAGE protein profiles for (A) *S. saprophyticus*168, (B) *S. saprophyticus*176 and (C) *B. thuringiensis*208 grown in the absence and presence of chromium. *Lanes* **M**, Medium range protein molecular marker (14.3-97.4kDa); **1**, isolate grown in absence of Cr^{6+}; **2**, isolate grown in 10µg mL^{-1} Cr^{6+}; **3**, isolate grown in 20µg mL^{-1} Cr^{6+}; **4**, isolate grown in 30µg mL^{-1} Cr^{6+}; **5**, isolate grown in 50µg mL^{-1} Cr^{6+}; **6**, isolate grown in 100µg mL^{-1} Cr^{6+}; **7**, isolate grown in 150µg mL^{-1} Cr^{6+}; **8**, isolate grown in 200µg mL^{-1} Cr^{6+}. Arrows indicate major proteins that are induced or repressed. Molecular weight of protein standards in kDa are shown on the left side of gel.

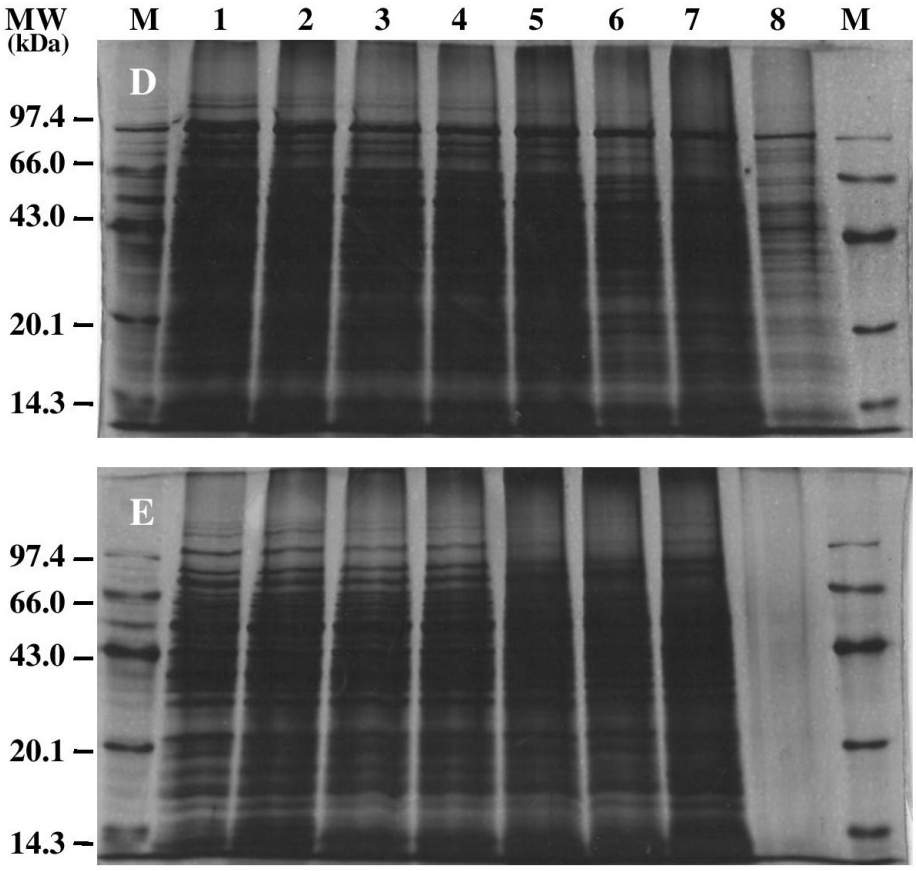


Figure 7.2.2 SDS-PAGE protein profiles for (D) *B. cereus*273 and (E) *S. arlettae*324 grown in the absence and presence of chromium. *Lanes* **M**, Medium range protein molecular marker (14.3-97.4kDa); **1**, isolate grown in absence of Cr; **2**, isolate grown in 10µg mL^{-1} Cr^{6+}; **3**, isolate grown in 20µg mL^{-1} Cr^{6+}; **4**, isolate grown in 30µg mL^{-1} Cr^{6+}; **5**, isolate grown in 50µg mL^{-1} Cr^{6+}; **6**, isolate grown in 100µg mL^{-1} Cr^{6+}; **7**, isolate grown in 150µg mL^{-1} Cr^{6+}; **8**, isolate grown in 200µg mL^{-1} Cr^{6+}. Arrows indicate major proteins that are induced or repressed. Molecular weight of protein standards in kDa are shown on the left side of gel.

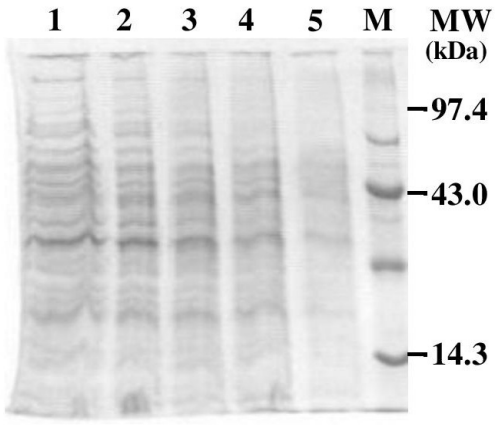


Figure 7.3 SDS-PAGE protein profiles for *S. xylosus*3 grown in the absence and presence of chromium. *Lanes* **M**, Medium range protein molecular marker (14.3-97.4kDa); **1**, isolate grown in absence of Cr^{6+}; **2**, isolate grown in 50µg mL^{-1} Cr^{6+}; **3**, isolate grown in 100µg mL^{-1} Cr^{6+}; **4**, isolate grown in 150µg mL^{-1} Cr^{6+}; **5**, isolate grown in 200µg mL^{-1} Cr^{6+} Arrows indicate major proteins that are induced or repressed. Molecular weight of protein standards in kDa are shown on the left

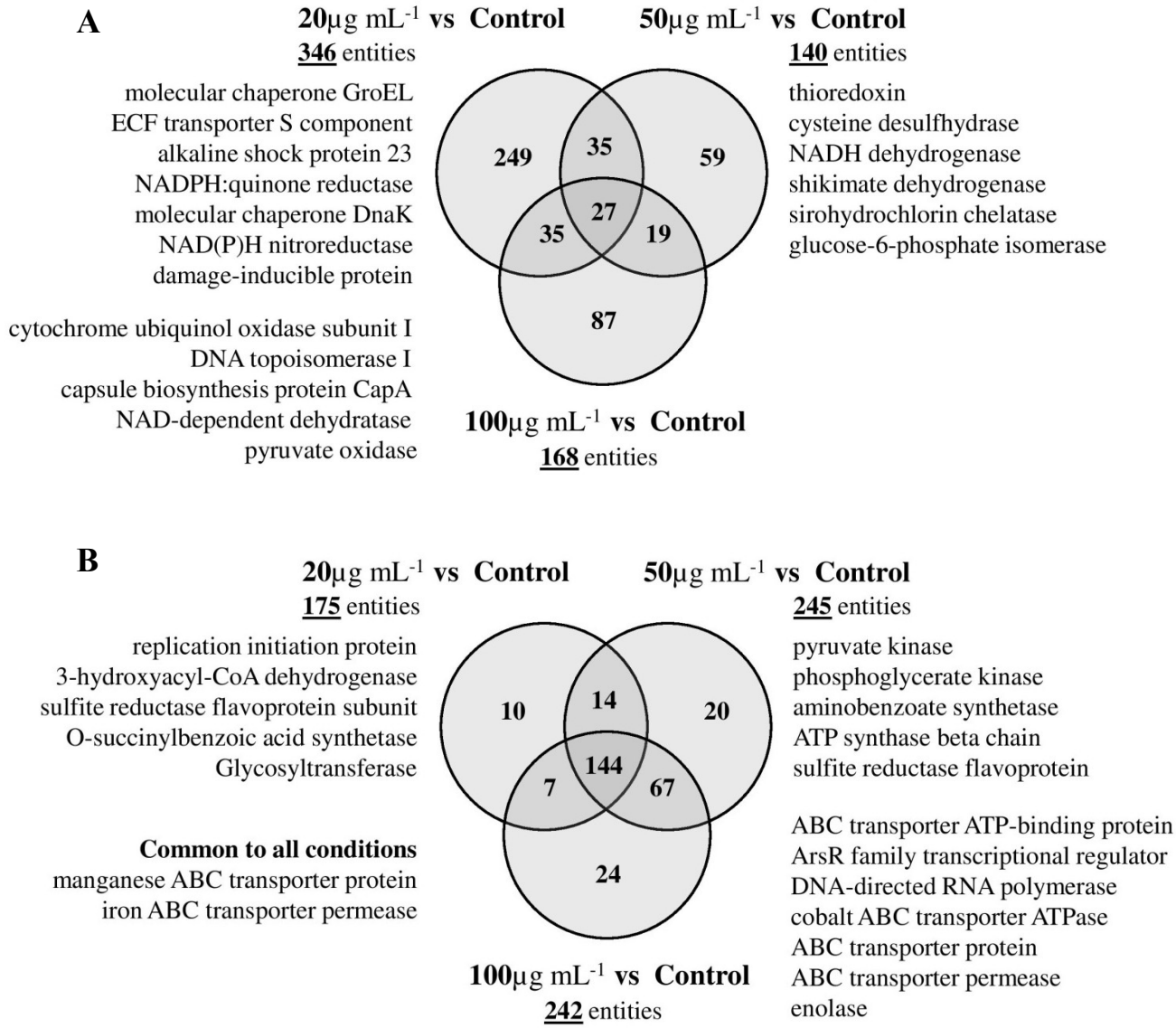


Figure 7.4.1 Distribution of normalized differentially expressed proteins in response to various chromate conditions. Venn diagram showing the number of (A) up-regulated and (B) down-regulated proteins in ***S. saprophyticus*168**; in response to different Cr^{6+} conditions. Examples of proteins unique to each condition (*relative compliment groups in all*) are provided

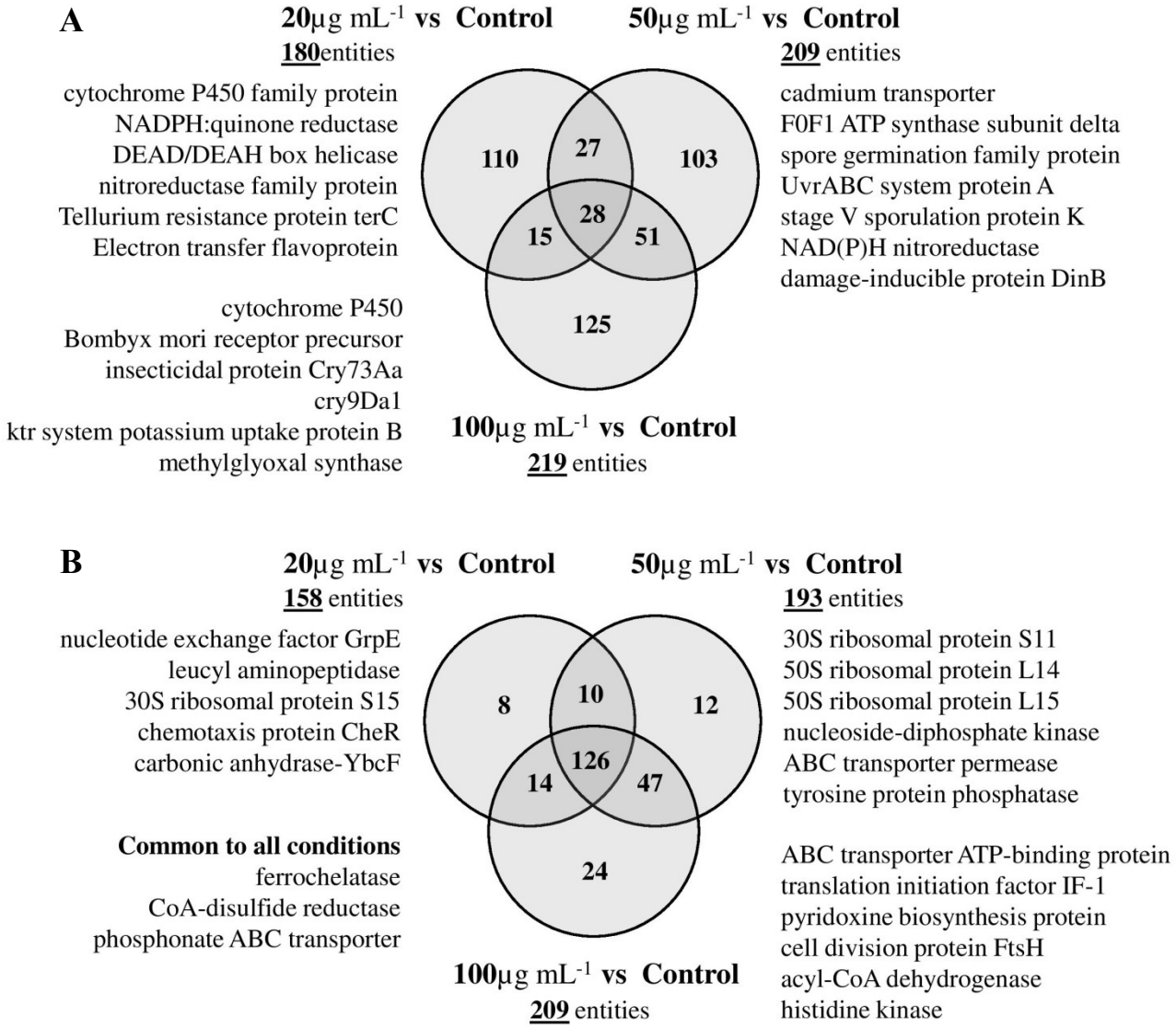


Figure 7.4.2 Distribution of normalized differentially expressed proteins in response to various chromate conditions. Venn diagram showing the number of (C) up-regulated and (D) down-regulated proteins in ***B. thuringiensis*208**; in response to different Cr^{6+} conditions. Examples of proteins unique to each condition (*relative compliment groups in all*) are provided

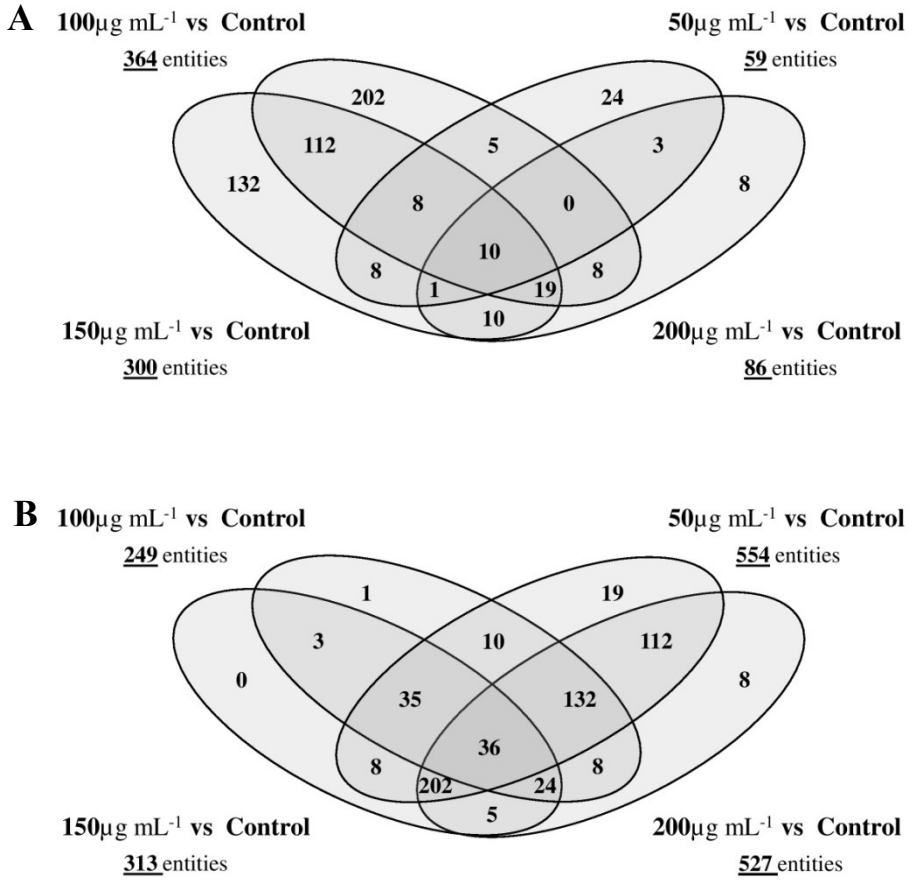


Figure 7.4.3 Distribution of normalized differentially expressed proteins in response to various Cr^{6+} conditions. Venn diagram showing the number of (A) up-regulated and (B) down-regulated proteins in ***S. xylosus*3**; in response to different Cr^{6+} conditions.

Table 7.1 List of proteins that may have role in Cr^{6+} resistance or reduction LANDSCAPE

NIOER168 (*Staphylococcus saprophyticus)*			**NIOER208** (*Bacillus thuringiensis*)		
20μg mL^{-1} Cr^{6+}	**50**μg mL^{-1} Cr^{6+}	**100**μg mL^{-1} Cr^{6+}	**20**μg mL^{-1} Cr^{6+}	**50**μg mL^{-1} Cr^{6+}	**100**μg mL^{-1} Cr^{6+}
Stress markers					
damage-inducible protein damage-inducible protein DinB alkaline-shock related protein competence/damage-inducible protein A alkaline shock protein 23	damage-inducible protein damage-inducible protein DinB alkaline-shock related protein	damage-inducible protein damage-inducible protein DinB alkaline-shock related protein competence/damage-inducible protein A	universal stress protein UspA putative universal stress protein general stress protein	putative universal stress protein general stress protein	universal stress protein UspA
Stress response					
thioredoxin reductase L-lactate dehydrogenase NAD(P)H nitroreductase	thioredoxin L-lactate dehydrogenase	L-lactate dehydrogenase	peroxiredoxin lipid hydroperoxide peroxidase [a] superoxide dismutase catalase	peroxiredoxin lipid hydroperoxide peroxidase [a] alkyl hydroperoxide reductase subunit F	peroxiredoxin lipid hydroperoxide peroxidase [a] alkyl hydroperoxide reductase subunit F
Biofilm and sporulation related					
stage V sporulation protein B putative biofilm-associated protein	putative biofilm-associated protein capsular biosynthesis protein	putative biofilm-associated protein capsular biosynthesis protein capsule biosynthesis protein CapA	stage V sporulation protein S stage V sporulation protein G spore germination protein S-layer protein capsule biosynthesis protein CapK	stage V sporulation protein S stage V sporulation protein G stage V sporulation protein K spore germination protein spore germination family protein S-layer protein	stage V sporulation protein G S-layer protein
DNA repair					
DNA repair	DNA repair	DNA repair	Ku protein	Ku protein	Ku protein

protein Rad50	protein Rad50	protein Rad50			
ABC-ATPase UvrA	ABC-ATPase UvrA	excinuclease ABC subunit A		UvrABC system protein A	DNA-damage repair protein (DNA polymerase IV) (plasmid)
excinuclease ABC subunit A	excinuclease ABC subunit A	restriction endonuclease subunit S DNA recombination/repair protein RecA			
Protein folding, transport and degradation					
molecular chaperone GroEL	molecular chaperone GroEL	molecular chaperone GroEL	chaperonin GroEL	chaperonin GroEL	chaperonin GroEL
molecular chaperone DnaJ	molecular chaperone DnaJ	molecular chaperone DnaJ	co-chaperone GroES	co-chaperone GroES	co-chaperone GroES
molecular chaperone DnaK	preprotein translocase subunit SecY	preprotein translocase subunit SecY	molecular chaperone DnaK	molecular chaperone DnaK	molecular chaperone DnaK
preprotein translocase subunit SecY	preprotein translocase subunit SecA	preprotein translocase subunit SecA	10 kDa chaperonin	10 kDa chaperonin	10 kDa chaperonin
preprotein translocase subunit SecD			ATP-dependent Clp protease proteolytic subunit	ATP-dependent Clp protease proteolytic subunit preprotein translocase subunit SecA	ATP-dependent Clp protease proteolytic subunit
Energy metabolism					
dihydrolipoamide dehydrogenase [b]	dihydrolipoamide dehydrogenase [b]	cytochrome ubiquinol oxidase subunit I	dihydrolipoyl dehydrogenase [b]	dihydrolipoyl dehydrogenase [b]	dihydrolipoyl dehydrogenase [b]
F0F1 ATP synthase subunit gamma			F0F1 ATP synthase subunit beta	F0F1 ATP synthase subunit beta	F0F1 ATP synthase subunit beta
ECF transporter S component			F0F1 ATP synthase subunit alpha	F0F1 ATP synthase subunit alpha	F0F1 ATP synthase subunit alpha
			electron transfer flavoprotein subunit beta	F0F1 ATP synthase subunit delta	electron transfer flavoprotein subunit beta
			electron transfer flavoprotein subunit alpha	electron transfer flavoprotein	cytochrome P450

				subunit beta	
			cytochrome P450 family protein	electron transfer flavoprotein subunit alpha	
Metal related					
ArsR family transcriptional regulator	ArsR family transcriptional regulator	ArsR family transcriptional regulator	Tellurium resistance protein terC	cadmium transporter	
arsenical pump-driving ATPase (plasmid)	arsenical pump-driving ATPase (plasmid)	copper-translocating P-type ATPase			
arsenate reductase (thioredoxin)	copper-translocating P-type ATPase				
copper-translocating P-type ATPase					
copper resistance protein CopC					
Transport/iron or sulphur metabolism					
Fe-S protein assembly chaperone HscA	Fe-S protein assembly chaperone HscA	Fe-S protein assembly chaperone HscA	Fe-S cluster assembly protein SufB	Fe-S cluster assembly protein SufB	Fe-S cluster assembly ATPase SufC
heme ABC transporter permease	heme ABC transporter permease	ferrous iron transporter A	Ferrichrome transport system permease fhuG	Fe-S cluster assembly ATPase SufC	thioredoxin-disulfide reductase
ferrous iron transporter A	sulfite reductase flavoprotein subunit	sulfite reductase flavoprotein subunit	thioredoxin-disulfide reductase	Fe-S cluster assembly protein SufD	
ferrichrome ABC transporter substrate-binding protein				ferrochelatase	
heme A synthase				thioredoxin-disulfide reductase	
sulfite reductase flavoprotein subunit					
cation-efflux system membrane protein (plasmid)					
Enymes of interest					
NADPH:quinone reductase	oxidoreductase	oxidoreductase	NAD(P)/FAD-dependent oxidoreductase	NAD(P)H nitroreductase	FAD dependent oxidoreductase

NAD(P)H nitroreductase FMN reductase FAD reductase oxidoreductase nitroreductase	nitroreductase	NADPH:quinone reductase nitroreductase family protein	NADH dehydrogenase-like protein YjlD	NADH dehydrogenase-like protein YjlD oxidoreductase

Table 7.2 Up-regulated proteins common to all Cr^{6+} stress conditions in *S. saprophyticu*168 and *B. thuringiensis*208. Genes are based on protein nomenclature with gene indicated in **bold** with species specific code beside it

Genes based on Protein	**Annotation**	**Known function**	Fold change in **20**μg mL^{-1} **Cr^{6+}** *	Fold change in **50**μg mL^{-1} **Cr^{6+}** *	Fold change in **100**μg mL^{-1} **Cr^{6+}** *
*Staphylococcus saprophyticu*168 (**NIOER168**)					
sufA	Fe-S protein assembly chaperone HscA	protein maturation	19.54	17.43	18.29
gapA1 SAMEA2297795_00329	glyceraldehyde-3-phosphate dehydrogenase	glycolysis	19.60	17.97	17.92
EP54_11100	ATP-dependent exonuclease V alpha subunit	helicase activity	20.26	18.34	17.25
aspC SAMEA2297795_02101	aspartate aminotransferase	pyridoxal phosphate binding	19.37	17.65	17.60
polC CD119_00260	DNA polymerase III alpha chain	transcription process	18.49	17.62	18.08
ASS94_12285	lantibiotic ABC transporter ATP-binding protein	ATPase activity	17.99	16.22	17.34
SE00_12840	hydrolase TatD	endodeoxyribonuclease activity	18.00	16.65	17.28
SSP2195	ABC transporter permease	transmembrane transport	4.14	3.71	5.57
alaS SSP1143	alanine--tRNA ligase	alanyl-tRNA aminoacylation	19.70	17.95	18.93
A0131_04815	LysR family transcriptional regulator	transcription regulation	17.91	17.30	18.48
SSP1389	4-oxalocrotonate tautomerase	isomerase activity	19.01	18.50	19.61
CD120_00065	copper-translocating P-type ATPase	metal related	20.42	19.14	20.08
carB SSP1569	carbamoyl phosphate synthase large subunit	Pyrimidine metabolism	19.20	18.63	18.21
A0131_01725	carnitine dehydratase	catalytic activity	19.57	18.27	18.89
***repB*_2** SAMEA2297795_02690	replication protein	DNA binding	17.13	16.33	16.43
*Bacillus thuringiensis*208 (**NIOER208**)					
***gap*1**	glyceraldehyde-3-phosphate dehydrogenase	glycolysis	3.23	2.70	2.45
C2L81_3012	alpha-keto acid	enzyme	2.30	2.86	1.42

0	dehydrogenase subunit E2				
COK72_32010	molecular chaperone DnaK	protein folding	3.80	3.24	2.32
rplD BC_0132	50S ribosomal protein L4	structural constituent of ribosome	1.05	2.19	2.88
glmS BC_0190	glutamine--fructose-6-phosphate transaminase	glutamine metabolism	5.02	3.35	2.42
BTG_04710	cysteine synthase A	pyridoxal phosphate binding	5.43	1.03	2.58
deoD BC_1463	purine nucleoside phosphorylase	nucleoside metabolism	1.82	2.08	3.94
***asd*2** BCA_3897	aspartate-semialdehyde dehydrogenase	L-methionine biosynthesis	7.64	6.14	6.28
glmM BC_0188	phosphoglucosamine mutase	phosphoglucosamine mutase activity	17.44	18.94	17.00
	lipid hydroperoxide peroxidase	antioxidant	17.07	20.06	18.29
CON36_04620	recombinase RecA	DNA repair	17.58	18.18	17.82
C2L96_27540	Ku protein	DNA repair	18.40	17.22	18.31
slp	S-layer protein	S-layer	20.13	19.91	19.74
trxB BCE33L4851	thioredoxin-disulfide reductase	removal of superoxide radicals	19.11	18.83	18.43
***gerAA*_5** BLX06_15150	spore gernimation protein	spore germination	2.66	3.01	3.48
AT272_14220	multidrug transporter AcrB	transporter activity	14.62	14.19	14.39

* in comparison to the fold change observed in control (**0**µg mL^{-1}), *system generated*

Table 7.3 Up-regulated proteins common to all Cr^{6+} stress conditions in *S. xylosus*3

Genes based on Protein ID	**Annotation**	**Known function**	Fold change in **50**µg mL^{-1} **Cr^{6+}** *	Fold change in **100**µg mL^{-1} **Cr^{6+}** *	Fold change in **150**µg mL^{-1} **Cr^{6+}** *	Fold change in **200**µg mL^{-1} **Cr^{6+}** *
tuf	Translation elongation factor Tu	Protein biosynthesis	1.138	6.853	5.394	2.057
SXYLSMQ121_1549	Translation elongation factor Ts	Protein biosynthesis	1.113	7.085	4.771	1.767
HMPREF1208_00854	DNA-binding protein HU	DNA condensation	1.296	1.148	1.037	7.573
***rsp*A**	30S ribosomal protein S1	Component of ribosom	1.283	7.014	6.029	2.101
-	Small GTP-binding protein	GTP binding	3.413	14.275	12.971	7.391
C273_07362	Carboxyl-terminal protease	membrane component with peptidase activity	-	-	-	-
groS	10 kDa chaperonin	stress response and chaperone	-	-	-	-
ERYG_03274	YSIRK family signal peptide	transmembrane transport	-	-	-	-
rplW	50S ribosomal protein L23		-	-	-	-
-	uncharacterized		-	-	-	-

* in comparison to the fold change observed in control (**0**µg mL^{-1}), *system generated*

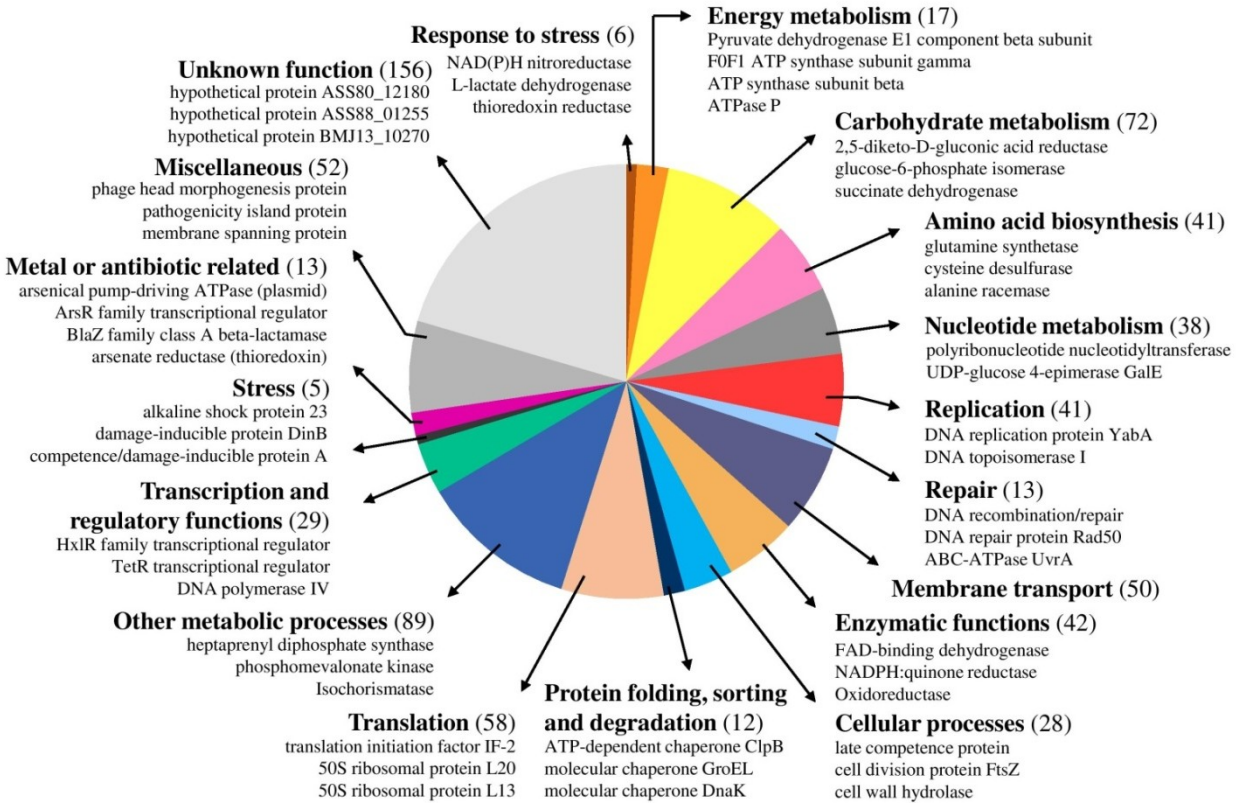


Figure 7.5A **Differentially expressed proteins in *S. saprophyticus*168** as a result of Cr^{6+} treatment were categorized into *carbohydrate metabolism*, *amino acid metabolism*, *nucleotide metabolism*, *replication*, *repair*, *membrane transport*, *enzymatic functions*, *cellular processes*, *protein folding and degradation*, *translation*, *transcription and regulatory functions*, *stress*, *metal or antibiotic related*, *energy metabolism*, *other metabolic processes*, *miscellaneous* and *unknown function* according to COG functional categories. The number in brackets indicates number of proteins

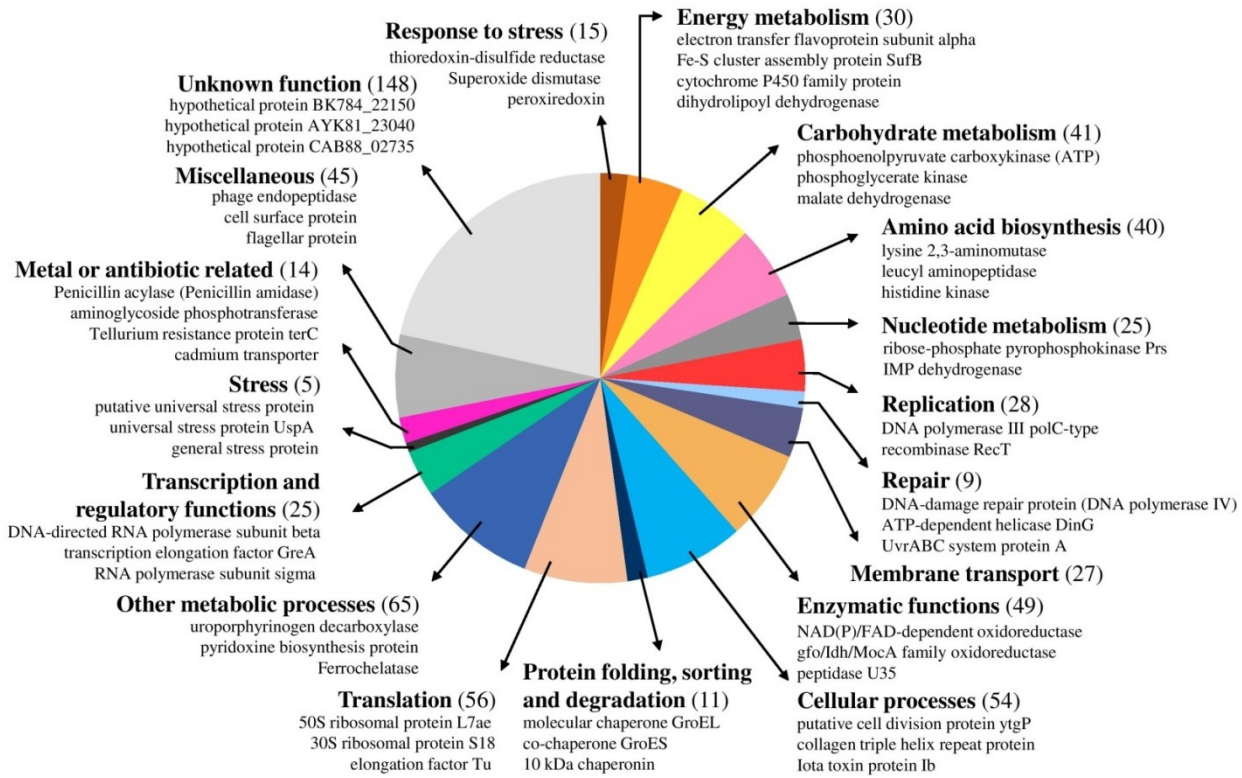


Figure 7.5B **Differentially expressed proteins in *B. thuringiensis*208** as a result of Cr^{6+} treatment were categorized into *carbohydrate metabolism*, *amino acid metabolism*, *nucleotide metabolism*, *replication*, *repair*, *membrane transport*, *enzymatic functions*, *cellular processes*, *protein folding and degradation*, *translation*, *transcription and regulatory functions*, *stress*, *metal or antibiotic related*, *energy metabolism*, *other metabolic processes*, *miscellaneous* and *unknown function* according to COG functional categories. The number in brackets indicates number of proteins

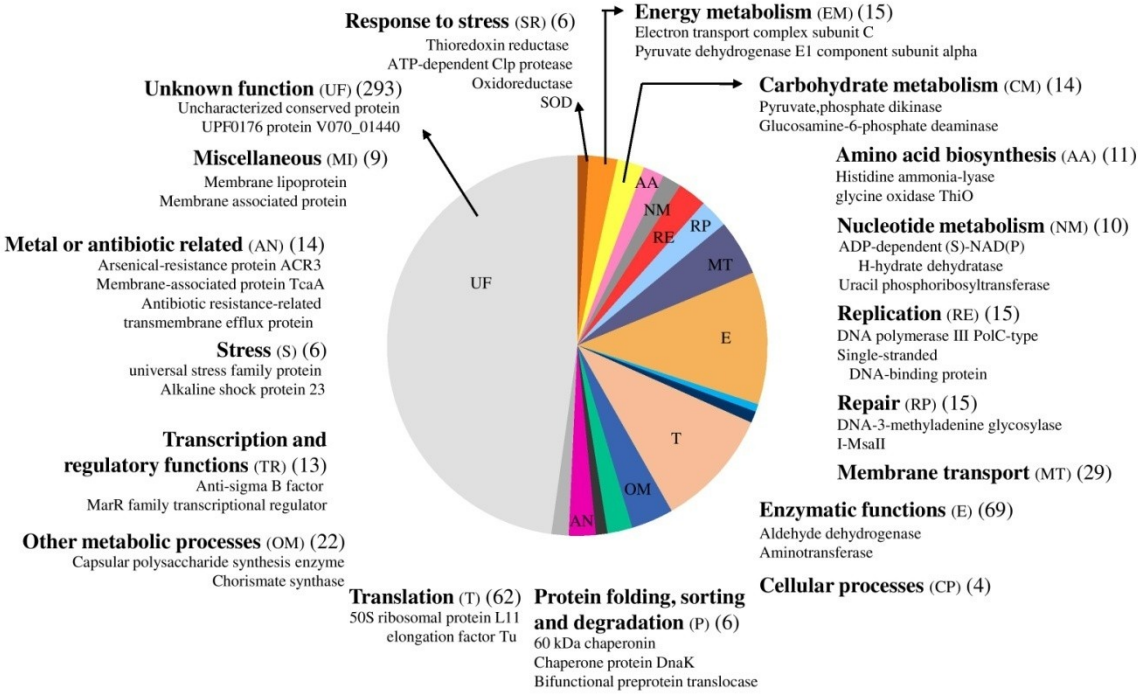


Figure 7.5C **Differentially expressed proteins in *S. xylosus*3** as a result of Cr^{6+} treatment were categorized into *carbohydrate metabolism*, *amino acid metabolism*, *nucleotide metabolism*, *replication*, *repair*, *membrane transport*, *enzymatic functions*, *cellular processes*, *protein folding and degradation*, *translation*, *transcription and regulatory functions*, *stress*, *metal or antibiotic related*, *energy metabolism*, *other metabolic processes*, *miscellaneous* and *unknown function* according to COG functional categories. The number in brackets indicates number of proteins

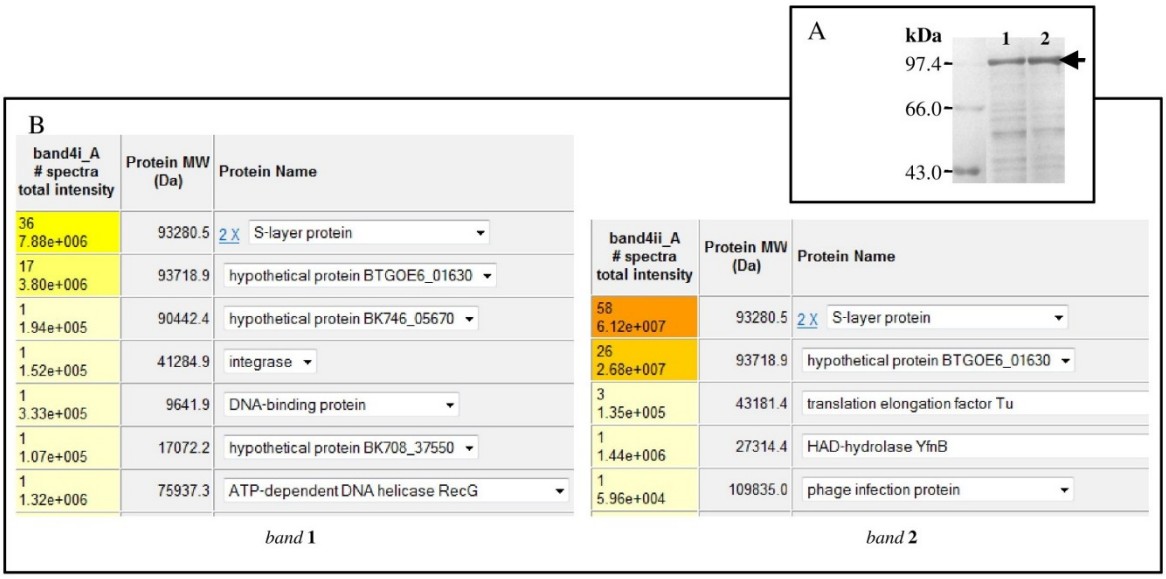


Figure 7.6 In-gel protein identification using peptide fragmentation fingerprint (PFF). The SDS-PAGE gel of isolate preparation *B. cereus*273 grown in Cr^{6+} is shown in (A) with the bands analyzed labeled with an arrow. (B) Spectrum mill searches based on the mass fingerprint spectra generated from MS/MS of in-gel digested bands **1** and **2**. MS/MS search was done against NCBI *Bacillus cereus* database acquired from NCBI protein database

7.4 Discussion

SDS-PAGE based analysis of expressional differences

In this study, expressional proteomics was used to examine chromium induced protein expression in select marine CRB. SDS-PAGE was performed on whole-cell protein extracts of *S. saprophyticus*168, *S. saprophyticus*176, *B. thuringiensis*208, *B.cereus*273 and *S. arlettae*324 grown in the absence and in the presence of 20, 50 and 100µg mL^{-1} Cr^{6+}. Concentrations of Cr^{6+} were chosen to reflect their ability to detoxify Cr^{6+} rather than maximum concentration tolerated (Pereira *et al.*, 2017b). SDS-PAGE results represent a preliminary indication of the suppression/repression of both high and low-molecular weight proteins. Most of the proteins are possibly of constitutive function and are suppressed rather than repressed. There have been reports of proteins that have been actively repressed by bacteria (Chandrangsu *et al*., 2017; Quintana *et al*., 2017) especially those involved in metal uptake and transport. This has been described as an adaptation mechanism to limit the entry of toxic ions intracellularly and it is likely that some of the proteins observed to be down-regulated in this study may have a similar role. A few proteins were also observed to be up-regulated representing a regulatory response of proteins that may confer either protection or response. In *B. thuringiensis*273, a considerable amount of polypeptide was expressed on 93kDa regardless of test Cr^{6+} concentration indicating that it/they may have a major role in resistance mechanism. Additionally, SDS-PAGE profiles revealed reproducible strain-specific patterns (Goris *et al*., 2001) that could be widely used to examine strain variation between bacterial species (Caugant *etal*., 1987; Davies *et al*., 1996). These distortions in *Staphylococci* indicate that most proteins of note had molecular weights between 20 to 65kDa. Polypeptides that

were anomalous (present in some, absent in others) could play a role in species specific functions.

In-gel based analysis of band 93kDa from SDS-PAGE of *B. cereus*273

Investigation of the major polypeptide band from SDS-PAGE of *B. cereus*273 with molecular weight of 93kDa, followed by in-gel digestion and LC-MS/MS revealed it to be "S-layer protein". "S-layer" or "surface layer proteins are exoproteins that form bi-dimensional crystalline arrays which cover bacterial surfaces and help participate in cellular protection or adhesion (Sara *et al.*, 2000). Cell walls and/or cell wall associated extracellular polymeric substance (EPS) such as capsules, slimes or S-layers form potent matrices with high affinities for dissolved metal, due to the abundant presence of metal-binding functional groups like carboxylates, sulfates phosphates etc. This phenomenon has been well reported in bacteria with metal biosorption properties (Green-Ruiz, 2006; Lo *et al*., 2003; Velásquez and Dussan, 2009). It is thus very likely that *B. cereus*273 also utilizes biosorption mechanism (in addition to others) to adsorb and sequester Cr^{6+} on to its surface to resist Cr^{6+}, and in the process remove it from liquid medium. The observation of remnants of an EPS layer shown in Figure **5.6**F, the positive detection of S-layer protein and its over-expression corroborates the presence of this mechanism. This is supported by Merroun *et al.* (2005) and François *et al.* (2012) who report of other bacilli producing S-layer proteins that complex metal ions. Additionally, in-solution digestion, followed by LC-MS/MS of *B. thuringiensis*208 whole-cell protein revealed the over-expression of proteins involved in capsule and S-layer formation (**Table 7.1**).

Although of a different *Bacillus* species, this indicates that it may be a vital resistance mechanism.

In-solution based analysis of expressional differences in *S. saprophyticus*168 and *B. thuringiensis*208

SDS-PAGE allowed for pattern discerning as well as visualization of whole-cell protein profiles. In solution LC-MS/MS was used to further that understanding and to examine and delineate expressional proteomic differences in response to varying Cr^{6+} concentrations. In this study, *S. saprophyticus*168 and *B. thuringiensis*208 were investigated to explore possible resistance and detoxification mechanisms. Both isolates showed decreased growth (OD_{600}) and biomass yield with increase in Cr^{6+} concentration. This is due to the toxicity of Cr, primarily attributable to increase in oxidative stress *via* reactive intermediates formed during intracellular reduction of Cr^{6+} to Cr^{3+}. This reduction, brought about by cytochrome P-450, cytoplasmic proteins and/or mitochondrial proteins generates short-lived intermediates of Cr (Cr^{+4} or Cr^{5+}). The reduction is accompanied by the reduction of molecular oxygen to peroxide, followed by the formation of hydrogen peroxide and reactive oxygen species (ROS). These ROS, including single oxygen (O) and superoxide (O^{2-}), hydroxyl (OH^{-}) and hydrogen peroxide (H_2O_2) radicals combine readily with DNA-protein complexes as well as other biomolecules, hindering normal physiological function (Pereira *et al*., 2017b). On comparative analysis, both isolates expressed stress indicators (**Table 7.1**) indicative of cellular damage. *S. saprophyticus*168 was particularly affected when grown in 20µg mL^{-1} Cr^{6+}. In response, multiple mechanisms of resistance to metals were predicted to be

activated with a particular focus on enzymes and pathways that performed cell-redox homeostasis, DNA repair and proteins involved in mis-folded proteins.

Many superoxide removal proteins like L-lactate dehydrogenase, superoxide dismutase, catalase etc. were up-regulated. They are known to catalyze decomposition of superoxide anion (O_{2*-}) to hydrogen peroxide and molecular oxygen, and may play a role in relieving the oxidative stress. Thompson *et al.*, (2010) as well as Ackerly *et al*., also detected similar proteins in Cr^{6+} challenged bacterial cells. Proteins such as lipid hydroperoxide peroxidase, alkyl hydroperoxide reductase, thioredoxin reductase etc were also up-regulated constituting proteins involved in cell-redox homeostasis as a response to oxidative stress. These were also observed by Carmel and Storz (2000) as well as in marine *S. xylosus* reported in Pereira *et al.*, (2017b).

Oxidative stress inevitably and undoubtedly results in DNA damage. Both isolates up-regulated multiple proteins with predicted functions in DNA repair and DNA repair complexes like ABC-ATPase UvrA, DNA-damage repair protein (DNA polymerase IV) , Ku protein etc. Many of these complexes form repair systems that scan DNA for abnormalities, recognize or process them. An increased DNA repair response observed in *S. saprophyticus*168 was consistent with the number of stress indicators implying that it was affected by Cr^{6+} by a greater extent when compared with *B. thuringiensis*208.

Chaperones and peptide transport related proteins play a role in folding and processing of mis-folded proteins. These proteins were observed to be in high demand to cope with the large number of mis-folded proteins generated directly as a consequence of oxidative based modification (Gregersen *et al*., 2006). Proteins such as preprotein translocase subunit SecA amd ATP-dependent Clp protease proteolytic subunit were up-

regulated in addition to molecular chaperones. Exogenous chromate (CrO_4^{2-}) shares structural similarity with sulfate (SO_4^{2-}) and enters via sulfate transporters. It was observed that some transport related proteins (ABC transporters) and proteins involved in iron and sulfur metabolism (ferrochelatase, CoA-disulphide reductase) were down-regulated. Thompson *et al.* (2010) observed a similar repression of certain proteins. It is likely that these proteins play an active role in Cr^{6+} uptake, and their repression would limit uptake.

In contrast to protein repression, other proteins were observed to be induced in Cr^{6+} conditions. Several proteins known to be components of the iron oxidation pathway as observed by (Felicio *et al.*, 2003) were up-regulated such as Fe-S protein assembly chaperone HscA, heme ABC transporter permease, ferrous iron transporter A, ferrichrome ABC transporter substrate-binding protein and heme A synthase. Many reports (summarized in Pereira *et al.*, 2017b) reveal a direct link between Cr^{6+} sensitivity and iron availability, also suggesting its vital role in maintaining intracellular redox environment and providing a defense against metal toxicity.

With up-regulation of metal and anti-biotic related proteins, some membrane proteins (also observed in SDS-PAGE) were down- regulated. Membrane proteins allow the passage of ions through the formation of hydrophilic channels (Achouak *et al.*, 2001) that span the membrane. Heavy metals, antibiotics, aromatic compounds and other xenobiotics can decrease the amounts of channels. This is supported by reports that demonstrate a relationship between membrane protein deficiency and an increase in metal resistance in bacterial isolates (Felicio *et al.*, 2003). Interestingly, no proteins were observed with direct connotation to CHR protein families or other proteins concerning

Cr^{6+}. Instead, proteins related to other metals were expressed with a large number attributed to arsenic. This suggests that proteins with functions related to chromium may be less well annotated in comparison to other metals. Another hypothesis may be, that these proteins are annotated, but not with functions concerning Cr^{6+} as observed by the large fraction of up-regulated oxidoreductases and nitroreductases. These enzymes may possess dual function possibly utilizing chromate as an electron donor and acting as chromate reductases. Sequence analysis as well as protein isolation may help in confirmation.

In addition to responses of known chromate reducing enzymes during oxidative stress, several other proteins and enzymes such as cytochrome-P450 (Mikalsen et al., 1991) have been implicated in Cr^{6+} reduction. These particular proteins form part of electron transport chains and may be active in the transport of electrons used for metal ion reduction as earlier postulated by Kwak *et al.*, (2003). These proteins were expressed in both isolates and constituted part of their energy metabolism. Although such proteins were expressed in both isolates, higher numbers were up-regulated in *B. thuringiensis*208. It is likely that its energy metabolism or shuttling of electrons may have a role to play in its survival, and/or in Cr^{6+} metabolism.

Claude and Adolphe (2012) reported the up-regulation of a putative pyruvate dehydrogenase E1 component beta subunit and a putative ribosome-associated protein Y in *S. saprophyticus* subsp. *saprophyticus* ATCC 15305. Both proteins were expressed during Cr^{6+} stress and were homologous to proteins reported to be functional in chromate (*S. oneidensis*) and osmotic (*S. aureus*) stress. Bar *et al*, (2007) described the response of *Klebsiella pneumonia* exposed to cobalt and lead, using a proteomic approach. They

found L-isoaspartate protein carboxymethyltransferase type II and DNA gyrase A to be differentially over-expressed under metal stress. Zhai *et al*., (2017) identified key proteins involved in the cadmium (Cd) tolerance of *Lactobacillus plantarum* strains. They postulated that tolerance was a result of (i) energy conservation survival mode, (ii) induction of cellular defenses and repair systems, (iii) increased synthesis of amino acids, (iv) Cd binding and cell wall biosynthesis ability, (v) regulation of ion transport and several other key proteins. Moore *et al.*(2005) found that control of metal ion uptake, storage and efflux genes in *Bacillus subtilis* strains allowed them to tolerate high concentrations of copper, cadmium, silver, nickel, zinc and arsenic ions. Additionally, reports of Lee *et al*., (2001), Wang and Crowley (2005), Chourey *et al.* (2006) and Kiliç *et al*. (2010) identified many proteins (using proteomics techniques) involved in protein biosynthesis, energy production, glutathione synthesis, sulfur assimilation, exopolysaccharide synthesis and ion transport; as well as metallothioneins and oxidoreductases that were up-regulated in metal stress conditions.

For *S. saprophyticus*168 and *B. thuringiensis*208 proteins with predicted adaptive functions can be grouped as oxidative stress response (with superoxide removal and enhancement of free-radical detoxifying activities), DNA repair response and activation of amino acid synthesis. Up-regulation of proteins involved in peptide transport (and folding), iron metabolism and energy metabolism suggest that different systems contribute simultaneously. The protein profiles generated using LC-MS/MS analysis reveal several insights into differential expression of proteins under Cr^{6+} stress. The proteins common to all conditions either up or down-regulated suggest that some

constitutive functions may be unaffected. This implies that both isolates are can grow under considerable Cr^{6+} stress.

In-solution based analysis of expressional differences observed in *S. xylosus*3

In response to oxidative stress generated when grown in medium with 50µg mL^{-1} Cr^{6+}, only one DNA repair and stress protein were up-regulated. This minor-defense implies that this concentration posed insignificant risk to growth and that damage caused may be minimal. As discussed earlier, oxidative stress is attributable to reactive intermediates formed during intracellular degradation of Cr^{6+} to Cr^{3+} (Pereira *et al*., 2017b) brought about by intracellular proteins or by mitochondrial action. Short lived Cr^{4+} and Cr^{5+} intermediates formed are accompanied by ROS formation. These highly reactive radicals readily bind to important biological molecules and impair their function. They form DNA adducts, protein complexes and damage lipid constructs largely affecting cellular function (Thompson *et al.*, 2010). When grown in higher concentrations of 100 and 150µg mL^{-1} Cr^{6+}, defense responses were increased with respect to the number of proteins expressed that confer protection. In addition to DNA repair proteins and stress proteins (which increased in number), peptide folding and degradation related proteins as well were up-regulated. DNA repair proteins form recognition complexes that detect, locate and repair lesions that form on DNA (Storz *et al.*, 2000). Similarly, peptide folding and degradation related proteins help to cope with the large number of mis-folded proteins as well as degrade ones that have been oxidatively modified by ROS damage. (Gregersen *et al*., 2006). An increase in both number as well as up-regulation of these related proteins implicates higher intracellular stress.

The 613 proteins were grouped into COG functional groups corresponding to 25% of the predicted 2499 protein-coding ORF's within *S. xylosus* proteome (Proteome ID: UP000027230). Around 48% proteins were without a predicted function which highlights that there may still be many proteins that are uncharacterized with their functions unknown. These proteins may have roles in functional responses to Cr^{6+} stress and characterizing them may offer suggestions. This is possible by improving MS/MS search parameters or by investing in the study of transcriptomics, which may offer keys to stress response (Cheung and Gu, 2003).

Extra and intracellular sequestration of metal, decreased permeability, efflux, and enzyme action to innocuous forms are strategies bacteria implement to survive metal stress (Thompson *et al*., 2007). Identifying proteins that partake in these responses to oxidative stress as cellular defense will allow for enhanced understanding of bacterial metal resistance. Proteins that are down-regulated are also important in contributing to this understanding. Response to Cr^{6+} by *S. xylosus*3 could be categorized as per dosage concentration, which varied accordingly. Stress responses could be grouped as the up-regulation of oxidative stress responses with superoxide removal, enhancement of free-radical detoxifying activities, and peptide transport and degradation. It is important to note that, although there was some inhibition of certain processes, many cellular processes continued unhindered implicating the resilience of *S. xylosus*3.

In summary, expressional proteomics allows for extensive examination of changes in the protein composition as well as identification of proteins part of the response in a given physiological state (Vasseur *et al*., 1999). Application of this technique provides a global

view and an approach to address molecular mechanisms of bioremediation, especially cellular responses to external stimuli/stresses. Key proteins identified in this study, as well as the responses of marine *S. saprophyticus*168, *B. thuringiensis*208 and *S. xylosus*3 will help better understanding of possible tolerance and detoxification strategies in marine bacterial communities. Exploration of their differential expression, detoxification function/s of up-regulated proteins with their regulatory factors would help gain insights into the possible adoption of proven Cr^{6+} biotransforming bacterial strains for bioremediation of Cr^{6+} laden effluents.

Chapter 8

Molecular characterization of chromate modulating genes

8.1 Introduction

The rise in environmental metal contamination has led to increased presence of autochthonous metal-tolerant bacteria. Many strains of various genera/species of bacteria have developed several strategies for survival and several mechanisms of Cr^{6+} resistance and reduction have been identified. These include proteins that have been purified from cellular cytoplasms or membranes (Patra *et al*., 2010). The genes or operons that encode these resistance factors may be present either chromosomally or extra chromosomally on plasmids. Genomic tools have allowed for extensive examination of determinants as well as identification of key responsive genomic elements. Thompson *et al.*, (2007), *Branco et al.* (2008) and Juhnke *et al.* (2002) reported Cr^{6+} related genes and how they contribute to resistance. Cr^{6+} resistance conferring genes have also been observed in multiple genera such as *Escherichia*, *Cupriavidus*, *Pseudomonas*, *Burkholderia Ralstonia*, *Bacillus* and *Acinetobacter*, and reported to be involved in one of, Cr^{6+} ion transport, Cr^{6+} resistance, Cr^{6+} efflux, Cr^{6+} reduction or sulphate reduction (Pradhan *et al.*, 2016). These genes can be detected by molecular characterization using primers designed to target and amplify template. Positive amplification confirms their presence. In this study, 25 isolates confirmed to possess the tolerance of very high concentrations of Cr^{6+} in the growth medium were tested for a few chromosomally encoded *chr* genes to understand mode of resistance and Cr^{6+} detoxification.

8.2 Methods

8.2.1 PCR amplification of chromate resistance genes

All 25 chromium resistant bacterial isolates (**Table 8.1**) were examined for the presence of genes involved in chromium resistance using specific PCR primers (**Table 8.2**) following Aguilar *et al.* (2008), Kamika *et al.* (2013) and Kouadjo *et al.* (2011, 2012). Genomic DNA extracted (extraction outlined in section **3.2.2**) was used as template (2µl, 30-50µg DNA μl^{-1}) with 1µl each of forward and reverse primer (10pmol) and 12µl of PCR Master Mix (Thermo, USA). The reaction volume was made up to 25µL with 9µL of Nuclease free water. Gene amplification was done following standard reaction conditions through a thermal cycler and detected through gel electrophoresis (1% agarose).

8.2.2 PCR amplification of chromate reduction genes

Isolates were also examined for genes encoding for chromium reduction. Primer sets are presented in **Table 8.2** and their amplification was carried out as described by Ackerley *et al.* (2006), He *et al.* (2015) Kouadjo *et al.* (2011) and Kwak *et al,* (2003). Genomic DNA extracted (extraction outlined in section **3.2.2** of chapter 3) was used as template (2µl, 30-50µg DNA μl^{-1}) with 1µl each of forward and reverse primer (10pmol) and 12µl of PCR Master Mix (Thermo, USA). The reaction volume was made up to 25µL with 9µL of Nuclease free water. Gene amplification was done following standard reaction conditions through a thermal cycler and detected through gel electrophoresis (1% agarose).

Table 8.1 Bacterial isolates screened for PCR amplification of chromate resistance and reduction related genes

S No.	Bacterial code	Bacteria identified by 16S rRNA gene	Source	Accession No.	MIC* (μg mL^{-1})
1	NIOER164	*Staphylococcus saprophyticus*	Divar^{W}	MG205890	200
2	NIOER165	*Staphylococcus xylosus*	Divar^{W}	MG205891	250
3	NIOER168	*Staphylococcus saprophyticus*	Divar^{W}	MG205894	450
4	NIOER176	*Staphylococcus saprophyticus*	Dias^{W}	MG205901	300
5	NIOER182	*Exiguobacterium aestuarii*	Dias^{W}	MG205907	200
6	NIOER185	*Staphylococcus cohnii*	Dias^{S}	MG205910	200
7	NIOER188	*Staphylococcus arlettae*	Dias^{S}	MG205912	200
8	NIOER191	*Staphylococcus saprophyticus*	Dias^{S}	MG205914	250
9	NIOER202	*Staphylococcus cohnii*	G5^{W}	MG205924	200
10	NIOER203	*Staphylococcus cohnii*	G5^{W}	MG205925	250
11	NIOER208	*Bacillus thuringiensis*	G5^{W}	MG205930	350
12	NIOER236	*Staphylococcus saprophyticus*	Divar^{W}	MG205956	300
13	NIOER261	*Staphylococcus arlettae*	Divar^{W}	MG205978	300
14	NIOER273	*Bacillus cereus*	Dias^{W}	MG205987	350
15	NIOER285	*Bacillus cereus*	Dias^{W}	MG205999	250
16	NIOER286	*Bacillus pseudomycoides*	Dias^{W}	MG206000	250
17	NIOER289	*Bacillus cereus*	Dias^{W}	MG206002	200
18	NIOER292	*Bacillus thuringiensis*	Dias^{W}	MG206005	200
19	NIOER299	*Bacillus pseudomycoides*	Dias^{W}	MG206012	200
20	NIOER303	*Bacillus pseudomycoides*	Dias^{W}	MG206015	250
21	NIOER307	*Bacillus thuringiensis*	Dias^{W}	MG206017	200
22	NIOER320	*Bacillus cereus*	Dias^{W}	MG206028	200
23	NIOER321	*Staphylococcus xylosus*	Dias^{W}	MG206029	200
24	NIOER324	*Staphylococcus arlettae*	Dias^{W}	MG206032	300
25	NIOER325	*Bacillus cereus*	Dias^{W}	MG206033	250

*MIC in liquid broth

W water, **S** sediment

Table 8.2 Complete list of primers used in this study to target chromate resistance and reduction related genes

Primer name	Mechanism involved	Primer sequence (5'-3') **F** forward, **R** reverse orientation	AT* (ºC)	Reference
chrA_1	CHR transporter (efflux/reduction)	**F** 5'-GCTCGATCATCTAGATTAACGCGCTTGGG-3' **R** 5'-CCTTTAGGTGCTGGATCCGACGATTCAGC-3'	55	Aguilar *et al.* (2008)
ChrB_1	CHR transporter (efflux/reduction)	**F** 5'-GTCGTTAGCTTGCCAACATC-3' **R** 5'-CGG AAAGCAAGATGTCGATCG-3'	57	Kamika *et al.* (2013)
ChrA_2	CHR transporter (efflux/reduction)	**F** 5'-AAAGGTACCTCGGTACATACCGCGCCCACT-3' **R** 5'-AAATCTAGATCAGTGATGCAACAACGGATA-3'	59	Kouadjo *et al.* (2012)
ChrB_2	CHR transporter (efflux/reduction)	**F** 5'-TGCGBGAYGGYGYYTAYCT-3' **R** 5'-SGCVCCRTCRAARTCRAA-3'	52	Kouadjo *et al.* (2012)
*Chr*C	chromium resistance	**R** 5'-GGGCAAGGCGCTCGGCGGCGG-3' **R** 5'-TGCGCCAGGCAGCCCCCGCAA-3'	60	Kouadjo *et al.* (2012)
chrR_1	*E. coli* chromium reductase gene	**F** 5'-CGCGGGGGCATATGTCTGAAAAATTGCAGGT-3' **F** 5'-TTTGGGATCCTTAGATCTTAACTCGCTGAA-3'	-	Ackerley *et al.*(2006)
chrR_2	*P. aeruginosa* chromium reductase gene	**F** 5'-GACTGGCAYCTSGTGCACCT **R** 5'-GGADGASACGTCGATCAGGT	60	He *et al.* (2015)
chrR_3	*P. putida* chromium reductase gene	**F** 5'-AAC GAA GAT ATC GAAGCC GA-3' **R** 5'-CCC TGT TCA ACT TCACCC AT -3'	58	Kouadjo *et al.* (2011)
ABC transporter	sulphate transport metabolism pathway	**F** 5'-CTATATCATATGTTAATTGACTTG-3' **R** 5'-CTCGAGCTACACATTATTCATAAA-3'	54	Kouadjo *et al.* (2011)
nfsA_1	*E. coli* nitroreductase (M)	**F** 5'-GTAGGATCCACGCCAACCATTGAAC-3' **R** 5'-ACTGAATTCTTAGCGCGTCGCCCAAC-3'	55	Kwak *et al.* (2003)
*nfs*B	*E. coli* nitroreductase (m)	**F** 5'-GTAGGATCCGATATCATTTCTGTCGC-3' **R** 5'-ACTGAATTCTTACACTTCGGTTAAGGTG-3'		
nfsA_2	*V. harveyi* nitroreductase (M)	**F** 5'-GTAGGATCCAACAATACGATTGAAAC-3' **R** 5'-ACTGAATTCTTAGCGTTTTGCTAGCC-3'		
chrR_4	*Staphylococcus* chromium reductase gene	**F** 5'-TTAYYTTTTVATWAATCCWGMWTYATTHAG-3' **R** 5'-DTGTCWGABYATGTWTAYRAHTTRWTGAARAA-3'	-	This study

AT- Annealing temperature

* PCR was performed under high stringency conditions

M- Major

m- minor

8.3 Results

To evaluate Cr^{6+} resistance and reduction ability, several chromate modulating genes were tested. Chromosomal DNA from all 25 CRB was used as template in conventional PCR. Of all the genes targeted in the gDNA, *Chr*A_1, *Chr*B_2 (*Chr*B), *Chr*R_1 and *Chr*R_3 (*Chr*R), *ABC* transporter, *Nfs*A_1 and *Nfs*A_2 (*Nfs*A), and *Nfs*B showed positive amplification.

Gene *chr*A_1 of size ~950bp (**Fig. 8.1**A) with primers of Aguilar-Barajas *et al.*(2008) for *Shewanella Chr* gene was amplified in *Bacillus cereus*273 and *B. cereus*285. This gene encoded a chromate efflux protein from the chromate ion transport family (CHR). Both isolates were *Bacilli* and there have been previous reports of the same (Ramirez-Diaz *et al.*, 2008). None of the isolates (**Table 3**) showed positive amplification for primer set of Kouadjo *et al.* (2012) designed for *Chr*A determinants distributed in *Cupriavidus metallidurans*.

The isolates differed with respect to the *Chr*B gene they carried as per two differently used primers. For primers from Kouadjo *et al.* (2012), multiple isolates of *Staphylococcus* sp. were positive for *Chr*B_2 gene (**Fig. 8.1**B) such as *S. saprophyticus*164, *S. xylosus*165, *S. saprophyticus*168, *S. cohnii*185, *S. gallinarum*188, *S. cohnii*202, *S. cohnii*NIOER203, *S. arlettae*261, *S. xylosus*321 and *S. arlettae*324. Bacilli such as *B. pseudomycoides*299, *B. pseudomycoides*303 and *B. cereus*325 were also positive for multiple Cr^{6+} genes. Primers for *Chr*B developed by Nies *et al.* (1990) utilized by both Kanika and Momba (2013) as well as Abou-Shanab *et al.* (2007) were negative for all isolates tested.

Presence of *Chr*R encoding genes was examined with different primers from

Ackerley *et al.* (2006), He *et al.* (2015) and Kouadjo *et al.* (2011). Different responses were observed as per the primer set. Isolate *S. arlettae*324 tested positive for *Chr*R_1 (**Fig. 8.1**C) using primers from Ackerly *et al.*(2006) A variety of isolates also tested positive (primers from Kouadjo *et al*., 2012) for *Chr*R_3 (**Fig. 8.1**D) such as *S. cohnii*203, *S. saprophyticus*236, *S. arlettae*261, *B. cereus*285, *B. cereus*289, *B. thuringiensis*292, *B. thuringiensis*307, *B. cereus*320, *S. xylosus*321 and *S. arlettae*324. The *Chr*R_4 primer sequence was deduced from multiple alignment of *Chr*R gene sequences harbored by several *Staphylococci* species. These were acquired from databases and primers were designed on the homologous regions in this gene. None of the bacteria tested positive for this primer set. Presence of *ABC* transporter gene was tested using primers of Kouadjo *et al.* (2011). Most *Staphylococci* isolates (*S. saprophyticus*164, *S. xylosus*165, *S. saprophyticus*168, *S. saprophyticus*176, *S. saprophyticus*191, and *S. cohnii*202) possessed this transporter (**Fig. 8.1**E). Interestingly, both *S. xylosus*321 and *S. saprophyticus*324 were not positive with this primer. Presence of nitroreductase genes was detected using primers from Kwak *et al,* (2003). *Nfs*A_1 was found to be dominant and positive in most of the isolates tested. Nitroreductase gene *Nfs*B and *Nfs*A_2 were positive only in few isolates (**Fig. 8.1**F, G, and H).

The only isolate belonging to *Exiguobacterium*, *Exiguobacterium aestuarii*182, tested positive for *Nfs*A_1. Isolates *S. cohnii*185 (*Chr*B_2), *S. gallinaram*188 (*Chr*B_2), *B. cereus*273 (*Chr*A_1), *B. pseudomycoides*299 (*Chr*B_2) and *B. pseudomycoides*303 (*Chr*B_2) all tested positive for one gene each. *B. cereus*286 tested negative for genes in the study. Nitrate reductases genes being distributed randomly, no apparent species-wise, sample-wise or location-wise pattern was observed.

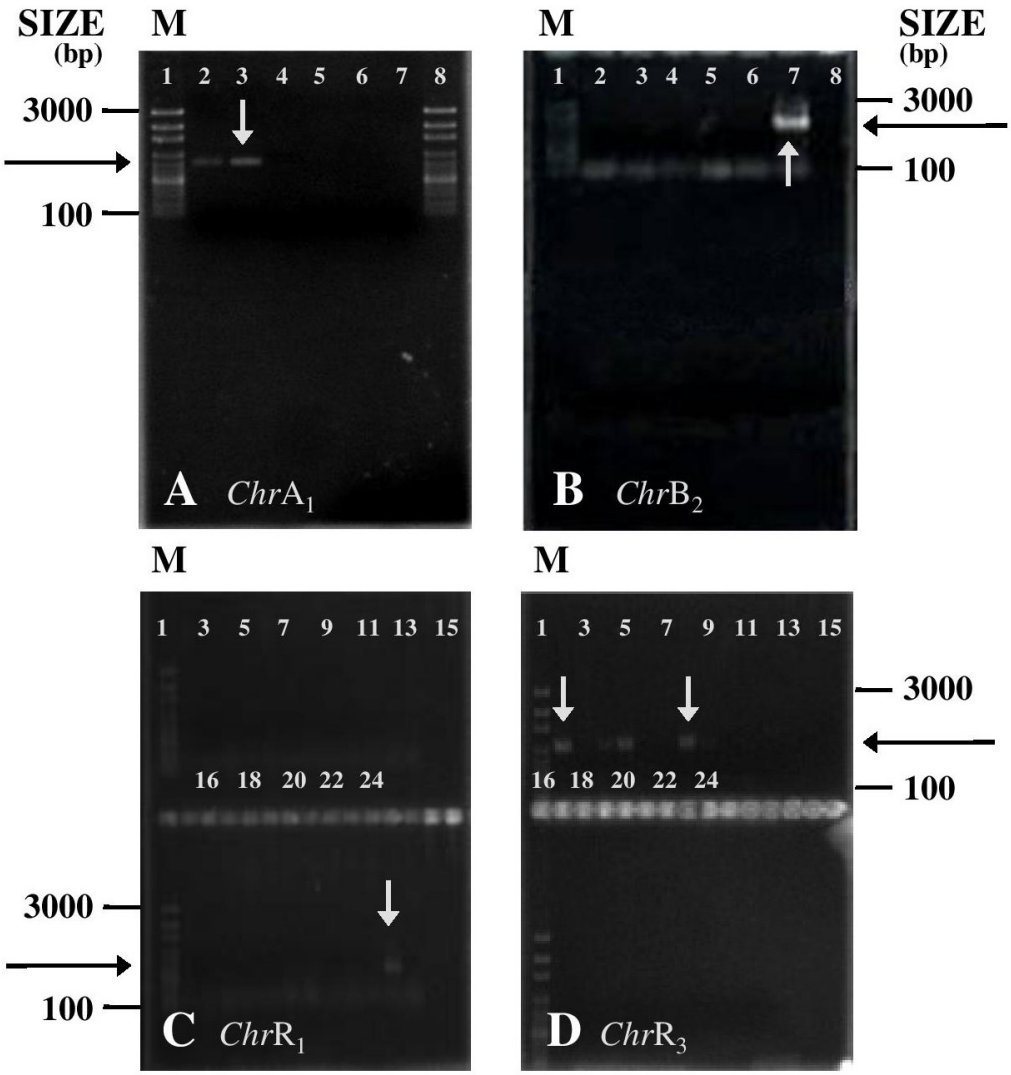


Figure 8.1 PCR detection of Cr^{6+} metal resistance/reduction genes in select chromate reducing bacteria. Agarose gel electrophoresis (1%) of PCR products of total genomic DNA from select CRB isolates with primer pairs for genes (A) *Chr*A_1, (B) *Chr*B_2, (C) *Chr*R_1 and (D) *Chr*R_3. *Lane* **M**, 100 bp DNA ladder (Marker); 1–5 or 1-25, amplified PCR product of (A) *lane* **2**, $ChrA_1$_NIOER273; (A) *lane* **3**, $ChrA_1$_NIOER285; (B) *lane* **7**, $ChrB_2$_NIOER168;(C) *lane* **25**, $ChrR_1$_NIOER324; (D) *lane* **2**, $ChrR_3$_NIOER203, *lane* **4**, $ChrR_3$_NIOER236, *lane* **5**, $ChrR_3$_NIOER289, *lane* **8**, $ChrR_3$_NIOER324

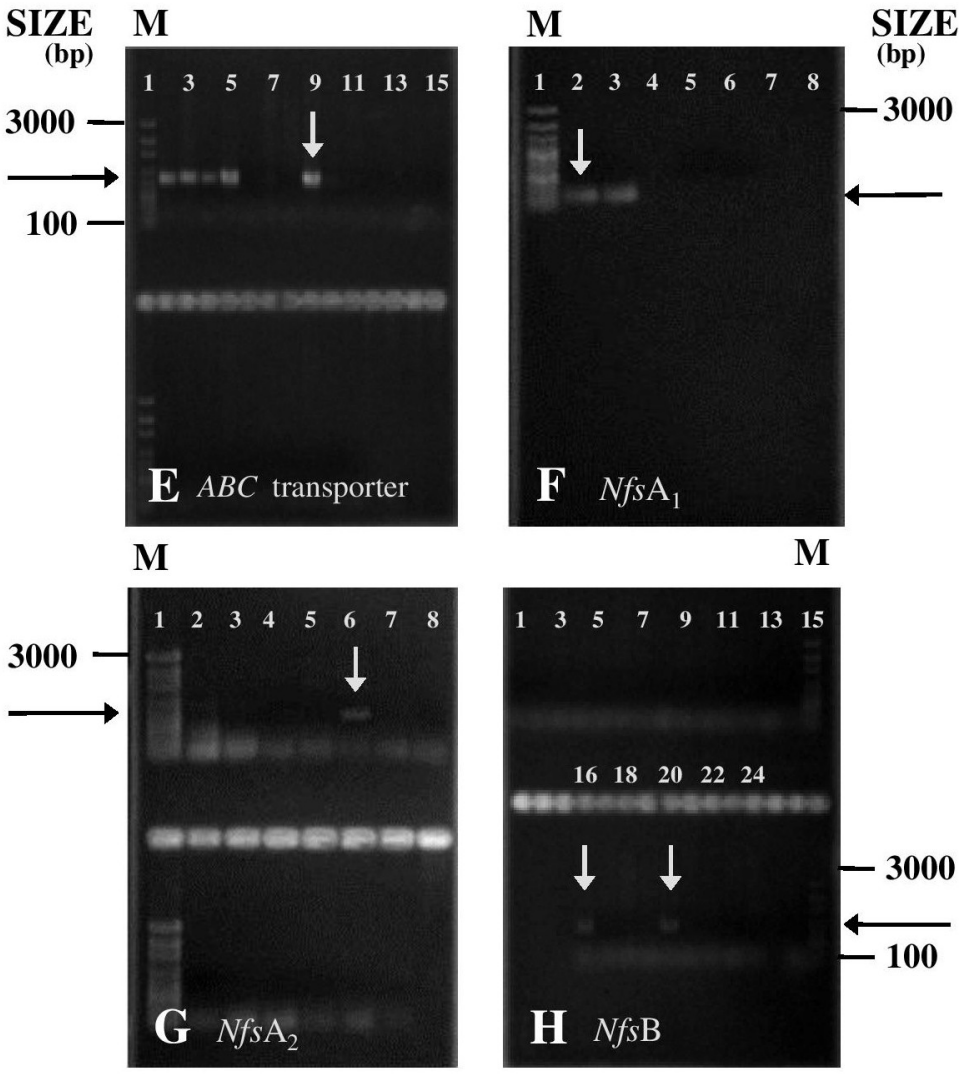


Figure 8.2 PCR detection of Cr^{6+} metal resistance/reduction genes in select chromate reducing bacteria. Agarose gel electrophoresis (1%) of PCR products of total genomic DNA from select CRB isolates with primer pairs for genes (E) *ABC* transporter, (F) *Nfs*A_1, (G) *Nfs*A_2 and (H) *Nfs*B. *Lane* **M**, 100 bp DNA ladder (Marker); 1–5 or 1-25, amplified PCR product of (E) *lane* **2**, ABC transporter_NIOER164, *lane* **3**, ABC transporter_NIOER165, *lane* **4**, ABC transporter_NIOER168, *lane* **5**, ABC transporter_NIOER176, *lane* **9**, ABC transporter_NIOER202; (F) *lane* **2**, $NfsA_1$_NIOER164, *lane* **3**, $NfsA_1$_NIOER168; (G) *lane* **6**, $NfsA_2$_NIOER176; (H) *lane* **16**, NfsB_NIOER168, *lane* **20**, Nfs_1_NIOER208

Table 8.3 List of isolates positive for chromate modulating genes

	ChrA_1	*Chr*B_1	*Chr*A_2	*Chr*B_2	*Chr*C	*Chr*R_1	*Chr*R_2	*Chr*R_3	*ABC**	*Nfs*A_1	*Nfs*B	*Nfs*A_2	*Chr*R_4
NIOER164	-	-	-	+	-	-	-	-	+	+	-	-	-
NIOER165	-	-	-	+	-	-	-	-	+	+	-	-	-
NIOER168	-	-	-	+	-	-	-	-	+	+	+	-	-
NIOER176	-	-	-	-	-	-	-	-	+	+	-	+	-
NIOER182	-	-	-	-	-	-	-	-	-	+	-	-	-
NIOER185	-	-	-	+	-	-	-	-	-	-	-	-	-
NIOER188	-	-	-	+	-	-	-	-	-	-	-	-	-
NIOER191	-	-	-	-	-	-	-	-	+	+	-	-	-
NIOER202	-	-	-	+	-	-	-	-	+	-	-	-	-
NIOER203	-	-	-	+	-	-	-	+	-	-	-	-	-
NIOER208	-	-	-	-	-	-	-	-	-	+	+	-	-
NIOER236	-	-	-	-	-	-	-	+	-	+	-	-	-
NIOER261	-	-	-	+	-	-	-	+	-	+	-	-	-
NIOER273	+	-	-	-	-	-	-	-	-	-	-	-	-
NIOER285	+	-	-	-	-	-	-	+	-	+	-	-	-
NIOER286	-	-	-	-	-	-	-	-	-	-	-	-	-
NIOER289	-	-	-	-	-	-	-	+	-	-	-	-	-
NIOER292	-	-	-	-	-	-	-	+	-	+	-	-	-
NIOER299	-	-	-	+	-	-	-	-	-	-	-	-	-
NIOER303	-	-	-	+	-	-	-	-	-	-	-	-	-
NIOER307	-	-	-	-	-	-	-	+	-	+	+	-	-
NIOER320	-	-	-	-	-	-	-	+	-	+	-	-	-
NIOER321	-	-	-	+	-	-	-	+	-	-	-	-	-
NIOER324	-	-	-	+	-	+	-	+	-	+	-	+	-
NIOER325	-	-	-	+	-	-	-	-	-	+	+	-	-

*** transporter**

\+ Positive

\- Negative

Key: NIOER164 *Staphylococcus saprophyticus*, **NIOER165** *S. xylosus*, **NIOER168** *S. saprophyticus*, **NIOER176** *S. saprophyticus*, **NIOER182** *Exiguobacterium aestuarii*, **NIOER185** *S. cohnii*, **NIOER188** *S. arlettae*, **NIOER191** *S. saprophyticus*, **NIOER202** *S. cohnii*, **NIOER203** *S. cohnii*, **NIOER208** *Bacillus thuringiensis* , **NIOER236** *S. saprophyticus*, **NIOER261** *S. arlettae*, **NIOER273** *B. cereus*, **NIOER285** *B. cereus*, **NIOER286** *B. pseudomycoides*, **NIOER289** *B. cereus*, **NIOER292** *B. thuringiensis*, **NIOER299** *B. pseudomycoides*, **NIOER303** *B. pseudomycoides*, **NIOER307** *B. thuringiensis*, **NIOER320** *B. cereus*, **NIOER321** *S. xylosus*, **NIOER324** *S. arlettae*, **NIOER325** *B. cereus*

8.4 Discussion

Cycling of Cr metal through redox reactions *in situ* is aided by indigenous bacteria, suggesting the role of genetic precursors (Patra *et al*., 2010) in chromium tolerant/resistant bacteria. The Cr^{6+} resistance (*Chr*A, *Chr*B, *Chr*C and *ABC* transporter) and reduction related (*Chr*R, *Nfs*A and *Nfs*B genes) genes tested for in 25 different Cr^{6+} resistant bacterial isolates show a strain specific response. The function of each gene is specific and has been previously reported.

In this study, detection of *Chr*B_2 (gene- *Chr*B) in multiple *Bacilli* and *Staphylococci* isolated from different locations is useful in describing its prevalence as well as importance in Cr^{6+} resistance in these isolates. *Chr*A is a well studied gene belonging to the chromate ion transporter family (CHR; Ramirez-Diaz *et al.*, 2008) found both chromosomally and extra-chromosomally. It is responsible for forming a transmembrane chemiosmotic pump. *Chr*B is closely related to *Chr*A since they usually occupy the same reading frame, but there have been reports of it being absent in constitutively expressed Cr^{6+} resistance determinants (Cervantes *et al.* 2001). *Chr*B structurally differs from *Chr*A and has been reported (Nies *et al*., 1989) to serve a dual function as a regulatory protein and a functional part of the efflux system, as reported by Nies *et al.* (1990). In this study, *Chr*B is expressed by many isolates, but the same cannot be said for the presence of *Chr*A (*Chr*A_1), which was only observed in two *Bacillus* isolates. This may be taken to indicate that *Chr*B might exist independently of *Chr*A and may have an additional role to play in Cr^{6+} resistance.

This hypothesis is supported by Kouadjo *et al.* (2012) who tested around 70 *Staphylococci* strains isolated from a fly ash dumping site to determine the genetic mechanisms responsible for chromate resistance. Detection of Chr determinants *ChrA*, C*hrB* and *ChrC* from plasmid pMOL28 that conferred resistance to *Cupriavidus metallidurans* (Rozycki and Nies,

2009) was performed. The study stated that all isolates tested, were positive for *ChrB* and *ChrC*, whereas *ChrA* was only found to be present in *S. aureus*. Interestingly, all isolates in this study were negative for the presence of *Chr*C.

Detection of Cr^{6+} reductase genes (*Chr*R) suggests its ability to confer Cr^{6+} reduction. Ackerly *et al.* (2004) studied purified soluble chromium reductases from *E. coli* (YieF) and *P. putida* (ChrR). They found that both contributed to some form of resistance to Cr^{6+} although the direct role of ChrR in protection against chromate toxicity was not ascertained. They also found that they were of inducible nature under the control of starvation promoters-as they were expressed in stationary phase. Their broad substrate range-meant that they could be used to remediate multiple pollutants. *Chr*R_3 encoded by gene *ChrR* is a soluble flavin mononucleotide-binding protein demonstrating quinone reductase activity. It was also observed by Kouadjo *et al.* (2011) who tested for *Chr*R, (based on ChrR from *P. putida*, Ackerly *et al.* 2004) using a primer pair designed for amplifying corresponding regions in Cr^{6+} resistant *Staphylococci* species. In this study, it was seen in at least ten marine originating isolates, most of which were identified to be bacilli. This suggests that there may be some genera specific factors at play in gene transfer between species.

It has been reported that nitroreductases possess oxidoreductase function and can utilize/biotransform Cr^{6+} as substrate (Pradhan *et al.*, 2016). Kwak *et al*. (2003) purified a protein with chromate reductase function from *P. ambugua* and found it to be homologous with other nitroreductases. The report also outlines the use of nitroreductases from *Vibrio harveyi* KCTC 2720 and *Escherichia coli* DH5α confirming that nitroreductases can also function as chromate reductases. Although they behave as ChrR, they are usually induced only in the presence of

nitrite. In this study, most isolates showed positive presence of *Nfs*A_1 suggesting that it might have a more important constitutive function than reported.

In addition to *Chr*A and *Chr*B, ABC transporters are also known to provide Cr^{6+} resistance. They are a group of integral membrane proteins that transport intracellular drug molecules as well as metal ions across the membrane. This translocation function is coupled by energy provided by hydrolysis of ATP as reported by Chang (2003). Studies of Kouadjo *et al*. (2011) on *Staphylococci* species also described other mechanisms used by Gram positive bacteria to survive Cr^{6+} stress. They found the presence of gene clusters associated in Cr^{6+} challenging systems part of a gene encoding sequence with high homology to ABC transporter ATP binding protein. This transporter was found to be a P-loop NTPase similar in function to P-loop ABC sulphate transporter; which was abundantly expressed during Cr^{6+} stress in *Arthrobacter* sp. (Kouadjo *et* al., 2011). Such proteins may have close links to the iron transport system or act as active importers of sulphate in sulphate limitation during Cr^{6+} stress. In this study, only isolates belonging to *Staphyloccoci* showed positive amplification. Besides the primer set being genera specific, it is likely that these transporters play a role in conferring Cr^{6+} resistance to *Staphyloccoci* species.

Recent work suggests that chromate resistance is controlled by diverse genes as all genes were not expressed in Cr resistant strains. The gene transfer process is heavily implied as it is the main basis of heavy metal resistance acquisition within bacterial communities (Mergeay, 2000 Nies, *et al.* 1999). It also highlights that *Chr* related genes, as well as genetic determinants for other metals in soil bacteria like *C. metallidurans* could be distributed to other bacteria of divergent lineage by horizontal gene transfer events (Kouadjo *et al.* 2012) as observed by Abou-Shanab *et al*. (2007). Importantly, there must be many other proteins like flavoproteins or

nitroreductases with unrelated metabolic function that could perform ChrR function or provide Cr^{6+} resistance.

Diffusion of toxic metals across sediments and aquifers is of environmental significance. As such, resistance to metals is commonly observed in bacteria, either isolated from contaminated or from metal-free environments. This was also observed by Nagevenkar and Ramaiah (2010) and De *et al.* (2008) who isolated arsenic and mercury resistant bacteria from slightly polluted (as per standard pollution index, (Yan *et al.*, 2015) coastal waters; and reported highly metal-resistant strains. Results in this study are indirect evidences to the pervasiveness of chromium or metal resistant genetic material in marine bacterial populations. Horizontal gene transfer across bacterial communities and subsequent natural selection in marine environs might explain how these strains acquired these resistance/removal genes (Abou-Shanab *et al*., 2007). Additionally, in Cr^{6+} detoxifying bacteria, the rate of Cr^{6+} removal as well as the ability to tolerate Cr^{6+} is a product of multiple regulatory sequences, each that contributes to survival/ detoxification. This altered expression of enzymatic activity or resistance can be studied by identifying which genetic determinants are contributing. Designed primers are also an effective tool to identify organisms either in culture, in consortium or in environmental samples having Cr^{6+} genetic potential. Although this may be outside the scope of this study, it is a promising method. The ability of bacterial isolates examined in this study to detoxify chromium and encode a few Cr^{6+} modulating genes indicate that these systems may have some function in the natural environment in addition to surviving Cr^{6+}. Knowledge of this will help in targeting isolates with better potential and possibly re-engineer their detoxifying functions.

Chapter 9

Summary

Studies on metal tolerant microbial flora are important for an understanding and development of microbe mediated solutions for metal pollution. Many basic studies carried out on metal tolerant bacterial species indicate the detoxification potential of a number of environmental isolates from disparate ecotypes. Indeed, all the toxic heavy metals are modulated either through reduction, oxidation and metal-non metal complexation by bacteria from natural habitats including those from marine ecosystems. From this study, it is evident that fractions of autochthonous bacteria capable of growing in medium amended with chromate are sizable and widespread in the marine ecosystem. As earlier reported, the environmental effects of pollution and metal related dumping are broad. Since coastal systems are at particular risk to the presence of toxicants, experiments were conducted to study whether marine bacteria were capable of surviving as well as detoxifying it. The hypothesis put forth was whether native isolates tolerant of high concentrations of chromate could detoxify it in a measurable manner.

The following are some of the major features from this work

- Parametric assessment of samples from the lower stretches of Mandovi estuary outline moderate pollution. The site at Divar mangroves is particularly affected by urban outfall. Such effects are also evident at Dias beach, the other of the three sampling locations off Goa. Offshore site G5 experiences very-low levels of pollution in comparison.
- Salinity, pH and temperatures were observed to be lower during monsoon season at sampling sites. In contrast, dissolved oxygen, nitrates and nitrites were found to be higher during monsoon seasons. Each parameter was intrinsically linked with another and formed negative and positive correlations, mostly signifying synergistic effects of those parameters on the abundance and variations in chromate tolerant bacteria. Spatially, Dias

and offshore sampling sites resembled each other following similar patterns in physico-chemical change.

- Divar mangrove environment is of poor quality as corroborated by physico-chemical parameters and bacterial abundance. Although, chromate pollution was non-evident, presence of chromate tolerant strains indicates that these bacteria are metabolically equipped to undergo stress challenge. This was evident from the comparable fractions of chromate resistant bacteria (CRB) populations from marine environs
- A relatively large fraction of native cultivable bacteria was found to resist chromate. Levels of environmental chromium reported by other studies are comparatively low and it is evident from this that prevalence of such metal tolerant bacteria is not necessary linked with current levels of chromium pollution in the locations off Goa. Not surprisingly, the abundance of CRB were invariably higher in the tannery effluent sample from Pallavaram.
- The DNA from 278 strains isolated which tolerated chromate (sequenced and analyzed for 16S rRNA gene) represented as many as 15 different genera.
- The community of CRB was found to be dominated by bacilli, regardless of the sampling location or season. This is possibly due to the physiological versatility of this group to chromate.
- Of the 278 CRB strains isolated, five capable of complete biotransformation of chromate, were submitted to extensive physiological and biochemical characterization. Two were found to be *Bacilli* and three, *Staphylococci*.

- Antibiotic resistance profiles highlighted the dominant mechanisms of co-selective resistance in both antibiotics and metals, as well as the prevalence of such traits in environments that lack sources of antibiotics.
- All five strains of *S. saprophyticus*168, *S. saprophyticus*176, *B. thuringiensis*208, *B. cereus*273 and *S arlettae*324 had chromate removal/biotranformation rates $\geq$0.2µg mL^{-1} h^{-1} and were able to tolerate $\geq$250µg mL^{-1} chromate.
- Marine *S. xylsous*3 and *S. gallinaram*8 were able to remediate chromate contaminated soil mitigating its toxic effects, enough so that *Vigna radiata* seeds were able to germinate and sprout. Without bacterial action, germination success as well as sprouting was severely affected by presence of chromate.
- From expressional proteomics, cellular responses to chromate stress were found to belong to protein moieties related to: oxidative stress response, superoxide removal, enhancement of free-radical detoxifying enzymes, DNA repair response, up-regulation of proteins involved in protein folding and degradation and activation of amino acid metabolism. The proteomic responses in the overall assert, marine bacterial responses to chromate stress. Iron metabolism and energy metabolism were also expressed as minor responses.
- Chromate modulating genes were detected in 25 representative strains shown to possess a variety of chromate reduction, resistance or efflux genes. Presence of these genes indicates the prevalence of genetic material pertaining metal resistance in native environments.

Future prospects

Although the potential for using marine microbes in remedial applications and biodiscovery is limited by the difficulty in true mimicking of natural conditions, marine bacteria offer several advantages. More detailed studies would give new insights on factors aiding resistance. Additionally, abundance studies especially enumeration of CRB on a regular basis might be vital in monitoring pollution levels in sensitive areas, as well as the obtaining of more robust candidates for chromium remediation. The fact that many genera contain strains of CRB is a useful indicator that the natural isolates carefully chosen based on rigorous screening exercises would lead to a bioresources-wealth useful for bioremediation. A more detailed study of proteins would help generate pathways that would be particularly helpful in differentiating marine bacterial responses to chromate and provide robust clues for evolving efficient microbial bioremediation steps.

Appendix

A. Media Composition

A.1 Nutrient Agar

Peptic digest of animal tissue	5.0g
Beef extract	1.5g
Yeast extract	1.5g
NaCl	5.0g
Agar	15g

A.2 Nutrient Broth

Peptone	10.0g
Beef extract	3.0g
NaCl	5.0g

Dissolved in 1000mL double distilled water (d/w)
pH was adjusted to 7.0 with 1N NaOH
pH was adjusted to 6.0 with 1N HCl (when amended with Cr^{6+})

B. Composition of stains, buffers and reagents

B.1 Stains

Gram stain reagents

Crystal violet

Solution A- 2g of crystal violet in 20mL ethanol
Solution B- 0.8g ammonium oxalate dissolved in 80mL d/w

Gram's Iodine

Dissolve 1g iodine and 2g Potassium iodide in 300mL d/w, filter through Whatman filter paper No. 1

Safranine

2.5g Safranine was dissolved in 10mL ethanol, and volume made up to 100mL with d/w and filter through Whatman filter paper No. 1

B.2 Buffers

Phosphate buffer (PB) (0.2M)

Solution **A** (0.2M monobasic di-hydrogen phosphate) 2.76g NaH_2PO_4 in 100mL d/w
Solution **B** (0.2M dibasic hydrogen phosphate): 2.84g Na_2HPO_4 in 100mL d/w

For pH 7.4
Solution **A** (19mL) + solution **B** (81mL) =100mL 0.2M phosphate buffer

TE buffer

10mL 0.11M Tris-HCl (pH 8.0) was diluted with 90mL d/w. To this 0.0372g EDTA was added. pH was adjusted to 80.

C. Chemical estimation

Winkler's Titration (Parsons et al. 1984)

Sodium thiosulphate solution (0.0025M)
Sulphuric acid *conc.*
Winkler solution 1 (manganous sulfate)
Winkler solution 2 (alkaline-iodide)

Winkler solution 1 and 2 were added to samples collected in bottles to sequester dissolved oxygen in the sample. The precipitate formed on inverting the stoppered bottle is dissolved with sulphuric acid and titrated against Sodium thiosulphate solution using starch solution as an indicator.

Biochemical oxygen demand (BOD)

Phosphate buffer: 8.5 g KH_2P0_4, 21.75 g K_2HP0_4, 33.4 g $Na_2HP0_4 \bullet 7H_20$, and 1.7 g NH_4Cl in approx was dissolved in 1L d/w

Magnesium sulfate: 22.5 g $MgSO_4 \bullet 7H_20$ in reagent water was dissolved 1 L.

Calcium chloride: 27.5 g $CaCl_2$ was dissolved in 1 L d/w

Ferric Chloride: Dissolve 0.25 g $FeCl_3 \bullet 6H_20$ in reagent water. Dilute to 1 L.

D. Preparations for molecular techniques

D.1 Protein extraction: Urea-Thiourea extraction buffer (5mL)

7M Urea	2.102g
2M Thiourea	0.761g
4% CHAPS	0.2g
0.8% IPG buffer	40 µL
20mM DTT	0.01542g
1M Tris HCl (pH 8.0)	100µL

CHAPS- Cholamidopropyl-dimethylammonio-1-propanesulfonate
DTT- Dithiothreitol
IPG- ampholyte containing buffer

Cell cultures were pelleted and the growth medium was discarded. To the pellet, 500µL of extraction buffer was added, homogenized and centrifuged. The supernatant was transferred to a fresh microfuge tube and held at 4°C.

D.2 Folin lowry's method for Proteins estimation (Lowry *et al*., 1951)

Reagents	*Preparation*
Reagent A	2% Na_2CO_3 in 0.1N NaOH
Reagent B	0.5% $CuSO_4$ in 1% potassium sodium tartarate
Reagent C	Mixture of Reagent A + Reagent B (50 + 1mL), made prior use
Reagent D	Folin and Ciocalteau's phenol reagent (1:2, with d/w)
Standard bovine serum albumin solution	0.1mg BSA in 1mL d/w

To 100µL of protein sample, 900µL d/w + 5mL of Reagent C was added and incubated at room temperature for 10min. To this mixture, 0.5mL of Reagent D was added and the tubes were incubated for 30min in dark. Optical densities were measured at 660nm with protein extraction buffer used as blank. Concentrations were determined from standard graph using Bovine serum albumin as the standard.

D.3 SDS-PAGE

Stock solutions

Reagents	*Preparation*
Acrylamide/Bis-acrylamide solution	29.2% acrylamide and 1.2% (w/v) was dissolved in d/w. pH was adjusted to 7.0 and stored at 4°C
Resolving gel buffer (1.5M TrisHCl, pH 8.8)	18.615g Tris base was dissolved in 100mL d/w. pH was adjusted to 8.8 with 6N HCl
Stacking gel buffer (0.5M TrisHCl, pH 6.8)	12.11g Tris base was dissolved in 100mL d/w. pH was adjusted to 6.8 with 6N HCl
APS (10%)	0.1g APS was dissolved in 1mL d/w (fresh)

APS- Ammonium persulphate

Electrophoresis buffer (1X)

Tris base	3.0g
Glycine	14.4g
SDS (10%)	10mL
d/w	1L
pH 8.3	

Sample buffer

0.5M Tris-HCl (1M, pH 6.8)	1.25mL
Glycerol	2.5mL
10% SDS	2mL
0.5%(w/v) bromophenol blue	0.2mL
d/w	3.55mL

Gel running buffer (10X)

Tris base	30.3g/L
Glycine	144g/L
SDS	10g/L

1X was used for electrophoresis

Gel monomer preparation (Monomer - Acrylamide/Bis-acrylamide solution)
Composition of resolving and stacking gel is as follows:

Gel %	d/w	Monomer	Gel buffer	10% SDS	10% APS	TEMED
Resolving gel (12%)	3.4mL	4mL	2.5mL resolving gel buffer	0.1mL	50µL	5µL
Stacking gel (6%)	5.4mL	2.0mL	2.5mL stacking gel buffer	0.1mL	50µL	10µL

Silver staining

Silver stain		Developer		Hydrator	
silver nitrate	0.2%	Na_2CO_3	6g	10% $Na_2S_2O_3$	1mL
formaldehyde	0.75µL	formaldehyde	50µL	d/w	49mL
d/w	100mL	10% $Na_2S_2O_3$	40µL		
		d/w	100mL		

Na_2CO_3- sodium carbonate
$Na_2S_2O_3$- sodium thiosulphate

D.4 Reagents for in solution digestion

Reagents	*Preparation*
6M urea in 50mM Tris-HCl (pH 8.0)	0.36g was dissolved in 1mL nanopure water
50mM Ammonium bicarbonate	0.395g of Ammonium bicarbonate was dissolved in 100mL nanopure water
200mM DTT	30.86mg DTT in 1mL of 50mM Ammonium bicarbonate
200mM IAA	22mg IAA was dissolved in 0.6mL 50mM Ammonium bicarbonate

1mM HCl, Trypsin (1µg/ µL), Formic acid (MS grade)

Methanol precipitated protein pellet was dissolved in urea, treated with DTT and IAA for reduction and alkylation, in dark. The mixture was then treated with trypsin to digest proteins/peptides in solution, overnight. The mixture was acidifed with formic acid and transferred in to a MS autosampler vial for LC-MS QToF.

D.5 Reagents for in-gel digestion

Reagents	*Preparation*
100mM Ammonium Bicarbonate	0.791g of Ammonium bicarbonate was dissolved in 100mL nanopure water
10mM Ammonium Bicarbonate w 10% (v/v)	2mL of 100mM Ammonium Bicarbonate + 16mL nanopure water + 2mL Acetonitrile(ACN) = 20mL
10mM DTT in 100mM Ammonium Bicarbonate	0.0077g was dissolved in 5mL of 100mM Ammonium Bicarbonate
55mM IAA in 100mM Ammonium Bicarbonate	0.0101g was dissolved in 1ml of 100mM Ammonium bicarbonate
5% Formic acid in water	200µl of Formic acid was dissolved in 2ml of nanopure water

100mM Ammonium bicarbonate/acetonitrile (1:1, v/v)	10ml of 100mM Ammonium bicarbonate was mixed with 10ml of Acetonitrile. Total volume 20ml
Trypsin, 20ng/ml	trypsin containing vial was dissolved in 10mM ammonium bicarbonate containing 10% (vol/vol) acetonitrile

The gel slab was rinsed thoroughly with water before the band was excised with a scalpel. Cut band was transferred to a microcentrifuge tube and processed for reduction and alkylation. The pieces are then saturated with trysin solution for peptide digestion. To the mixture formic acid/ACN extraction buffer is added. Peptides in solution are withdrawn and transferred to a MS autosampler vial for LC-MS QToF.

E. Preparation of metal stock solution

Metal salt	*For stock solution* (1000µg mL^{-1})- *dissolved in* 1L d/w	
Potassium dichromate	$K_2Cr_2O_7$ (**2.829**g)	*M.W- 294.189*
Mercuric chloride	$HgCl_2$ (**1.353**g)	*M.W- 271.52*
Cadmium chloride	$CdCl_2$ (**1.631**g)	*M.W- 183.317*
Copper sulfate	$CuSO_4$ (**2.512**g)	*M.W- 159.609*
Zinc chloride	$HgCl_2$ (**2.085**g)	*M.W- 136.286*
Arsenic trioxide	As_2O_3 (**1.319**g)	*M.W- 197.841*
Nickel chloride	$NiCl_2$ (**2.208**g)	*M.W- 129.599*
Chromium chloride	$CrCl_3 \cdot 6H_2O$ (**5.124**g)	*M.W- 266.45*

All solutions were filter sterilized and stored in dark.

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Publications

Pereira E J, Fonseca S, Meena R M and Ramaiah N (2017) *Improved sprouting and growth of mung plants in chromate contaminated soils treated with marine strains of Staphylococcus species*. Indian Journal of Microbiology **57** (4): 400-408

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Conference proceeding and presentations

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Authors: Pereira E J and Ramaiah N.

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Title: *Differential protein analysis of chromate stress response in marine Staphylococcus xylosus and its remediation application*.

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