

*Università degli Studi di Firenze*

Dottorato di Ricerca in

*"Scienza del Suolo e Climatologia"*

Ciclo XXV

*Coordinatore* Prof. Luca Calamai

**Chromate resistance and biofilm development in  
*Pseudomonas alcaliphila* 34: molecular bases**

Settore Scientifico Disciplinare AGR/16



UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

*Dottoranda*

Dott.ssa Luisa Santopolo

*Tutori*

Prof.ssa Luciana Giovannetti

Dott. Carlo Viti

UNIVERSITA' DEGLI STUDI DI FIRENZE  
DISPAA

DIPARTIMENTO DI SCIENZE DELLE PRODUZIONI  
AGROALIMENTARI E DELL'AMBIENTE

Piazzale delle Cascine, 18 - 50144 Firenze (FI)

Anni 2010/2013

*A mia madre e mio padre*

# Table of contents

## Chapter 1

|                                                                   |    |
|-------------------------------------------------------------------|----|
| <b>Introduction</b>                                               | 1  |
| 1. Chromium                                                       | 2  |
| 1.1. Properties and anthropic use of chromium                     | 3  |
| 1.2. Chromium occurrence in environment                           | 4  |
| 1.3. Chromium toxicity                                            | 7  |
| 1.4. Mechanisms of bacterial tolerance and resistance to chromate | 8  |
| 1.4.1. Reduction of hexavalent chromium                           | 11 |
| 1.4.2. Efflux of chromate                                         | 12 |
| 1.4.3. Other mechanisms of chromate tolerance                     | 14 |
| 1.5. Bioremediation of chromium                                   | 15 |
| 2. Biofilm                                                        | 20 |
| 2.1. Biofilms: adaptable structures                               | 21 |
| 2.2. The biofilm life cycle                                       | 22 |
| 2.3. Biofilm resistance                                           | 25 |
| 2.4. Biofilm application in bioremediation                        | 29 |
| 3. References                                                     | 32 |

## Chapter 2

|     |    |
|-----|----|
| Aim | 45 |
|-----|----|

## Chapter 3

|                                                                                                                                                                 |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| A novel approach combining the Calgary Biofilm Device and Phenotype<br>MicroArray for the characterization of the chemical sensitivity of<br>bacterial biofilms | 48 |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|----|

## Chapter 4

|                                                                                                               |    |
|---------------------------------------------------------------------------------------------------------------|----|
| Draft Genome Sequence of Chromate-Resistant and Biofilm-Producing<br>Strain <i>Pseudomonas alcaliphila</i> 34 | 60 |
|---------------------------------------------------------------------------------------------------------------|----|

## **Chapter 5**

Molecular bases of chromate resistance and biofilm development in

*Pseudomonas alcaliphila* 34

68

## **Chapter 6**

Conclusions and future works

91

# **Chapter 1**

## **Introduction**

## 1. Chromium

The presence of contaminated sites is a common problem in all industrialized countries and stems from human activities, such as factories, mines, landfills and other facilities that can lead to contamination of soil owing to spills, leaks of equipment/tanks, improper waste management, etc. As far as Italy is concerned, according to the Environmental Data Yearbook 2011 compiled by the Institute for Environmental Protection and Research (ISPRA), over 15,000 potentially contaminated sites have been reckoned and only a negligible minority of them (about 3,000) have been so far



**Figure 1.** Contaminated Sites of National Interest (SIN) [(44), modified].

reclaimed. Among these assessed sites and on the basis of characteristics of the polluted area, amounts and harmfulness of pollutants, health and ecological risks, 57 areas have been included in the list of contaminated Sites of National Interest (SIN) (44) (Fig. 1).

The typology of pollutants that are found in a given contaminated site obviously depend on both kind of industrial activity (e.g. petrol-chemical plants and chemical, steel and mining industries) and on the disposal procedures of by-products arising from the

related industrial processes. According to the United States Agency for Toxic Substances

and Disease Registry (ATSDR), the Priority List of Hazardous Substance 2011 (also called the Substance Priority List) names As, Pb, Hg and Cd as 4 of the top 10 most prevalent environmental toxins that are hazardous to public health (5). Similarly, a European Commission on heavy metals in waste also identified these compounds as hazardous, but highlighted chromium as the most widespread toxic heavy-metal pollutant in the European Union (38). The strong impact of hexavalent chromium on environment and on human health demands suitable technologies to neutralize its hazard.

### 1.1. Properties and anthropic use of chromium

Chromium is a transition metal located in group VI-B of the periodic table. It naturally occurs in rocks, soils, plants, and volcanic emissions. Chromium is the seventh most abundant element on earth, but since most chromium is in the earth's mantle, it results being only the twenty-first most abundant element in the crust (11). Chromium is rarely found as a free metal in nature. It was discovered in Siberian red lead ore by Nicolas-Louis Vauquelin in 1797. In 1798, the new metal was isolated by reduction of chromium oxide with charcoal at high temperature (72). Noting the many colors produced by the compounds, Fourcroy (1755 to 1809) and Haüy (1743 to 1822) suggested the name chromium from the Greek word χρώμα (chroma) meaning color, reflecting the brilliant hues of reds, yellows, and greens of its compounds. Indeed chromium is one of the chief ingredients in mineral and metallic colors, being responsible for the color of some gem stones (41).

Commercial chromite deposits are found mainly in two forms: stratiform seams in basin-like intrusions, often multiple seams through repeated igneous injections and the more irregular podiform or lenticular deposits. The best known example of a stratiform deposit is the Bushveld Igneous Complex of South Africa. This complex contains the most of the world's chromite reserves. The Great Dyke



Figure 2. Chromium.

of Zimbabwe, traversing nearly the length of the country, is very similar and has been linked to the Bushveld in geological history. Other stratiform deposits occur in Madagascar and in the Orissa district of India (42).

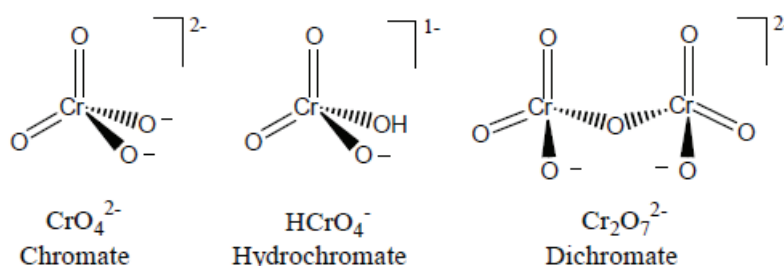
Chromium is a hard metal that takes a high polish and has a high melting point. It is also odorless and tasteless. A clean surface of chromium metal reacts strongly with atmospheric oxygen. However, the reaction stops quickly owing to the formation of a strong, dense and nonporous Cr(III) oxide ( $\text{Cr}_2\text{O}_3$ ) surface layer, which is estimated to be one to three formula units thick.  $\text{Cr}_2\text{O}_3$  passivates the metal from any further reaction with oxygen. This is why chromium does not corrode and why it retains its metallic sheen (41).

For its properties chromium is one of the most widely used metals in a variety of industrial processes. The world production of chromium is in the order of  $10^7$  tons per

year; chromium is used in alloys, including stainless steel, and in chemical industrial processes, mainly leather tanning, pigments and electroplating (12). The widespread use of chromium in diverse industrial processes has converted it into a serious contaminant of air, soil and water.

## 1.2. Chromium occurrence in environment

Chromium occurs in environment in the trivalent Cr(III) and hexavalent Cr(VI) oxidation state that are the most stable and abundant forms (12). Cr(VI) is a strong oxidizing agent, commonly present in solution as the hydrochromate ( $\text{HCrO}_4^-$ ), chromate ( $\text{CrO}_4^{2-}$ ) or dichromate ( $\text{Cr}_2\text{O}_7^{2-}$ ) oxyanions (Fig. 3), depending on the pH. Cr(VI) exists as water-soluble anions that may persist in water for long periods and is considered as a key contaminant (51).



**Figure 3.** Main aqueous forms of Cr(VI).

Cr(III) derivatives are much less mobile and exist in the environment mostly forming stable complexes with both organic and inorganic ligands (125). In addition, Cr(III) is less toxic because it is less soluble at physiological pH. At neutral pH, Cr(III) tends to precipitate as hydroxide [ $\text{Cr}(\text{OH})_3$ ] or hydrated oxide ( $\text{Cr}_2\text{O}_3 \cdot \text{H}_2\text{O}$ ) (26,88). The low solubility of Cr(III) is likely the major reason why Cr(III) makes up a small percentage of the total chromium concentration in polluted groundwater. Mobilisation of the  $\text{Cr}(\text{OH})_3$  precipitate is slow, unless enhanced by dissolution in strongly acidic environments or by being complexed with organic compounds (88). In waters chromium originates from natural sources, such as weathering of rock constituents, wet precipitation and dry fallout from the atmosphere and run-off from the terrestrial systems. Chromium concentration in rivers and lakes is usually limited to  $0.5 \pm 100$  nM, whereas in sea waters it varies from 0.1 to 16 nM (54). The presence and ratio between



these two forms [Cr(III) and Cr(VI)] depend on various processes, which include chemical and photochemical redox transformation, precipitation/dissolution and adsorption/desorption reactions.

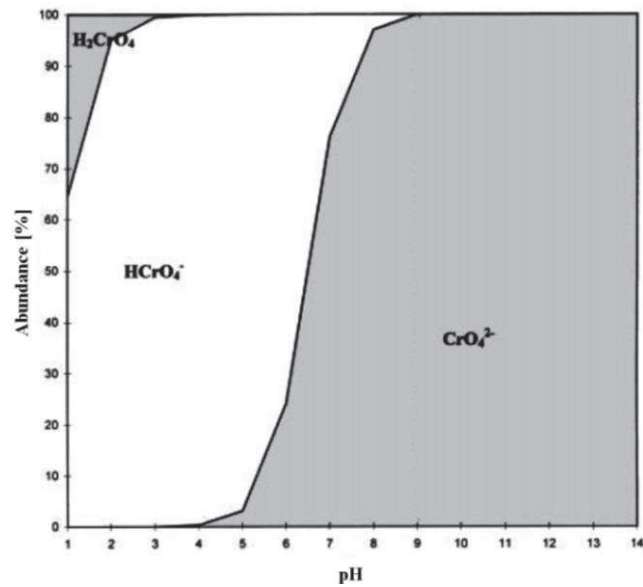
Chromium present in the atmosphere originates from anthropogenic sources, accounting for  $60\pm 70\%$ , as well as from natural sources, which account for the remaining  $30\pm 40\%$  (98). The lowest concentrations of chromium ( $5\pm 13\text{ pg/m}^3$ ) have been observed over the South Pole. Average atmospheric concentrations of this metal are, however, much higher: they range from  $1\text{ ng/m}^3$  in rural to  $10\text{ ng/m}^3$  in polluted urban areas (75). In air, chromium compounds are present mostly as fine dust particles. This dust eventually settles over land and water. The amount of chromium at any particular time and location depends on the intensity of industrial processes, proximity to the sources, the amount of chromium released and meteorological factors (9).

The average amount of chromium in various kinds of soils ranges from 0.02 to 58  $\mu\text{mol/g}$  and the main natural source is weathering of their parent materials. The levels of both Cr(III) and Cr(VI) in soil increase mainly from disposal of commercial products containing chromium, chromium waste from industry, and coal ash from electric utilities (54). Chromium present in soil is mostly trivalent; it is adsorbed to soil component and relatively little absorbed by the plants (9). In neutral-to-alkaline soils, Cr(VI) exists mostly in soluble but also in moderately-to-sparingly soluble chromates (e.g.  $\text{CaCrO}_4$ ,  $\text{BaCrO}_4$ ,  $\text{PbCrO}_4$ ). In more acidic soils ( $\text{pH}<6$ )  $\text{HCrO}_4^-$  becomes the dominant form (45) (Fig. 4).

Oxidation and reduction reactions can convert Cr(III) to Cr(VI) and *vice versa*. These processes depend on pH, oxygen concentration, presence of appropriate reducers and mediators acting as ligands or catalysts (54). The reduction of Cr(VI) to Cr(III) is of great environmental importance, since Cr(III) is less hazardous. Living organisms, ferrous iron, sulfide and organic matter, have the capacity to reduce hexavalent chromium (51). Losi et al. (1994) have demonstrated that organic matter of the soil plays an important role in the reduction of Cr(VI) to Cr(III) by creating reducing conditions, increasing activities of microbial communities, acting as an electron donator and indirectly lowering the oxygen level of the soil (oxygen is depleted through an increase of microbial respiration) (61).

The opposite oxidation process of Cr(III) to Cr(VI) proceeds readily in soils with oxidized Mn. Oxidized Cr(III) is proportional to the soil content of Mn oxide and to

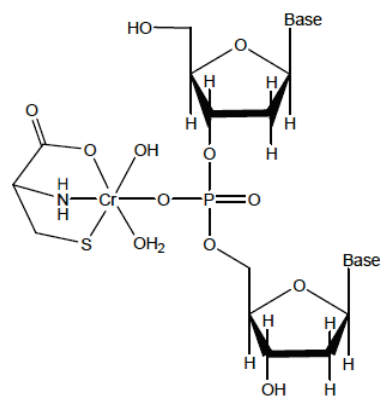
reduced Mn oxide (45). Although in soils the reduction of Cr(VI) to Cr(III) is favored compared to the Cr(III) oxidation, high concentrations of Cr(VI) may overcome the reducing capability of the environment and thus Cr(VI) may persist in potentially toxic levels (12).



**Figure 4.** Calculated abundance of Cr(VI) species in aqueous solution at total Cr(VI) concentration  $1 \times 10^{-6}$  M and within pH 1-14 [(54), modified].

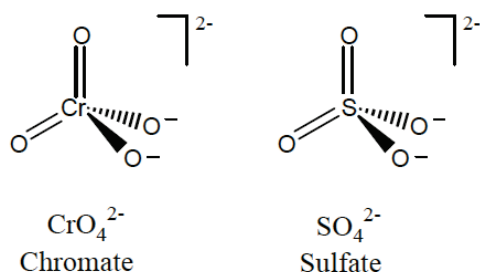
### 1.3. Chromium toxicity

Biological effects associated with chromium exposure are various, since the nature of these effects depends upon the metal speciation. Cr(III) is an essential trace element that is well-known for its role in the maintenance of normal carbohydrate metabolism in mammals and yeasts (65) through the enhancement of insulin activity (102). At the extracellular level, Cr(III) is relatively innocuous as a consequence of its insolubility and subsequent inability to cross cell membranes. Inside the cell, Cr(III) may generate toxic effects by its ability to bind to phosphates in DNA (86). The main forms of chromium-DNA adducts in mammalian cells are ternary complexes generated by cross-linking of cysteine and histidine to DNA via a phosphate-bound Cr(III) atom (126) (Fig.5). Cr(III) may exert



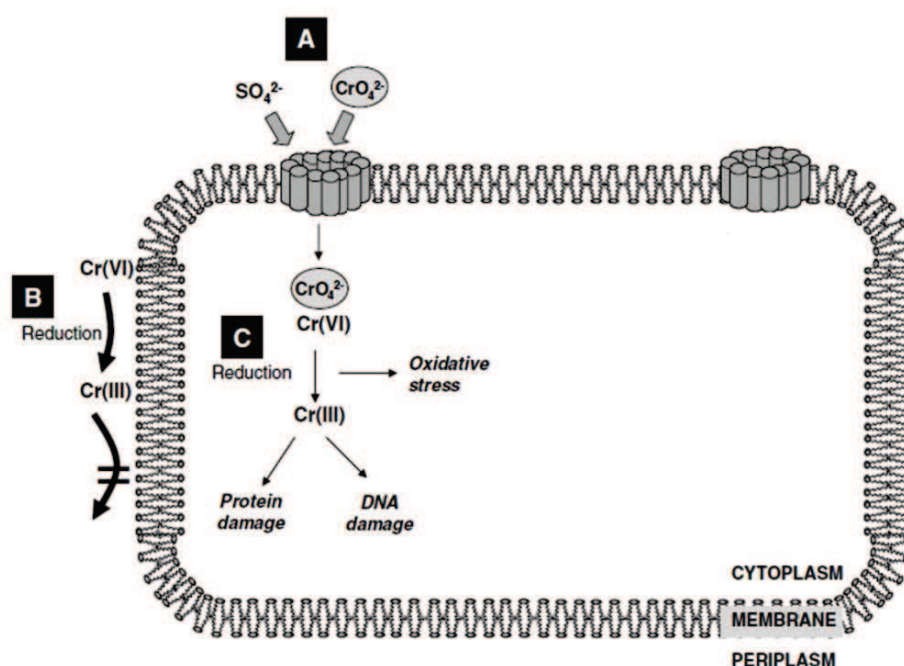
**Figure 5.** Proposed structure cysteine-DNA cross-link [(126), modified].

additional toxic effects by its ability to bind to carboxyl and sulfhydryl groups in proteins, and in human cells by competing with the transport of iron by transferrin (94,104). Oxidative damage to proteins has been established as a central mechanism of chromium toxicity in *Saccharomyces cerevisiae* (105). Cr(VI) is more bioavailable due to its high permeability of biomembranes and it is considered the most toxic form of chromium. Indeed, chromate chronic exposure leading to mutagenesis, carcinogenesis and teratogenesis (113). In bacteria chromate actively crosses biological membranes by means of the sulfate uptake pathway, which reflects the chemical analogy between these



**Figure 6.** Chemical structure of chromate and sulfate ions.

two oxyanions (11) (Fig. 6). In the cytoplasm, chromium toxicity is mainly related to the process of reduction of Cr(VI) to lower oxidation states [*i.e.*, Cr(III) and Cr(V)]. This reduction processes may generate free radicals (50). Oxidative damage to DNA is probably responsible for the genotoxic effects caused by chromate (Fig. 7).



**Figure 7.** Mechanisms of chromate transport and toxicity in bacterial cells. (A) Chromosome-encoded sulfate uptake pathway which is also used by chromate to enter the cell (B) Extracellular reduction of  $\text{Cr(VI)}$  to  $\text{Cr(III)}$  which does not cross the membrane. (C) Intracellular  $\text{Cr(VI)}$  to  $\text{Cr(III)}$  reduction may generate oxidative stress, as well as protein and DNA damage. [(91), modified].

#### 1.4. Mechanisms of bacterial tolerance and resistance to chromate

Arbitrary terms such as 'resistance' and 'tolerance' which are used rather loosely and often interchangeably in the literature are generally based on the ability to grow on a certain metal concentration in laboratory media. According to Gadd (1993), it is probably more appropriate to define 'resistance' as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned (*e.g.*, synthesis of metallothioneins or  $\gamma$ -glutamyl peptides), while 'metal tolerance' may be defined as the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity (29). Intrinsic properties that can determine survival include possession of extracellular polysaccharide and metabolite excretion, especially where this leads to detoxification of the metal species, *e.g.*, by binding or precipitation. However, in many cases distinctions are difficult because of the involvement of several direct and indirect physico-chemical

and biological mechanisms. A particular organism may directly and/or indirectly rely on several survival strategies.

A variety of Cr(VI) resistant bacteria has been reported, and the mechanisms of resistance to this ion may be encoded either by plasmids or by chromosomal genes. Usually, the genes located in plasmids encode membrane transporters, which directly mediate efflux of chromate ions from the cell's cytoplasm. On the other hand, resistance systems encoded within bacterial chromosomes are generally related to strategies such as specific or unspecific Cr(VI) reduction, free-radical detoxifying activities, repairing of DNA damage and processes associated with sulfur or iron homeostasis (91). Table 1 summarizes the bacterial strategies that have been related to chromate tolerance.

**Table 1.** Bacterial system related to chromate tolerance [(91), modified].

| Enzyme/system                       | Species                          | Function                        | Ref   |
|-------------------------------------|----------------------------------|---------------------------------|-------|
| <b>Transport</b>                    |                                  |                                 |       |
| ChrA transporter                    | <i>Pseudomonas aeruginosa</i>    | Efflux of cytoplasmic Cr(VI)    | (4)   |
| Cys operon products                 | <i>Shewanella oneidensis</i>     | Sulfate transport               | (10)  |
| TonB receptor, hemin transporter    | <i>Shewanella oneidensis</i>     | Iron transport                  | (10)  |
| <i>oscA</i>                         | <i>Pseudomonas corrugata</i>     | Sulfate transport               | (117) |
| <b>Reduction</b>                    |                                  |                                 |       |
| Chromate reductases                 | <i>Diverse species</i>           | Reduction of Cr(VI) to Cr(III)  | (12)  |
| <b>General and oxidative stress</b> |                                  |                                 |       |
| SOD, catalase                       | <i>Escherichia coli</i>          | Combat of oxidative stress      | (2)   |
| SOD, glutathione transferase        | <i>Caulobacter crescentus</i>    | Combat of oxidative stress      | (39)  |
| Outer membrane proteins             | <i>Caulobacter crescentus</i>    | General stress response         | (39)  |
| Fe-SOD ChrC                         | <i>Cupriavidus metallidurans</i> | Detoxification of free radicals | (49)  |
| STH                                 | <i>Pseudomonas corrugata</i>     | Redox state regulation          | (19)  |
| Malic enzyme                        | <i>Pseudomonas corrugata</i>     | Repair oxidative damage         | (20)  |
| <b>DNA repair</b>                   |                                  |                                 |       |
| SOS response                        | <i>Escherichia coli</i>          | Repair of DNA damage            | (60)  |
| RuvB DNA helicases                  | <i>Pseudomonas aeruginosa</i>    | Repair of DNA damage            | (66)  |
| RecG                                | <i>Pseudomonas corrugata</i>     | Repair of DNA damage            | (20)  |
| SO0368, UvrD, and HrpA helicases    | <i>Shewanella oneidensis</i>     | Repair of DNA damage            | (15)  |
| <b>Other mechanisms</b>             |                                  |                                 |       |
| Cys operon products                 | <i>Shewanella oneidensis</i>     | Sulfur metabolism               | (10)  |
| Adenylyl sulfate kinase             | <i>Shewanella oneidensis</i>     | Sulfur metabolism               | (10)  |
| Sulfite reductase                   | <i>Shewanella oneidensis</i>     | Sulfur metabolism               | (10)  |
| Ferritin                            | <i>Shewanella oneidensis</i>     | Iron binding                    | (10)  |

#### 1.4.1. Reduction of hexavalent chromium

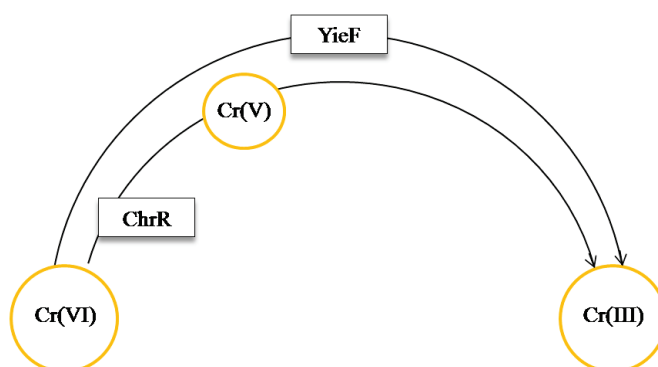
Under certain environmental conditions chromium can be interconverted between Cr(III) and Cr(VI) by oxidation-reduction reactions of biotic and abiotic nature. Microbial reduction of Cr(VI) to Cr(III) is not a plasmid-associated trait and can be considered as an additional chromate resistance mechanism. Three Cr(VI) reduction mechanisms have been described: *i*) chromate reduction under aerobic conditions is commonly associated with soluble chromate reductases that use NADH or NADPH as cofactors (81); *ii*) under anaerobic conditions, some bacteria can use Cr(VI) as an electron acceptor in the electron transport chain (108); *iii*) Cr(VI) can be also reduced by unspecific reactions associated with organic compounds such as amino acids, nucleotides, sugars, vitamins, organic acids, or glutathione (69).

Chromate reduction is carried out by diverse bacterial species; it may occur under aerobic or anaerobic conditions and may be associated with the cell membrane or with the soluble fraction. The first analyzed chromate reductase has been a membrane-associated enzyme from *Enterobacter cloacae* HO1 that transfers electrons to Cr(VI) by NADH-dependent cytochromes (78,119). Cr(VI) reductases have been primarily characterized in the context of alternative substrates; these enzymes commonly show a NADH:flavin oxidoreductase activity and can also act as chromate reductases. An example is the reductases NfsA/NfsB from *Vibrio harveyi*, with a nitrofurazone nitroreductase property as a primary activity and chromate reductase as a secondary function (55), and the ferric reductase FerB from *Pseudomonas denitrificans* that uses Fe(III)-nitrilotriacetate and chromate as substrates (63).

The currently best studied chromate reductase is ChrR from *Pseudomonas putida*, a soluble flavin mononucleotide-binding enzyme able to catalyze the reduction of Cr(VI) to Cr(III) (81). The ChrR enzyme functions as a NADH-dependent reductase activity. Studies with purified *P. putida* ChrR revealed a quinone reductase activity that generates a flavin semiquinone during chromate reduction; this reaction transfers NADH electrons to superoxide anion and probably produces the Cr(V) species transiently. ChrR activity seems to provide an antioxidant defense mechanism to *P. putida* by protecting cells against H<sub>2</sub>O<sub>2</sub> stress (30). ChrR in one pathway reduces Cr(VI) to Cr(III), generating intermediary Cr(V) and superoxide anion and by an additional

mechanism reduces quinones, which provide shielding from reactive oxygen species (ROS) (11).

The *Escherichia coli* YieF protein shares sequence homology with the *P. putida* ChrR enzyme. Both soluble enzymes are members of a widespread family of proteins and show similar properties but different reduction process. Under aerobic conditions, ChrR of *P. putida* catalyzes a combination of one- and two-electron transfers to Cr(VI) with the transient formation of Cr(V), while YieF has a different reaction mechanism that involves an obligatory four-electron reduction of chromate in which the enzyme simultaneously transfers three electrons to chromate to produce Cr(III) and one electron to molecular oxygen generating ROS (2) (Fig. 8).



**Figure 8.** Plausible mechanisms of enzymatic Cr(VI) reduction. ChrR of *P. putida* MK1 catalyzes a combination of one- and two-electron transfers to Cr(VI) with the transient formation of Cr(V), while YieF of *E. coli* mediates a four electron shuttle for the direct reduction of Cr(VI) to Cr(III) with the remaining electron transferred to oxygen [(14), modified].

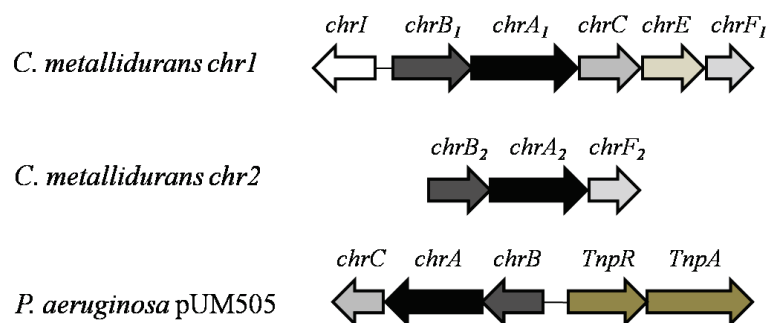
Cr(VI) reduction seems to be an efficient system of resistance to chromate in bacteria. Actually, Cr(VI) reductase may have a primary role other than Cr(VI) reduction (43). Cr(VI) reductases may be related to the recent introduction of Cr(VI) to the environment by anthropogenic activities.

#### 1.4.2. Efflux of chromate

The efflux of chromate is a Cr(VI)-resistance mechanism conferred by the ChrA protein. ChrA from *Pseudomonas aeruginosa* displays a topology of 13 transmembrane segments (TMS) and functions as a chemiosmotic pump that effluxes chromate from the cytoplasm using the proton-motive force. Efflux of chromate is inhibited by sulfate,



suggesting that this analog oxyanion may also bind to the ChrA protein (85). In fact, it has been proposed that ChrA may function as a chromate/sulfate antiporter (74). Nevertheless, sulfate transport by the ChrA protein has not yet been determined. The ChrA protein from *Cupriavidus* displays a different topology of 10 TMS and shows 29% of identical amino acids with respect to ChrA from *Pseudomonas*. The resistance mechanism, however, seems to be the efflux of chromate ions like in *Pseudomonas* (74). In addition, the *C. metallidurans* chromosome contains the *chrA2* gene, encoding a protein 84% identical to the product of its plasmid-encoded ChrA homolog (*chrA1*). The *chrA* gene from *C. metallidurans* plasmid pMOL28 forms part of the *chr1* operon that includes the *chrI*, *chrB*, and *chrF* genes, proposed to play regulatory roles for the expression of ChrA (49) (Fig. 9). It had been previously demonstrated that ChrB in presence of chromate has a function in the inducibility of the *chrA* gene (74). The complete sequencing of plasmid pB4 from *Pseudomonas* sp. B13 strain revealed the presence of the *chrBAC* gene cluster, which showed sequence similarity with the chromate resistance determinants from plasmids pUM505 and pMOL28 (37) (Fig. 9).



**Figure 9.** Comparison of genetic determinants of chromate resistance. *C. metallidurans chr1* and *chr2*, chromate resistance determinants 1 (plasmid pMOL28) and 2 (chromosomal); *P. aeruginosa* plasmid pUM505. Drawing not to scale [(37), modified].

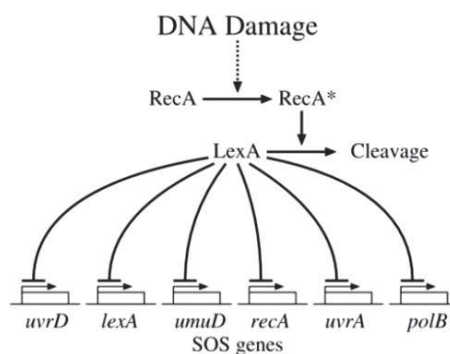
The CHR family of transporters is as a small group of prokaryotic proteins involved in chromate or sulfate transport, and includes proteins encoded in chromosomes and in plasmids (74). Phylogenetic analysis suggests that the CHR proteins may have derived from a gene duplication event, as occurred with members of other families of transporters. Besides, CHR gene clusters from different sources differ in their possession of associated potential regulatory genes: an example is the regulatory protein ChrB, which is present only in some gene clusters (11).

### 1.4.3. Other mechanisms of chromate tolerance

Besides Cr(VI) reduction and chromate efflux, above reported, bacteria display other mechanisms to deal with Cr(VI). Bacterial reduction of Cr(VI) may also result in the concomitant generation of free radicals and imposes oxidative damage on DNA (59).

The *chr1* operon from *C. metallidurans* plasmid pMOL28, which expresses the chromate efflux pump ChrA, is also constituted by *chrC* and *chrE* genes that seem to be involved in the chromate resistance (Fig. 10). ChrC protein shows homology to iron-containing SOD enzymes able to detoxify superoxide radicals, while protein ChrE may cleave some chromium-glutathione-complexes (49).

Protection of bacterial cells from DNA damage caused by Cr(VI) is another defensive mechanism. DNA helicases RecG and RuvB, components of the recombinational DNA repair system, were shown to participate in the response to DNA damage caused by chromate in *P. aeruginosa* (20,66). Ackerley *et al.* (2006) found that chromate exposure of *E. coli* cells led to the depletion of the pools of glutathione and other thiols, suggesting an important detoxifying role of these compounds against Cr(VI) (1). These authors have found that *E. coli* displays additional protective systems, including the induction of the SOS response and the activation of superoxide dismutase and catalase (Fig. 10).



**Figure 10.** The bacterial SOS DNA repair system. DNA damage is sensed by RecA, which induces autocleavage of the repressor LexA. LexA binds to the promoters of the SOS operons, including its own promoter and that of RecA.

Genome-wide analysis of cellular responses to chromate stress in *Caulobacter crescentus*, a bacterium able to survive in polluted habitats, revealed the up-regulation of genes encoding products involved in protection against oxidative stress and in repair

of DNA damage (39). *C. crescentus* seems not to have a chromate efflux system but Cr(VI) stress down-regulates a sulfate transport system, probably to reducing chromate uptake (39). A more complex response was found in the metal-reducing bacterium *Shewanella oneidensis* when exposed to chromate. Besides the aforementioned induction of genes involved in DNA repair and cellular detoxification, *S. oneidensis* showed the up-regulation of genes implicated in sulfur metabolism, iron transport, and transcriptional regulation (10).

### 1.5. Bioremediation of chromium

The remediation of Cr(VI)-contaminated soils, today, is essentially based on physical and chemical approaches, which include excavation or pumping of contaminated material, followed by the addition of reducing chemicals that lead to the precipitation and/or sedimentation of reduced chromium (118). Table 2 shows the advantages and disadvantages of the main traditional approaches for the remediation of soils contaminated with Cr(VI).

Bioremediation is an emerging *in situ* technology for the clean-up of environmental pollutants using microorganisms. By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade or transform the environmental contaminants into less toxic forms. It uses naturally occurring bacteria, fungi and algae to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes (116).

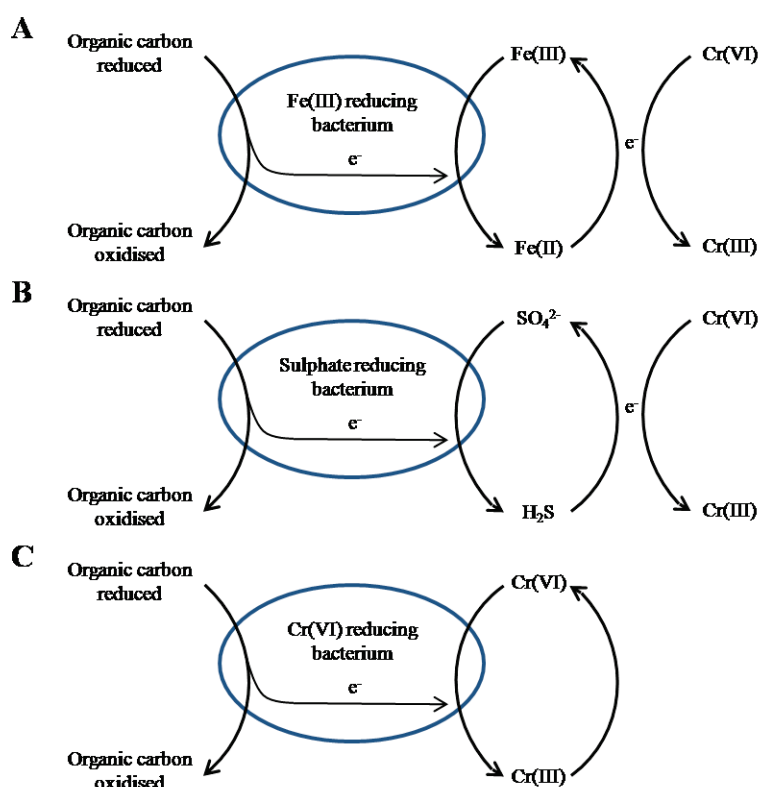
The chromium bioremediation approach offers some advantages compared with traditional techniques: *i*) it can be performed *in situ* without excavation of contaminated soils, *ii*) it can be applied to sites with high water table, *iii*) it can allow a continuous Cr(VI) stable reduction process, *iv*) it does not destroy the site that is to be detoxified (118).

**Table. 2.** Advantages and disadvantages of the main traditional approaches for the remediation of soil contaminated with Cr(VI) (118).

| Approach                                                | Advantages                                                                                                                                                           | Disadvantages                                                                                                                                                                                                                                                      |
|---------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Excavation and off-site disposal                        | Quick and appropriate for small volumes of soil, completely removes the contaminant                                                                                  | Expensive, may cause health hazard during the excavation, removed soil may need treatment, destroys the site but does not destroy the contaminant                                                                                                                  |
| Soil washing                                            | Reduces the volume of contaminated material that requires treatment, washing solution may be reused after decontamination treatment                                  | Needs the excavation of soil, may cause health hazard during the excavation, destroys the site but does not destroy the contaminant, generates contaminated water, is not appropriate for all soils                                                                |
| Soil flushing                                           | <i>In situ</i> technology that does not require excavation, its efficiency may be improved through electrokinetics                                                   | Does not destroy the contaminant, generates contaminated water                                                                                                                                                                                                     |
| Solidification/stabilisation <i>ex situ</i>             | Is relatively inexpensive                                                                                                                                            | May cause health hazard during the excavation, does not destroy the contaminant, increases the volumes of disposal material                                                                                                                                        |
| Solidification/stabilisation <i>in situ</i>             | Applicable to sites with high water tables                                                                                                                           | Does not remove the contaminant, before the treatment Cr(VI) may need to be reduced to Cr(III) in order to minimise potential leaching of pollutants                                                                                                               |
| Vitrification ( <i>in situ</i> or <i>ex situ</i> )      | Should reduce toxicity, mobility contaminant and volume of polluted soils; the final product can be demonstrated to be nonhazardous, may be performed <i>in situ</i> | Demands high energy and technology, requires significant amounts of additive                                                                                                                                                                                       |
| Chemical reduction ( <i>in situ</i> or <i>ex situ</i> ) | Reduces toxicity and mobility of the Cr(VI), may be performed <i>in situ</i>                                                                                         | Does not remove from the soil the Cr(III) produced, which may be oxidised to Cr(VI); is not appropriated for sites where the level of Cr is to be reduced, requires the control of chemical-physical characteristics of soil, the process of reduction may be slow |

A wide range of microorganisms, including bacteria, yeasts, and algae, with exceptional ability to reduce Cr(VI) to Cr(III) anaerobically and/or aerobically, has been isolated from Cr(VI)-contaminated and noncontaminated soils and water. Apart from enzymatic

reduction, nonenzymatic reduction of Cr(VI) can also be common and widespread in the environment. For instance, biotic-abiotic coupling reactions involving the microbially formed products, H<sub>2</sub>S (the end product of sulfate reduction) and Fe(II) (formed by Fe(III) reduction), can mediate the dissimilatory reduction of Cr(VI) (Fig.11). Despite the dominant occurrence of enzymatic and nonenzymatic reduction of Cr(VI), natural attenuation of Cr(VI) is a very slow process (51).



**Figure 11.** Bacterial reduction of Cr(VI) mediated by A) iron-reducing bacteria; B) sulfur-reducing bacteria; C) chromate-reducing bacteria [(118), modified].

Thus, there is a need to improve *in situ* bioreduction of Cr(VI) to Cr(III). The Cr(VI)-remediation efficiency can be enhanced by introducing in soils selected strains with intrinsic properties, such as high Cr(VI)-resistance and Cr(VI)-reduction capability (bioaugmentation) or stimulating the activity of indigenous Cr(VI)-reducers (biostimulation). In both cases, a strong limitation is that contaminated sites are usually lacking in nutrients and do not permit rapid growth of selected and/or indigenous bacteria and, therefore, their potential bioremediation activities are not fully expressed (118). A strategy to stimulate the metabolism and proliferation of bacterial Cr(VI)-reducers *in situ* may be the addition of nutrients to the environment (92,110). For

example, Jeyasingh and Philip (2005) reported that a bacterial concentration of  $15 \pm 1.0$  mg/g of soil (wet weight) and 50 mg of molasses/g of soil as carbon source were required for the maximum Cr(VI) reduction (47). However, the addition of nutrients to Cr(VI)-contaminated soils is a laborious and expensive approach and it may cause problems, because it results in the production of considerable biomass (112). In several studies, the use of bioreactors or biofilms for *ex situ* Cr(VI)-bioremediation of soils or soil wash effluents was assumed (24,71,106). The possibility to use bioreactor systems for Cr(VI)-bioremediation is cost-effective, but its success has been limited to large-scale decontamination projects (51). Cr(VI)-bioremediation *ex situ*, such as *in situ*, can be performed using microbial pure cultures or microorganisms consortia. Smith (2001) proposed sulphate-reducing bacteria (mixed-culture) biofilms to treat Cr(VI)-contaminated waterways and soils, suggesting that this system can be used to recover Cr(III) from the reduction and precipitation of Cr(VI) (100). Rama Krishna and Philip (2005) developed a novel *ex situ* treatment technology in which the leached Cr(VI) from a contaminated soil was treated in a bioreactor for Cr(VI) reduction, followed by a biosorption column for Cr(III) removal (90).

Though many studies have been reported on Cr(VI) contaminated soil remediation, most of them were confined to laboratory scale systems. It is therefore essential to conduct a pilot scale study and evolve an appropriate management strategy before starting the field remediation. Parameters such as moisture content, initial biomass concentration and electron donor play a significant role in achieving the remediation in the shortest possible time, with least cost and this would be possible through the development of a management model based on pilot scale experimental data. Several mathematical models are available for performance evaluation and management of Cr(VI) contaminated wastewaters and aquifers. Jeyasingh *et al.* (2010) isolate Cr(VI) reducing microorganisms for the remediation of solid waste containing Cr(VI) and evaluate the performance of the process in a pilot scale system. Further they developed a mathematical model for simulating the process and find the optimal strategy for cost-effective remediation of large scale Cr(VI) contaminated dump sites (48).

In order to move from the potential and/or laboratory scale systems phase to the applied one we need *i)* to have bacterial strains belonging to different species, selected for Cr(VI)-resistance and capability to Cr(VI)-reduction; *ii)* to increase knowledge on the mechanisms involved in the processes of resistance and reduction of Cr(VI); *iii)* to

understand how the bacterial kinetics of Cr(VI)-reduction are affected by abiotic factors, such as pH, temperature, electron acceptors and organic substrates (118).

Finally, in a pollute soil, in addition to Cr, could be other toxic substances that inhibit metabolic activity and growth of Cr(VI)-reducing bacteria. Therefore it would be desirable that microorganisms exploit in the process of Cr(VI)-bioremediation are able to tolerate also these toxic substances.

## 2. Biofilm

For more than a century, there has been a strong tradition for studies of microorganisms as free-floating (planktonic) liquid cultures. This has imprinted the view that microorganisms live as unicellular organisms. It is now clear that microorganisms do not live as pure cultures of dispersed single cells but instead accumulate at interfaces to form polymicrobial aggregates such as films, mats, flocks, sludge or 'biofilms'. Biofilms can be defined as a microbial aggregates that usually accumulate at a solid-liquid interface and are encased in a matrix of highly hydrated extracellular polymeric substance (EPS). Included in this definition are cell aggregates such as flocs (floating biofilms) and sludge, which are not attached to an interface but which share the characteristics of biofilms (27). Biofilms can be composed of a population that developed from a single species or a community derived from multiple microbial species, and they can form on a vast array of abiotic and biotic surfaces.

The strategy of microbial growth that leads to biofilm formation, compared with planktonic growth, results in the development of a microbial community that is characterized by a marked resistance to conventional biocides, antibiotics and to the action of environmental stresses, including those due to heavy metals (95,109). The mechanisms underlying tolerance of biofilms to toxic agents have not been fully elucidated. However, it has been suggested that the sequestration of toxicants by the extracellular polymeric matrix and the involvement of specialized cells termed "persistors" might be factors equally contributing to the aforementioned tolerance to potentially toxic agents (32). Furthermore, biofilms provide an effective environment for the degradation of organic contaminants, since diffusion processes occurring within these systems result in the development of redox gradients due to the combination of both anaerobic and aerobic processes. In addition, the heterogeneity of the biofilm matrix leads to the development of niches allowing the survival of slow-growing pollutant-degrading microbes (99).

Microorganisms are known to undergo through profound changes upon the transition from planktonic growth to biofilm that involve both gene expression and biochemical properties. However, despite the significant progress in this area, there is scant

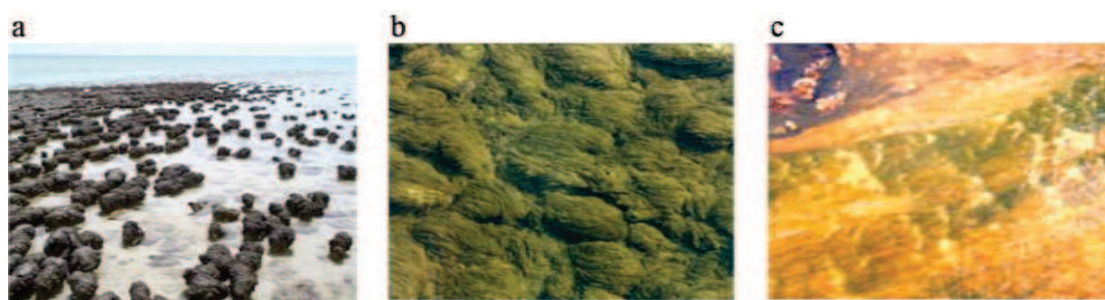


information on the mechanisms governing growth within biofilms and on the relationships of biofilms with the surrounding environment and with pollutants.

## 2.1. Biofilms: adaptable structures

Biofilm formation appears early in the fossil record (~3.25 billion years ago) and is common throughout a diverse range of organisms in both the Archaea and Bacteria lineages (31). In the context of evolution and adaptation it is likely that biofilms provided homeostasis in the face of the fluctuating and harsh conditions of the primitive earth (*i.e.*, extreme temperatures, extreme value of pH, exposure to ultraviolet light), thereby facilitating the development of complex interactions between individual cells and providing an environment which was sufficient for the development of signaling pathways and chemotactic motility (31).

Intriguingly, the visual characteristics of biofilms growing in diverse environments are strikingly similar, indicating there are important convergent survival strategies. Biofilms growing in fast-moving water tend to form filamentous streamers regardless of whether they occur in the drainage run-off from acid mines or in hydrothermal photosynthetic mats. They can also growth as an assemblage of organisms attached to and living on submerged solid surfaces in rivers or other natural environments (31) (Fig. 12).

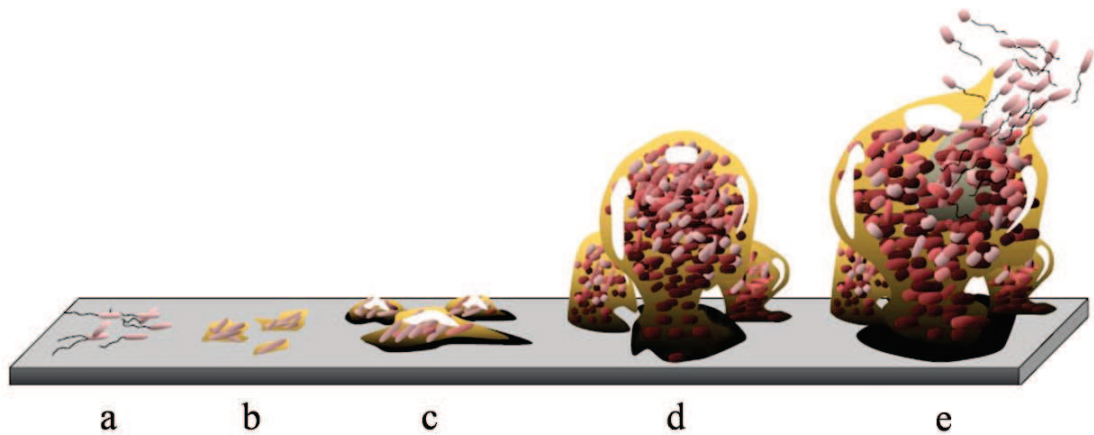


**Figure 12.** Biofilm structures. In quiescent waters biofilms tend to form mushroom or mound-like structures that are similar to those of stromatolites (a), while in fast-moving water biofilms tend to form filamentous streamers (b) and ripple structures (c) [(31), modified].

The complex interactions among the various species in multispecies biofilms affect the structure and function of these communities, with competition and synergism (metabolic cooperation) being common themes (84). The structures of biofilms are also known to depend on the carbon source provided to the microbe (67).

## 2.2. The biofilm life cycle

Microscopic analysis indicated that the transition from a free-swimming existence to a surface attached community-based lifestyle proceeded via distinct steps, culminating in the formation of a complex structural arrangement of cells (16). Commonly, it is recognized that the stages of biofilm development include: *i*) reversible and irreversible attachment; *ii*) surface motility and initiation of microcolony formation; *iii*) maturation, ageing and differentiation of microcolonies; and *iv*) biofilm dissolution and generation of specialized dispersal cells (53) (Fig. 13).



**Figure 13.** Biofilm development. a, initial attachment; b, irreversible attachment; c, microcolony formation; d, maturation; e, dispersion [(68), modified].

Biofilm formation is thought to begin when bacteria sense environmental conditions that trigger the transition to life on a surface. *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* form biofilms under almost any conditions that allow growth (77). On the other hand, some strains of *Escherichia coli* K-12 and *Vibrio cholerae* not form biofilms in minimal medium unless supplemented with amino acids (87,120), while *E. coli* O517:H7 is reported to make a biofilm only in low-nutrient media (21). In addition to the nutritional content of the medium, other environmental cues that can influence biofilm formation include temperature, osmolarity, pH, iron, and oxygen (76). The initial cell attachment is based on electrostatic attraction and physical forces. Some of reversible adsorbed cells begin to make preparations for a lengthy stay by forming structures which may then permanently bind to the surface. Although the initial contact of a bacterial cell with a surface is not necessarily regulated, there is evidence for regulation of the formation of stable cell-surface interactions. Some of the best evidence

to indicate that bacteria can sense contact with a surface and, in response, alter gene expression to promote stable cell-surface interactions comes from studies with the Cpx signalling system of *E. coli*. The Cpx signalling system is a typical two-component signalling pathway and is composed of CpxA, a sensor kinase and phosphatase, and CpxR, a response regulator (89). The genes regulated by the Cpx pathway that are required to enable stable cell-surface interactions are as yet undefined. However, it is known that the Cpx signalling pathway positively regulates P-pili, which may play a role in surface adhesion (40). In fact, a *cpxR* mutant strain forms less stable cell-surface interactions in comparison with the wild-type strain (79).

Cells irreversibly adsorbed on the surface proceed to reproduce and the daughter cells, which form microcolonies, begin to produce a polymer matrix (80). An important step in biofilm development is the formation of a characteristic biofilm architecture. Although numerous techniques have been utilized to document the biofilm architecture of bacteria, until recently it was not clear if this structural complexity was regulated or the consequence of stochastic processes. The observation that a mutant of *P. aeruginosa* unable to synthesize the major quorum-sensing (QS) molecules acylhomoserine lactones (acyl-HSLs) was radically altered in biofilm architecture clearly demonstrated that these molecules regulate the formation of biofilm structures in this organism (18). In *P. aeruginosa*, the depth of the mature biofilm structure is also reduced by the transcription factor RpoS, which directs entry into stationary phase in many Gram-negative bacterial species. Mutants of *P. aeruginosa* that lack RpoS formed biofilms of greater depth under flowing conditions (122). RpoS production is regulated at multiple levels, including transcription, translation and proteolysis, in response to different stress conditions including nutrient limitation (115). Thus, activation of RpoS would signal that nutrients are limiting in *P. aeruginosa* biofilms. This is another example of how biofilms are regulated in response to nutrient levels in the environment.

The biofilm life cycle culminates in the dispersal of physiologically differentiated free-living cells that can colonize new locations. Detachment can be caused by external perturbations, such as increased fluid shear, by internal biofilm processes, such as endogenous enzymatic degradation, or by the release of EPS or surface-binding proteins (64). There are several bacterially derived signals that induce dispersal, including acyl-homoserine lactones, cell-cell autoinducing peptides, diffusible fatty acids and D-amino acids. The fact that dispersal is induced by many different cues or signals potentially

allows precise regulation of the attached and planktonic phenotypes in response to changes in environmental conditions (64). In general, in the dispersal cells, genes that regulate features of the sessile biofilm phenotype, such as EPS and fimbriae, are down-regulated, whereas genes that encode factors which are important for a motile lifestyle, including flagella and proteins involved in chemotaxis, are up-regulated (93). Further, both increases and decreases in nutrient concentration and low, non-toxic concentrations of NO (nitric oxide), derived endogenously or from external sources, have been correlated with biofilm dispersal. Low NO concentrations regulate a widely conserved genetic pathway that controls the conversion between the planktonic and surface-attached lifestyles through the action of cyclic diguanylate (c-di-GMP) (8).

Although QS-based extracellular cell-cell signalling systems have been primarily linked to biofilm formation, QS also has an important role in dispersal from biofilms. In *S. aureus*, the accessory gene regulator (*agr*) QS system has variable effects on biofilm development, depending on the medium and the flow conditions. This system is repressed in biofilms but is actively expressed in the dispersal population (124). A consistent theme in the regulation of biofilm formation and dispersal is the central role of the intracellular second messenger c-di-GMP. The intracellular level of c-di-GMP regulates the transition from biofilm to planktonic phenotypes in response to various environmental cues and cell-cell signals in bacteria. Although the genes encoding the diguanylyl cyclases (DGCs) that synthesize c-di-GMP and the phosphodiesterases (PDEs) that degrade it are redundant and their expression is not well understood in all cases, the outcome is the same: a decrease in the intracellular c-di-GMP level leads to dispersal (64).

One of the most controversial aspects of a developmental model of biofilm formation is the requirement for independent and dedicated gene networks that have evolved specifically for the regulation of the biofilm developmental cycle. Numerous microarray studies have now been published analyzing the changes in gene expression that are correlated with biofilm formation. From these studies has emerged the presence of several metabolic and regulatory systems that have involved in biofilm development process. Therefore, biofilm formation, even for a single organism, does not constitute an independent genetic program but is the results of multiple, convergent genetic pathways (67). Patell *et al.* (2010) have carried out a meta-analysis of several *P. aeruginosa* microarray analysis of gene expression in biofilm and planktonic cultures (83). They

have found that although the total number of transcripts modulated in the biofilms was large within the individual studies, the overlap between the data sets was small; they did not identify any obvious global regulatory genes in the core biofilm-associated transcriptome (83). Therefore, their finding suggest that biofilm formation is unlikely to follow a generic pre-determined developmental pathway. Instead, biofilms are part of a continuum of growth modes, that responds to specific environmental demands.

### **2.3. Biofilm resistance**

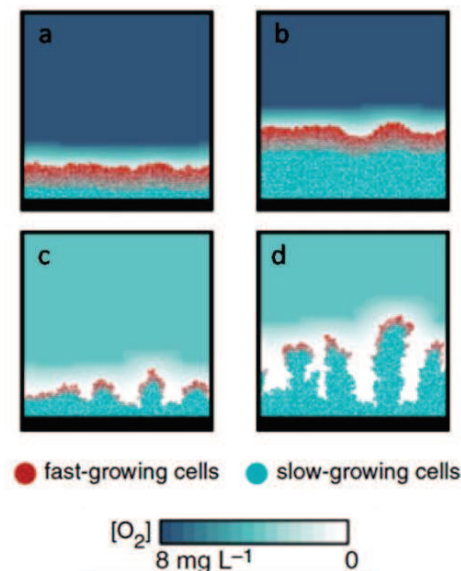
Bacteria embedded within biofilms exhibit unusual phenotypic features, and growth within this heterogeneous, multilayered, cellular matrix-entrapped community, and its eventual dispersal, requires the coordinated expression of an array of genes. In the literature there are several examples of biofilm that can tolerate levels of antimicrobial agents at least an order of magnitude higher than the minimum inhibitory concentrations (MICs) of genetically equivalent planktonic bacteria (46,109). Several explanations have been proposed for the enhanced resistance of biofilm-associated cells to both metals and antibiotics. For instance, both metal and antibiotic sequestration in the biofilm matrix and the presence of a small population of ‘persister’ cells might be contributing factors in the time-dependent tolerance of both planktonic cells and biofilms to high concentrations of antimicrobial agents (35).

Antibiotic resistance of planktonic bacteria usually involves inactivation of the antibiotic, modification of targets, and exclusion of the antibiotic (82). These actions typically require the acquisition of specific genetic factors, such as genes for  $\beta$ -lactamase or efflux pumps. As regards biofilm, it is possible to identified some general mechanisms involved in biofilm antibiotic resistance. These mechanisms include *i*) physical or chemical diffusion barriers to antimicrobial penetration into the biofilm, *ii*) slow growth of the biofilm owing to nutrient limitation, *iii*) activation of the general stress response and *iv*) the emergence of a biofilm-specific phenotypes (62).

The diffusion barriers consists mainly of the EPS matrix. It has been suggested that this matrix, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community. Either reaction of the compound with, or sorption to, the components of the biofilm can limit the transport of antimicrobial agents to the cells

within the biofilm (62). Mathematical modeling has predicted that while limited antibiotic diffusion may lead to the death of the outer layer of bacteria, it also stimulates subpopulations of bacteria put deeper within the biofilm to trigger adaptive changes (107). In some cases, biofilm survival may be affected by a diminish penetration and diffusion of antimicrobials through the biofilm matrix. For example, at sub-MIC concentrations of  $\beta$ -lactam antibiotics an increased alginate synthesis in *P. aeruginosa* biofilms was induced (6,123).

The slow growth of cells in the biofilm also contributes to antibiotics biofilm resistance. Several studies have highlighted the presence of hypoxic zones deep within biofilm, due to limited oxygen diffusion through biofilm matrix (82). The depletion of oxygen and nutrients inside biofilms might cause an altered metabolic activity and lead to slow growth of bacterial cells. Likewise, growth, protein synthesis and metabolic activity are stratified in biofilms. For example, there is a high level of activity at the surface but a low level in the centre, resulting in slow or no growth cells (103) (Fig. 14).



**Figure 14.** Diffusion limitation of a growth-limiting nutrient in a biofilm. (a–b) When environmental nutrient (e.g. oxygen) concentration is high, nutrients penetrate well into the biofilm, creating a thick layer of fast-growing cells; biofilm surface irregularities are not amplified, and the biofilm remains smooth. (c–d) When environmental nutrient concentration is low, nutrients are quickly depleted, creating a thin layer of actively growing cells. Therefore, cells residing in the peaks of irregularities continue to grow, while cells residing in the troughs of irregularities cease to grow and random irregularities in the biofilm surface are amplified [(70), modified].

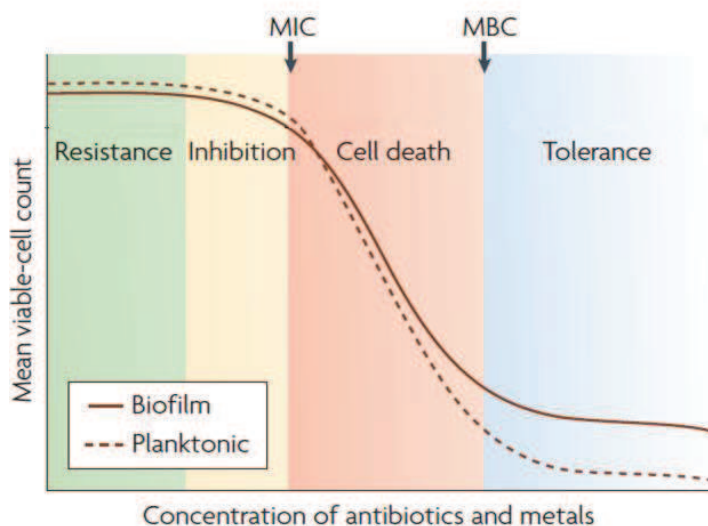


This may be one of the factors that contribute to the increased resistance of biofilms to antibiotics. In general all antimicrobials are more effective in killing rapidly growing cells, therefore slow growth of cells within the biofilm undoubtedly contributes to biofilm resistance. In addition slow growth is a major factor in the increased resistance of stationary planktonic cells to being killed.

Persister cells are considered to be either non-growing or slow-growing cells, and also have a greatly reduced susceptibility to antibiotics (56). In the persister's theory, these small subpopulations of bacteria within the biofilms differentiate into dormant cells, are able to survive extreme antibiotic treatment and have been hypothesized to be the result of phenotypic variations rather than due to stable genetic changes (52). Indeed, the formation of persisters might represent a common mechanism used by a wide range of bacteria during biofilm formation. This persistent population within biofilms may drastically inhibit the complete eradication of the biofilms, even after a prolonged high-level antibiotic treatment (80). In their study, Spoering and Lewis (2001) performed a detailed comparative examination of tolerance of biofilms versus stationary- and logarithmic-phase planktonic cells with four different antimicrobial agents (101). They found that, at least for *P. aeruginosa*, the notion that biofilms have greater resistance than do planktonic cells is unwarranted and they suggest that tolerance to antibiotics in stationary-phase or biofilm cultures is largely dependent on the presence of persister cells. However, it is unclear what relationship exists between planktonic persisters and biofilm resistance, and the mechanisms by which persisters form and/or confer increased antibiotic tolerance are still unknown.

General stress due to growth within a biofilm may be another reason of slow growth rate of some cells within it (62). The central regulator of stress response is the alternate  $\sigma$  factor, RpoS, originally thought to be expressed only in stationary phase (36). Cells growing at high densities undergo to general stress response and induce RpoS. Biofilm is characterized by an high cell densities, and therefore RpoS may be expressed (3). Finally, biofilm multidrug-resistance is also imputable to increased frequencies of horizontal gene transfer that occur in biofilm-growing bacteria and to the release of defensive enzymes into the extracellular space (*e.g.*,  $\beta$ -lactamase in biofilms has been considered to come from the layer of lysed bacteria resulting from exposure to antibiotic) (80).

The effect of toxic metals on biofilm is different from that related to antibiotics because to the different chemistry and function of metal species. However, similar to studies of biofilm antibiotic susceptibility (101), simple time- and dose-dependent killing assays have shown that in biofilms there are subpopulations of cells that die at different rates after the exposure of the entire population to metal ions (32,34) (Fig. 15).



**Figure 15.** Time- and dose-dependent killing of biofilms by metals. In general, both antibiotics and toxic metal species kill biofilm populations in a time- and concentration-dependent manner. This is observed as biphasic population killing, in which most of the growing population is rapidly killed by a low concentration of the antimicrobial, while a larger portion of biofilm population is able to withstand these lethal factors for exposure durations and at concentrations that exceed that which is lethal to the corresponding planktonic form [(33), modified].

As aforementioned, the limited diffusion of oxygen, nutrients and metabolites through the biofilm matrix generates an heterogeneous distributions in extracellular pH and redox gradients, that may also explain, in part, the tolerance of bacterial biofilms to metal ions. This features may influence metal-ion speciation, as well as certain constituents of the biofilm matrix can chelate and react with these compounds. Moreover, in a hypoxic environment, bacteria have a decreased metabolic ROS production and metal-catalysed Fenton-type reactions, which require oxygen. Biofilm is also composed by dead cells, that may contribute to pH heterogeneity and physiological microenvironments, and constitute a chemically reactive biomass able to drive the formation of metal precipitates or chelates through biosorptive sites (33). Other contributors to metal sequestration in biofilms are cell membranes and cell walls, which



offer many additional cationic and anionic sites for interactions with metal ions (114). Siderophores, which can bind to many kinds of transition metal ions and sequester them, can represent another mechanism by which microorganisms can interact with metals (33).

In biofilm, as well as in every microbial population, microorganisms produce a diverse array of metabolic end-products, many of which can bind and/or react with metal species, resulting in the precipitation or reduction of metal complexes. For example, biotic-abiotic coupling reactions involving the microbially formed products, H<sub>2</sub>S (the end product of sulfate reduction) and Fe(II) (formed by Fe(III) reduction), can mediate the dissimilatory reduction of Cr(VI) (118). Another example is the co-precipitation of metals with the carbonates (HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup>) that are produced during microbial respiration (25).

Collectively, metal-ion immobilization by biosorption and bio-inorganic reactions might permit cells to enter a protected physiological state by allowing them time to adapt (33). Like for antibiotics, persister cells contribute to biofilm heavy metal tolerance. In their work, Keren et. al. (2004) have characterized persister cells of *E. coli* (52). They have suggested that this cellular population is metabolically quiescent and expresses several genes that are associated with stress tolerance, such as chaperones, the multiple antibiotic resistance (*marRAB*) regulon and cold-shock proteins. It still is possible to isolate other types of phenotypic variants from biofilm populations and, as persister cells can only explain multidrug and multimetal tolerance, there might be other cell types that mediate biofilm resistance (33).

## **2.4. Biofilm applications in bioremediation**

Biofilm structure confers to the cells trapped inside several advantages compared to those living as planktonic cells: bacteria in biofilms show resistance to certain physical forces, such as shear flow, desiccation, and grazing (28). Furthermore, as above, one of the most important properties of biofilm-associated bacteria is tolerance to antimicrobial agents, making bacterial biofilms a major problem in clinical and industrial settings (17). In industry, biofilms cause corrosion and biofouling of equipment with consequent loss of efficiency (57,58). These same properties have beneficial aspects in

bioremediation, where bacterial biofilms are more proficient than planktonic microorganisms mainly because of their increased tolerance to toxic substances and of the close microorganisms interactions within biofilm, which make faster the degradation of pollutants (99). Within a biofilm the mineralization process is facilitate by the high cell densities and biomass, that localized solute concentrations and redox potential in the vicinity of the cells, as well as maintaining optimal conditions of pH (33).

Large volumes of dilute aqueous solutions such as industrial and municipal wastewaters are commonly treated through biofilm-based reactors. The main biofilm reactors are categorized according to the methods they use, such as the upflow sludge blanket (USB), biofilm fluidized bed (BFB), expanded granular sludge blanket (EGSB), biofilm airlift suspension (BAS) and internal circulation (IC) methods (73). Another promising application of biofilms is in heavy metal remediation (Table 3).

**Table 3.** Bioremediation of heavy metals using biofilms in bioreactors (99).

| Reactor or experimental conditions               | Methods of remediation        | Heavy metals remediated           | Refs    |
|--------------------------------------------------|-------------------------------|-----------------------------------|---------|
| Anaerobic–anoxic– oxic biofilm process           | Biosorption                   | Zn, Cd, Ni                        | (13)    |
| Biofilm formed on moving bed sand filter         | Biosorption, bioprecipitation | Cu, Zn, Ni, Co                    | (22)    |
| Rotary biofilm reactor for algae immobilization  | Immobilization                | Co                                | (111)   |
| Biofilm developed over granular activated carbon | Adsorption                    | Cd, Cu, Zn, Ni                    | (96,97) |
| Bacteriaimmobilized composite membrane reactor   | Bioprecipitation              | Cd, Zn, Cu, Pb, Y, Co, Ni, Pd, Ge | (23)    |

Like for chromate bioremediation, general heavy metal bioremediation can be achieved by immobilization, reduction/oxidation, concentration and partitioning to an environmental compartment to minimizing hazards (7). White and Gadd (2000) analysed the effect of copper on sulphate-reducing bacterial biofilms grown in continuous culture (121). They have observed a metal accumulation in the form of copper sulfide, as well as an increase in the EPS content of the biofilm. This evidence has confirmed the role of EPS in the entrapment of metal precipitates.

Sundar *et al.* (2011) have analyzed the applicability of bacterial biofilms for the bioremoval of trivalent chromium from tannery effluents: they have designed a continuous flow reactor for the development of biofilms on different substrates like

glass beads, pebbles and coarse sand. Biofilms have been developed using consortium of *Bacillus subtilis* and *Bacillus cereus* on coarse sand and have been able to remove 98% of Cr(III) (106).

A molecular approaches may enable the construction of improved strains with specific metal-binding and/or –reducing properties through the expression of specific proteins and molecules, which can improvement metal precipitation processes, as well as introduce transformation activities in resistant environmental strains.

### 3. References

1. **Ackerley, D. F., Y. Barak, S. V. Lynch, J. Curtin, and A. Martin.** 2006. Effect of chromate stress on *Escherichia coli* K-12. *J. Bacteriol.* **188**:3371-3381.
2. **Ackerley, D. F., C. F. Gonzalez, C. H. Park, R. Blake, M. Keyhan, and A. Matin.** 2004. Chromate-reducing properties of soluble flavoproteins from *Pseudomonas putida* and *Escherichia coli*. *Appl. Environ. Microbiol.* **70**:873-882.
3. **Adams, J. L. and R. J. McLean.** 1999. Impact of *rpoS* deletion on *Escherichia coli* biofilms. *Appl. Environ. Microbiol.* **65**:4285-4287.
4. **Alvarez, A. H., R. Moreno-Sanchez, and C. Cervantes.** 1999. Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa*. *J. Bacteriol.* **181**:7398-7400.
5. **ATSDR.** 2011. Priority list of hazardous substance 2011, Atlanta.
6. **Bagge, N., M. Schuster, M. Hentzer, O. Ciofu, M. Givskov, and E. P. Greenberg.** 2004. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and  $\beta$ -lactamase and alginate production. *Antimicrob. Agents Chemother.* **48**:1175-1187.
7. **Barkay, T. and J. Schaefer.** 2001. Metal and radionuclide bioremediation: issues, considerations and potentials. *Curr. Opin. Microbiol.* **4**:318-323.
8. **Barraud, N., D. J. Hassett, S. H. Hwang, S. A. Rice, S. Kjelleberg, and J. S. Webb.** 2006. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**:7344-7353.
9. **Bielicka, A., I. Bojanowska, and A. Wisniewski.** 2005. Two faces of chromium - pollutant and bioelement. *Pol. J. Environ. Stud.* **14**:5-10.
10. **Brown, S. D., M. R. Thompson, N. C. Verberkmoes, K. Chourey, M. Shah, J. Zhou, R. L. Hettich, and D. K. Thompson.** 2006. Molecular dynamics of

the *Shewanella oneidensis* response to chromate stress. Mol. Cell Proteomics. **5**:1054-1071.

11. **Cervantes, C. and J. Campos-García.** 2007. Reduction and efflux of chromate by bacteria. Microbiol. Monogr. **6**:407-419.
12. **Cervantes, C., J. Campos-Garcia, S. Devars, F. Gutierrez-Corona, H. Loza-Tavera, J. C. Torres-Guzman, and R. Moreno-Sanchez.** 2001. Interactions of chromium with microorganisms and plants. FEMS Microbiol. Rev. **25**:335-347.
13. **Chang, W. C., G. S. Hsu, S. M. Chiang, and M. C. Su.** 2006. Heavy metal removal from aqueous solution by wasted biomass from a combined AS-biofilm process. Bioresource Technol. **97**:1503-1508.
14. **Cheung, K. H. and J. Gu.** 2007. Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: A review. Int. Biodeter. Biodegr. **59**:8-15.
15. **Chourey, K., M. R. Thompson, J. Morrell-Falvey, N. C. Verberkmoes, S. D. Brown, M. Shah, J. Zhou, M. Doktycz, R. L. Hettich, and D. K. Thompson.** 2006. Global molecular and morphological effects of 24-hour chromium(VI) exposure on *Shewanella oneidensis* MR-1. Appl. Environ. Microbiol. **72**:6331-6344.
16. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappinscott.** 1995. Microbial Biofilms. Ann. Rev. Microbiol. **49**:711-745.
17. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284**:1318-1322.
18. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280**:295-298.
19. **Decorosi, F., L. Lori, L. Santopolo, E. Tatti, L. Giovannetti, and C. Viti.** 2011. Characterization of a Cr(VI)-sensitive *Pseudomonas corrugata* 28 mutant

- impaired in a pyridine nucleotide transhydrogenase gene. *Res. Microbiol.* **162**:747-755.
20. **Decorosi, F., E. Tatti, A. Mini, L. Giovannetti, and C. Viti.** 2009. Characterization of two genes involved in chromate resistance in a Cr(VI)-hyper-resistant bacterium. *Extremophiles* **13**:917-923.
  21. **Dewanti, R. and A. C. L. Wong.** 1995. Influence of culture conditions on biofilm formation by *Escherichia coli* 0157:H7. *Int. J. Food Microbiol.* **26**:147-164.
  22. **Diels, L., P. H. Spaans, S. Van Roy, L. Hooyberghs, A. Ryngaert, H. Wouters, E. Walter, J. Winters, L. E. Macaskie, J. Finlay, B. Pernfuss, H. Woebking, T. Pümpel, and M. Tsezos.** 2003. Heavy metal removal by sand filters inoculated with metal sorbing and precipitating bacteria. *Hydrometallurgy* **71**:235-241.
  23. **Diels, L., S. Van Roy, K. Somers, I. Willems, W. Doyen, M. Mergeay, D. Springael, and R. Leysen.** 1995. The use of bacteria immobilized in tubular membrane reactors for heavy metal recovery and degradation of chlorinated aromatics. *J. Memb. Sci.* **100**:249-258.
  24. **Dogan, N. M., C. Kantar, S. Gulcan, C. J. Dodge, B. C. Yilmaz, and M. A. Mazmanci.** 2011. Chromium(VI) bioremoval by *Pseudomonas* bacteria: role of microbial exudates for natural attenuation and biotreatment of Cr(VI) contamination. *Environ. Sci. Technol.* **45**:2278-2285.
  25. **Dupraz, C. and P. T. Visscher.** 2005. Microbial lithification in marine stromatolites and hypersaline mats. *Trends Microbiol.* **13**:429-438.
  26. **Ehrlich, H. L.** 2002. How microbes mobilize metals in ores: a view of current understandings and proposals for further research. *Miner. Metall. Process.* **19**:220-224.
  27. **Flemming, H. C. and J. Wingender.** 2010. The biofilm matrix. *Nat. Rev. Microbiol.* **8**:623-633.

28. **Fux, C. A., J. W. Costerton, P. S. Stewart, and P. Stoodley.** 2005. Survival strategies of infectious biofilms. *Trends Microbiol.* **13**:34-40.
29. **Gadd, G. M.** 1993. Interaction of fungi with toxic metals. *New Phytol.* **124**:25-60.
30. **Gonzalez, C. F., D. F. Ackerley, S. V. Lynch, and A. Martin.** 2005. ChrR, a soluble quinone reductase of *Pseudomonas putida* that defends against H<sub>2</sub>O<sub>2</sub>. *J. Biol. Chem.* **280**:22590-22595.
31. **Hall-Stoodley, L., J. W. Costerton, and P. Stoodley.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2**:95-108.
32. **Harrison, J. J., H. Ceri, N. J. Roper, E. A. Badry, K. M. Sproule, and R. J. Turner.** 2005. Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. *Microbiol.-Sgm* **151**:3181-3195.
33. **Harrison, J. J., H. Ceri, and R. J. Turner.** 2007. Multimetal resistance and tolerance in microbial biofilms. *Nat. Rev. Microbiol.* **5**:928-938.
34. **Harrison, J. J., R. J. Turner, and H. Ceri.** 2005. High-throughput metal susceptibility testing of microbial biofilms. *Bmc Microbiol.* **5**:1-11.
35. **Harrison, J. J., R. J. Turner, and H. Ceri.** 2005. Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa*. *Env. Microbiol.* **7**:981-994.
36. **Hengge-Aronis, R.** 1996. Regulation of gene expression during entry into stationary phase. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*. ASM Press.
37. **Henne, K. L., C. H. Nakatsu, D. K. Thompson, and A. E. Konopka.** 2009. High-level chromate resistance in *Arthrobacter* sp. strain FB24 requires previously uncharacterized accessory genes. *Bmc Microbiol.* **9**:1-14.

38. **Holm, O., E. Hansen, C. Lassen, F. Stuer-Lauridsen, and J. Kjolholt.** 2002. Heavy metals in waste - Final Report, Denmark.
39. **Hu, P., E. L. Brodie, Y. Suzuki, H. H. McAdams, and G. L. Andersen.** 2005. Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. J. Bacteriol. **187**:8437-8449.
40. **Hung, D. L., T. L. Raivio, C. H. Jones, T. J. Silhavy, and S. J. Hultgren.** 2001. Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili. EMBO J. **20**:1508-1518.
41. **Indipendent Environmental Technical Evaluation Group (IETEG).** 2005. Hexavalent chromium handbook. CRC PRESS, USA.
42. **International Chromium Development Association.** 2012. <http://www.icdacr.com>.
43. **Ishibashi, Y., C. Cervantes, and S. Silver.** 1990. Chromium reduction in *Pseudomonas putida*. Appl. Environ. Microbiol. **56**:2268-2270.
44. **ISPRA.** 2012. 10° Annuario dei dati ambientali 2011, Roma.
45. **James, B. R.** 1996. The challenge of remediating chromium-contaminated soil. Environ. Sci. Technol. **30**:248-251.
46. **Jefferson, K. K., D. A. Goldmann, and G. B. Pier.** 2005. The use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. Antimicrob. Agents Chemother. **49**:2467-2473.
47. **Jeyasingh, J. and L. Philip.** 2005. Bioremediation of chromium contaminated soil: optimization of operating parameters under laboratory conditions. J. Hazard. Mater. **118**:113-120.
48. **Jeyasingh, J., V. Somasundaram, L. Philip, and S. M. Bhallamudi.** 2010. Bioremediation of Cr(VI) contaminated soil/sludge: experimental studies and development of a management model. Chem. Eng. J. **160**:556-564.



49. **Juhnke, S., N. Peitzsch, N. Hübener, C. Große, and D. H. Nies.** 2002. New genes involved in chromate resistance in *Ralstonia metallidurans* strain CH34. Arch. Microbiol. **179**:15-25.
50. **Kadiiska, M. B., Q. H. Xiang, and R. P. Mason.** 1994. In vivo free radical generation by chromium(VI): an electron spin resonance spin-trapping investigation. Chem. Res. Toxicol. **7**:800-805.
51. **Kamaludeen, S. P., M. Megharaj, A. L. Juhasz, N. Sethunathan, and R. Naidu.** 2003. Chromium-microorganism interactions in soils: remediation implications. Rev. Environ. Contam Toxicol. **178**:93-164.
52. **Keren, I., N. Kaldalu, A. Spoering, Y. P. Wang, and K. Lewis.** 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett. **230**:13-18.
53. **Kjelleberg, S. and M. Givskov.** 2007. The biofilm mode of life: mechanisms and adaptation. Horizon Bioscience, Norfolk, U.K.
54. **Kotas, J. and Z. Stasicka.** 2000. Chromium occurrence in the environment and methods of its speciation. Environ. Poll. **107**:263-283.
55. **Kwak, Y. H., D. S. Lee, and H. B. Kim.** 2003. *Vibrio harveyi* nitroreductase is also a chromate reductase. Appl. Environ. Microbiol. **69**:4390-4395.
56. **Lewis, K.** 2005. Persister cells and the riddle of biofilm survival. Biochemistry **70**:267-274.
57. **Little, B., P. Wagner, W. G. Characklis, and W. Lee.** 1990. Microbial corrosion, p. 635-670. In W. G. Characklis and K. C. Marshall (ed.), Biofilm. Wiley, New York.
58. **Little, B., P. Wagner, R. Ray, and M. Mcneil.** 1990. Microbiologically influenced corrosion in copper and nickel seawater piping systems. Mar. Technol. Soc. J. **24**:10-17.

59. **Liu, K. J. and X. Shi.** 2001. In vivo reduction of chromium (VI) and its related free radical generation. *Mol. Cell Biochem.* **222**:41-47.
60. **Llagostera, M., S. Garrido, R. Guerrero, and J. Barbe.** 1986. Induction of SOS genes of *Escherichia coli* by chromium compounds. *Environ. Mutagen.* **8**:571-577.
61. **Losi, M. E., C. Amrhein, and W. T. Frankenberger.** 1994. Bioremediation of chromate-contaminated groundwater by reduction and precipitation in surface soils. *J. Environ. Qual.* **23**:1141-1150.
62. **Mah, T. F. C. and G. A. O'Toole.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **9**:34-39.
63. **Mazoch, J., R. Tesarik, V. Sedlacek, I. Kucera, and J. Turanek.** 2004. Isolation and biochemical characterization of two soluble iron (III) reductases from *Paracoccus denitrificans*. *Eur. J. Biochem.* **271**:553-562.
64. **McDougald, D., S. A. Rice, N. Barraud, P. D. Steinberg, and S. Kjelleberg.** 2012. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat. Rev. Microbiol.* **10**:39-50.
65. **Mertz, W.** 1993. Chromium in human nutrition. *J. Nutr.* **123**:626-633.
66. **Miranda, A. T., M. V. Gonzalez, G. Gonzalez, E. Vargas, J. Campos-Garcia, and C. Cervantes.** 2005. Involvement of DNA helicases in chromate resistance by *Pseudomonas aeruginosa* PAO1. *Mutat. Res.* **578**:202-209.
67. **Monds, R. D. and G. A. O'Toole.** 2009. The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol.* **17**:73-87.
68. **Monroe, D.** 2007. Looking for chinks in the armor of bacterial biofilms. *Plos Biol.* **5**:2458-2461.
69. **Myers, C. R., B. P. Carstens, W. E. Antholine, and J. M. Myers.** 2000. Chromium(VI) reductase activity is associated with the cytoplasmic membrane

- of anaerobically grown *Shewanella putrefaciens* MR-1. J. Appl. Microbiol **88**:98-106.
70. **Nadell, C. D., J. B. Xavier, and K. R. Foster.** 2008. The sociobiology of biofilms. FEMS Microbiol. Rev. **33**:206-224.
  71. **Nancharaiah, Y. V., C. Dodge, V. P. Venugopalan, S. V. Narasimhan, and A. J. Francis.** 2010. Immobilization of Cr(VI) and its reduction to Cr(III) phosphate by granular biofilms comprising a mixture of microbes. Appl. Environ. Microbiol **76**:2433-2438.
  72. **National Research council (U.S.).** 1974. Committee on biological effects of atmospheric pollutants, Chromium. Academy of Science, Washington.
  73. **Nicolella, C., M. C. van Loosdrecht, and S. J. Heijnen.** 2000. Particle based biofilm reactor technology. Trends Biotechnol. **18**:312-320.
  74. **Nies, D. H., S. Koch, S. Wachi, N. Peitzsch, and M. H. Saier.** 1998. CHR, a novel family of prokaryotic proton motive force-driven transporters probably containing chromate/sulfate antiporters. J. Bacteriol. **180**:5799-5802.
  75. **Nriagu, J. O. and E. Nieboer.** 1988. Chromium in natural and human environments. Wiley Interscience, New York.
  76. **O'Toole, G., H. B. Kaplan, and R. Kolter.** 2000. Biofilm formation as microbial development. Ann. Rev. Microbiol. **54**:49-79.
  77. **O'Toole, G. A. and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol **30**:295-304.
  78. **Ohtake, H., E. Fujii, and K. Toda.** 1990. Reduction of toxic chromate in an industrial effluent by use of a chromate-reducing strain of *Enterobacter cloacae*. Environ. Technol. **11**:663-668.

79. **Otto, K. and T. J. Silhavy.** 2002. Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. *Proc. Natl. Acad. Sci. U. S. A* **99**:2297-2292.
80. **Paraje, M. G.** 2011. Antimicrobial resistance in biofilms, p. 736-744. *In* A. Méndez-Vilas (ed.), *Science against microbial pathogens: communicating current research and technological advances*. FORMATEX.
81. **Park, C. H., M. Keyhan, B. Wielinga, S. Fendorf, and A. Matin.** 2000. Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl. Environ. Microbiol.* **66**:1788-1795.
82. **Patel, R.** 2005. Biofilms and antimicrobial resistance. *Clin. Orthop. Relat. Res.* 41-47.
83. **Patell, S., M. Gu, M. G. Davenport, R. D. Waite, and M. Welch.** 2010. Comparative microarray analysis reveals that the core biofilm-associated transcriptome of *Pseudomonas aeruginosa* comprises relatively few genes. *Environ. Microbiol. Reports* **2**:440-448.
84. **Periasamy, S. and P. E. Kolenbrander.** 2009. *Aggregatibacter actinomycetemcomitans* builds mutualistic biofilm communities with *Fusobacterium nucleatum* and *Veillonella Species* in saliva. *Infect. Immun.* **77**:3542-3551.
85. **Pimentel, B. E., R. Moreno-Sánchez, and C. Cervantes.** 2002. Efflux of chromate by cells of *Pseudomonas aeruginosa* expressing the ChrA protein. *FEMS Microbiol. Lett.* **212**:249-254.
86. **Plaper, A., S. Jenko-Brinovec, A. Premzl, J. Kos, and P. Raspor.** 2002. Genotoxicity of trivalent chromium in bacterial cells. Possible effects on DNA topology. *Chem. Res. Toxicol.* **15**:943-949.
87. **Pratt, L. A. and R. Kolter.** 1999. Genetic analyses of bacterial biofilm formation. *Curr. Opin. Microbiol.* **2**:598-603.

88. **Rai, L. C. and M. Raizada.** 1987. Toxicity of nickel and silver to *Nostoc muscorum*: interaction with ascorbic acid, glutathione, and sulfur-containing amino acids. *Ecotoxicol. Environ. Saf.* **14**:12-21.
89. **Raivio, T. L. and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *J. Bacteriol.* **179**:7724-7733.
90. **Rama Krishna, K. and L. Philip.** 2005. Bioremediation of Cr(VI) in contaminated soils. *J. Hazard. Mater.* **B121**:109-117.
91. **Ramirez-Diaz, M. I., C. az-Perez, E. Vargas, H. Riveros-Rosas, J. Campos-Garcia, and C. Cervantes.** 2008. Mechanisms of bacterial resistance to chromium compounds. *Biometals* **21**:321-332.
92. **Reddy, K. R., S. Chinthamreddy, R. E. Saichek, and T. J. Cutright.** 2003. Nutrient amendment for the bioremediation of a chromium-contaminated soil by electrokinetics. *Energ. Sources* **25**:931-943.
93. **Rollet, C., L. Gal, and J. Guzzo.** 2009. Biofilm-detached cells, a transition from a sessile to a planktonic phenotype: a comparative study of adhesion and physiological characteristics in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **290**:135-142.
94. **Salnikow, K. and A. Zhitkovich.** 2008. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: Nickel, arsenic, and chromium. *Chem. Res. Toxicol.* **21**:28-44.
95. **Sauer, K.** 2003. The genomics and proteomics of biofilm formation. *Genome Biol.* **4**.
96. **Scott, J. A. and A. M. Karanjkar.** 1998. Immobilized biofilms on granular activated carbon for removal and accumulation of heavy metals from contaminated streams. *Water Sci. Technol.* **38**:197-204.

97. **Scott, J. A., A. M. Karanjkar, and D. L. Rowe.** 1995. Biofilms covered granular activated carbon for decontamination of streams containing heavy metals and organic chemicals. *Minerals Eng.* **8**:221-230.
98. **Seigneur, C. and E. Constantinou.** 1995. Chemical kinetics mechanism for atmospheric chromium. *Environ. Sci. Technol.* **29**:222-231.
99. **Singh, R., D. Paul, and R. K. Jain.** 2006. Biofilms: implications in bioremediation. *Trends Microbiol.* **14**:389-397.
100. **Smith, W. L.** 2001. Hexavalent chromium reduction and precipitation by sulphate- reducing bacterial biofilms. *Environ. Geochem. Hlth* **23**:297-300.
101. **Spoering, A. L. and K. Lewis.** 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J. Bacteriol.* **183**:6746-6751.
102. **Sreekanth, R., V. Pattabhi, and S. S. Rajan.** 2008. Molecular basis of chromium insulin interactions. *Biochem. Biophys. Res. Commun.* **369**:725-729.
103. **Stewart, P. S. and M. J. Franklin.** 2008. Physiological heterogeneity in biofilms. *Nat. Rev. Microbiol.* **6**:199-210.
104. **Sugden, K., C. K. Campo, and B. D. Martin.** 2001. Direct oxidation of guanine and 7,8-dihydro-8-oxoguanine in DNA by a high-valent chromium complex: A possible mechanism for chromate genotoxicity. *Chem. Res. Toxicol.* **14**:1315-1322.
105. **Sumner, E. R., S. Shanmuganathan, T. C. Sideri, S. A. Willets, J. E. Houghton, and S. V. Avery.** 2005. Oxidative protein damage causes chromium toxicity in yeast. *Microbiol.* **151**:1939-1948.
106. **Sundar, K., M. Sadiq, A. Mukherjee, and N. Chandrasekaran.** 2011. Bioremoval of trivalent chromium using *Bacillus* biofilms through continuous flow reactor. *J. Hazard. Mater.* **196**:44-51.

107. **Szomolay, B., I. Klapper, J. Dockery, and P. S. Stewart.** 2005. Adaptive responses to antimicrobial agents in biofilms. *Environ. Microbiol.* **7**:1186-1191.
108. **Tebo, B. M. and A. Y. Obraztova.** 1998. Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors. *FEMS Microbiol. Lett.* **162**:193-198.
109. **Teitzel, G. M. and M. R. Parsek.** 2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **69**:2313-2320.
110. **Tokunaga, T. K., J. Wan, M. K. Firestone, T. C. Hazen, K. R. Olson, D. J. Herman, S. R. Sutton, and A. Lanzirotti.** 2003. In situ reduction of chromium(VI) in heavily contaminated soils through organic carbon amendment. *J. Environ. Qual.* **32**:1641-1649.
111. **Travieso, L., A. Pellón, F. Benítez, E. Sánchez, R. Borja, N. O'Farrill, and P. Weiland.** 2002. BIOALGA reactor: preliminary studies for heavy metals removal. *Biochem. Eng. J.* **12**:87-91.
112. **Tseng, J. K. and A. R. Bielefeldt.** 2002. Low-temperature chromium(VI) biotransformation in soil with varying electron acceptors. *J. Environ. Qual.* **31**:1831-1841.
113. **US Environmental Protection Agency.** 1998. Toxicological review of hexavalent chromium, Washington, DC.
114. **van Hullebusch, E. D., M. H. Zandvoort, and P. N. L. Lens.** 2003. Metal immobilization by biofilms: mechanisms and analytical tools. *Rev. Environ. Sci. Biotechnol.* **2**:9-33.
115. **Venturi, V.** 2003. Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? *Mol. Microbiol.* **49**:1-9.
116. **Vidali, M.** 2001. Bioremediation. an overview. *Pure Appl. Chem.* **73**:1163-1172.

117. **Viti, C., F. Decorosi, A. Mini, E. Tatti, and L. Giovannetti.** 2008. Involvement of the *oscA* gene in the sulphur starvation response and in Cr(VI) resistance in *Pseudomonas corrugata* 28. Microbiol. **155**:95-105.
118. **Viti, C. and L. Giovannetti.** 2007. Bioremediation of soils polluted with hexavalent chromium using bacteria-the challenge , *In* S. N. Singh and R. D. Tripathi (ed.), Environmental Bioremediation Technologies. Springer, Berlin.
119. **Wang, P. C., T. Mori, K. Toda, and H. Ohtake.** 1990. Membrane-associated chromate reductase activity from *Enterobacter cloache*. J. Bacteriol. **172**:1670-1672.
120. **Watnick, P. I., K. J. Fullner, and R. Kolter.** 1999. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. J. Bacteriol. **181**:3606-3609.
121. **White, C. and G. M. Gadd.** 2000. Copper accumulation by sulfate-reducing bacterial biofilms. FEMS Microbiol. Lett. **183**:313-318.
122. **Whiteley, M., M. G. Banger, R. E. Burngarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg.** 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature **413**:860-864.
123. **Wood, L. F., A. J. Leech, and D. E. Ohman.** 2006. Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. Mol. Microbiol. **62**:412-426.
124. **Yarwood, J. M., D. J. Bartels, E. M. Volper, and E. P. Greenberg.** 2004. Quorum sensing in *Staphylococcus aureus* biofilms. J. Bacteriol. **186**:1838-1850.
125. **Zayed, A. M. and N. terry.** 2003. Chromium in the environment: factors affecting biological remediation. Plant and Soil **249**:139-156.
126. **Zhitkovich, A.** 2005. Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium(VI). Chem. Res. Toxicol. **18**:3-11.



## **Chapter 2**

### **Aim**

Contamination of the environment with heavy metals is a serious problem because human activities such as industrial and sewage sludge application, have largely contributed to a wide spread of heavy metals in the terrestrial environment.

In this context, the development of biologically-assisted reclamation procedures is rather appealing because these techniques are environmentally sustainable, applicable to the treatment of large areas and competitive from an economic viewpoint with respect to physico-chemical approaches. With few exceptions, most of information concerning the application of microorganisms to contaminated habitats have been so far derived from planktonic cultures although in terrestrial ecosystems microorganisms are generally found as aggregated and complex communities encased within extracellular biopolymers, the so-called biofilms. For this reason, it is not appropriate to use information arising from only planktonic cultures in order to both plan and accomplish bioremediation processes. Moreover, biofilms for their main prominent features as high microbial density and ability to either deplete contaminants (*e.g.*, via mineralization) or to immobilize them (*e.g.*, via bioadsorption and bioaccumulation) could be suggested for the remediation of sites characterized by the presence of heavy metals, since they show capacity to adhere to substrates, and resistance to heavy metals and other environmental stressors.

Among the heavy metals, chromium is one of the most used because it is employed in several user industries (metallurgical, chemical, refractory, etc), and it is considered one of the priority pollutants, both at the Italian and the International level. In order to plan and apply an effective system for large-scale chromate bioremediation, a better understanding of the complex microorganism-metal interactions is needed. The present work fitted in this context and was mainly addressed to give a significant contribution for the comprehension of the molecular bases concerned chromate resistance and biofilm formation in a bacterium belongs to *Pseudomonas alcaliphila*. *P. alcaliphila* 34 is a Cr(VI) hyper-resistant [Cr(VI)-MIC 40 mM] and biofilm producing bacterium isolated from a soil artificially polluted with 1000 mg Cr(VI) kg<sup>-1</sup>. It was selected for this study because it has previously been thoroughly characterized in terms of hundreds of biochemical attributes, and its chromate reducing capability in the presence of different carbon/energy sources, and has been found to be suitable in developing strategies for chromate remediation.

The general objective of the project was reached with the achievement of the following intermediate objectives:

- i. The development of a procedure, combining the Calgary Biofilm Device (MBEC device) and Phenotype MicroArray (PM) technology, for a wide-scale analysis of toxic chemicals susceptibility of biofilm and planktonic culture.
- ii. *Pseudomonas alcaliphila* 34 genome sequencing, assembly and annotation.
- iii. A transcriptome analysis of *P. alcaliphila* 34 strain to investigate molecular bases implicated in chromate resistance and biofilm development.

Altogether, this work has provided insights into the specific properties related to biofilm development and chromate resistance processes, which are fundamental in bioremediation of chromate contaminated soils.

## **Chapter 3**

### **A novel approach combining the Calgary Biofilm Device and Phenotype MicroArray for the characterization of the chemical sensitivity of bacterial biofilms**

Published on *Biofouling: The Journal of Bioadhesion and Biofilm Research*, 2012, 28:9,  
1023-1032

## A novel approach combining the Calgary Biofilm Device and Phenotype MicroArray for the characterization of the chemical sensitivity of bacterial biofilms

L. Santopolo, E. Marchi, L. Frediani, F. Decorosi, C. Viti\* and L. Giovannetti

Dipartimento di Biotecnologie Agrarie - sezione di Microbiologia and Laboratorio Genexpress, Università degli Studi di Firenze, Florence, Italy

(Received 18 May 2012; final version received 28 August 2012)

A rapid method for screening the metabolic susceptibility of biofilms to toxic compounds was developed by combining the Calgary Biofilm Device (MBEC device) and Phenotype MicroArray (PM) technology. The method was developed using *Pseudomonas alcaliphila* 34, a Cr(VI)-hyper-resistant bacterium, as the test organism. *P. alcaliphila* produced a robust biofilm after incubation for 16 h, reaching the maximum value after incubation for 24 h ( $9.4 \times 10^6 \pm 3.3 \times 10^6$  CFU peg<sup>-1</sup>). In order to detect the metabolic activity of cells in the biofilm, dye E (5×) and menadione sodium bisulphate (100 µM) were selected for redox detection chemistry, because they produced a high colorimetric yield in response to bacterial metabolism ( $340.4 \pm 6.9$  Omnilog Arbitrary Units). This combined approach, which avoids the limitations of traditional plate counts, was validated by testing the susceptibility of *P. alcaliphila* biofilm to 22 toxic compounds. For each compound the concentration level that significantly lowered the metabolic activity of the biofilm was identified. Chemical sensitivity analysis of the planktonic culture was also performed, allowing comparison of the metabolic susceptibility patterns of biofilm and planktonic cultures.

**Keywords:** Phenotype MicroArray; Calgary Biofilm Device; biofilm; planktonic culture; *Pseudomonas*; chemical sensitivity analysis

### Introduction

Biofilms are the most common form of bacterial growth in nature (Harrison et al. 2007). They are defined as microbial communities attached to a surface and embedded in a self-produced matrix consisting of extracellular polymeric substances (EPS). This structure provides the cells present in the biofilm with several advantages compared to those living as planktonic cells. Bacteria in biofilms show resistance to certain physical forces, such as shear flow, desiccation, and grazing (Sutherland 2001; Fux et al. 2005; Matz et al. 2005). Furthermore, one of the most important properties of biofilm-associated bacteria is tolerance to antimicrobial agents (antibiotics, disinfectants, heavy metals, and biocides), making bacterial biofilms a major problem in clinical and industrial settings (Costerton et al. 1999). Eighty percent of bacterial infections are caused by bacteria in biofilms (Costerton 2004; Sung et al. 2006), which may be difficult to eradicate even after prolonged antibiotic therapy. In industry, biofilms cause corrosion and biofouling of equipment, with consequent loss of efficiency (Little et al. 1990a, 1990b). These same properties have beneficial aspects in bioremediation, where bacterial biofilms are more proficient than

planktonic microorganisms (Singh et al. 2006) because of their increased tolerance to toxic substances (such as heavy metals) present in waste waters (Harrison et al. 2007). For all of these reasons, studying the tolerance of bacterial biofilms to toxic compounds is a highly pertinent issue for clinical, industrial and environmental microbiology.

Susceptibility testing of planktonic bacteria often fails to predict the resistance levels of the same bacteria in the biofilm state. Better laboratory procedures are needed to test biofilm susceptibility and resistance levels. Currently, one of the most widely used methods for testing the susceptibility of microbial biofilm to toxic compounds is the Calgary Biofilm Device (MBEC device) (Ceri et al. 1999; De Kievit et al. 2001; Harrison et al. 2005; Hill et al. 2005; Irias-Moliz et al. 2010). The MBEC device consists of two parts: the top forms a lid with 96 pegs, which sit in the wells of a standard 96-well microtiter plate. The MBEC device thus facilitates the production of 96 equivalent biofilms grown on pegs (Ceri et al. 2001). These biofilms are used in standard experiments for biofilm susceptibility assays. After exposure to toxic chemicals, biofilm quantification is conducted by conventional plating after disruption of the biofilms by sonication. However, biofilm disruption is sometimes incomplete

---

\*Corresponding author. Email: carlo.viti@unifi.it



or can kill the cells, so the number of colonies obtained does not necessarily reflect the number of viable bacteria in the biofilm. Indirect methods for detecting biofilm activity have been used to overcome this limit (Peeters et al. 2008a, 2008b; Mariscal et al. 2009; Smith et al. 2009), but these assays are not yet considered reliable enough for routine application.

Phenotype MicroArray (PM), a high-throughput technology for the phenotypic analysis of microorganisms, enables a rapid and extensive analysis of microbial susceptibility to toxic chemical compounds (Decorosi et al. 2009; Viti et al. 2009). PM uses 96-well plates, and each well of a plate is designed to test a different phenotype with tetrazolium redox dyes used as a reporter of active metabolism (Bochner et al. 2001). Chemical sensitivity panels (PM11-20) produced by Biolog contain 240 toxic chemical compounds (eg antibiotics, antimetabolites, heavy metals, and chelators) at four different concentrations. The plates are inoculated with a standardized cell suspension together with an added redox dye and then incubated under static conditions in the OmniLog apparatus, which functions both as an incubator and as a plate reader. The reduction of the dye leads to the formation of a purple color that is recorded by the OmniLog – CCD camera every 15 min providing quantitative and kinetic information about microbial activity in each tested condition (Bochner et al. 2008; Viti et al. 2008).

Integration of the MBEC device and PM technologies could offer the possibility of obtaining an extensive characterization of the metabolic sensitivity of bacterial biofilms to toxic compounds. The objective of the present study was to integrate the MBEC device and PM technologies for high-throughput chemical susceptibility testing of biofilm cultures of *P. alcaliphila* 34, a biofilm-producing bacterium that is hyper-resistant to Cr(VI) characterization (Viti et al. 2007). The metabolic susceptibility of planktonic cultures to toxic compounds was also evaluated.

## Materials and methods

### Bacterial strains, media and growth conditions

*Pseudomonas alcaliphila* 34, isolated from a soil artificially polluted with 1000 mg Cr(VI) kg<sup>-1</sup> (Viti et al. 2007), was selected for study because it has previously been thoroughly characterized in terms of hundreds of biochemical attributes, and its chromate-reducing capability in the presence of different carbon/energy sources, and has been found to be suitable in developing strategies for chromate remediation (Viti et al. 2007). These features, and the capability of the strain to produce biofilm, suggested its use as the test organism to validate the integration of MBEC device

and PM technologies. *P. alcaliphila* 34 was grown on plates containing LB (Sambrook et al. 1989) together with 5 mM K<sub>2</sub>CrO<sub>4</sub> at 30°C.

Toxic compound exposure was performed in Luria Bertani medium (LB) for antibiotics and biocides, or in Tris-minimal medium (TMM) (Mergeay 1995) for heavy metals, to minimize complexation and precipitation.

### Biofilm cultivation

Biofilms were grown in the MBEC-physiology and genetics (P&G) device, in compliance with the manufacturer's instructions (MBEC Bioproducts, Edmonton, Alberta, Canada, <http://www.mbec.ca>). The device consists of a lid with 96 pegs, each peg providing a surface for bacteria to adhere to and colonize, and where they can form a biofilm. Wells of the microtiter plate were inoculated with 150 µl of a *P. alcaliphila* 34 suspension containing ~10<sup>7</sup> CFU ml<sup>-1</sup>, prepared from bacterial colonies collected from the surface of an agar plate [LB + 5 mM Cr(VI)]. The lid of the MBEC device was placed into the 96-well plate and then the device was placed on a gyrorotary shaker at 150 rpm and 95% relative humidity, and incubated at 30°C.

Biofilm development was quantified by viable cell count as previously described (Ceri et al. 2001). Pegs were detached from the lid of the MBEC device and rinsed twice using 0.9% NaCl to remove planktonic cells and placed in 200 µl of 0.9% NaCl. Biofilms adhering to the pegs were disrupted by sonication for 15 min, as reported by Harrison et al. (2010). After sonication the cellular suspensions were diluted serially in 0.9% NaCl and 20 µl of each dilution were plated on LB agar. To determine the number of CFU per peg, colonies were counted after incubation for 24 h at 30°C.

### Redox potential indicators and electron carriers for metabolic activity assay of biofilms

Six redox potential indicators, dye A, dye D, dye E, dye F, dye G and dye H (Biolog Inc.), along with menadione (MD), phenazine ethosulfate (PES), phenazine methosulfate (MES) and menadione sodium bisulphite (MSB) as electron carriers, were used to measure the metabolic activity of *P. alcaliphila* 34. Dye A, dye D, dye E, dye F, dye G and dye H were purchased from Biolog as stock 100 × concentration water-solutions, as opposed to the standard working solution (1 × concentration). Stock solutions of the electron carriers, PES, MES and MSB, were prepared in water at 10 mM concentration. Since MD is water insoluble, stock solutions were prepared in acetone or

ethanol (EtOH) or dimethyl sulfoxide (DMSO) at a concentration of 10 mM.

Combinations of electron carrier (0  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M) and redox potential indicator were used to test the metabolic activity of *P. alcaliphila* 34 biofilms grown on the peg lid of the MBEC device for 24 h in LB, after washing twice with 0.9% saline to remove adherent planktonic bacteria. The metabolic activity of biofilms was measured using the OmniLog instrument (Biolog) as described below.

#### **Detection of the metabolic activity of biofilms**

The color change of the redox potential indicators due to the metabolic activity of biofilms was monitored and measured by Phenotype MicroArray (Biolog) technology using an OmniLog instrument, which has a dual function as an incubator and a plate reader. The reduction of the dye causes the formation of a purple color which is recorded by a CCD camera every 15 min (Bochner et al. 2001). The colorimetric signal intensity was represented as Arbitrary OmniLog Units (AOU) and the kinetic colorimetric data were stored in computer files.

#### **Set up of microplates for chemical sensitivity assays**

A preliminary experiment was performed in order to establish the minimal inhibition concentration (MIC) of each chemical. In a 96-well microtiter plate, *P. alcaliphila* 34 was subjected to an array of toxic compounds at different concentrations in LB (antibiotics and biocides) or TMM (heavy metals). Each well was inoculated with a bacterial culture in the late exponential phase with an optical density at 600 nm ( $OD_{600}$ ) of 0.1. Growth data were collected after incubation for 24 h at 30°C using a microtiter plate spectrophotometer (programmable MPT reader DV 990 BV5, GDV). Each experiment was performed in triplicate. The toxic chemicals tested were:  $AlK(SO_4)_2$  (MIC 0.3 mM),  $CdSO_4$  (MIC 0.8 mM),  $CoCl_2$  (MIC 0.2 mM),  $K_2CrO_4$  (MIC 50 mM),  $MnSO_4$  (MIC 12.5 mM),  $NiCl_2$  (MIC 0.3 mM),  $CuCl_2$  (MIC 0.8),  $VOCl_2$  (MIC 1.5 mM),  $ZnCl_2$  (MIC 0.2 mM), ethylenediaminetetraacetic acid (EDTA) (MIC 40 mM), gentamicin B (MIC 1  $\mu$ g  $ml^{-1}$ ), kanamycin (MIC 625  $\mu$ g  $ml^{-1}$ ), streptomycin (MIC 10  $\mu$ g  $ml^{-1}$ ), polymyxin B (MIC 1.6  $\mu$ g  $ml^{-1}$ ), doxycycline hyclate (MIC 2  $\mu$ g  $ml^{-1}$ ), cephalothin sodium salt (MIC 500  $\mu$ g  $ml^{-1}$ ), penicillin G sodium salt (MIC 80  $\mu$ g  $ml^{-1}$ ), carbenicillin disodium salt (MIC 500  $\mu$ g  $ml^{-1}$ ), oxacillin (MIC 500  $\mu$ g  $ml^{-1}$ ), protamine sulfate salt (MIC 60  $\mu$ g  $ml^{-1}$ ), benzalkonium chloride (MIC 0.8  $\mu$ g  $ml^{-1}$ ).

Toxic chemicals were prepared as solutions in distilled water at 10 times the highest concentration desired in the working plate and sterilized by filtration. Subsequently, each solution was serially diluted along four wells of a 96-deep-well plate (Sarstedt) with deionized sterile water in order to obtain a 10 $\times$  solution series (four concentration points). For each concentration series, a negative control without a toxic chemical was also included. Plates were sealed with parafilm and stored at room temperature (heavy metals and biocides) or at -20°C (antibiotics). Working solutions (1 $\times$ ) were freshly prepared by diluting the stock solutions (10 $\times$ ) with the appropriate medium (LB or TMM for antibiotics/biocides and heavy metals, respectively) into 96-wells standard microplates (Sarstedt) Figure S1 [Supplementary material is available via a multimedia link on the online article webpage].

#### **Assay of the metabolic sensitivity of biofilms to toxic compounds**

Biofilms grown on the lid of the MBEC device for 15 h were washed twice with 0.9% NaCl to remove adherent planktonic bacteria. The peg lid was then transferred into a 96-well microplate with toxic compounds, set up as described above. The plate was incubated at 30°C, 150 rpm, 95% relative humidity, for 6 h. After exposure to the toxic compounds, *P. alcaliphila* 34 biofilms were assessed for metabolic activity. The lid of the MBEC device was removed, washed twice with a 0.9% NaCl solution and then placed into a 96-well microplate containing 200  $\mu$ l of LB plus the redox potential indicator and the electron carrier. The microplate was incubated for 5 h at 30°C in the OmniLog apparatus (Biolog), each with triple replicates.

#### **Assay of the metabolic sensitivity of planktonic cultures to toxic compounds**

A 96 Multiply PCR plate was inoculated with a bacterial culture grown overnight at 30°C to reach an  $OD_{600}$  of 0.1 ( $\sim 10^7$  CFU  $ml^{-1}$ ). LB and TMM were used as media for biocide/antibiotic sensitivity assays and heavy metal sensitivity assays, respectively. Toxic chemicals obtained from stock solutions were added to the bacterial cultures at four different concentrations (the same as used in the biofilm assays). The plate was incubated at 30°C, 150 rpm, 95% relative humidity for 6 h. As with the biofilms, after exposure to the toxic compounds, *P. alcaliphila* 34 planktonic cultures were washed twice with a 0.9% NaCl by centrifugation (3,500 rpm, for 15 min, at room temperature) and finally the pellets were resuspended in 200  $\mu$ l of LB together with the redox potential indicator and the electron carrier. The microplate was incubated for 5 h



at 30°C in the OmniLog instrument (Biolog), each with triple replicates.

### Statistical analysis

The metabolic activities of biofilms and planktonic cells, evaluated by reduction of the redox potential indicator, were also expressed as percentage activity compared to the control (absence of toxic compound). Each toxic compound was tested in a single plate at four different concentrations in triplicate (Figure S1) [Supplementary material is available *via* a multimedia link on the online article webpage]. For each plate the colorimetric intensity of the 12 negative controls (no toxic compound) were averaged, and used as a reference to calculate the percentage metabolic activity for each well containing the toxic compound. The percentage metabolic activity for each concentration (three replicates) was averaged and the standard deviation (SD) was calculated.

To analyze the effect of each chemical assayed on the metabolic activity of *P. alcaliphila* 34, two-way ANOVA was performed on results obtained in the presence of the different chemicals and for the different culture type (biofilm/planktonic). Interaction between the two factors was also evaluated. When the ANOVA analysis was significant, the Bonferroni *post hoc* test was performed to locate the significant differences ( $\alpha = 0.05$ ).

### Results and discussion

#### Biofilm cultivation and choice of the redox potential indicator/electron carrier couple for the metabolic activity assay

The MBEC device was introduced to quickly determine the antibiotic susceptibility of biofilms. In this

system, biofilms grow on 96 identical pegs, attached to the top lid of a microtiter plate (Ceri et al. 1999). PM, a high-throughput technology, was developed to analyze the phenotypic characteristics of microorganisms in microtiter plates using a redox potential indicator as a reporter of metabolic activity (Bochner et al. 2001). The combined use of these two technologies permitted easy phenotypic characterization of bacterial biofilm (Figure 1). Nevertheless, the inability to adequately lodge the pegs of the MBEC device in the wells of PM plates (the PM plates wells are smaller in volume than those of the MBEC device) was an obstacle to combining the two technologies. The problem was overcome by manually dispensing toxic compounds in microplates compatible with the MBEC device. The advantage of this operation, which is repeatable and can be speeded up by using some basic automation procedures, is that the plate can be designed according to the researcher's specific needs.

Figure 2 shows *P. alcaliphila* 34 biofilm development on the peg lid of the MBEC device. Biofilm began to form after incubation for 6 h. After 15 h, the biofilm was well-formed and adherent to the peg surface, and at 24 h had reached the maximum density. After growth for 24 h the number of CFU decreased, suggesting that biofilm had entered into the detachment and dispersion phase or that the release of biofilm cells by the sonication step had decreased. Mature biofilm sometimes has more EPS and thus the cells are more adherent. After establishing the development conditions for biofilm, experiments were performed to find an efficient and suitable redox indicator to quantify the metabolic activity of *P. alcaliphila* 34 biofilm and planktonic cultures using the OmniLog instrument. Six redox potential indicators provided by Biolog (dye A, dye D, dye E, dye F, dye G

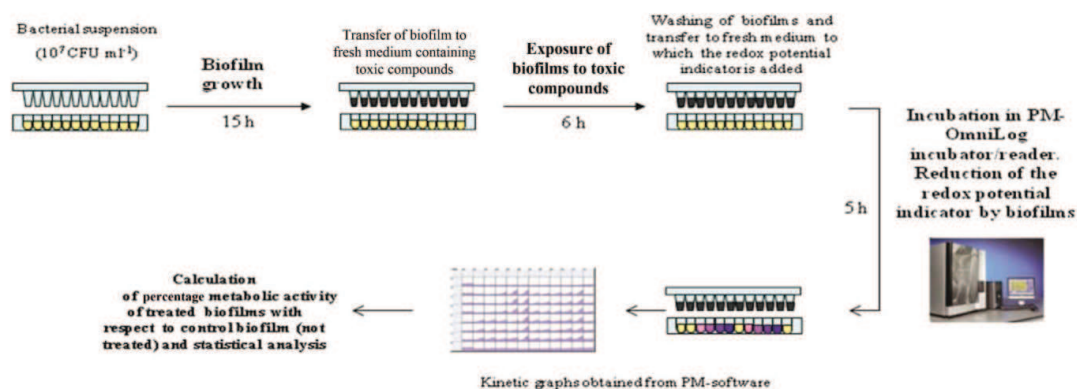


Figure 1. Flowchart of the high-throughput protocol for chemical susceptibility testing using the combined MBEC device/PM approach.



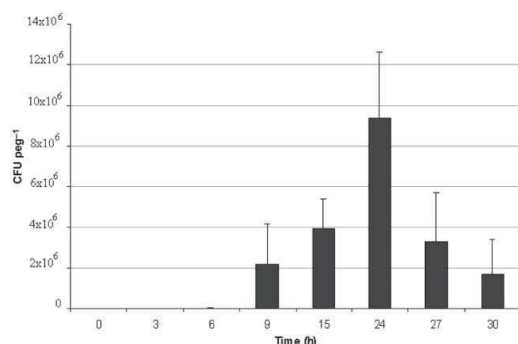


Figure 2. *P. alcaliphila* 34 biofilm development (CFU peg<sup>-1</sup>) on the peg lid of the MBEC device. Data are expressed as means  $\pm$  SD (n = 4).

and dye H) were tested in combination with the following electron carriers: menadione (MD), phenazine ethosulfate (PES), phenazine methosulfate (MES) and menadione sodium bisulphite (MSB) (Table S1) [Supplementary material is available *via* a multimedia link on the online article webpage].

High colorimetric yields (>75 OAU) were obtained with the following redox potential indicator/electron carrier combinations: dye D – 100  $\mu$ M MSB, dye E – 100  $\mu$ M MSB, dye D – 10  $\mu$ M PMS, dye E – 10  $\mu$ M PMS, dye D – 100  $\mu$ M PMS, dye E – 100  $\mu$ M PMS, dye F – 100  $\mu$ M PMS, dye G – 100  $\mu$ M PMS, dye H – 100  $\mu$ M PMS. The combinations dye D – 100  $\mu$ M MSB, dye E – 100  $\mu$ M MSB, dye D – 10  $\mu$ M PMS and dye E – 100  $\mu$ M PMS showed the lowest coefficients of variation (6.6%, 6.3%, 10.5% and 11.1%, respectively). Higher coefficients of variations were obtained for the remaining combinations which consequently were deemed not suitable for the set up of the method. The choice among combinations dye D – 100  $\mu$ M MSB, dye E – 100  $\mu$ M MSB, dye D – 10  $\mu$ M PMS and dye E – 100  $\mu$ M PMS was made on the basis of financial criteria. Dye D and dye E have a similar cost, but PMS is much more expensive than MSB, thus the latter is preferable when several metabolic activity assays need to be performed. Dye E was used for the test.

Experiments carried out to evaluate the influence of dye E concentration on the sensitivity assay showed that there was a linear relationship ( $R^2$  0.99 and 0.98 in the presence and absence of MSB, respectively) between the increase in the dye concentrations and the increase in the colorimetric assay yields (Table 1). Therefore, 100  $\mu$ M MSB plus 5  $\times$  dye E was a suitable combination of electron carrier and redox potential indicator for assaying the metabolic activity in *P. alcaliphila* 34.

Table 1. Colorimetric yield of metabolic activity assay obtained using different dye E concentrations with 0  $\mu$ M or 100  $\mu$ M MSB.

| Dye E concentration | MSB concentration                    |                                       |
|---------------------|--------------------------------------|---------------------------------------|
|                     | 0 $\mu$ M<br>Colour intensity (OAU*) | 100 $\mu$ M<br>Colour intensity (OAU) |
| 1 $\times$          | 39.5 $\pm$ 7.4                       | 78.7 $\pm$ 9.1                        |
| 2 $\times$          | 92.6 $\pm$ 2.2                       | 146.2 $\pm$ 11.7                      |
| 3 $\times$          | 101.0 $\pm$ 11.5                     | 221.3 $\pm$ 6.1                       |
| 4 $\times$          | 151.5 $\pm$ 10.8                     | 292.3 $\pm$ 6.1                       |
| 5 $\times$          | 197.2 $\pm$ 2.6                      | 340.4 $\pm$ 6.9                       |

Note: \*Data are expressed as OmniLog Arbitrary Units (OAU) recorded after incubation for 5 h and represent average values  $\pm$  SDs (n = 4).

#### *P. alcaliphila* 34 biofilm and planktonic biochemical sensitivity assay

Preliminary tests were performed to verify the repeatability of the MBEC device/PM approach. In 10 different experiments on planktonic cultures no significant differences were found (Chi square test,  $P > 0.05$ , data not shown), indicating a high repeatability. As regards the biofilm cultures, the data obtained were consistent with the reproducibility limit associated with the MBEC device approach (Chi square test,  $P > 0.1$ , data not shown) (Ceri et al. 1999).

Biofilms, after growth for 15 h, were used to evaluate metabolic sensitivity to toxic compounds. Twenty-two different antimicrobials (antibiotics, biocides and heavy metals) at lower and higher concentrations than the MIC (reported in Materials and methods) were used. The incubation with toxic compounds was stopped after exposure for 6 h, at which time the biofilms were transferred to new plates containing LB to which 100  $\mu$ M MSB and 5  $\times$  dye E were added, before incubation in the OmniLog instrument for 5 h to measure metabolic activity. For the assays to ascertain the metabolic sensitivity of planktonic cultures to toxic compounds, stationary phase cultures were treated similarly to the biofilms. Cultures in the stationary phase were chosen because they, like bacterial biofilms, consist mainly of slow-growing and non-growing cells (persisters) that are tolerant to stressors. Persisters are present in low numbers in the log phase of growth, whereas their frequency increases with the culture entry into the stationary phase and during biofilm formation (Lewis 2010). Persisters are a subpopulation genetically identical to but phenotypically different from other cells, having a low or inactive metabolism that makes them unaffected by or tolerant to toxic compounds (Patel 2005; Anderson and O'Toole 2008).

The results of the biofilm and planktonic culture metabolic activity assays are given as metabolic activity percentages relative to the controls (biofilm or planktonic activity without toxic compounds) (Figures 3 and 4, which show the effects of some toxic compounds, and Table S2) [Supplementary material is available *via* a multimedia link on the online article webpage]. Two-way ANOVA, performed on data obtained using different chemical concentrations and culture types (biofilm/planktonic), revealed a significant effect of chemical concentrations. The Bonferroni test showed that chemical exposure significantly lowered the metabolic activity both for biofilms and planktonic cultures, suggesting that the method described here is able to detect metabolic effects induced by toxic chemicals (Table S2). Furthermore,

two-way ANOVA revealed a significant effect of culture type (biofilm/planktonic) on the percentage metabolic activity in response to chemical treatment. It is important to underline that planktonic culture in the stationary phase and biofilm are not equivalent. Even though a large proportion of genes were found to be consistently regulated in *Pseudomonas aeruginosa* in both the stationary phase and biofilm cultures in contrast to the late exponential growth phase cultures, the global biofilm gene expression pattern was clearly distinct from the stationary phase culture (Dötsch et al. 2012). The Bonferroni test (Table S2) showed that biofilms were significantly less active than planktonic cultures after exposure to cephalotin, doxycycline, gentamycin, streptomycin, benzalkonium chloride, EDTA, cadmium, cobalt, nickel, vanadium and zinc.

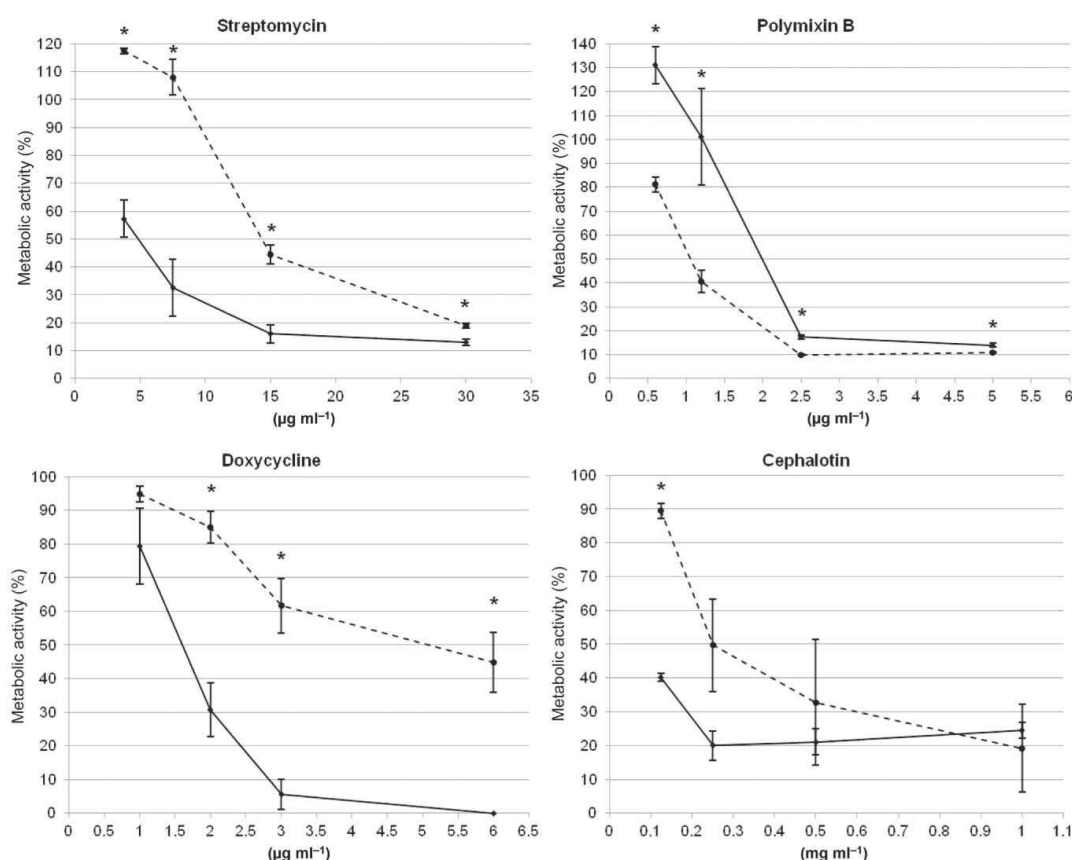


Figure 3. Metabolic activity (percentage activity compared to the control) tested by the combined MBEC device/PM approach of biofilm and planktonic cultures of *P. alcaliphila* 34 in the presence of streptomycin, polymyxin B, doxycycline and cephalotin. - - - = planktonic culture, — = biofilm. \* = statistically significant difference (two way ANOVA followed by the Bonferroni *post hoc* test,  $P < 0.05$ ).

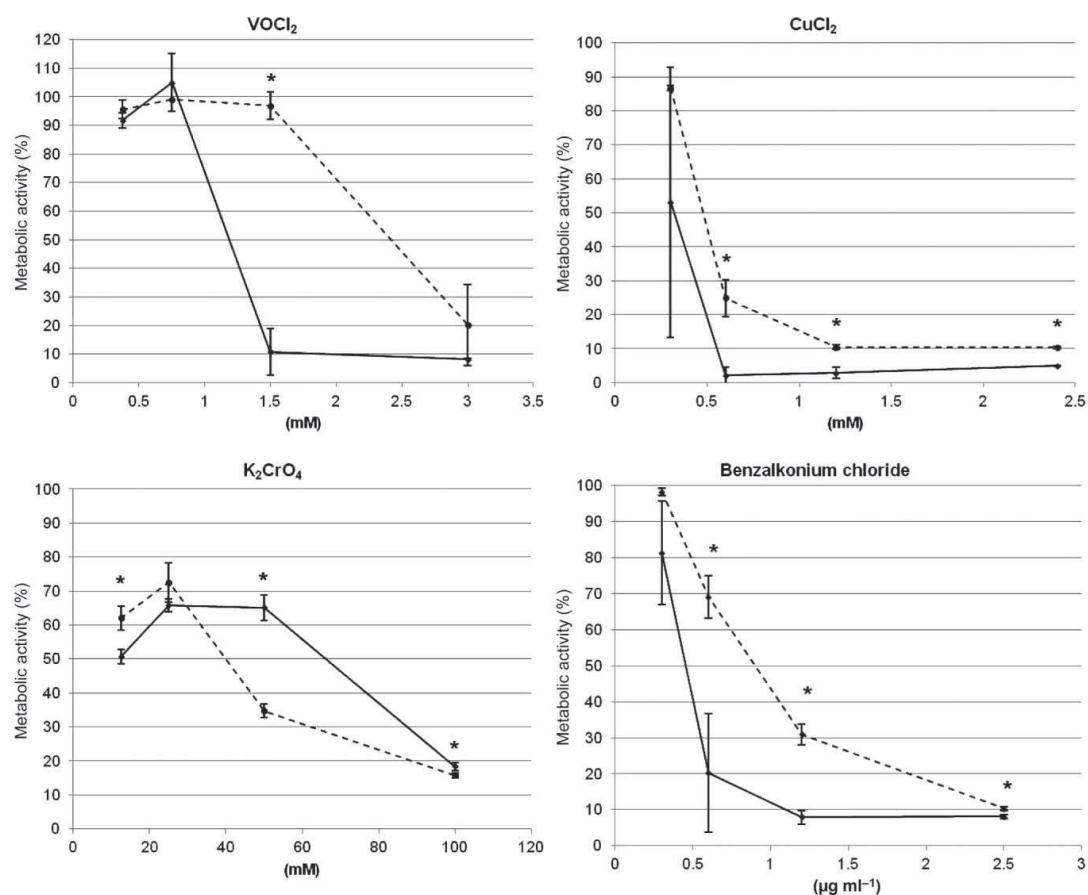


Figure 4. Metabolic activity (percentage activity compared to the control) tested by the combined MBEC device/PM approach of biofilm and planktonic cultures of *P. alcaliphila* 34 in the presence of VOCl<sub>2</sub>, CuCl<sub>2</sub>, K<sub>2</sub>CrO<sub>4</sub>, and benzalkonium chloride. - - - = planktonic culture, — = biofilm. \* = statistically significant difference (two way ANOVA followed by the Bonferroni *post hoc* test T,  $P < 0.05$ ).

This finding was not unexpected because stationary phase cultures are in general more tolerant to toxins than exponentially growing cells and sometimes can be even more resistant than biofilm bacteria (Spoering and Lewis 2001). The common assumption that biofilms are more tolerant than planktonic cells has already been confuted by Lewis (2001). Studying *P. aeruginosa* biofilms and planktonic cultures, Lewis (2001) found that cultures in the stationary phase were not only more tolerant than logarithmic-phase planktonic cultures but also than biofilms, in all of the cases examined. Therefore, any conclusions regarding biofilm and planktonic culture resistance should take into account the growth phase of planktonic cultures (Desai et al. 1998). For example antibiotics such as  $\beta$ -lactams and aminoglycosides kill bacteria during the growth

phase, whereas slow-growing or non-growing bacteria cells are tolerant to these toxins. Therefore, the highest activity found here for *P. alcaliphila* 34 stationary phase cultures could be due to a higher number of non-growing or slow growing cells in stationary phase cultures with respect to biofilm cultures (Lewis 2008). Regarding the action of toxic metal cations (cadmium, cobalt, nickel and zinc) on biofilm and stationary phase *P. alcaliphila* 34 cultures the authors hypothesize that the effect of metal cations also depends on the size of the subpopulations that cope with stress through their reduced growth rate.

*P. alcaliphila* 34 biofilms had a higher metabolic activity than planktonic cultures after exposure to polymyxin B, protamine and chromate (Figures 3 and 4, Table S2) [Supplementary material is available *via* a



multimedia link on the online article webpage]. Protamine and polymyxin B are two highly cationic antimicrobial peptides (CAPs) that alter membrane structure and permeability. The mechanisms of bacterial killing are not fully understood, but are thought to involve initial contact through electrostatic interactions between the positively charged peptide and the negatively charged bacterial envelope, followed by a general disruption of the membrane, leakage of  $K^+$ , ATP and other cellular components, and inhibition of metabolic processes, including protein synthesis (Johansen et al. 1997). There is now evidence that CAPs such as polymyxin B interact with ds-DNA (Kong et al. 2011). The structural characteristic of these compounds include the presence of a fatty acid portion that dissolves in the hydrophobic region of the cytoplasmic membrane and disrupts cell membrane integrity. Some components of biofilm EPS can interact with CAPs. In *P. aeruginosa*, it has been suggested that alginate, a component of biofilm, binds CAPs with the formation of CAP-alginate complexes due to the charge neutralization between the two species (Chan et al. 2005; Kuo et al. 2007). This interaction gives biofilm EPS a protective role possibly by trapping and aggregating the peptides before they reach the target bacteria (Chan et al. 2004).

*P. alcaliphila* 34 biofilms exposed to Cr(VI) concentrations  $>50$  mM were more active than planktonic cultures (Figure 4). The higher tolerance of biofilms with respect to planktonic stationary phase cultures could be associated with the reduction of Cr(VI) to Cr(III) by constitutive reductase released by cellular lysis or secretion as well, as was reported for *Pseudomonas putida* (Priester et al. 2006). It is possible that the EPS plays a role in the protection of extracellular reductase and therefore in Cr(VI) detoxification.

To conclude, a significant interaction between toxic chemical concentration and culture type (biofilm/planktonic) was detected by two-way ANOVA, confirming, as has been extensively reported (Aaron et al. 2002; Teitzel and Parsek 2003; Folsom et al. 2006; Caraher et al. 2007; Shapiro et al. 2011), that the toxic compound-induced effect on biofilm and planktonic cultures can not be equal and that one culture type may be more or less sensitive to the toxic compound than another. As the reported results indicate, the methods developed in the present study can be used to detect the effect of toxic compounds on biofilms and planktonic cultures. The method proposed here offers, for the first time, the possibility of applying a combined approach using PM technology and the MBEC device to the chemical sensitivity analysis of biofilms. It is important to remember that the proposed method, though it can give important

information about the cellular activity, should not be regarded as a definitive indicator of cell viability, since, like other approaches (ie viable counts), it measures only an aspect of cell physiology.

The MBEC device/PM approach is a reliable, repeatable, accurate, and quick method of determining the effect of toxic chemicals on the metabolic activity of biofilm and planktonic cultures. To the best of the authors' knowledge this is the first report of the testing of a wide range of redox potential indicators and electron carriers to determine metabolic activity in bacterial biofilm. The combined use of MSB together with 5× dye E offers the possibility to markedly increase the sensitivity of the MBEC device/PM approach. This approach, which provides a quick and easy assay enabling the quantification of the metabolic activity in *P. alcaliphila* 34, could be used on other bacteria and especially for screening. The only obstacle to the complete combination of the MBEC device and PM technologies lies in the need to set up microplates for exposure to toxic compounds since the wells of the PM panels are too small to serve as suitable receptacles for the pegs of the MBEC device. Although this step can be accomplished with basic automated liquid-handling procedures, an improvement to the Biolog microplates is desirable to allow the full integration of the MBEC device and PM technology.

#### Acknowledgments

This work was supported by the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) (PRIN, 2008). The authors thank Barry R. Bochner for the critical revision of the manuscript.

#### References

- Aaron SD, Ferris W, Ramotar K, Vandemheen K, Chan F, Saginur R. 2002. Single and combination antibiotic susceptibilities of planktonic, adherent, and biofilm-grown *Pseudomonas aeruginosa* isolates cultured from sputa of adults with cystic fibrosis. *J Clin Microbiol* 40:4172–4179.
- Anderson GG, O'Toole GA. 2008. Innate and induced resistance mechanisms of bacterial biofilms. *Curr Top Microbiol Immunol* 322:85–105.
- Bochner BR, Giovannetti L, Viti C. 2008. Important discoveries from analysing bacterial phenotypes. *Mol Microbiol* 70:274–280.
- Bochner BR, Gadzinski P, Panomitros E. 2001. Phenotype MicroArrays for high-throughput phenotypic testing and assay of gene function. *Genome Res* 11:1246–1255.
- Caraher E, Reynolds G, Murphy P, McClean S, Callaghan M. 2007. Comparison of antibiotic susceptibility of *Burkholderia cepacia* complex organisms when grown planktonically or as biofilm in vitro. *Eur J Clin Microbiol* 26:213–216.
- Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* 37:1771–1776.

- Ceri H, Olson M, Morck D, Storey D, Read R, Buret A, Olson B. 2001. The MBEC assay system: multiple equivalent biofilms for antibiotic and biocide susceptibility testing. *Methods Enzymol* 337:377–385.
- Chan C, Burrows LL, Deber CM. 2004. Helix induction in antimicrobial peptides by alginate in biofilms. *J Biol Chem* 279:38749–38754.
- Chan C, Burrows LL, Deber CM. 2005. Alginate as an auxiliary bacterial membrane: binding of membrane-active peptides by polysaccharides. *J Peptides Res* 65:343–351.
- Costerton B. 2004. Microbial ecology comes of age and joins the general ecology community. *P Natl Acad Sci USA* 101:16983–16984.
- Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318–1322.
- Decorosi F, Tatti E, Mini A, Giovannetti L, Viti C. 2009. Characterization of two genes involved in chromate resistance in a Cr(VI)-hyper-resistant bacterium. *Extremophiles* 13:917–923.
- De Kievit TR, Parkins MD, Gillis RJ, Srikumar R, Ceri H, Poole K, Iglewski BH, Storey DG. 2001. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 45:1761–1770.
- Desai M, Buhler T, Weller PH, Brown MRW. 1998. Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during exponential growth. *J Antimicrob Chemother* 42:153–160.
- Dötsch A, Eckweiler D, Schniederjans M, Zimmermann A, Jensen V, Scharfe M, Geffers R, Häussler S. 2012. The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing. *PlosOne* 7:e31092.
- Folsom JP, Siragusa GR, Frank JF. 2006. Formation of biofilm at different nutrient levels by various genotypes of *Listeria monocytogenes*. *J Food Protect* 69:826–834.
- Fux CA, Costerton JW, Stewart PS, Stoodley P. 2005. Survival strategies of infectious biofilms. *Trends Microbiol* 13:34–40.
- Harrison JJ, Ceri H, Turner RJ. 2007. Multimetal resistance and tolerance in microbial biofilms. *Nat Rev Microbiol* 5:928–938.
- Harrison JJ, Ceri H, Roper NJ, Badry EA, Sproule KM, Turner RJ. 2005. Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. *Microbiology-UK* 151:3181–3195.
- Harrison JJ, Stremick CA, Turner RJ, Allan ND, Olson ME, Ceri H. 2010. Microtiter susceptibility testing of microbes growing on peg lids: A miniaturized biofilm model for high-throughput screening. *Nat Protoc* 5:1236–1254.
- Hill D, Rose B, Pajkos A, Robinson M, Bye P, Bell S, Elkins M, Thompson B, MacLeod C, Aaron SD, Harbour C. 2005. Antibiotic susceptibilities of *Pseudomonas aeruginosa* isolates derived from patients with cystic fibrosis under aerobic, anaerobic, and biofilm conditions. *J Clin Microbiol* 43:5085–5090.
- Johansen C, Verheul A, Gram L, Gill T, Abee T. 1997. Protamine-induced permeabilization of cell envelopes of gram-positive and gram-negative bacteria. *Appl Environ Microbiol* 63:1155–1159.
- Kong L, Liu Z, Hu X, Liu S. 2011. Interaction of polymyxin B with ds-DNA, and determination of DNA or polymyxin B via resonance Rayleigh scattering and resonance non-linear scattering spectra. *Microchim Acta* 173:207–213.
- Kuo HH, Chan C, Burrows LL, Deber CM. 2007. Hydrophobic interactions in complex of antimicrobial peptides with bacterial polysaccharides. *Chem Biol Drug Des* 69:405–412.
- Little B, Wagner P, Characklis WG, Lee W. 1990a. Microbial corrosion. In: Characklis WG, Marshall KC, editors. *Biofilms*. New York (NY): Wiley. p. 635–670.
- Little B, Wagner P, Ray R, McNeil M. 1990b. Microbiologically influenced corrosion in copper and nickel seawater piping systems. *Mar Technol Soc J* 24:10–17.
- Lewis K. 2001. Riddle of biofilm resistance. *Antimicrob Agents Chemother* 45:999–1007.
- Lewis K. 2008. Multidrug tolerance of biofilm and persister cells. In: Romeo T, editor. *Current topics in microbiology and immunology*. Berlin/Heidelberg (Germany): Springer-Verlag. p. 107–131.
- Lewis K. 2010. Persister cells. *Annu Rev Microbiol* 64:357–372.
- Mariscal A, Lopez-Gigosos RM, Carnero-Varo M, Fernandez-Crehuet J. 2009. Fluorescent assay based on resazurin for detection of activity of disinfectants against bacterial biofilm. *Appl Microbiol Biotechnol* 82:773–783.
- Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S. 2005. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *P Natl Acad Sci USA* 102:16819–16824.
- Mergeay M. 1995. Heavy metal resistances in microbial ecosystems. In: Akkermans ADL, van Elsas JD, de Bruij FJ, editors. *Molecular microbial ecology manual*. Dordrecht (The Netherlands): Kluwer Academic. p. 1–17.
- Patel R. 2005. Biofilms and antimicrobial resistance. *Clin Orthop Relat Res* 437:41–47.
- Peeters E, Nelis HJ, Coenye T. 2008a. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J Microbiol Meth* 72:157–165.
- Peeters E, Nelis HJ, Coenye T. 2008b. Evaluation of the efficacy of disinfection procedures against *Burkholderia cenocepacia* biofilms. *J Hosp Infect* 70:361–368.
- Priester JH, Olson SG, Webb SM, Neu M, Hersman LE, Holden PA. 2006. Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. *Appl Environ Microbiol* 72:1988–1996.
- rias-Moliz MT, Ferrer-Luque CM, Gonzalez-Rodriguez MP, Valderrama MJ, Baca P. 2010. Eradication of *Enterococcus faecalis* biofilms by cetrimide and chlorhexidine. *J Endodont* 36:87–90.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbour (NY): Cold Spring Harbor Laboratory. Vol. 3, p. A.1.
- Shapiro JA, Nguyen VL, Chamberlain NR. 2011. Evidence for persisters in *Staphylococcus epidermidis* RP62a planktonic cultures and biofilms. *J Med Microbiol* 60:950–960.
- Singh R, Paul D, Jain RK. 2006. Biofilms: implications in bioremediation. *Trends Microbiol* 14:389–397.
- Smith K, Perez A, Ramage G, Gemmell CG, Lang S. 2009. Comparison of biofilm-associated cell survival following in vitro exposure of methicillin-resistant *Staphylococcus aureus* biofilms to the antibiotics clindamycin, daptomycin, linezolid, tigecycline and vancomycin. *Int J Antimicrob Ag* 33:374–378.
- Spoering AL, Lewis K. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* 183:6746–6751.



- Sung BH, Lee CH, Yu BJ, Lee JH, Lee JY, Kim MS, Blattner FR, Kim SC. 2006. Development of a biofilm production-deficient *Escherichia coli* strain as a host for biotechnological applications. *Appl Environ Microbiol* 72:3336–3342.
- Sutherland IW. 2001. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology-UK* 147:3–9.
- Teitzel GM, Parsek MR. 2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 69:2313–2320.
- Viti C, Bochner B, Giovannetti L. 2008. Florence conference on phenotype microarray analysis of microorganisms – the environment, agriculture, and human health. 19–21 March 2008, Florence, Italy. *Ann Microbiol* 58:347–349.
- Viti C, Decorosi F, Tatti E, Giovannetti L. 2007. Characterization of chromate-resistant and -reducing bacteria by traditional means and by a high-throughput phenomic technique for bioremediation purposes. *Biotechnol Progr* 23:553–559.
- Viti C, Decorosi F, Mini A, Tatti E, Giovannetti L. 2009. Involvement of the *oscA* gene in the sulphur starvation response and in Cr(VI) resistance in *Pseudomonas corrugata* 28. *Microbiology-UK* 155:95–105.

## ADDITIONAL FILES

### **Additional file, Figure S1:**

Challenge plate scheme.

### **Additional file, Table S1:**

Table of colorimetric yield of the assays for measuring biofilm metabolic activity using different combinations of redox potential indicators and electron carriers. Biofilms of *P. alcaliphila* 34 grown on the pegs of Calgary Device in LB medium for 24 hours were transferred in a new microplate containing LB added with different couples of redox potential indicators and electron carriers. The biofilms were incubated at 30°C into the Omnilog instrument for 5 hours.

### **Additional file, Table S2:**

Table of metabolic susceptibility values for biofilm and planktonic culture of *P. alcaliphila* 34 exposed to toxic compounds. For each toxic compound concentration biofilm activity was calculated compared to the untreated condition (absence of the toxic compound); for each toxic compound concentration planktonic culture activity was calculated compared to the untreated condition (absence of the toxic compound).



## **Chapter 4**

### **Draft Genome Sequence of Chromate-Resistant and Biofilm-Producing Strain *Pseudomonas alcaliphila* 34**

Submitted to *Genome announcement*

## **Draft Genome Sequence of Chromate-Resistant and Biofilm-Producing Strain *Pseudomonas alcaliphila* 34**

Luisa Santopolo<sup>1</sup>, Emanuela Marchi<sup>1</sup>, Francesca Decorosi<sup>1</sup>, Marco Galardini<sup>2</sup>, Matteo Brilli<sup>3</sup>, Luciana Giovannetti<sup>1</sup> and Carlo Viti<sup>1</sup>

<sup>1</sup>Dipartimento di Biotechnologie Agrarie - sezione di Microbiologia, Università di Firenze, Firenze, Italy

<sup>2</sup>Dipartimento di Biologia Evoluzionistica, Università di Firenze, Firenze, Italy

<sup>3</sup>UMR CNRS 5558 LBBE "Biométrie et Biologie Évolutive", Lyon, France

### **ABSTRACT**

We report the draft genome sequence of *Pseudomonas alcaliphila* 34, a Cr(VI) hyper-resistant and biofilm-producing bacterium that could be used for bioremediation of chromate polluted soils. The genome sequence might be helpful to explore the mechanisms involved in chromium resistance and biofilm formation.

*Pseudomonas alcaliphila* 34, previously identified as *P. mendocina* with Biolog-ID System (Biolog, Hayward, CA) (1) is a Cr(VI) hyper-resistant and biofilm-producing bacterium isolated from a soil artificially polluted with chromate (2). The strain has been thoroughly characterized in terms of hundreds of biochemical attributes and its chromate-reducing capability in the presence of different carbon/energy sources (1), and in terms of its biofilms development capability and activity in presence of several stressors (3). Therefore, *P. alcaliphila* 34 does indeed have exceptional abilities and it is a prospective candidate for remediation in environments where chromate contamination occurs together with other stressors (1).

Here we present the draft genome sequence of *P. alcaliphila* 34, and provide information on the genetic bases that establish its high-level chromium tolerance and biofilm formation. Furthermore, its genome sequence might provide insight into the biotechnological exploitation of the organism for the remediation of chromium-contaminated sites (4).

*P. alcaliphila* 34 genome was sequenced using Illumina HiSeq2000 technology. A total of 101,323,720 reads (101 bp long) were assembled using the CLC Genomic Workbench assembler (CLC Bio, Denmark), resulting in 1,718-fold coverage of a 5.44 Mb genome distributed in 277 unoriented contigs, and with an overall G+C content of 62%. Blast (5) analysis on the nonredundant (NR) database allowed us to discard contaminant sequences from organisms unrelated to *Pseudomonas*, such as those from plants and insects.

Contigs were ordered using CONTIGuator 2.3 (6) with the *P. mendocina* NK01 genome, which is the closest available as a reference (GenBank accession no. [CP002620.1](#)). Fifty contigs, for a total of 5.27 Mb, were aligned with the reference genome, allowing us to define their relative order; sequences not mapped on the reference genome, corresponding to 13 contigs and a total of 144,392 nucleotides (nt), were added later on to the draft genome. Gaps between contigs were identified and closed using PCR followed by Sanger sequencing and, in some cases, primer walking sequencing of PCR products. The final draft genome of *P. alcaliphila* 34 consists of eight supercontigs plus 10 single contigs, with a total length of 5,445,828 bp.

Genome annotation was performed with the Rapid Annotations using Subsystems Technology (RAST) pipeline (7), allowing for the identification of 4,983 protein-coding

sequences, 61 tRNAs, and 4 copies of the genes for 5S, 16S, and 23S rRNA, as described for other *Pseudomonas* species (8, 9).

Genome analysis indicated that *P. alcaliphila* 34 possesses a putative *chrBACF* operon (10) that might be responsible for the high chromate resistance of the bacterium. Mercuric and arsenic resistance operons and many genes encoding putative multidrug resistance efflux systems were also identified on the genome. Additionally, genes known to be involved in biofilm formation, including the genes implicated in flagellar and type IV pilus biogenesis and motility (11, 12), plus two alginate biosynthesis gene clusters (13, 14) were identified.

A more detailed analysis of this genome and a comparative analysis with other Cr(VI)-resistant and biofilm producing bacteria will provide further insight into the specific properties related to these processes.

**Nucleotide sequence accession numbers.** The draft genome sequence of strain 34 has been deposited at DDBJ/EMBL/GenBank under the accession number ANGB000000000. The version described in this paper is the first version, ANGB010000000.

## ACKNOWLEDGMENT

This work was supported by the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) (PRIN, 2008).

## REFERENCES

- 1 **Viti C, Decorosi F, Tatti E, Giovannetti L.** 2007. Characterization of chromate-resistant and -reducing bacteria by traditional means and by a high-throughput phenomic technique for bioremediation purposes. *Biotechnol. Progr.* **23**:553-59.
- 2 **Viti C, Mini A, Ranalli, G, Lustrato G, Giovannetti L.** 2006. Response of microbial communities to different doses of chromate in soil microcosms. *Appl. Soil Ecol.*, **34**:125-139.
- 3 **Santopolo L, Marchi E, Frediani L, Decorosi F, Viti C, Giovannetti L.** 2012. A novel approach combining the Calgary Biofilm Device and Phenotype MicroArray for the characterization of the chemical sensitivity of bacterial biofilms. *Biofouling* **28**:1023-1032.
- 4 **Viti C, Giovannetti L.** 2008. Bioremediation of soils polluted with hexavalent chromium using bacteria: a challenge. *In: Environmental Bioremediation Technologies*, edited by S.N. Singh, R. D. Tripathi, pp. 57-76.
- 5 **Altschul SF, Madden TL, Schäffer AA, Zhanq J, Zhanq Z, Miller W, Lipman DJ.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**(17): 3389-402.
- 6 **Galardini M, Biondi EG, Bazzicalupo M, Mengoni A.** 2011. CONTIGuator: a bacterial genome finishing tool for structural insights on draft genomes. *Source Code Biol. Med.* **6**:11.
- 7 **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics*

9:75.

- 8 **Coenye T, Vandamme P.** 2003. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiol. Lett.* 228:45-49.
- 9 **Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenner P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK-S, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959-964.
- 10 **Branco R, Chung AP, Johnston T, Gurel V, Morais P, Zhitkovich A.** 2008. The chromate-inducible *chrBACF* operon from the transposable element Tn*OtChr* confers resistance to chromium(VI) and superoxide. *J. Bacteriol.* 190:6996-7003.
- 11 **Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJM, Klausen, Givskov M, Whitchurch CB, Engel JN, Tolker-Nielsen T.** 2008. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ. Microbiol.* 10:2331-2343.
- 12 **Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S, Tolker-Nielsen T.** Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. 2003. *Mol. Microbiol.* 48:1511-1524.
- 13 **Davies DG, Chakrabarty AM, Geesey GG.** 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 59:1181-1186.
- 14 **Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, Parsek**



**MR.** 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. J. Bacteriol. 183:5395-5401.

## **ADDITIONAL FILES**

### **Additional file 1:**

*P. alcaliphila* 34 draft genome sequence.

### **Additional file, Figure S1:**

*P. alcaliphila* 34 draft genome scheme. Each arrow represents a contig.

### **Additional file, Figure S2:**

*P. alcaliphila* 34 RAST genome annotation chart.

### **Additional file, Table S1:**

Table of *P. alcaliphila* 34 genome annotation performed with RAST pipeline.

## **Chapter 5**

### **Molecular bases of chromate resistance and biofilm development in *Pseudomonas alcaliphila* 34**

*Manuscript to be submitted*

## **Molecular bases of chromate resistance and biofilm development in *Pseudomonas alcaliphila* 34**

Santopolo L.<sup>1</sup>, Marchi E.<sup>1</sup>, Decorosi F.<sup>1</sup>, Viti C.<sup>1</sup>, Giovannetti L.<sup>1</sup>

<sup>1</sup>Dipartimento di Biotecnologie Agrarie - sezione di Microbiologia and Laboratorio Genexpress, Università degli Studi Firenze, Florence, Italy

### **ABSTRACT**

*Pseudomonas alcaliphila* 34 is a Cr(VI) hyper-resistant and biofilm producing bacterium. The biological impact of an acute chromate stress exposure, and the molecular mechanisms underlying the early stages of biofilm formation were assessed by whole genome microarrays. A time series transcriptome profiles of *P. alcaliphila* 34 planktonic cultures treated with 1 mM K<sub>2</sub>CrO<sub>4</sub> were compared to untreated planktonic cultures grown in parallel after 5, 30, 60, 90 and 120 min post-chromate addiction. Temporal gene expression profile of cells exposed to Cr(VI) showed an up-regulation of genes encoding for proteins implicated in oxidative stress (an alkyl hydroperoxide reductase and a redox-sensitive transcriptional activator SoxR) and in DNA repair (Lex A and RecA). Genes involved in sulfur metabolism were also induced, as well as genes related to flagellar motility and EPS production resulted differentially expressed. Genes related to the last two features, which have been shown to be partially involved in biofilm formation, were also differentially regulated in 7-h developing *P. alcaliphila* 34 biofilm, suggesting that chromate addiction could induce a markedly biofilm production as a defense mechanism.

## INTRODUCTION

Potentially hazardous levels of heavy metals have dispersed into subsurface sediment and groundwater in a number of metal-contaminated sites and represent a challenge for environmental remediation. Hexavalent chromium [Cr(VI)], in the form of chromate ( $\text{CrO}_4^{2-}$ ) or dichromate ( $\text{Cr}_2\text{O}_7^{2-}$ ) species, is among the widespread toxic heavy-metal pollutants (4,14) due to its prevalent use in many industrial activities (15,18). Cr(VI) is highly soluble and toxic, with chronic exposure leading to mutagenesis and carcinogenesis (33). The strong impact of Cr(VI) on the environment and on the human health demands suitable technologies to neutralize its hazard. Cr(VI) detoxification of contaminated soils is essentially based on physical and chemical approaches, which include excavation or pumping of contaminated material, followed by the addition of reducing chemicals that lead to the precipitation and/or sedimentation of reduced chromium [Cr(III)], less toxic than Cr(VI) and greatly insoluble (36).

Effective bioremediation of Cr(VI) polluted sites requires the knowledge of genetic pathways for resistance and biotransformation by component organisms within a microbial community. Microorganisms exhibiting Cr(VI)-reducing and -resistance activities have been detected in chromate-contaminated sites as well as in uncontaminated ecosystems (32,37). To cope with Cr(VI) microorganisms employed diverse mechanisms, consisting of precipitation and reduction of Cr(VI) to the less toxic and mobile Cr(III), obstruction of the ion uptake system or activation of the chromate efflux to diminish intracellular accumulation of Cr(VI) (7). For example, the hydrophobic protein ChrA was found to be responsible for the plasmid-specified resistance phenotype in various bacterial species and appears to function as a secondary transport system for the extrusion of chromium ions (3), while additional protective strategies are related to oxidative stress, DNA repair mechanisms and/or sulfur or iron metabolism (7).

In bioremediation processes to metal removal cells immobilized as a biofilm on inert supports can be used (9,30). The formation of biofilms can be considered as a development cycle. It begins when free-swimming (planktonic) bacteria recognize a surface and firmly attach themselves to it. Subsequently, the attached cells grow and divide, and at the same time recruit additional planktonic cells that attach to the cells already on the surface (23). Growth as a biofilm may confer more tolerance to

environmental stress factors and elevated levels of organic and inorganic pollutants, compared with the planktonic mode of growth (9,21). Biofilms can also influence the sorption, mobilization and decomposition of metal, organic and radionuclide pollutant species (12,31). Understanding the fundamental processes contributing to biofilm formation is beneficial to anyone involved with natural or industrial systems, where biofilms may play a significant role in determining variables such as bulk water quality, product quality or toxic compounds biodegradation.

*Pseudomonas alcaliphila* 34 is a Cr(VI) hyper-resistant and biofilm producing bacterium, isolated from a soil artificially polluted with chromate (37). The strain has been thoroughly characterized in terms of hundreds of biochemical attributes and its Cr(VI)-reducing capability in the presence of different carbon/energy sources (35), and in terms of biofilms development capability and activity in presence of several stressors (28). Therefore, *P. alcaliphila* 34 has exceptional abilities and it is a prospective candidate for remediation in environments where chromate contamination occurs together with other stressors (35). Its potential utility in the bioremediation of chromate contaminated sites prompted the complete sequencing of *P. alcaliphila* 34 genome (27). The goal of the study described here was to obtain global insight into temporal alterations in mRNA expression that occur in response to an acute chromate stress in the *P. alcaliphila* 34 midexponential planktonic culture and biofilm development processes. Altogether, this data made a contribution to the knowledge of molecular processes and cellular pathways enabling chromate resistance and biofilm formation.

## MATERIALS AND METHODS

### *Bacterial strain, media and growth conditions*

*P. alcaliphila* 34, isolated from a soil artificially polluted with 1000 mg Cr(VI) kg<sup>-1</sup> (37), was used for microarray experiment. The strain has previously been thoroughly characterized in terms of hundreds of biochemical attributes, and its chromate reducing capability in the presence of different carbon/energy sources (35). *P. alcaliphila* 34 was maintained on LB medium supplemented with 5 mM K<sub>2</sub>CrO<sub>4</sub> at 30°C in aerobic conditions. The TMM medium (22) with 0.2% sodium gluconate and 1 mM Na<sub>2</sub>SO<sub>4</sub> was used for biomass production in the chromate time series microarray experiment, in



order to minimize metal complexation and precipitation. Six 250-ml side-arm Pyrex flasks containing TMM medium were inoculated with fresh overnight cultures of *P. alcaliphila* 34 until an optical density of 20-25 klett units. The cultures were grown to the midexponential phase (110 klett units), at 30°C with shaking (150 rpm) and then three of the six cultures were supplemented with 1 mM K<sub>2</sub>CrO<sub>4</sub>. The remaining three cultures served as the control samples and were grown in parallel with the treated cells. For total cellular RNA extraction, the control and treated cultures were harvested in parallel at different time points (5, 30, 60, 90 and 120 min post-chromate addition). Samples for each conditions were directly collected in RNA protect (Qiagen) and stored at -80°C.

For biofilm and planktonic culture expression analysis, wells of three microtiter plate were inoculated with 150 ml of a *P. alcaliphila* 34 suspension containing  $\sim 10^7$  CFU ml<sup>-1</sup> in LB, prepared from bacterial colonies collected from the surface of an agar plate [LB + 5 mM Cr(VI)]. The 96-well plates were placed on a gyratory shaker at 150 rpm and incubated at 30°C. After 7 hours of incubation, planktonic cultures were removed from the plates and an aliquot (500 µl) for each plate was directly collected in RNA protect (Qiagen) and stored at -80°C. These planktonic cultures served as the control samples for biofilm expression analysis. After planktonic cultures removal, the microtiter plates wells were washed three time with LB medium. The biofilms formed on the walls of the wells were collected using sterile cotton swabs, resuspended directly in RNA protect (Qiagen) and stored at -80°C.

#### *RNA Isolation and Preparation of Fluorescein-labeled cDNA*

Total RNA was isolated with hot acid phenol method (5). RNA samples were treated with RNase-free DNase Set (5 Prime) to remove residual genomic DNA and subsequently further purified using isopropanol precipitation according to the manufacturer's RNA cleanup protocol. To verify the absence of genomic DNA, on RNA samples aliquots were performed PCR using universal 16S rRNA primers (17). The integrity of all RNA samples was assessed visually using 1% agarose gel electrophoresis and ethidium bromide staining and then the quantity and purity of the RNA were measured using NanoDrop ND- 1000 UV-VIS Spectrophotometer version 3.2.1.

cDNA probes for cDNA microarray analysis were prepared using FairPlay III Labeling kit (Agilent Technologies): reverse-transcription RNA was followed by coupling to Cy3 dye or Cy5 dye, according to the manufacturer's protocol. The Cy3 or Cy5-labeled cDNA probes were purified using MinElute Spin columns (Qiagen).

*Microarray Hybridization, Scanning, Image Quantification, and Data Analysis*

The Agilent eArray platform was used to design a microarray based on ORF identified in *P. alcaliphila* 34 genome (27). The slide layout consisted of eight arrays of 15,000 elements with each probe presents in triplicate (8x15K).

The two separately labeled cDNA pools (*e.g.* the Cr(VI)-treated and the corresponding control time point) to be compared were mixed together and hybridized on the microarray using FairPlay III Labeling kit (Agilent Technologies) according to the manufacturer's protocol. Fluorescent cDNA bound to the microarray was detected with a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA), using the GenePixPro6.1 software package to quantify microarray fluorescence. Data analysis was carried out using the LIMMA Bioconductor package on R. Locally weighed linear regression (LOWESS, locally weighted scatterplot smoothing) analysis was performed to normalize within arrays, and quantile normalization was carried out among arrays. Differentially expressed genes were calculated by fitting a linear model to the data, followed by empirical Bayes statistics. Genes with  $p < 0.05$  were considered to be significantly differentially expressed compared to the treatment condition. Data were expressed as FC (Fold Change) in base 2 logarithmic scale ( $\log_2FC$ ). Genes exhibiting significant changes in expression at a degree of 2-fold or higher ( $\log_2FC \geq 1.0$ ,  $\log_2FC \leq -1.0$ ) were further analyzed using the program Multi Experiment Viewer Version 4.8.1 (<http://www.tm4.org/mev/>) using Euclidean distance.

## RESULTS AND DISCUSSION

### Transcriptome analysis of Cr(VI)-treatment planktonic cultures *versus* control planktonic cultures

#### *General transcriptome patterns in response to acute chromate stress*

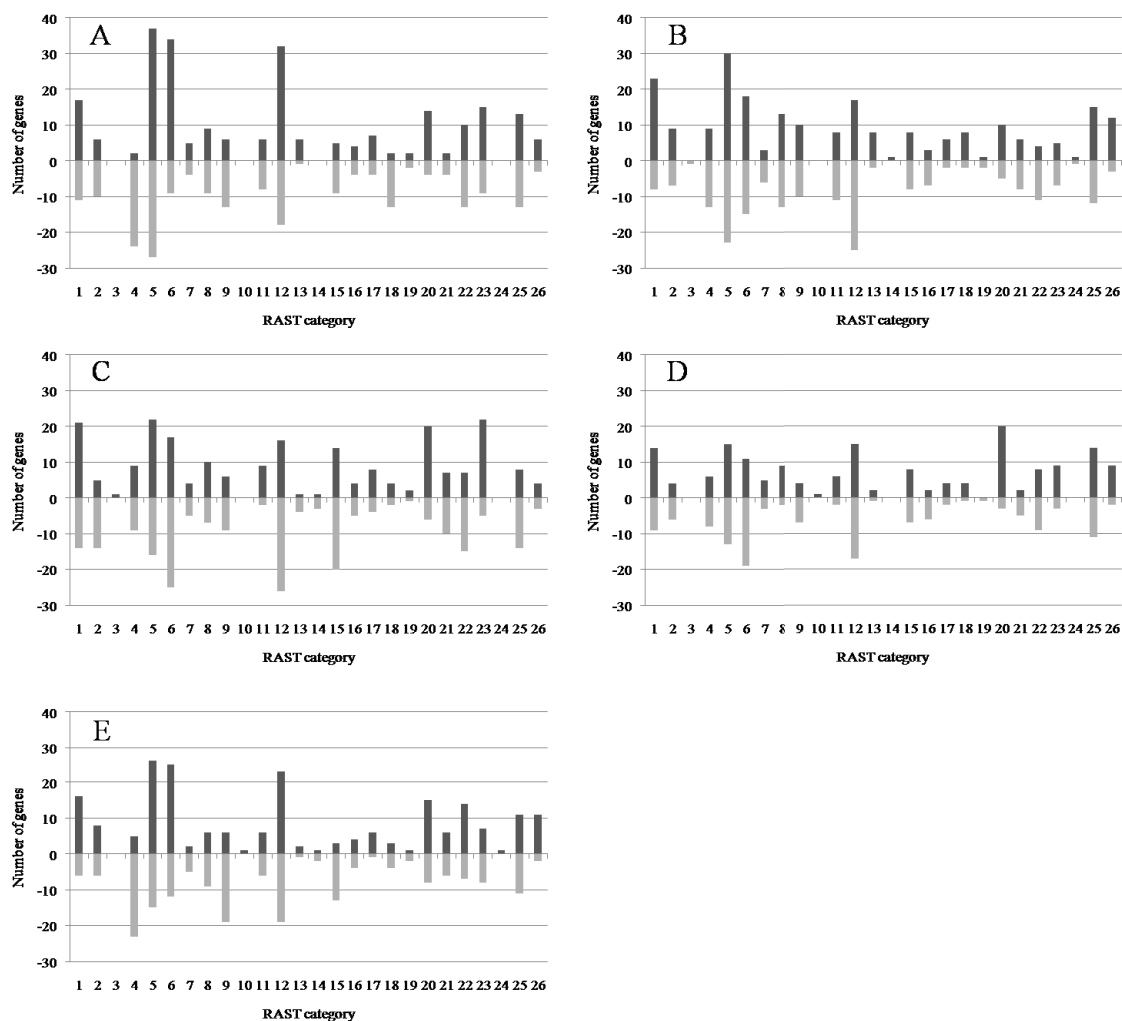
To investigate the effect of an acute stress exposure on global gene expression of *P. alcaliphila* 34 planktonic cultures, a time-series microarray experiments were performed. Global transcriptional profile of the treated and control cultures grown in parallel were compared at 5, 30, 60, 90, 120 min after Cr(VI) addition, using whole genome microarray.

Overall, approximately 17% of the total predicted *P. alcaliphila* 34 genes ( $n = 4,983$ ) spotted on the array showed a statistically significant ( $p < 0.05$ ) change in expression for at least one of the time points during chromate stress exposure. Generally the number of up- and down-regulated genes decreased slightly with time of exposure to chromate with the exception of the genes related to 120-min after Cr(VI) addition (Tab. 1).

**Table 1.** Differentially expressed genes number in the time-series microarray experiments at different intervals after Cr(VI)-treatment and relative number of annotated genes in RAST Categories.

| Time (min) after addition<br>of 1 mM K <sub>2</sub> CrO <sub>4</sub> | Differentially Expressed Genes (DEGs) |         |           | Annotated in<br>RAST<br>Categories |
|----------------------------------------------------------------------|---------------------------------------|---------|-----------|------------------------------------|
|                                                                      | Total                                 | Induced | Repressed |                                    |
| 5                                                                    | 874                                   | 432     | 442       | 670                                |
| 30                                                                   | 869                                   | 420     | 449       | 651                                |
| 60                                                                   | 855                                   | 438     | 417       | 666                                |
| 90                                                                   | 622                                   | 311     | 311       | 476                                |
| 120                                                                  | 763                                   | 376     | 387       | 599                                |

Chromate-responsive genes displayed a wide distribution among functional classes, based on *P. alcaliphila* 34 genome annotation carried out with RAST (6), across the entire course of exposure (Fig. 1).



**Figure 1.** Number of differentially expressed genes at various time-points (5-min, *A*; 30-min, *B*; 60-min, *C*; 90-min, *D* and 120-min, *E*) after acute chromate stress exposure. Genes are grouped by functional classification according to *P. alcaliphila* 34 genome RAST annotation. *Column 1*, cofactors, vitamins, prosthetic groups, pigments; *column 2*, fatty acids, lipids and isoprenoids; *column 3*, phages, prophages, transposable elements, plasmids; *column 4*, virulence, disease and defense; *column 5*, aminoacids and derivatives; *column 6*, carbohydrates; *column 7*, cell division and cell cycle; *column 8*, cell wall and capsule; *column 9*, DNA metabolism; *column 10*, dormancy and sporulation; *column 11*, iron acquisition and metabolism; *column 12*, membrane transport; *column 13*, metabolism of aromatic compounds; *column 14*, miscellaneous; *column 15*, motility and chemotaxis; *column 16*, nitrogen metabolism; *column 17*, nucleoside and nucleotides; *column 18*, phosphorus metabolism; *column 19*, potassium metabolism; *column 20*, protein metabolism; *column 21*, regulation and cell signaling; *column 22*, respiration; *column 23*, RNA metabolism; *column 24*, secondary metabolism; *column 25*, stress response; *column 26*, sulfur metabolism. The black (up-regulated) or gray (down-regulated) bars represent the numbers of genes with statistically significant ( $p < 0.05$ ) change in expression.

The early response (5-min) was characterized by a marked up-regulation of genes whose products mostly belonged to the categories of amino acids and derivatives, carbohydrates and membrane transport (Fig. 1A). During the middle time intervals (30, 60 and 90-min) of chromate exposure the number of differentially expressed genes (DEGs) in these categories gradually decreased, while during the late phase (120-min) the mRNA abundance levels relatives to the categories above increased. At post-treatment time interval of 30-min, compared to the early response (5-min), there was an increase in the induced genes involved in phosphorous and sulfur metabolism (Fig. 1B). 60-min time-point was characterized by an increased in the DEGs number related to motility and chemotaxis and RNA metabolism (Fig. 1C). At the 90-min post-Cr(VI) addiction there was a general decrease in the DEGs number, with the exception of genes involved in stress response and sulfur metabolism, which were mostly up-regulated (Fig. 1D). At 120-min, the general trend in the number of DEGs was similar to that of the early response (5-min), with the exception of genes related to phosphorus and sulfur metabolism. The number of down-regulated genes related to phosphorus metabolism was greater at 5-min time point compared to 120-min, while at 120-min induced genes that were implicated in sulfur metabolism were more numerous that at 5-min post Cr(VI) addiction (Fig. 1A and 1E). During the late phase, a general increase in the number of down-regulated genes related to DNA metabolism was also observed. Finally, at 90-min and 120-min post-Cr(VI) addiction time point there was an up-regulation of genes involved in dormancy and sporulation.

The transcriptome data was carried out by cluster analysis of microarray expression profile patterns for genes that were differentially regulated more than 2-fold ( $\log_2FC \geq 1.0$ ,  $\log_2FC \leq -1.0$ ) (Fig. S1). The pairwise complete linkage analysis was lacking in gene expression values that either showed a non-statistically significant ( $p < 0.05$ ) change in expression or were not differentially expressed. The analysis revealed a small subgroup, comprising four genes, that exhibited substantially greater expression levels in all time-points (in some cases  $\log_2FC > 4$ ) compared with the other induced Cr(VI)-responsive genes. These genes encoded for hypothetical protein (23630.peg.1748, 23630.peg.767), thermostable hemolysin delta-VPH (23630.peg.252) and transporter DME (drug/metabolite exporter) family (23630.peg.3977). On the contrary, five genes encoding for sensory box histidine kinase/response regulator (23630.peg.2738 and 23630.peg.4979), transcriptional regulator AraC family (23630.peg.582), chemotaxis

protein MotB-related protein (23630.peg.2719), copper-sensing two-component system response regulator CusR (23630.peg.1053) were down-regulated during the entire exposure treatment (Fig. S1).

#### *DEGs involved in oxidative stress and DNA repair*

Some bacteria exposed to Cr(VI) can reduce the highly toxic and mobile Cr(VI) to the harmless and less mobile Cr(III) (8). Bacterial reduction of Cr(VI) may also result in the concomitant generation of reactive oxygen species (ROS) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (2). These ROS have been demonstrated to induce a variety of DNA lesions such as single strand breaks and DNA-DNA or DNA-protein cross-links (19).

Cr(VI)-stressed *P. alcaliphila* 34 showed an induction of some genes implicated in oxidative stress: an alkyl hydroperoxide reductase subunit C-like protein (also named sulfate starvation-induced protein 8, 23630.peg.1341), a primary scavenger for hydrogen peroxide in *E. coli* (29), was highly up-regulated ( $\log_2\text{FC} \geq \pm 1.0$ ) at 5, 30, 90 and 120-min post-Cr(VI); a redox-sensitive transcriptional activator SoxR, that activates the transcription of the soxS gene (which controls the superoxide response regulon) was induced at 5 and 30-min time intervals, while it was down-regulated at 120-min (Tab. S2). The expression trend of this two genes suggested that the putative alkyl hydroperoxide reductase subunit C-like protein, which was induced during the entire time of exposure, may be activated by oxidative stress connected with sulfur starvation (26), while the SoxR activator was induced during the early stages of treatment as a result of the oxidative stress generated by chromate reduction.

A glutaredoxin (23630.peg.1271) and a rubredoxin (23630.peg.1825), two protein implicated in oxidative stress, were also up-regulated at 5, 30, 60, 90-min and 5, 30, 90-min after Cr(VI)-addiction, respectively (Tab S1).

In *E. coli* oxidative stress activates the SOS response (1). Since oxidative stress has been established as a central mechanism of chromium toxicity, the SOS response appeared to be a likely defensive strategy. Indeed, we observed an up-regulation of LexA protease (23630.peg.3892) and RecA protein (23630.peg.4570), implicated in the SOS response (Tab. S2). The induction of this two genes suggested the activation of an SOS like pathway of DNA repair in response to chromate. A DNA-3-methyladenine glycosylase (23630.peg.1656), which is part of the broader helix-hairpin-helix DNA repair glycosylase supefamily, was also induced during all time points (Tab. S1). Two

pegs encoding for a FMN-dependent NADH-azoreductase (23630.peg.2816 and 23630.peg.4187) were overall up-regulated at all time points with the exception of 5-min (Tab. S1).

#### *Induction of sulfur metabolism genes*

Chromate actively crosses biological membranes by means of the sulfate transport system, which reflects the chemical analogy between these two oxyanions (8). When the external chromate concentration is high, competition for sulfate transport takes place between the sulfate and chromate. In the case in which the amount of sulfate that crosses the membrane is inadequate for the sulfate assimilation pathway, a sulfate starvation condition occurs (34). In our array experiments several genes involved in sulfur metabolism were induced. Pegs 23630.peg.2835 and 23630.peg.2831, encoding for a ABC-type sulfonate transport systems and an alpha-ketoglutarate-dependent taurine dioxygenase respectively, were up-regulated during the entire time intervals (Tab. S1). An acyl-CoA dehydrogenase (23630.peg.1355) was up-regulated during the entire exposure period, while a FMN reductase (23630.peg.1340), involved in alkanesulfonate assimilation, and a Cys regulon transcriptional activator CysB (23630.peg.3551) were significantly induced at all time point (Tab. S1). Interestingly, a region of the hypothetical protein 23630.peg.1369, that was highly induced during almost all intervals, was similar to *oscA* sequence of *P. corrugata* 28 (34). Furthermore, like in *P. corrugata* 28, this gene was located upstream of a gene cluster that encodes for sulfate ABC transporter components (CysP, CysT, CysW and CysA). Like gene 23630.peg.1369, CysP was strongly expressed at 5, 30, 90 and 120-min after chromate addiction, while CysT was induced at 5, 30 and 120-min time points (Tab. S1). In *P. corrugata* 28 the *oscA-sbp* transcriptional unit was strongly and quickly overexpressed after chromate exposure, suggesting a similar organization and regulation in *P. alcaliphila* 34.

#### *Iron acquisition and metabolism*

Among the genes that were induced more than 2-fold ( $\log_2FC \geq 1.0$ ,  $\log_2FC \leq -1.0$ ) several genes encoding products with function in iron acquisition and metabolism were identified. A ferrichrome transport ATP-binding protein FhuC (23630.peg.4736), an iron-regulated protein A precursor (23630.peg.316), an hypothetical protein related to



heme utilization (23630.peg.1530) and a ferric iron ABC transporter (23630.peg.2008) (Tab. S2). Two genes encoding for a bacterioferritin-associated ferredoxin (23630.peg.4094) and for a putative iron-regulated membrane protein (23630.peg.4647) were induced throughout the time course of Cr(VI) exposure, while a ferrichrome-iron receptor (23630.peg.411 and 23630.peg.4729) was induced at 90-min and down regulated during the other Cr(VI) post-treatment time intervals (Tab. S2). Similarly, a periplasmic hemin-binding protein (23630.peg.566) and an ABC transporter (iron.B12.siderophore.hemin, 23630.peg.1580) were down-regulated in all time points with the exception of 60-min (Tab. S2). Overall, about thirty genes, whose products are predicted to be involved in iron sequestering and metabolism, were differentially expressed in at least one time point after Cr(VI) addiction (Tab. S1). Regulation of iron uptake, which was already observed in *Shewanella oneidensis* response to an acute chromate shock (7), may serve to sequester iron in order to prevent the generation of highly reactive hydroxyl radicals via the Fenton reaction.

#### *DEGs involved in virulence, disease and defense*

*P. alcaliphila* 34 response to chromate displayed an intriguing temporal expression pattern regarding to the genes classified as belonging to the RAST category of virulence, disease and defense (Fig. 1). At 5 min post-Cr(VI) addiction an increase in the number of down-regulated genes, following by an increase in the number of induction genes at 30, 60 and 90-min were observed, while at 120-min post-treatment there was a further increase in the number of down-regulated genes implicated in virulence, disease and defense. This expression trend could suggest an activation of mechanisms involved in chromate defense (*e.g.* multidrug resistance efflux pumps and cobalt-zinc-cadmium resistance) during the middle time intervals (30, 60 and 90-min). In particular, genes involved in copper homeostasis (a copper resistance protein B, a multicopper oxidase, a copper chaperone and a copper tolerance protein) were substantially down-regulated ( $\log_2\text{FC} \leq -1.0$ ) at 5 and 120 min time points, while a copper-sensing two-component system response regulator CusR (23630.peg.1053) and a cation efflux system protein CusA (23630.peg.1008) were down-regulate during the entire time of exposure (Tab. S2). These results could be an evidence of a relationship between the presence of chromate and the copper metabolism.

*Genes involved in motility and biofilm formation in P. alcaliphila 34 planktonic cultures exposed to chromate*

From genes that were differentially regulated more than 2-fold ( $\log_2\text{FC} \geq 1.0$ ) the presence of several genes involved in flagellar motility emerged (Tab. S2). The flagellar motor switch protein FliM (23630.peg.1441) was induced during the entire time of exposure, while genes encoding for other flagellar components showed an heterogeneous expression pattern (Tab. S2). For example, gene encoding for the flagellar assembly protein FliH (23630.peg.4771) was down-regulated at 60 and 120-min, while the flagellar regulatory protein FleQ (23630.peg.1440) displayed pattern of temporal expression with an initial induction at the 5-min time interval followed by a second induction at 60 and 90-min (Tab. S2). The substantial presence of differentially expressed flagellar motility genes could be correlated with biofilm formation. In *P. aeruginosa* PA14 flagella or flagellum-driven motility is also required for biofilm formation (16,24).

Hallmarks of a mature biofilm include the production of an extracellular matrix or EPS (extracellular polymeric substance) and an increased resistance to antimicrobial stress (21). EPS composition presumably varies from organism to organism. *Pseudomonas aeruginosa* has been long thought to produce alginate, a polymer of the uronic acids, as the primary secreted polysaccharide in biofilms (38). In this regard, several genes involved in alginate metabolism were identified as differentially expressed: the outer membrane protein AlgE (23630.peg.4443); the alginate biosynthesis protein AlgX (23630.peg.4699); AlgJ (23630.peg.4701), which is required for alginate O-acetylation; an alginate biosynthesis transcriptional activator (23630.peg.3770); an alginate lyase precursor (23630.peg.2117) and a RNA polymerase sigma-H factor AlgT (23630.peg.4012). The first three genes (AlgE, AlgX and AlgJ) were up-regulated in at least three time intervals, while the remaining genes were down-regulated at 30-min (the transcriptional activator was down-regulated also at 120-min, while the RNA polymerase sigma-H factor AlgT was down-regulated also at 5 and 120-min) (Tab. S1). Three genes, *pelA*, *pelB* and *pelC*, involved in the production of a glucose-rich matrix material in *P. aeruginosa* strain PA14 (11), were also down-regulated in at least one time-point after chromate treatment. Interestingly, a cell division inhibitor (23630.peg.272), putative responsible for persister cell characteristic, was up-regulated at 120 min post-Cr(VI) treatment (Tab. S1).

Overall, the significant presence of several differentially expressed genes related to biofilm development in *P. alcaliphila* 34 chromate stressed planktonic cultures suggested that chromate addition could induce a markedly biofilm production as a defense mechanism.

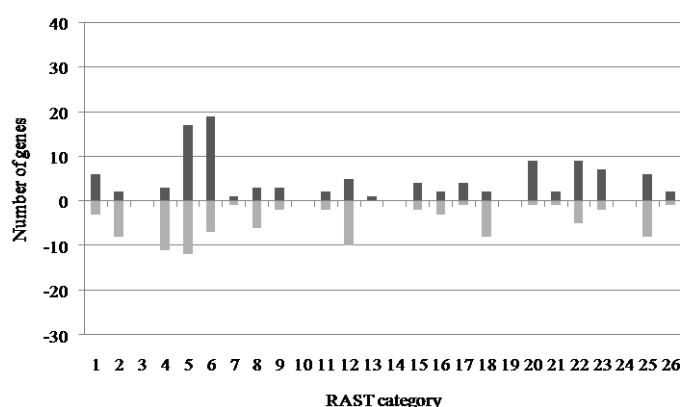
### Transcriptome analysis of 7-h developing biofilms *versus* planktonic cultures

Transcriptome analysis related to biofilm developmental processes provided for a comparison of gene expression profile of biofilms and relative planktonic cultures at the beginning (7 h) of biofilm formation (28). Only 7% of the total predicted *P. alcaliphila* 34 genes ( $n = 4,983$ ) represented on the microarray showed a statistically significant ( $p < 0.05$ ) change in expression in a 7-h biofilm (Tab. 2).

**Table 2.** Differentially expressed genes number in transcriptome profiling experiments in 7-h developing biofilm and relative number of annotated genes in RAST Categories.

|             | Differentially Expressed Genes (DEGs) |         |           | Annotated in RAST Categories |
|-------------|---------------------------------------|---------|-----------|------------------------------|
|             | Total                                 | Induced | Repressed |                              |
| 7-h biofilm | 372                                   | 187     | 185       | 310                          |

In 7-h developing biofilm, majority of differentially expressed genes belongs to aminoacids and derivatives, carbohydrates and membrane transport RAST categories (Fig. 2). Genes involved in cell wall and capsule and in motility and chemotaxis were also differentially regulated. Interestingly, these categories included genes responsible of alginate metabolism and flagellar motility, processes which has been shown to be partially involved in biofilm formation (13,16,24). Particularly, an alginate biosynthesis protein AlgJ (23630.peg.4701), which is required for alginate O-acetylation, and a probable poly(beta-D-mannuronate) O-acetylase (23630.peg.4702) were down-regulated (Tab. S1). The presence of O-acetyl groups plays an important role in physical and chemical alginate properties, including its viscosity and ability to bind to calcium ions; O-acetylation of alginate is also required for *P. aeruginosa* to form microcolonies in a biofilm (10).



**Figure 2. Number of differentially expressed genes in 7-h developing biofilm. Genes are grouped by functional classification according to *P. alcaliphila* 34 genome RAST annotation. Column 1, cofactors, vitamins, prosthetic groups, pigments; column 2, fatty acids, lipids and isoprenoids; column 3, phages, prophages, transposable elements, plasmids; column 4, virulence, disease and defense; column 5, aminoacids and derivatives; column 6, carbohydrates; column 7, cell division and cell cycle; column 8, cell wall and capsule; column 9, DNA metabolism; column 10, dormancy and sporulation; column 11, iron acquisition and metabolism; column 12, membrane transport; column 13, metabolism of aromatic compounds; column 14, miscellaneous; column 15, motility and chemotaxis; column 16, nitrogen metabolism; column 17, nucleoside and nucleotides; column 18, phosphorus metabolism; column 19, potassium metabolism; column 20, protein metabolism; column 21, regulation and cell signaling; column 22, respiration; column 23, RNA metabolism; column 24, secondary metabolism; column 25, stress response; column 26, sulfur metabolism. The black (up-regulated) or gray (down-regulated) bars represent the numbers of genes with statistically significant ( $p < 0.05$ ) change in expression.**

These results could suggest that during the early phase of *P. alcaliphila* 34 biofilm development the alginate acetylation was reduced and probably microcolonies have not yet formed. The list of genes differentially regulated more than 2-fold ( $\log_2FC \geq 1.0$ ) in 7-h developing biofilm contains five genes involved in flagellar biosynthesis and motility (FliE, FliN, FlgF, FlgA) (20) (Tab. S3). These genes were all induced, while the RNA polymerase sigma factor for flagellar operon (23630.peg.1819) was down-regulated ( $\log_2FC = -1.0$ ). A type IV pilus biogenesis protein PilE (23630.peg.4474) and a twitching motility protein PilG (23630.peg.2083) were also down-regulated (Tab. S3). As previously noted, flagella and motility have been implicated as important for biofilm formation: before the biofilm can form, bacteria must attach to the surface and/or to each other. Attachment is mediated by cell-surface organelles, such as flagella and type I fimbriae, which are characteristic of the first and second stages of biofilm formation, reversible and irreversible attachment (25). Particularly, flagella or flagella-mediated

motility appears to be important for the formation of a bacterial monolayer on a surface. Type IV pili appear to play a role in downstream events such as microcolony formation (24). Accordingly, the up-regulation of genes involved in flagellar motility and the down-regulation of a type IV pilus biogenesis protein could confirm that *P. alcaliphila* 34 biofilm, after 7 h of incubation in a microplate, was in the early developmental phase, as previously demonstrated (28).

Both in chromate and biofilm transcriptome analysis about the 16% of the total number of induced genes and repressed genes were functionally classified as hypothetical or conserved hypothetical proteins, whereas poorly characterized genes (putative genes or genes not included in RAST categories) constituted 50% of the total number of differentially expressed genes at each time points. Large portion of genes with unassigned cellular functions among induced or repressed genes reveals how much of *P. alcaliphila* 34 molecular basis implicated in chromate resistance and biofilm development remains to be explored.

General overview of the transcriptome data showed a decrease in the number of up-regulated genes with a putative function in aminoacids and derivatives, and carbohydrates metabolism, especially at 60 and 90-min post-chromate addiction, and a parallel increase in the number of induced genes implicated in sulfur metabolism. This result could indicate that due to competitive inhibition of sulfate uptake by chromate, *P. alcaliphila* 34 attempted to cope with sulfur starvation activating sulfur metabolism. The induction of genetic pathways relative to iron acquisition and metabolism, and the down-regulation of copper metabolism were also observed, indicating a correlation between chromate exposure and these two metabolic pathways. A central mechanism of chromate toxicity is the oxidative damage to proteins; indeed, chromate-stressed *P. alcaliphila* 34 showed an induction of both oxidative stress response systems and mechanisms related to DNA repair.

In *P. alcaliphila* 34 7-h developing biofilm an induction of genetic pathway involved in flagellar motility and the down-regulation of a type IV pilus metabolic system were observed. Flagella and motility are partially responsible of biofilm formation, since before the biofilm can form, bacteria must attach to the surface and/or to each other. Attachment is mediated by cell-surface organelles, such as flagella and pilus: flagella appears to be important for the formation of a bacterial monolayer of the abiotic surface,

while type IV pili could play a role in downstream events such as microcolony formation.

Global data analysis showed an interestingly feature: in *P. alcaliphila* 34 chromate-treated planktonic cultures gene networks related to biofilm development were differentially expressed, indicating that biofilm formation could be a defense mechanism to deal with chromate. Overall, this study suggested that biofilm of the highly Cr(VI)-resistant *P. alcaliphila* strain could be used as an interesting model for additional studies as well as an opportunity to be further exploited for bioremediation of Cr(VI) from contaminated environments.

#### **ACKNOWLEDGMENT**

This work was supported by the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) (PRIN, 2008).

## REFERENCES

1. **Ackerley, D. F., Y. Barak, S. V. Lynch, J. Curtin, and A. Martin.** 2006. Effect of chromate stress on *Escherichia coli* K-12. *J. Bacteriol.* **188**:3371-3381.
2. **Ackerley, D. F., C. F. Gonzalez, C. H. Park, R. Blake, M. Keyhan, and A. Matin.** 2004. Chromate-reducing properties of soluble flavoproteins from *Pseudomonas putida* and *Escherichia coli*. *Appl. Environ. Microbiol* **70**:873-882.
3. **Alvarez, A. H., R. Moreno-Sanchez, and C. Cervantes.** 1999. Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa*. *J. Bacteriol.* **181**:7398-7400.
4. **ATSDR.** 2011. Priority List of Hazardous Substance 2011, Atlanta.
5. **Ausbel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Current protocols in molecular biology. John Wiley & Sons Inc., New York City, NY.
6. **Aziz, R. K., D. Bartels, A. A. Best, M. DeJongh, T. Disz, R. A. Edwards, K. Formsma, S. Gerdes, E. M. Glass, M. Kubal, F. Meyer, G. J. Olsen, R. Olson, A. L. Osterman, R. A. Overbeek, L. K. McNeil, D. Paarmann, T. Paczian, B. Parrello, G. D. Push, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke, and O. Zagnitko.** 2008. The RAST server: Rapid Annotations using Subsystems Technology. *BMC Genomics* **9**:1-15.
7. **Brown, S. D., M. R. Thompson, N. C. Verberkmoes, K. Chourey, M. Shah, J. Zhou, R. L. Hettich, and D. K. Thompson.** 2006. Molecular dynamics of the *Shewanella oneidensis* response to chromate stress. *Mol. Cell Proteomics.* **5**:1054-1071.
8. **Cervantes, C., J. Campos-Garcia, S. Devars, F. Gutierrez-Corona, H. Loza-Tavera, J. C. Torres-Guzman, and R. Moreno-Sanchez.** 2001. Interactions of chromium with microorganisms and plants. *FEMS Microbiol. Rev.* **25**:335-347.



9. **Costerton, J. W., Z. Lewandowski, D. Debeer, D. Caldwell, D. Korber, and G. James.** 1994. Biofilms, the customized microniche. *J. Bacteriol.* **176**:2137-2142.
10. **Franklin, M. J. and D. E. Ohman.** 2002. Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. *J. Bacteriol.* **184**:3000-3007.
11. **Friedman, L. and R. Kolter.** 2003. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol. Microbiol.* **51**:675-690.
12. **Harrison, J. J., H. Ceri, and R. J. Turner.** 2007. Multimetal resistance and tolerance in microbial biofilms. *Nat. Rev. Microbiol.* **5**:928-938.
13. **Hentzer, M., G. M. Teitzel, G. J. Balzer, A. Heydorn, S. Molin, M. Givskov, and M. R. Parsek.** 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J. Bacteriol.* **183**:5395-5401.
14. **Holm, O., E. Hansen, C. Lassen, F. Stuer-Lauridsen, and J. Kjolholt.** 2002. Heavy metals in waste - final report, Denmark.
15. **James, B. R.** 1996. The challenge of remediating chromium-contaminated soil. *Environ. Sci. Technol.* **30**:248-251.
16. **Klausen, M., A. Heydotn, P. Ragas, L. Lambertsen, A. Aaes-Jørgensen, S. Molin, and T. Tolker-Nielsen.** 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol. Microbiol.* **48**:1511-1524.
17. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and Goodfellow.M. (ed.), *Nucleic acid techniques in bacteria systematics*. John Wiley & Sons, Chichester, UK.
18. **Langård, S.** 1980. *Metals in the environment*. Academy Press Inc., New York.
19. **Liu, K. J. and X. Shi.** 2001. In vivo reduction of chromium (VI) and its related free radical generation. *Mol. Cell Biochem.* **222**:41-47.

20. **Macnab, R. M.** 2003. How bacteria assemble flagella. *Ann. Rev. Microbiol* **57**:77-100.
21. **Mah, T. F. C. and G. A. O'Toole.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **9**:34-39.
22. **Mergeay, M.** 1995. Heavy metal resistances in microbial ecosystems, p. 1-17. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruij (ed.), *Molecular microbial ecology manual*. Kluwer Academic, Netherlands.
23. **O'Toole, G., H. B. Kaplan, and R. Kolter.** 2000. Biofilm formation as microbial development. *Ann. Rev. Microbiol.* **54**:49-79.
24. **O'Toole, G. A. and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* **30**:295-304.
25. **Prüß, B. M., K. Verma, P. Samanta, P. Sule, S. Kumar, J. Wu, D. Christianson, S. M. Horne, S. Stafslie, A. J. Wolfe, and A. Denton.** 2010. Environmental and genetic factors that contribute to *Escherichia coli* K-12 biofilm formation. *Arch. Microbiol.* **192**:715-728.
26. **Quadroni, M., P. James, P. Dainese-Hatt, and A. Kertesz.** 1999. Proteome mapping, mass spectrometric sequencing and reverse transcription-PCR for characterization of the sulfate starvation-induced response in *Pseudomonas aeruginosa* PAO1. *Eur. J. Biochem.* **266**:986-996.
27. **Santopolo, L., E. Marchi, F. Decorosi, M. Galardini, M. Brilli, L. Giovannetti, and C. Viti.** 2013. Draft genome sequence of chromate-resistant and biofilm producing strain *Pseudomonas alcaliphila* 34. *Genome Announcements* submitted.
28. **Santopolo, L., E. Marchi, L. Frediani, F. Decorosi, C. Viti, and L. Giovannetti.** 2012. A novel approach combining the Calgary Biofilm Device and Phenotype MicroArray for the characterization of the chemical sensitivity of

- bacterial biofilms. *Biofouling: The Journal of Bioadhesion and Biofilm Research* **28**:1023-1032.
29. **Seaver, L. C. and J. A. Imlay.** 2001. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J. Bacteriol.* **183**:7173-7181.
  30. **Singh, R., D. Paul, and R. K. Jain.** 2006. Biofilms: implications in bioremediation. *Trends in Microbiol.* **14**:389-397.
  31. **Teitzel, G. M. and M. R. Parsek.** 2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **69**:2313-2320.
  32. **Turick, C. E., W. A. Apel, and N. S. Carmiol.** 1996. Isolation of hexavalent chromium-reducing anaerobes from hexavalent-chromium-contaminated and non contaminated environments. *Appl. Microbiol. Biotechnol.* **44**:683-688.
  33. **US Environmental Protection Agency.** 1998. Toxicological review of hexavalent chromium, Washington, DC.
  34. **Viti, C., F. Decorosi, A. Mini, E. Tatti, and L. Giovannetti.** 2008. Involvement of the *osca* gene in the sulphur starvation response and in Cr(VI) resistance in *Pseudomonas corrugata* 28. *Microbiology* **155**:95-105.
  35. **Viti, C., F. Decorosi, E. Tatti, and L. Giovannetti.** 2007. Characterization of chromate-resistant and -reducing bacteria by traditional means and by a high-throughput phenomic technique for bioremediation purposes. *Biotechnol. Prog.* **23**:553-559.
  36. **Viti, C. and L. Giovannetti.** 2007. Bioremediation of soils polluted with hexavalent chromium using bacteria-the challenge , *In* S. N. Singh and R. D. Tripathi (ed.), *Environmental bioremediation technologies*. Springer, Berlin.
  37. **Viti, C., A. Mini, G. Ranalli, G. Lustrato, and L. Giovannetti.** 2006. Response of microbial communities to different doses of chromate in soil microcosms. *Appl. Soil Ecol.* **34**:125-139.

38. **Wozniak, D. J., T. J. O. Wyckoff, M. Starkey, R. Keyser, P. Azadi, G. A. O'Toole, and M. R. Parsek.** 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. PNAS **100**:7907-7912.

## ADDITIONAL FILES

### **Additional file, Figure S1:**

Cluster Analysis of Microarray dataset. Linkage clustering analysis of statistically significant ( $p < 0.05$ ) *P. alcaliphila* 34 genes exhibiting  $\log_2FC > 1.0$  or  $\log_2FC < -1.0$  at least at one time points during Cr(VI) stress exposure. Transcriptional profiles, based on the Euclidean similarity, are shown at 5, 30, 60, 90 and 120 min post-chromate addition.

### **Additional file, Table S1:**

Table with expression ratios ( $\log_2FC$ ) for all *P. alcaliphila* 34 genes exhibiting statistically significant ( $p < 0.05$ ) change in expression and classified according to RAST annotation.

### **Additional file, Table S2:**

Table with *P. alcaliphila* 34 statistically significant ( $p < 0.05$ ) genes exhibiting  $\log_2FC \geq 1.0$  or  $\log_2FC \leq -1.0$  at least at one time points during Cr(VI) stress exposure.

### **Additional file, Table S3:**

Table with expression ratios ( $\log_2FC \geq 1.0$  or  $\log_2FC \leq -1.0$ ) of 7-h developing biofilm genes exhibiting statistically significant ( $p < 0.05$ ) change in expression compared to planktonic culture.

## **Chapter 6**

### **Conclusions and future works**

A phenotypic and molecular characterization of the bacterium *P. alcaliphila* 34 has been carried out in the present work. The development of an integrated system combining two high-throughput technologies, the Calgary Biofilm Device (MBEC device) and Phenotype MicroArray (PM), has offered the possibility to obtain an extensive characterization of toxic compounds susceptibility of *P. alcaliphila* 34 biofilm and planktonic culture. The common assumption that biofilms are more tolerant than planktonic cells was confuted and was showed that cultures in the stationary phase were not only more tolerant than logarithmic-phase planktonic cultures, but also than biofilms in presence of several chemicals. Therefore, any conclusions regarding biofilm and planktonic culture resistance should take into account the growth phase of planktonic cultures and the nature of chemicals.

To provide insights into the biotechnological exploitation of the organism for remediation of chromium-contaminated sites, *P. alcaliphila* 34 genome has been sequenced using a next generation sequencing technology. Genome annotation allowed the identification of 4,983 protein-coding sequences and 61 tRNAs. Based on ORFs that have been identified in *P. alcaliphila* 34 genome, a microarray for transcriptomic analysis of molecular mechanisms responsible for chromate resistance and biofilm formation, has been designed. From temporal genomic profiling of *P. alcaliphila* 34 planktonic cultures exposed to an acute chromate stress the overexpression of genes involved in sulfur metabolism was highlighted, as well as the activation of oxidative stress response system and mechanism related to DNA repair. The chromate shock response of *P. alcaliphila* 34 was also characterized by the enhanced expression of genetic pathways relative to iron acquisition and metabolism, and the down-regulation of copper metabolism, suggesting a correlation between chromate exposure and the pathways of these two metals.

The analysis of differentially expressed genes related to the early developing *P. alcaliphila* 34 biofilm revealed the induction of the genetic pathway involved in flagellar motility and the down-regulation of a type IV pilus metabolic system. Interestingly, from the transcriptomic analysis of *P. alcaliphila* 34 response to an acute chromate challenge the presence of genetic pathways involved in biofilm formation has emerged. This observation suggests that biofilm formation may be a survival strategy to deal with chromate.



In conclusion, obtained data have showed that the MBEC device/PM approach is a reliable, repeatable, accurate, and quick method. In particular, this integrated system has enabled to evaluate, for the first time, the effect of toxic chemicals (*i.e.*, antibiotics, biocides, heavy metals) on metabolic activity of microbial biofilm. The acquired know-how indicates that, before proceeding to a bioremediation process, it is necessary to determine at what stage of growth the microorganism shows the best fitness in presence of a given pollutants, while transcriptomic analysis provides insights into the molecular mechanisms related to biofilm development and chromate resistance. Furthermore, the obtained information may be used to design a biological system for bioremediation of chromate contaminated soils using biofilm of the highly Cr(VI)-resistant *P. alcaliphila* 34 strain.

## *Ringraziamenti*

*Giunta al termine di questo percorso, desidero ringraziare la Prof.ssa Luciana Giovannetti e il Dott. Carlo Viti che durante questi tre anni mi hanno permesso di lavorare in modo costruttivo e sereno.*

*Un sincero e affettuoso grazie va a Francesca, Emanuela, Grazia e Giulia che mi hanno indirizzata, supportata, sopportata e senza le quali questo lavoro non sarebbe stato possibile.*

*Grazie anche ad Emanuela ed Alessia e alle nostre chiacchierate sulla vita.*

*Infine, un grazie infinito alla mia grande e bella famiglia (inclusi gli ultimi arrivati), riferimento sicuro della mia vita, e a Luca, che mi aiuta a dare il giusto senso alle cose.*