CHROMATE RESISTANCE OF Bacillus firmus
I. INTRODUCTION

METAL ION RESISTANCE

In nature, microorganisms are ubiquitous and so are the metal ions, which either occur in ores or natural rocks and minerals, or else are released to the environment via industrial and household uses of their compounds. As these metal ions, especially the heavy metals, are toxic at high concentrations, microorganisms have evolved various biological systems to cope with their interactions with living cells. One such system has led to the development of resistant cells, i.e., they require higher concentrations of the toxicant than the parent culture before being affected. These cells can convert reactive forms of the metals to the less reactive ones.

Microorganisms can be isolated that exhibit high levels of resistance against some metal ions in their naturally occurring state. It has also been observed that microorganisms can develop resistance against high, toxic concentrations of many metal ions, when adapted in presence of increasingly high concentrations. Thus it is possible to develop a resistant strain of bacterium by the adaptation process.
Biochemical and Genetic bases of Bacterial Resistance to Heavy Metal Ions

The development of resistant microbial cells assures the involvement of mechanisms which bars the heavy metals from imparting toxic effects to the living cells. One such system inactivates the metals to the less reactive forms, which can then be volatilized off or stored within the cells. Resistance may also arise from an alteration in the permeability properties of the cell membrane so that cellular uptake of the specific metal ion is diminished. In addition, certain microbes are known to elaborate proteins which keep the metal in an innocuous form.

Different mechanisms have been suggested, which are used by the bacteria for achieving resistance. These are:-

1. Reduction to Free Metal

This mechanism of resistance is most frequently observed in the case of mercury, and has been widely studied both from genetic as well as enzymatic points of view. It has been observed by different workers that most bacteria develop resistance against mercury salts and organomercurials by reducing them to metallic mercury, which can then be volatilized off from the cells. The genes specifying such enzymes have been shown to be plasmid-borne, and a number of such plasmids have been characterized in Escherichia coli.
Staphylococcus aureus and Pseudomonas (Summers and Lewis, 1973; Schottel et al., 1974). These plasmids determine resistance to mercuric ion as well as to a range of mercurials. The enzyme systems specified by such plasmids generally consist of a reducing enzyme and hydrolases, and are inducible in nature. The properties of such enzymes have been reviewed by Chakrabarty (1976).

Oxyanions of heavy metals, such as selenium and tellurium, can also be reduced microbially (Silverberg et al., 1976), and the reducing enzymes involved have been known, in many cases, to be plasmid-specified. Resistance to borates and chromates can also be determined by plasmids (Summers and Jacoby, 1978), but the specific chemical mechanisms involved in these cases are unknown.

2. Reduced Uptake of Metal

It has been seen that the resistance against metal ions, in some cases, is brought about by a mechanism, in which there is reduction in the uptake of the ions into the cells. This process takes place in the case of Cd\(^{2+}\) (Chopra, 1971). In E. coli, accommodated to Cd\(^{2+}\), the resistance involved exclusion of the ion from the cell, together with reversal of damage caused by prior exposure (Mitra et al., 1975). This sort of resistance is known to be specified by a plasmid, at least in the case of S. aureus (Novick and Bouanchaud, 1971; Smith and Novick, 1972; Kondo et al.,...
1974; Chopra, 1975). In these cases, a permeability change occurs in the cells, so that resistant cells harboring the plasmid do not accumulate the toxic levels of cadmium normally accumulated by the sensitive cells. Thus, a mercury-sensitive Enterobacter aerogenes strain, derived from a mercury resistant strain by treatment with Mitomycin C, exhibited a 6-fold higher Hg content than the parent strain (Yoshio and Imura, 1980).

Normally, cadmium uptake by sensitive cells is temperature-dependent and inhibited by inhibition of energy metabolism.

3. Intracellular Accumulation of Metal

In some cases, resistance against metal ions can be manifested by an efficient intracellular accumulation of the ions (Tornabene and Edwards, 1972), which then appears to be stored in apparently innocuous forms. This mode of resistance has been observed to be plasmid-mediated, in certain cases. Thus, Kondo et al. (1974) have demonstrated that more than 90% of Hg$^{2+}$ ions present in a test medium can be taken up by S. aureus cells harboring the penicillinase plasmid. This mechanism was inducible in nature, and appears to be independent of temperature. Similar accumulation of Hg$^{2+}$ has been demonstrated for a resistant strain of Enterobacter aerogenes, which was shown to concentrate 91% of the total $^{203}$Hg in the intracellular protein fraction in 3 hours (Hamdy
and Noyes, 1975). Sayler et al. (1975) have reported some Pseudomonas strains, similarly capable of accumulating and volatilizing mercury. The use of a Pseudomonas culture, harboring aggregates of mercury resistance and other plasmids, has been recommended for removal and recovery of mercury from industrial wastes (Chakrabarty et al., 1975).

In a strain of Pseudomonas ambigua G-1, resistant to chromate, it was found that the chromate present in the culture fluid was taken up within the cells (Horitsu et al., 1978). As regards the fate of the intracellular metal ions, it has been suggested that they are bound to some intracellular components, which renders them harmless. The nature of binding of Hg$^{2+}$ to the intracellular protein is not known, but in case of Cd, highly specific metal-binding metallothionein-like proteins have been described. Mitra et al. (1975) have observed a prolonged lag phase in batch cultures of E. coli supplemented with cadmium and ascribed this mainly to the time needed for induction of a unique cadmium-binding protein that was virtually absent in cultures without added cadmium.

4. Biochemical Transformations of Metals

Many microbes have adopted the process of modifying the chemical nature of the heavy metals, by transforming their compounds, e.g., methylation, formation of sulphides, change of valence states by oxidation-reduction, etc. After
transformation, these compounds are generally eliminated by volatilization after methylation, or precipitation after sulphide formation. They may also exist in a less toxic form by changing valence states.

The methylation of certain heavy metals by microorganisms is well-established (Wood, 1974; Ridley et al., 1977). Such methylated derivatives are often quite toxic, e.g., methyl mercury and di-methyl mercury. Anaerobic bacteria in lake or river sediments, as well as some aerobic mercury-resistant bacteria can methylate Hg$^{2+}$. Thus, some Pseudomonas strains have been found to produce methyl mercury from Hg$^{2+}$ (Silver et al., 1976). Similar methylation of other metals by microorganisms have been observed, the metals involved being selenium (Chau et al., 1976), lead (Wong et al., 1975), tellurium (Summers and Jacoby, 1977), tin (Wood, 1974), cadmium (Huey et al., 1975) and chromium (Ridley et al., 1977). Transmethylation reactions, where methyl groups are transferred to mercury ions from biologically methylated tin compounds, are also known (Brinckman et al., 1976). In some Pseudomonas strains, the genes specifying methylation of Hg$^{2+}$ are known to be specified by plasmids pMGl and pMG2 (Silver et al., 1976), but the disposition of methylation genes in other bacteria has not been investigated.

In Klebsiella aerogenes growing in continuous culture in the presence of high concentration of cadmium, growth started after an extended lag phase. This indicated a
physiological adaptation process, and growth was accompanied by a formation of cadmium sulphide (Aiking et al., 1982). Pan-Hou and Imura (1981) reported the formation of sulphide of mercury by a mercury resistant strain of Clostridium cochlearium T-2.

The chemolithotropic bacterium, Thiobacillus ferrooxidans, is known to withstand high concentrations of ferrous iron, which is transformed to ferric iron by the microorganism. The bacterium derives its energy from this oxidation process.

**INVOLVEMENT OF PLASMIDS IN RESISTANCE MECHANISMS**

Review on the mechanisms of tolerance in microorganisms point out that this process is frequently linked to plasmids, the extrachromosomal genetic elements. It is a well-documented fact that antibiotic resistance, in most of the cases, is plasmid mediated. This, from recent researches, is also appearing to be true, in the case of metals. Numerous works have been reported which suggest the involvement of plasmids in resistance mechanisms. These plasmids are often referred to as the resistance factors (R-factors) or R-plasmids. Resistance factors have been reviewed and discussed in Meynell et al. (1968), Campbell (1969), Novick (1969), Watanabe (1963), Anderson (1968) and Novick and Bouanchaud (1971).
Plasmid-mediated resistance mechanisms have been reported by various workers, both in gram-positive and gram-negative microorganisms. In *Staphylococcus aureus*, plasmid-encoded resistance to erythromycin (Mitsuhashi *et al.*, 1963), penicillin (Peyru *et al.*, 1969), methicillin (Dornbush *et al.*, 1969), lincomycin (Bastos *et al.*, 1980) and multiple resistance to erythromycin, penicillin, tetracycline (Bastos and Penido, 1981) have been reported. Resistance to macrolide, lincosamide and streptogramin has been reported to be specified by plasmids in *Staphylococcus epidermidis* (Parisi *et al.*, 1981). Zaharieva and Valerianov (1980) have observed plasmid-linked resistance to erythromycin and lincomycin, in streptococci. Plasmid-coded antibiotic resistance has also been observed in thermophilic bacilli (Imanaka *et al.*, 1981).

Plasmid-specified resistance in *E. coli* to gentamicin (Ike *et al.*, 1981) and in other enterobacteria (Parkhomenko and Lukach, 1980) has been reported. Non-enzymatic chloramphenicol resistance is also found to be specified by plasmids in *E. coli* (Dorman and Foster, 1982). Involvement of plasmids in antibiotic resistance has also been established in *Haemophilus influenzae*, for chloramphenicol (van Klingeren *et al.*, 1977), kanamycin (Dang Van *et al.*, 1975), ampicillin (Elwell *et al.*, 1975; Saunders and Sykes, 1977); in *Haemophilus ducreyi* for ampicillin (Thomson and Bilgeri, 1982); in *Haemophilus pleuropneumoniae*, for ampicillin, streptomycin and sulphadiazine (Hiresh *et al.*, 1981). This phenomena has also been observed in *Pseudomonas aeruginosa*, where plasmids
encode resistance to gentamicin (Jacoby, 1974) and carbenicillin (Michel-Briand et al., 1977). Peng (1982) reported that the plasmid pMR9 in Pseudomonas maltophilia governs multiple resistance to a number of antibiotics. Apart from these, resistance specified by plasmids has been reported in Serratia marcescens for gentamicin (John and William, 1981) and in Campylobacter jejuni for tetracycline resistance (Taylor et al., 1980).

The involvement of bacterial plasmids in resistance mechanisms is not limited to antibiotics. The R-plasmids are also known to confer resistance to toxic heavy metal ions. Penicillinase plasmids of Staphylococcus aureus are known to provide resistance to As, Bi, Cd, Hg, Pb, Sb and Zn compounds (Richmond and John, 1964; Novick and Roth, 1968; Peyru et al., 1969; Dyke et al., 1970; Kondo et al., 1974; Chopra, 1975; Silver et al., 1981). Lactose plasmids of group N streptococci provide resistance to compounds of arsenic and chromium (Efstatthiou and McKay, 1977). In enterobacteria, plasmids determine resistance to As, Ag, Co, Hg, Ni and Te compounds (Smith, 1967; Summers and Silver, 1972; Hedges and Baumberg, 1973; Summers and Lewis, 1973; Schottel et al., 1974; McHugh et al., 1975; Summers and Jacoby, 1977; Yoshio and Imura, 1980). Again, in Escherichia coli, plasmid-determined resistance to arsenate, arsenite and antimony (III) has been reported by Silver et al. (1981).
Resistance to compounds of mercury and tellurium in *Pseudomonas aeruginosa* is plasmid-mediated (Summers and Lewis, 1973; Clark *et al.*, 1977; Summers and Jacoby, 1977). In addition, two new resistances were detected (Summers and Jacoby, 1978) in *P. aeruginosa* plasmids, which were already found to be responsible for other metal resistances. These were, resistance against the trivalent compounds of boron, borate and metaborate and resistance to the hexavalent compounds of chromium, chromate and dichromate.

However, genetic modification governing resistance mechanism is not always plasmid linked, but occasionally chromosomally linked (Dyke *et al.*, 1970). In a clinically isolated *P. aeruginosa*, resistance to streptomycin, kanamycin, chloramphenicol, sulphanilamide and HgCl₂ was noticed, but there was no detectable plasmid DNA (Anisimova *et al.*, 1982). There are more reports on antibiotic resistance which are unrelated to plasmids, as in the case of tetracycline resistance in *Bacillus thuringiensis* var. *galleriae* (Belykh *et al.*, 1982), chloramphenicol and tetracycline resistance in *Citrobacter freundii* (Austen and Trust, 1981) and chloramphenicol resistance in some gram-negative bacteria (Gaffney *et al.*, 1981).
Table 2.1  Plasmid-specified resistance to toxic elements

<table>
<thead>
<tr>
<th>Element Resisted</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co, Hg, Ni</td>
<td>Smith, 1967.</td>
</tr>
<tr>
<td>As, Cu, Cd, Hg, Pb, Sb, Zn</td>
<td>Novick and Roth, 1968.</td>
</tr>
<tr>
<td>As, Cu, Cd, Hg, Pb, Sb</td>
<td>Novick and Bouanchaud, 1971.</td>
</tr>
<tr>
<td>As</td>
<td>Hedges and Baumberg, 1973.</td>
</tr>
<tr>
<td>Hg</td>
<td>Schottel et al., 1974.</td>
</tr>
<tr>
<td>Ag, Hg, Te</td>
<td>McHugh et al., 1975.</td>
</tr>
<tr>
<td>Hg</td>
<td>Chakrabarty, 1976.</td>
</tr>
<tr>
<td>As, Cr</td>
<td>Efstathiou and McKay, 1977.</td>
</tr>
<tr>
<td>As, Hg, Te</td>
<td>Summers and Jacoby, 1977.</td>
</tr>
<tr>
<td>Hg</td>
<td>Yoshio and Imura, 1980.</td>
</tr>
<tr>
<td>As, Sb</td>
<td>Silver et al., 1981.</td>
</tr>
<tr>
<td>Hg</td>
<td>Pan-Hou and Imura, 1980.</td>
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</tbody>
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Recognition and Identification of Extrachromosomal Elements

The involvement of plasmids, in various activities of microorganisms, has led to the need for development of suitable methods for the recognition and identification of these elements.

There are different ways of determining the involvement of plasmids in specifying phenotypic characters of microorganisms. Novick (1969), in a review, has laid down some criteria.
for establishing plasmid linkage of a certain trait. One of the methods for establishing plasmid-linkage is by "curing".

Bacterial strains carrying plasmids often throw off plasmid-negative variants as a result of occasional errors in plasmid replication or segregation. The frequency of such variants can often be increased by certain physical or chemical agents, e.g., elevated temperature, thymine starvation, novobiocin, mitomycin-C, sodium dodecyl sulphate, acriflavine, acridine and other intercalating dyes like ethidium bromide. This effect is often referred to as "curing" of plasmids, by which it is implied that the plasmid is selectively inactivated or inhibited in replication.

Acriflavine and acridine dyes as "curing" agents were successfully applied and reported by Hirota (1960), Watanabe and Fukasawa (1961) and Hashimoto et al. (1964). The effective use of acridine orange in elimination of plasmids in Bacillus sp., has also been reported (Hara et al., 1982).

Novobiocin is also reported to be acting as a curing agent (McHugh and Swartz, 1977).

Plasmid curing due to incubation at high temperatures, is also an established phenomenon (Zamenhof and Greer, 1958; May et al., 1964; Asheshov, 1966; Terawaki et al., 1967).
TOXICITY OF CHROMIUM

Environmental contamination by certain heavy metal compounds are bringing about problems to human health, including genetic hazards. It has been reviewed by De Vogt et al. (1980), Jenette (1980), Martell (1980), Bigaliev (1982), Krigman (1982) and Pawelczak (1982).

The present investigation focuses specifically on the metal chromium. Research on toxic effects of chromium, and pollutions caused by it have been reported by different workers (Berbenni and Ariati, 1980; Mahbubani, 1981). Research on the toxicology of chromium has focused primarily on human beings (Langard, 1980) and the microbiota (EPA, 1979), with some research on plants (Huffman and Allaway, 1973). Epidemiological evidence for an excess risk of lung cancer in chromate workers has accumulated in the past decades and various chromium compounds have been found to be carcinogenic for rodents.

There are also reports of evaluating the toxicity of chromium to microbiota. For example, 1.6 to 3.2 ppm Cr$^{6+}$ inhibited growth of the algae, *Chlorella variegatus* and *Chlorococcum humicola* (Hervey, 1949). In the fungus *Gnomononion platani*, conidia production was reduced by 2.8 ppm Cr$^{3+}$ (Staskawicz and Smith, 1977). Ten to twelve ppm Cr$^{6+}$ inhibited growth of soil bacteria and actinomycetes (Ross et al., 1981); the numbers of actinomycetes, bacteria
and fungi isolated from soil were decreased by 1, 10 and 100 ppm Cr$^{6+}$, respectively (Drucker et al., 1979).

Although chromium can occur in different valence states, the trivalent (Cr$^{3+}$) and hexavalent (Cr$^{6+}$) are commonly encountered in the environment. Of these, Cr$^{6+}$ is highly toxic to biological systems, even in very low concentrations, and is highly carcinogenic (Anderson, 1981). It is the hexavalent chromium which, on exposure, is responsible for abnormal changes in the respiratory tracts (Mizutani et al., 1982).

The highly toxic and mutagenic nature of hexavalent chromium, as compared to the trivalent state, has also been reported in the case of bacteria. Cr$^{3+}$ was less toxic than Cr$^{6+}$ to growth of Klebsiella pneumoniae (Baldry et al., 1977) and to fermentation by a mixed rumen microbiota (Forsberg, 1978). Furthermore, Cr$^{6+}$ but not Cr$^{3+}$, was mutagenic to Salmonella typhimurium (Petrilli and De Flora, 1978), and Escherichia coli (Venitt and Levy, 1974). Hexavalent chromium was more inhibitory for Rec$^{-}$ (recombination-deficient strain) than for Rec$^{+}$ (wild strain) cells of Bacillus subtilis, thus exhibiting strong rec-effect, which suggests mutagenicity based on its DNA-damaging capacity (Nishioka, 1975). Similar results were also observed with fungi, hexavalent chromium was more toxic than equivalent levels of Cr$^{3+}$ to mycelial growth rates, spore formation and spore germination of fungi (Babich et al., 1982).
The foregoing discussion points out that chromium, in the hexavalent state, is highly toxic and the effects imparted by it are often mutagenic, causing damages to the chromosome. The carcinogenic nature of Cr$^{6+}$ is also relevant from the reports, thus posing a serious risk of health hazard in the environment wherever the release and existence of chromium, in the hexavalent form, is probable. Thus, the mechanisms involved in processes where biological systems tolerate toxic concentrations of chromium, have aroused interest, and is a phenomenon worth probing.

**RESISTANCE TO CHROMIUM**

The review on the works related to heavy metal resistance reveals the extensive research that has been going on in this particular field. But reports on the resistance to chromium is comparatively rare. A comprehensive discussion would make it clear.

It has already been mentioned in connection with discussions on plasmid-mediated resistance to heavy metals in bacteria, that chromium resistance has been observed in certain bacteria, e.g., in streptococci (Efstathiou and McKay, 1977) and *Pseudomonas aeruginosa* (Summers and Jacoby, 1978). In another report, Marques et al., (1979) isolated 71 strains of *P. aeruginosa*, of which 100% were resistant to chromium and to other metals. There is another report from the same laboratory that gram-negative bacteria isolated from a river
were resistant to CrO$_4^{2-}$ (Simon-Pujol et al., 1979). These strains were also resistant to a number of antibiotics, and the antibiotic resistances were associated with tolerance to high CrO$_4^{2-}$ concentrations. Morozzi et al. (1982) observed resistance to chromium, and other heavy metals, in a strain of *E. coli*. Growth in presence of the metal lengthened the lag phase. Cells adapted to the presence of chromium lost the adaptation property after growing in a metal-free medium.

Regarding the biochemical basis of tolerance to chromium, the reports suggest various mechanisms for its detoxication. In studying the toxicity of chromium to soil bacterial isolates, Ross et al. (1981) have reported that chromium (VI) supplemented into the medium decreased, indicating that reduction of the added Cr (VI) was occurring. In another report, Horitsu et al. (1978) have isolated chromate resistant bacterium from activated sludge which was characterized as *Pseudomonas ambigua* G-1. The authors found that this chromate-resistant strain was capable of intracellular accumulation of chromate salts from the culture medium. The intracellular chromium is bound to a protein component in the soluble fraction, as evidenced by disc electrophoresis.

Chromium resistant mutants of the yeast *Saccharomyces cerevisiae* was obtained by UV mutagenesis (Ono and Weng, 1982), and these were able to grow in the presence of 1000 μg CrO$_3$/ml. Analyses of recombination suggested that the chromium
resistance mutation was determined by single mutation located on a certain region of the yeast genome. Although it was observed that the mutants had slightly reduced rates of \( \text{Cr}^{6+} \) uptake, the exact mechanism of resistance was not discovered.

Chromate, being a potent carcinogen, has also been studied in relation to the detoxication mechanisms in mammalian systems. It has been reported (Gruber and Jenette, 1978) that rat liver microsomes, in presence of NADPH, enzymatically reduced chromate \( \text{Cr}^{6+} \) to \( \text{Cr}^{3+} \) and that the electron-transport cytochrome P-450 system was responsible for the chromate-reductase activity of microsomes (Garcia and Jenette, 1981). Further studies have revealed (Jenette, 1982) that a stable reactive intermediate, chromium (V), is formed upon metabolism of the inorganic carcinogen chromate by rat liver microsomes in the presence of NADPH. Goodgame et al. (1982) had discussed that carcinogenic \( \text{Cr} \) (VI) forms \( \text{Cr} \) (V) with ribonucleotides, but not with deoxyribonucleotides. In vitro, BHK (Syrian hamster fibroblast) cells were found to bring about reduction of soluble \( \text{Cr} \) (VI) to \( \text{Cr} \) (III) by cell metabolites, accompanied by decreased cytotoxicity (Levis and Majone, 1981).

The present work is concerned with chromate resistance by the process of adaptation, in a soil-isolated strain of \textit{Bacillus firmus}. In studying the resistance mechanism, it is necessary to establish the genetic linkage of the resistance. There are a number of reports on the occurrence of plasmids
Schlessinger (1976) has reviewed on plasmid DNA occurring in bacilli. Other reports on plasmids in Bacillus sp. are by, Carlton and Helsinki (1969), Henneberry and Carlton (1973), Lovett and Bramucci (1974), Lovett and Bramucci (1975), Tanaka et al. (1977), Le Hégarat and Anagnostopoulos (1977), Marahiel et al. (1981). Toxin production mediated by plasmids in Bacillus anthracis has been reported (Mikesell et al., 1983). Resistance to antibiotics has been observed to be specified by plasmids in Bacillus cereus and Bacillus subtilis (Bernherd et al., 1978), and in thermophilic bacilli (Imanaka et al., 1981). Thus, possibility exists for the involvement of plasmids in specifying the chromate-tolerance exhibited by the isolated strain Bacillus firmus.

The present investigation deals with chromate resistance in the bacterium Bacillus firmus. This microorganism, isolated from soil, has been adapted to toxic concentrations of hexavalent chromium, and the nature of its resistance property has been investigated.

The work is being reported in this part of the dissertation in the following steps -

1) Development of a chromate-resistant strain of B. firmus and studies on its characteristics.

2) Preliminary investigations on the mechanism involved in chromate-resistance in B. firmus.
These are followed by a critical discussion of the results obtained.