III. PRELIMINARY OBSERVATION ON MECHANISMS INVOLVED IN CHROMATE-TOLERANCE IN Bacillus firmus

INVOLVEMENT OF PLASMIDS

The introductory discussion on the review of research done on bacterial mechanism of resistance suggest that the genetic control of this sort of phenomenon is frequently specified by plasmids, the extrachromosomal elements. The involvement of plasmids in various functions in the Bacillus sp. is often reported and it was well justified to speculate a role of plasmids in chromate resistance of B. firmus.

One of the methods for investigating the involvement of plasmids in a certain phenotypic character, is by "curing" (Novick, 1969). Curing assures the selective removal of plasmids, thus causing loss of plasmid-linked phenotypic characters.

Bacillus firmus Crr (the chromate-resistant strain) was cured with different curing agents to prove the involvement of plasmids in chromate resistance mechanism. The DNA was extracted and electrophoresed on agarose gel to show the absence of plasmid DNA.

MATERIALS AND METHODS

Growth medium: Nutrient broth and nutrient agar.

Incubation: All incubations were at 37°C, unless
A. Curing

The chromate resistant strain Crr was subjected to a physical agent and two chemical agents for curing.

a. Curing by growth at elevated temperature

This was done according to the method of Asheshov (1966).

B. firmus Crr, was grown in nutrient broth at 42°C on a rotary shaker. One ml was transferred to 10 ml of fresh broth and incubated for 24 h. The process was repeated twice.

Finally, the culture, after proper dilution, was plated to obtain single isolated colonies, which were subcultured on agar slants.

b. Curing with acriflavine and novobiocin

Treatment with acriflavine was done according to Watanabe and Fukasawa (1961).

The resistant strain was grown overnight in presence of 1.5, 2.0 and 3.0 μg/ml of acriflavine, in shaking condition.

The same procedure was repeated with novobiocin at concentrations of 100, 150 and 200 μg/ml.
After incubation, the cultures were plated to obtain single colonies. Each of the single colonies obtained after curing were tested for their ability to tolerate the same level of chromate.

B. Isolation of plasmid DNA

Plasmid DNA was isolated, to clarify the presence of plasmid-linked chromate resistance, by exhibiting absence of plasmid DNA in cured strains, with concomitant loss in the resistance property.

Bacterial strains: (1) Bacillus firmus, (2) Bacillus firmus Crr, derived from (1), and its four cured strains.

Preparation of cleared lysate

Cleared lysate was prepared by the SDS-NaCl precipitation method of Guerry et al. (1973). Forty ml late logarithmic-phase cells, at a concentration of about $6 \times 10^8$ cells/ml, were collected, washed with 0.1 M sodium phosphate buffer (pH 7.2) and suspended in 1.5 ml of 25% sucrose in 0.05 M Tris, pH 8.0. The subsequent mixing steps were done by gentle inversions of the tube. To this suspension was added, 0.2 ml of a lysozyme solution (5 mg/ml in 0.05 M Tris, pH 8.0), kept on ice for 5 min, and then 0.5 ml EDTA (0.25 M, pH 8.0) and again chilled in ice for 5 min. Complete cellular
lysis was achieved by the addition of sodium dodecyl sulphate (SDS, Sigma Chemical Co., U.S.A.) to a final concentration of 2%. The lysate was heat shocked for 15 min at 55°C, with intermittent inversions of the tube. After cellular lysis, 5 M NaCl was added to the viscous solution with gentle mixing, to a final concentration of 1 M. The lysates were stored at 4°C overnight, after which they were spun-down at 15,000 r.p.m. for 30 min at 4°C. The supernatant served as the 'cleared lysate', rich in plasmid DNA.

Concentration of DNA

The DNA in the supernatant was concentrated with polyethylene glycol (PEG), according to Humphreys et al. (1975).

The cleared lysates were transferred to plastic tubes. The volume of the supernatants were measured and PEG 6000 (Sigma Chemical Co., U.S.A.) was added to 10% end concentration (from a stock solution of 42% w/v PEG 6000 in 0.01 M sodium phosphate buffer, pH 7.0). These were mixed by gentle inversions, kept on ice overnight, spun-down in a swing-out rotor for 5 min at 3,000 r.p.m. at 4°C. The DNA pellets were resuspended in TE buffer (0.05 M Tris, 0.02 M EDTA; pH 8.0).

Agarose gel electrophoresis

Resolution of the DNA extract was performed according to Meyers et al. (1976).
Electrophoresis was done in 0.7% agarose (Sigma Chemical Co., U.S.A.) dissolved in Tris-borate buffer (80 mM Tris-base, 2.5 mM disodium EDTA and 8.2 mM boric acid) pH 8.5. Gels were set in vertical gel tubes. 80-90 µl of the DNA extract was layered on top of the gel. A tracking dye, consisting of 30% sucrose and 0.005% bromophenol blue, was added, about 20 µl for each sample.

Electrophoresis was carried out at 50V for 6 h at room temperature. The gels were removed from their tubes onto a large petri dish. These were soaked in Tris-borate buffer containing 0.5 µg/ml of ethidium bromide (from a stock solution of 100 µg/ml). When the gels were placed under a short wave UV lamp, the DNA bands could be visualized because of the fluorescence of the intercalated ethidium bromide.

The different steps adopted for the isolation of plasmid DNA, are summarized in Table 2.5.

Table 2.5 Steps of plasmid isolation

<table>
<thead>
<tr>
<th>Step</th>
<th>Object</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cell growth</td>
<td>40 ml of cells in nutrient broth medium, about 6 x 10^8 cells/ml.</td>
</tr>
<tr>
<td>2.</td>
<td>Cell lysis</td>
<td>Washed cells resuspended at high osmolarity, cold; addition of lysozyme, Na_2EDTA, SDS to 2%; 55°C pulses.</td>
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Table 2.5 (Continued......)

<table>
<thead>
<tr>
<th>Step</th>
<th>Object</th>
<th>Method</th>
</tr>
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<tbody>
<tr>
<td>3.</td>
<td>Removal of membrane-chromosome</td>
<td>Addition of NaCl to 1 M; overnight storing at 4°C; centrifugation,</td>
</tr>
<tr>
<td></td>
<td>complexes</td>
<td>30 min at 15,000 r.p.m.</td>
</tr>
<tr>
<td>4.</td>
<td>Concentration of remaining DNA</td>
<td>Addition of PEG 6000 to 10%, refrigeration for 6 h or longer;</td>
</tr>
<tr>
<td></td>
<td>from supernatant</td>
<td>centrifugation at 3,000 r.p.m.*, 5 min; resuspension in about</td>
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<tr>
<td></td>
<td></td>
<td>1/40th the volume.</td>
</tr>
<tr>
<td>5.</td>
<td>Resolution of plasmid DNA</td>
<td>Electrophoresis through agarose gels.</td>
</tr>
</tbody>
</table>

*Treatment methods are summarized. For details, see text.

RESULTS

On screening the cells treated with different curing agents, it was found that, 20 out of 150 (in case of culture incubated at 42°C), 32 out of 150 (in case of acriflavin treatment) and 1 out of 150 (in case of novobiocin treatment) were found to exhibit a loss in resistance to chromate. These strains retained their sensitivity to chromate, even after repeated culturing.

The percentage of curing by novobiocin (0.06%) was so low, that it may be considered to be a case of spontaneous curing, rather than specifically by the agent.
Agarose gel electrophoresis

Fig. 2.5 represents the electrophoretic patterns of the DNA extracts of the parent, resistant and cured strains. In the parent and resistant strains (lanes 1 and 2), two bands could be seen, the upper being a contamination of chromosomal DNA fragments, and the lower, plasmid DNA. Lanes 3 and 4 showed the DNA bands of the single colony isolates of the acriflavine treated strain, lane 5 showed that of strain treated with novobiocin, while lane 6 showed the DNA band pattern of the cured strain obtained after growth at elevated temperature. The last 4 lanes exhibit only one DNA band, corresponding to the position of chromosomal DNA, thus proving that curing of the plasmid, existing in the parent strain, has occurred due to the effect of treatment with acriflavine and growth at elevated temperature.

BIOCHEMICAL MECHANISMS INVOLVED IN CHROMATE-TOLERANCE IN Bacillus firmus

To determine the probable mechanism adopted by the resistant strain of Bacillus firmus to tolerate toxic concentrations of chromate, some experiments were done, the chromate in the culture filtrate was estimated to find out whether there was any change in its concentration, and, the transformation of the chromate, Cr$^{6+}$, to Cr$^{3+}$ was studied in vitro.
MATERIALS AND METHODS

Growth

The chromate resistant strain, *E. firmus* Crr, was inoculated, about 4 - 6 x 10^16 cells into 10 ml of medium containing 40 mg/ml K_2CrO_4. At 24 and 48 h, aliquots were removed, the cells were spun down, and the culture supernatant tested for its chromate content.

The parent strain was grown in nutrient broth as control.

Estimation of chromate (hexavalent chromium, Cr^{6+})

This was done by the *s*-diphenylcarbazide method (Vogel, 1961).

A 0.25% solution of diphenylcarbazide was prepared in 50% acetone. The culture filtrate was diluted 10^4 times. To 5 ml of this, 6 N sulphuric acid was added to make the concentration to 0.2 N, when subsequently diluted to 25 ml. One ml of the diphenylcarbazide reagent was added, and made up to 25 ml with water. The pink colour produced was estimated at 540 nm. A calibration curve was prepared with potassium chromate.
Fractionation of cell components

The chromate resistant strain (Crr) of Bacillus firmus was grown in medium containing 40 mg/ml \( \text{K}_2\text{CrO}_4 \). The late log phase cells were collected by centrifugation, washed in 0.01 M phosphate buffer (pH 7.2), and suspended in 0.05 M Tris-HCl buffer (pH 7.4). This was sonicated in Braun-sonic 1510 sonicator at an output frequency of 20 KHz at 100W for 20 min, with intermittent pulses, duration of each pulse being not more than 30 sec. This was centrifuged at 10,000 r.p.m. for 15 min at 4°C. The supernatant obtained, was the cell-free extract (CFE, Fraction I).

The CFE was then centrifuged at 1,05,000 x g at 4°C, in a Beckman ultracentrifuge. The supernatant thus obtained was the soluble fraction (Fraction II), and the pellet was suspended in Tris-HCl buffer, pH 7.4 (Fraction III).

Protein concentrations of the Fractions I, II and III were determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as the standard.

Transformation of chromate by cell fractions

Chemicals

Chromate was in the form of \( \text{K}_2\text{CrO}_4 \) (Analar grade), obtained from the local market.
Nicotinamide adenine dinucleotide, reduced (NADH) was obtained from Sigma Chemical Co., U.S.A.

Incubation of chromate with cellular fractions and determination of chromate after in vitro incubation, was done as reported by Gruber and Jenette (1978).

All solutions were buffered at pH 7.4 with 0.05 M Tris-HCl. The initial concentration of chromate in the assay system was $4 \times 10^{-5}$ M, and that of NADH was $4 \times 10^{-4}$ M. The desired concentration of NADH was achieved by adding the correct amount of a freshly prepared $4 \times 10^{-3}$ M NADH solution to the reaction mixture. The reaction was started by adding aliquots of the standard NADH solution to the assay system to give the desired final concentration of NADH. Final volume of the reaction mixture was 5.0 ml. The mixtures were shake incubated at 37°C for 18 h.

Following incubation, the reaction was stopped by removal of the protein by its extraction from the aqueous solution by vigorously vortexing the mixture with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuging for 15 min at 3,000 r.p.m. The upper aqueous phase was carefully removed and extracted thrice more, after which no protein precipitate was visible in the organic layer.
Chromate and NADH determination

Following removal of protein, the concentration of chromate and the concentration of NADH in the aqueous solution were determined spectrophotometrically by measuring the absorbance of each solution at 340 nm and 400 nm on a Shimadzu UV-vis double beam spectrophotometer (UV-200S), in 1 cm cuvette. The absorbance readings were corrected by subtracting readings determined for solutions which originally contained protein, but no chromate or NADH, and which had been incubated and extracted as above. The extinction coefficients of Cr\(^{6+}\) and NADH at 340 and 400 nm were determined by measurements on standard solutions. Using these extinction coefficients (chromate, \(\varepsilon_{340} = 1.58 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\), \(\varepsilon_{400} = 1.64 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\); NADH, \(\varepsilon_{340} = 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\), \(\varepsilon_{400} = 1.1 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}\) and the corrected absorbances of each solution at the two wavelengths, the concentration of chromate was calculated.

In all experiments, the values given represent the mean of duplicate determinations.

RESULTS

Determination of chromate in culture filtrate

The amount of chromate (Cr\(^{6+}\)) in the culture fluid of the resistant strain was found to decrease after an incubation
of 48 h (Table 2.6), by an amount of 0.5 μg/ml (at $10^{-4}$ dilution), which amounts to about 5 mg/ml in the original culture.

Table 2.6  Determination of Cr$^{6+}$ in the culture filtrate of *Bacillus firmus*

<table>
<thead>
<tr>
<th>Incubation period (days)</th>
<th>Amount of Cr$^{6+}$* (mg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Resistant strain</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
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</table>

*mg/ml culture filtrate.

Table 2.6 showed that the amount of chromate (Cr$^{6+}$) decreased in the culture medium of the resistant strain, but not in that of the parent strain. This suggests a mechanism which has brought about reduction in the amount of Cr$^{6+}$ in the culture filtrate.

**In vitro transformation of Cr$^{6+}$**

The cellular fraction obtained in the pellet after centrifugation of the CFE at 1,05,000 x g, when incubated with chromate in the presence of NADH, brought about reduction of a portion of the hexavalent chromium (Table 2.7).
Table 2.7  \textit{In vitro transformation} of \(\text{Cr}^{6+}\) by Bacillus \textit{firmus} Crr

<table>
<thead>
<tr>
<th>Set No.</th>
<th>Constituents of reaction mixture</th>
<th>Protein concentration of reaction mixture (mg/ml)</th>
<th>% (\text{Cr}^{6+}) present after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fraction I(^b) + (\text{K}_2\text{CrO}_4) + NADH</td>
<td>1.25</td>
<td>98.6</td>
</tr>
<tr>
<td>II</td>
<td>Fraction II(^b) + (\text{K}_2\text{CrO}_4) + NADH</td>
<td>0.96</td>
<td>97.4</td>
</tr>
<tr>
<td>III</td>
<td>Fraction III(^b) + (\text{K}_2\text{CrO}_4) + NADH</td>
<td>3.50</td>
<td>82.1</td>
</tr>
<tr>
<td>IV</td>
<td>Fraction III(^b) + (\text{K}_2\text{CrO}_4)</td>
<td>3.50</td>
<td>99.2</td>
</tr>
<tr>
<td>V</td>
<td>(\text{K}_2\text{CrO}_4) + NADH</td>
<td>0.00</td>
<td>97.1</td>
</tr>
</tbody>
</table>

\(^a\)Incubation for 18 h, at 37°C.

\(^b\)Fraction I = Cell-free extract.

Fraction II = Soluble fraction after centrifugation at 1,05,000 x g.

Fraction III = Pellet after centrifugation at 1,05,000 x g.

Hexavalent chromium gives an absorption band at 373 nm, trivalent chromium is absorbed at \(\sim 570 - 580\) nm.

In the present experiment, the reaction mixture after incubation, showed an absorbance of 0.04 at the 570 - 580 nm region, but the control set (incubated without cell fraction) failed to show any absorption, the absorbance being 0.00 at the 570 - 580 nm region. This indicated that reduction of a certain
amount of the hexavalent chromium (Cr\(^{6+}\)) to trivalent chromium (Cr\(^{3+}\)), must have occurred. The simultaneous decrease in absorbance at 370 nm from the reaction mixture after 18 h incubation and appearance of an absorbance at 570 - 580 nm region, further strengthened the view that Cr\(^{6+}\) has been reduced to Cr\(^{3+}\).

It is evident from the Table 2.7, that the conversion of Cr\(^{6+}\) by cell protein occurred only in the presence of NADH (conversion 17.9%, Set III), and not in absence of it (Set IV). Chemical reduction by NADH was ruled out, incubation of K\(_2\)CrO\(_4\) with NADH for 18 h bringing about 3% reduction (Set V), in contrast to about 18% in presence of cell-protein (Set III). The soluble fraction (Fraction II) obtained after centrifugation of CFE at 1,05,000 x g was not very efficient in transforming Cr\(^{6+}\) (Set II).