CHAPTER 4: RESPONSE OF HR TO Zn^{2+} AND Cu^{2+}
Globally, urbanisation and rapid industrialisation in coastal countries has lead to the pollution of the environment. Estuarine regions, in particular act as sinks to heavy metal ions. A few reports indicated tolerance of haloarchaea to heavy metal ions (259,260). Studies evaluating the effect of heavy metal ions of Cu$^{+2}$ and Zn$^{+2}$ on growth and cellular characteristics of HR are discussed in this chapter.

METHODOLOGY

4.1 DETERMINATION OF GROWTH OF HR IN MINERAL SALTS MEDIUM CONTAINING GLUCOSE AS SOLE SOURCE OF CARBON

Seed culture of HR was inoculated to a final concentration of 5% into a 150 ml flask containing 50 ml sterile mineral salts medium (NSM) (Appendix I) containing 20% NaCl, pH 7 and 0.2% glucose (NGSM). The flask was incubated at RT on a rotary shaker at 150 rpm. The changes in absorbance were monitored by withdrawing aliquots at pre-decided intervals of time and observing the absorbance at 600 nm, using a Shimadzu UV-240 spectrophotometer.

4.1.1 Effect of Cu$^{+2}$/Zn$^{+2}$ on growth of HR in NGSM

To study the effect of heavy metal ions of Cu$^{+2}$/Zn$^{+2}$ on growth of HR, metal salts of CuSO$_4$·5H$_2$O / ZnSO$_4$·5H$_2$O were weighed according to the required concentration and dissolved in sterile mineral medium before inoculation of the culture.
4.1.2 Effect of continuous subculture in Cu$^{+2}$/Zn$^{+2}$ on growth of HR

Seed culture of HR was inoculated into NGSM containing Cu$^{+2}$ or Zn$^{+2}$ at 1mM and the amount of growth was measured by determining the absorbance at 600 nm at the end of 7 d, during the stationary phase of growth. The effect of serial exposure to metal ions on the growth was studied by serially transferring HR grown in the presence of metal ions, to NGSM containing Cu$^{+2}$/Zn$^{+2}$, at 1 mM, for four subsequent subcultures.

4.2 DETERMINATION OF CHEMICAL COMPOSITION OF CELL ENVELOPES AND MEMBRANES OF HR GROWN IN PRESENCE OF METAL IONS

4.2.1 Isolation of cell envelopes and cell membranes of HR grown in NGSM with and without Cu$^{+2}$/Zn$^{+2}$

Resting cells of HR ($A_{600}$~2) were prepared from culture grown in NGSM with or without the metal ions for 7 d. and used to prepare envelopes and membranes fractions, as described earlier. The envelope membrane preparation in each case, was suspended in 10 ml of 20% NaCl and used for further determination of chemical constituent analysis.

4.2.2 Protein

To 100 µl of envelope or membrane sample, 1 N NaOH was added to make the volume up to 1 ml. 5 ml of Folin reagent 'C' (Appendix II) was then added and the tubes were incubated at RT for 20 min (329). 0.5 ml of Folin-Ciocalteaux reagent (Appendix II) was then added and the tubes were incubated in dark at RT for 15 min and the absorbance was measured at 660 nm against a reagent blank. The protein content was determined from a standard curve prepared using bovine serum albumin as a standard.
4.2.3 Lipid

Lipids were extracted from 1 ml of the envelope or membrane sample using 5 ml Chloroform: Methanol (2:1 v/v) mixture (79). The chloroform layer was separated using a separating funnel after extraction, and placed in a clean pre-weighed glass flask, and was evaporated to dryness using N₂ gas and the weight of the lipid determined.

4.2.4 Total Carbohydrate

100 µl of envelope or membrane samples was taken in a test tube and the volume was made to 1 ml using distilled water. It was then mixed thoroughly with 50 µl of 80% of aqueous phenol (330). After an addition 5 ml of concentrated H₂SO₄ the tubes were incubated at RT for 5 min, cooled under running tap water and the absorbance was measured at 480 nm. The total carbohydrate content was measured from a standard curve prepared using D-glucose as a standard (Appendix V).

4.3.4 Hexosamine

1 ml of envelope or membrane sample was hydrolysed using 2 ml of 6 N HCl in a glass stoppered Pyrex tube at 100°C for 12 h. The HCl was then evaporated to dryness using a sand bath and the volume was made to 1 ml with distilled water. To 400 µl of the above hydrolysate, was added 0.05 ml Reagent A (Appendix II) mixed and allowed to stand at RT for 5 min (331). An aliquot of 0.15 ml of Reagent B (Appendix II) was then added, the tubes stoppered and heated in a boiling water bath for 3 min and allowed to cool at RT. Thereafter, 0.3 ml of Reagent C (Appendix II) and 2.7 ml of glacial acetic acid were added and the tubes were incubated at 37°C for 20 min. Absorbance was measured at 585 nm, on cooling the
reaction mixture at RT. The hexosamine content was obtained from a standard curve obtained using glucosamine as a standard (Appendix V).

4.3.5 Total Sulphate

One ml samples of envelope or membrane were hydrolysed using 6 N HCl for 12 h. The hydrolysate was evaporated on a sand bath and the volume was made to 1 ml with distilled water. To 1 ml of the above samples, 1.4 ml of 4% TCA and 0.5 ml barium chloride-gelatin reagent were added. The tubes were allowed to stand for 20 min and the absorbance was measured at 360 nm (332) and the sulphate content was determined from a standard curve using K₂SO₄ as a standard (Appendix V).

4.3 DETERMINATION OF Cu²⁺ / Zn²⁺ IONS IN CELLS OF HR

4.3.1 Incubation of HR with metal ions

HR cells grown in NGSM were harvested at the late log phase and the pellet obtained was washed with 20% NaCl. The washed pellet was resuspended in 20% NaCl to an absorbance of 2 at 600 nm. To 100 ml of the above cell suspension metal ions of Cu²⁺ / Zn²⁺ in the form of CuSO₄·5H₂O / MgSO₄·H₂O were added to a final concentration of 5 mM. Also, in a separate set, HR cells having absorbance of 2 at 600 nm were incubated with 0.5 mm of Cu²⁺ / Zn²⁺ for 30 min at RT on a rotary shaker at 150 rpm before the addition of Cu²⁺ / Zn²⁺ at 5 mM concentration. 5ml samples from each flask were immediately withdrawn and the flasks were incubated at RT on a rotary shaker at 150 rpm. Aliquots of 5ml were removed after intervals of 0.5, 1, 2, 4, 8 and 16 h from individual flasks, centrifuged separately at 12,000 rpm for 10 min. The pellet thus obtained in each case was washed in mineral salts with 20% NaCl and analysed for metal ions.
4.3.2 Atomic absorption spectrophotometric analysis of Cu$^{+2}$ / Zn$^{+2}$ in cell digests

Wet digestion of cell pellet

The washed pellet, dispersed in 2 ml of de-ionized water was mixed with 5 ml of HNO$_3$ : H$_2$SO$_4$ mixture (2:1 v/v) in glass stoppered test tube and digested in a boiling water bath for 18 h or till the suspension became clear.

Estimation of metal ions

The digested samples were made up to 10 ml with de-ionized water and were analysed by the atomic absorption spectrophotometer (GBC 932 AA) for the content of Cu$^{+2}$ or Zn$^{+2}$ ions.

4.4 SCREENING FOR PRESENCE OF PLASMID IN HR

HR cells grown in NTYE, NGSM and in NGSM with Cu$^{+2}$ / Zn$^{+2}$ (1mM) individually, were harvested at the late log phase and each cell mass was washed with 20 % NaCl. The washed cells were suspended in 20 % NaCl to an absorbance of 2 at 600 nm.

4.4.1 Alkali-lysis method

4 ml of each of the above cell suspension was centrifuged at 10,000 rpm at 4°C for 20 min and the supernatant was decanted. To the pellet was added 100 μl of ice-cold solution I (Appendix II) at RT and mixed by vigorous vortexing. 200 μl of freshly prepared solution II (Appendix II) was then added and mixed the contents by gently inverting the eppendorf tube 5 to 6 times. The tubes were incubated at 0°C for 10 min and 150 μl of ice-cold solution III (Appendix II) was then added. The contents were thoroughly mixed by gently shaking the tube. The tubes were then
incubated 0°C for 10 to 15 min. The supernatant was decanted after centrifuging the tubes at 12000 rpm for 10 min into a clean, dry eppendorf tube and 2 volumes of chilled absolute alcohol (900 µl) was added. The tubes were incubated at −70°C for 10 min and were centrifuged at 12,000 rpm for 20 min at 0°C to precipitate the plasmid DNA. Alcohol was removed using a micropipette and the pellet was air-dried for 30 min. The pellet was then dissolved in 50 µl of TE buffer (Appendix II) and checked for absorbance at 260, 256 and 280 nm. 50 µl of the dissolved sample along with 2 µl of the dye solution (Appendix II) was loaded on an agarose gel (0.8%) prepared in T.A.E. buffer (pH 8.0) (Appendix II) and the electrophoresis was carried out at 70 volts for 8 h. The gel was observed, for the presence of plasmid on UV Photodyne and photographed.

4.4.2 Slot lysis method

2 ml each of the above cell suspension described above was centrifuged at 12,000 rpm, for 10 min at 4°C. The pellet was then mixed with 30 µl of lysis solution (Appendix II) and loaded into the wells of 0.5% agarose gel with a double comb system (Appendix IV). Electrophoresis was carried out at 8 mA for 1.5 h and at 40 mA for 15 h (119).

RESULTS

4.5 GROWTH OF HR WITH GLUCOSE AS SOLE SOURCE OF CARBON

HR on inoculation into NGSM with 20% NaCl and 0.2% glucose and incubation at RT at 150 rpm, grew with an initial lag of one d, and reached a maximum absorbance of 1.3 at 600 nm by the 5th d at a doubling time of 1.1 d. Stationary phase followed thereafter and persisted till 7th d, with no
change in absorbance as depicted in Fig. 34. During this growth, the culture attained a pink pigmentation, by the 4th d. The pink colour darkened with further incubation up to 6 d. Colonies on NGSM agar were translucent and attained pink pigmentation by the 6th d (Plate 4.1).

4.6 TOLERANCE TO Zn$^{2+}$ DURING GROWTH OF HR

During the growth of HR in NGSM containing different concentration of Zn$^{2+}$, the turbidity of HR steadily increased with time at 1, 2 or 3 mM as see in Fig. 35. However, no increase in absorbance was observed at 4 or 5 mM concentration. HR grew with a time lag of one d at 1mM concentration, reaching a maximum absorbance of 0.96 within 6 d, with a doubling time of 1.4 d. A two-d lag period was observed during growth at concentration of 2 mM or 3 mM that reached a maximum absorbance of 0.45 and 0.3 respectively on the 5th d, with no further increase on incubation up to 7 d. The doubling times were 2 and 2.5 d during growth of HR at concentration of 2 / 3 mM of Zn$^{2+}$ respectively.

4.7 TOLERANCE TO Cu$^{2+}$ DURING GROWTH OF HR

As seen from the Fig. 36, at concentration of 1 or 1.5 mM of Cu$^{2+}$, the turbidity increased with time, where as no increase was observed at concentration of 2.5 or 3 mM up to 7 d of incubation. During the growth of HR at a concentration of 2 mM of Cu$^{2+}$, there was an initial increase in absorbance after 2 d, which, however, declined on the 3rd d and did not increase further, as seen in Fig. 36. HR grew with an initial time lag of 1 and 2 d reaching a maximum absorbance of 0.76 and 0.51 on the 5th d with doubling times of 1.25 and 1.5 d at concentration of 1 and 1.5 mM respectively. No increase in absorbance was observed when HR was inoculated into NGSM containing Cu$^{2+}$ at concentrations of 2.5 or 3 mM.
Fig. 34 Growth of HR in NGSM
Plate 4.1. HR grown on NGSM agar
Fig. 35 Growth of HR in NGSM with different concentrations of Zn\(^{12}\) (ZnSO\(_4\).5H\(_2\)O)

- ● 1mM
- 2mM
- ▼ 3mM
- ■ 4mM
- ○ 5mM
- Control
Fig. 36 Growth of HR in NGSM with different concentrations of Cu$^{2+}$ (CuSO$_4$.5H$_2$O)

- Control
- 1mM
- 1.5mM
- 2mM
- 2.5mM
- 3 mM
4.8 RESPONSE OF HR TO CONTINUOUS SUBCULTURE IN Cu\(^{2+}\) / Zn\(^{2+}\)

Growth response obtained on serial transfer of HR, grown in the presence of metal ions to NGSM containing metal ions of 1 mM Cu\(^{2+}\) / Zn\(^{2+}\) over four subsequent subcultures is shown in Fig. 37. HR growing in the presence of Zn\(^{2+}\) during each of the subcultures showed a steady decrease in maximum absorbance. A 16.1% decrease in maximum absorbance was observed during growth on second exposure to Zn\(^{2+}\) as compared to that of the first exposure, which further decreased by 24.5% and 31.2% on the 3\(^{rd}\) and 4\(^{th}\) exposures, respectively.

In the case of Cu\(^{2+}\) ions, the maximum absorbance obtained decreased by 15.8% at the end of 2\(^{nd}\) exposure as compared to the 1\(^{st}\) exposure. As seen from Fig. 37, this was followed by a further decrease of 31.6% and 40.8% respectively after 3\(^{rd}\) and 4\(^{th}\) exposures to Cu\(^{2+}\) ions.

4.9 EFFECT OF METAL IONS ON PIGMENTATION OF HR

During growth in NGSM liquid medium without metal ions, HR developed faint pink pigmentation on the 4\(^{th}\) d, which darkened on further incubation up to 6 d. Also, a dull pink pigmentation was observed when grown in NGSM containing Zn\(^{2+}\) at 1 mM concentration on the 4\(^{th}\) d, which did not intensify on further incubation up to 6 d to the extent seen in HR growing in glucose alone. (Plate 4.2).

In the case of HR grown in the presence of Cu\(^{2+}\) (1 mM) in NGSM, there was no visual discernable pigmentation. NGSM with Cu\(^{+2}\), however, had a light blue colouration brought about by the Cu\(^{2+}\) ions (Plate 4.2). Sedimentation of the cells by centrifugation at 8000 rpm for 20 min revealed the presence of whitish cells with a faint blue tinge. On NGSM agar containing 1 mM Cu\(^{2+}\) HR grew as translucent colonies by the end of 3 d, at RT. These colonies turned light orange by 6 d; dull orange in 8 d
Fig. 37 Growth of HR on serial exposure to Zn$^{2+}$ (1 mM) or Cu$^{2+}$ (1 mM)
Plate 4.2. Growth of HR
A) NGSM without metal ions
B) NGSM with 1mM Zn$^{+2}$
C) NGSM with 1mM Cu$^{+2}$
D) Uninoculated NGSM
and creamish thereafter. Finally, after 12 d colonies attained a bluish tinge (Plate 4.3). The dull orange colonies present at the end of 6 d, when subcultured on to NGSM agar containing 1 mM Cu²⁺, grew with a light orange tinge and attained creamish colour at the end of 10 d. However, these creamish colonies failed to grow when sub-cultured, further.

The acetone extract of pigment from HR grown in NGSM, on spectral analysis showed absorbance in the region of 300 nm to 600 nm, revealing 8 absorption maxima at 350; 368; 386; 426; 496; 528 and 600 nm (Fig. 38a) that corresponded to phytofluenes (350 & 368 nm); retinal (386 nm); β-carotene (426 nm); lycopene (468 nm) and bacterioruberin (496 and 528 nm), respectively.

As seen in Fig. 38b, the spectral analysis of cell extracts grown with metal indicated the presence of 8 peaks of absorption maxima at 350; 368; 386; 426; 468; 496 and 528 nm. These peaks matched with the λ max and some of them differed in their intensities with the peaks observed for pigment of HR cells grown in the absence of metal ions (Fig. 38a). For example, the intensity of the peak at 600 nm increased by 33% and peaks at 528; 496; 468 and 426 nm decreased by 15.6; 15.8; 15.1 and 4.2% respectively in the case of acetone extracts of HR cells grown in the presence of Zn²⁺, as compared to those of the cells grown without metal ions.

Spectral analysis of acetone extracts of HR grown in the presence of Cu²⁺ revealed four additional absorption maxima at 660; 640; 560 and 540 nm beside the 6 peaks, normally observed between 300 – 700 nm when HR was grown in glucose alone. The peak at 600 nm in Cu²⁺ showed a 62.5% increase in intensity while the peaks at 426; 386; 368 and 350 nm increased by 40; 37.5; 39.1 & 65.2% in comparison with those of the cells grown without metal ions. The peaks at 528 nm and 468 nm were
Plate 4.3. HR grown on NGSM agar with 1mM Cu²⁺
Fig. 38 Pigment profiles of HR cells grown in
a) NGSM     b) NGSM with 1 mM Zn$^{12+}$
c) NGSM with 1 mM Cu$^{12+}$
absent in acetone extracts of HR grown in the presence of Cu$^{+2}$ ions (Fig. 38c).

4.10 CHANGES IN CELL SURFACE HYDROPHOBICITY OF HR GROWN WITH Zn$^{+2}$/Cu$^{+2}$

Resting cells of HR grown in NGSM with either 1 mM Zn$^{+2}$/Cu$^{+2}$ when mixed with n-hexadecane for MATH assay, moved to hexadecane phase the absorbance of aqueous layer declined by 50 - 58% as seen in Table 4.1. In contrast, the resting cells of HR prepared by growing in NGSM without Zn$^{+2}$/Cu$^{+2}$ and treated likewise, showed a very little difference (Table 4.1). The percent hydrophobicity calculated on the basis of absorbance were 3, 58 and 50 % for the cells grown in NGSM and in the presence of Zn$^{+2}$/Cu$^{+2}$ respectively.

4.11 CHANGES IN CELLULAR LIPIDS OF HR GROWN WITH Zn$^{+2}$/Cu$^{+2}$

HR grown in NGSM gave a total yield of 52.1mg/L of whole cell lipids by modified Bligh & Dyers method. The yield of total lipids decreased to 40.4 mg/L and 38.2 mg/L in cells grown in the presence of Zn$^{+2}$ and Cu$^{+2}$, respectively.

The total lipids, on fractionation yielded A$_1$ & A$_2$ fractions in a ratio of 1:2. The A$_1$ fraction on TLC analysis in Petroleum ether : Diethyl ether : Acetic acid (90 : 10 : 1v/v/v) in case of HR grown in NGSM without Cu$^{+2}$/Zn$^{+2}$, resolved into a total of 6 spots with Rf values of 0.86; 0.76; 0.57; 0.36; 0.12 and 0.05 that corresponded to S$_1$, S$_2$, S$_3$, X$_1$, X$_2$ and X$_3$ respectively of the A$_1$ fraction of cells grown in NTYE medium (Fig. 39). However, only five spots corresponding to S$_1$, S$_3$, X$_1$, X$_2$ and X$_3$ respectively were observed on TLC analysis of A$_1$ fractions from HR cells grown in the presence of either Cu$^{+2}$ or Zn$^{+2}$ ions (Fig. 39).
Table 4.1 Hydrophobicity of HR grown in NGSM with and without metal ions

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>OD of cell suspension</th>
<th>Calculated % affinity to n-hexadecane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_1$</td>
<td>$A_2$</td>
</tr>
<tr>
<td>NGSM with 20% NaCl</td>
<td>1.00</td>
<td>0.97</td>
</tr>
<tr>
<td>NGSM with 20% NaCl and 1 mM Cu$^{2+}$</td>
<td>1.00</td>
<td>0.50</td>
</tr>
<tr>
<td>NGSM with 20% NaCl and 1 mM Zn$^{2+}$</td>
<td>1.00</td>
<td>0.42</td>
</tr>
</tbody>
</table>

$A_1$: Initial OD of HR cell suspension in PUM-NaCl buffer

$A_2$: OD of HR cell suspension after interaction with n-hexadecane
Fig. 39 Thin layer chromatogram of A1 fraction of Lipid of HR grown in NTYE (NT), NGSM (NG), NGSM + Cu$^{2+}$ (Cu) and NGSM + Zn$^{2+}$ (Zn)
The $A_2$ fractions on TLC analysis in Chloroform : Methanol : Acetic acid : $H_2O$ (80 : 22.5 : 10 : 4 v/v/v), and on spraying with phosphomolybdate, showed the presence of 2 spots with Rf values of 0.84 and 0.61, that corresponded to Archaeotidylglycerol and Archaeotidylglycerol phosphate respectively in all cases i.e. HR grown in NGSM with and without metal ions and in NTYE. However, the intensity of the spots decreased by approximately 10 fold in the cells grown in NGSM and in NGSM with Cu$^{+2}$/Zn$^{+2}$. Duplicate thin layer chromatogram of $A_2$ fractions when sprayed with $\alpha$ - naphthol reagent revealed the presence of 2 pink coloured spots with Rf values of 0.66 and 0.47 that correspond to Diglycosyl archaeol and Sulphated diglycosyl archaeol, respectively in the case of HR grown in NGSM with or without Cu$^{+2}$ ions. Whereas, the $A_2$ fraction from HR grown in NGSM containing Zn$^{+2}$ ions, showed only a single pink spot at Rf 0.47, corresponding to Sulphated diglycosyl archaeol (Plate 4.4).

4.12 CHANGES IN CHEMICAL COMPOSITION OF CELL ENVELOPE AND PLASMA MEMBRANE OF HR GROWN WITH Zn$^{+2}$/Cu$^{+2}$

As depicted in Fig. 40, cell envelopes of HR contained 61% protein, 30% lipid and 4.4% carbohydrate on per cent dry weight basis. Minor quantities of hexosamine and sulphate were also found to be present. However, when grown in presence of Zn$^{+2}$ or Cu$^{+2}$, the protein content of envelopes increased by 14.5 & 16.4% and the lipid content decreased by 16.2 & 19.6% respectively as seen in Fig. 41 with no significant changes in the other components.

The plasma membranes of HR cells grown in NGSM consisted of 56% protein, 37% lipid and 2% carbohydrate with minor quantities of sulphate and hexosamine, as depicted in Fig. 42. The variations observed in case of cells grown with metal ions, in protein content (12.0 & 14.2%
Plate 4.4. Thin layer chromatogram of A_2 fraction of HR grown in NTYE (NT); NGSM (NG); NGSM+Cu^{2+} (Cu) and NGSM+Zn^{2+} (Zn) on spraying with α-napthol
Fig. 40 Composition of cell envelopes of HR grown in NGSM (as mg/100 mg dry weight)

- Protein
- Hexosamine
- Carbohydrate
- Lipid
- Sulphate
- Others

Fig. 41 Variation in cell envelope composition of HR grown in presence of metal ions

- Increase in protein
- Decrease in lipid
Fig. 42 Composition of plasma membranes of HR grown in NGSM (as mg/100mg dry weight)

- Protein
- Hexosamine
- Sulphate
- Lipid
- Others
- Carbohydrate

Fig. 43 Variation in plasma membrane composition of HR grown in presence of metal ions

- Increase in protein
- Decrease in lipid
increase for Zn\textsuperscript{+2} and Cu\textsuperscript{+2} respectively) and the lipid content (10.4 & 11.3% decrease in Zn\textsuperscript{+2} and Cu\textsuperscript{+2} respectively) are presented in Fig. 43.

4.13 ACCUMULATION OF Zn\textsuperscript{+2} / Cu\textsuperscript{+2} BY RESTING CELLS OF HR

Resting cells of HR grown in NGSM were used either directly or after a pre incubation with 0.5 mM Zn\textsuperscript{+2} / Cu\textsuperscript{+2}, for studying the sorption of individual ions, separately. The initial uptake of 31 ppm of Zn\textsuperscript{+2}, remained almost constant up to 4 h in case of HR cells directly incubated with metal or up to 8 h in case of HR cells that were prior incubated with metal ion. A maximum sorption of 75 ppm after 16 h of incubation with the metal ions was observed in both cases as depicted in Fig. 44. Resting cells took up to 60 ppm of Cu\textsuperscript{+2} in 16 h. However, as seen in Fig. 45. the uptake of Cu\textsuperscript{+2} by the cells pre-incubated with Cu\textsuperscript{+2} was 44 ppm in 16 h.

4.14 OCCURRENCE OF PLASMID IN HR

60 μl each, of extracted DNA from HR grown in NTYE. NGSM and in NGSM with Cu\textsuperscript{+2} / Zn\textsuperscript{+2} by alkali-lysis, showed a single band with Ethidium bromide on electrophoresis. The position of this band corresponded with that of the first band of \(\lambda\) DNA/Hind III digest (23.13 Kbp), run along with the samples (Plate 4.5).

Whole cell lysates of HR, when directly electrophoresed on a 0.5% agarose gel at 8 mA for 1h 30 min, gave a single band very close to the wells in each case, i.e. HR grown with and with out metal ions. On continuation of electrophoresis at 40 mA for 15 h, this band resolved into 2 faint bands at a position about 8 mm from the loaded well. The first band of \(\lambda\) DNA/Hind III digest (23.13 Kbp) and the band observed in alkali lysis DNA extract, run on the same gel, moved further, to ~ 3 cm from the bottom of the gel as depicted in Fig. 46.
Fig. 44 Sorption of Zn$^{+2}$ by HR

- □ Resting cells
- ■ Resting cells prior incubated with 0.5 mM Zn$^{+2}$
Fig. 45 Sorption of Cu$^{+2}$ by HR

- Resting cells
- Resting cells prior incubated with 0.5 mM Cu$^{+2}$
Plate 4.5. Agarose gel electrophoresis of DNA extracted by alkali-lysis from HR grown in:

a: NTYE  
b: NGSM  
c: DNA/HindIII-digest

e: NGSM with Zn$^{2+}$

f: NGSM with Cu$^{2+}$ and c,d: λ DNA/HindIII-digest
Fig. 46 Agarose gel electrophoresis of whole cell lysates of HR grown in:
1) NTYE 2) NGSM 3) NGSM with Cu$^{+2}$ 4) NGSM with Zn$^{+2}$ and
5) DNA extracted by alkali lysis method
6) Hind III digest of λ DNA
DISCUSSION

Environmental Pollution arising from heavy industrialisation and other anthropogenic activities has been a major concern in recent times throughout the world (248-250). Estuarine regions, especially act as sinks for these pollutants due to the discharge of un/ill-treated effluents containing organic and heavy metal toxicants of industrial and domestic origin (343). Microflora, resident to these eco-niches, therefore would be exposed to these toxicants and often develop resistance mechanisms to survive the stress. HR, originally isolated from an estuarine salt pan, showed resistance to a number of heavy metals during an earlier study in the laboratory (344).

Heavy metal ions of Cu$^{+2}$ and Zn$^{+2}$ play an important role as cofactors for many enzymes and are essentially required in nanomolar concentrations for growth. Yet, at higher concentrations, these metal ions are highly toxic to microbial cells causing alteration of enzyme active sites, oxidation of membrane components etc. (334).

In view of this, effect of heavy metal ions of Cu$^{+2}$ and Zn$^{+2}$ on HR was studied, herein. Presence of nutrient rich growth factors such as peptone and yeast extract have been shown to bind the metal ions in the medium and does not allow the true interaction of organisms with the metal ions (14). Hence, the response of HR to Cu$^{+2}$ / Zn$^{+2}$ has been studied in mineral salts medium (NGSM) containing 20% NaCl and 0.2% Glucose. Growth of HR in presence of Cu$^{+2}$ / Zn$^{+2}$ at 1 mM (Plate 4.2) indicates the tolerance of HR to these metal ions. However, increase in concentration of metal ions, above 1mm, decreased the growth of HR as seen in Fig. 35 for Zn$^{+2}$ and Fig. 36 for Cu$^{+2}$ and resulted in total inhibition of growth at 4mm for Zn$^{+2}$ and 2mm for Cu$^{+2}$, thus indicating the MIC (minimum inhibitory concentration) levels for growth of HR. Studies done
on some other haloarchaea, reported the MIC levels of 0.5 & 2.5 mM of 
Zn$^{2+}$ and Cu$^{2+}$ respectively (260).

Although, HR grew fairly well at 1mm concentration of Cu$^{2+}$/Zn$^{2+}$, 
repeated exposure of these metal ions, decreased the growth as seen in 
Fig. 37, suggesting that continuous exposure to metal ions, if results in 
accumulation in the cell, may possibly reach toxicity levels affecting the 
metabolism and decreasing the growth. Heavy metal ions are largely 
reported to interfere with metabolic activities such as photosynthesis, N$_2$
fixation, and denitrification in microbiota (261,264), thus establishing 
toxicity. In the light of this, response of to heavy metal ions was studied by 
determining the cellular characteristics of HR, grown in presence of Cu$^{2+}$/
Zn$^{2+}$ in glucose mineral salts medium. Although, some workers reported 
the MIC levels of heavy metals to haloarchaea (260, 261), no reports are 
available on the effect of these heavy metal ions on cellular features of 
haloarchaea.

As indicated in Plate 4.2, HR grew as dull pink pigmented cells in 
case Zn$^{2+}$, in contrast to the bright pink pigmentation observed in mineral 
salts medium without metal ions. The UV spectra of acetone extracts of 
these cells, indicated variations in intensities (in case of Zn$^{2+}$), and / or 
absorption maxima (in case of Cu$^{2+}$), as compared to those of cells grown 
without metal ions (Fig. 38). However, the increase in intensities of 
phytofluenes (350 & 368 nm), retinal (386 nm) and of an unidentified 
component (600 nm) in HR cells grown with Cu$^{2+}$ ions, (Fig. 38) as 
compared to their intensities in HR grown without metal ions, indicate that 
these components may possibly be involved in resistance mechanism 
employed by HR to, subvert the stress exerted by Cu$^{2+}$ ions. Such reports 
of metal ion induced changes in pigmentation of haloarchaea have not 
been reported. Cd$^{2+}$ ions, however, are known to delay the pigmentation i 
the eubacterium S. marcescens (257).
The high increase in surface hydrophobicity of HR cells grown in the presence of Zn\(^{2+}\) or Cu\(^{2+}\) ions, accounting to 50-58% as compared to the 3% of cells grown without metal ions, indicates the importance of surface hydrophobicity, especially under conditions of stress. Increase in surface hydrophobicity, as discussed by Vreeland (210), generally decreases the cell permeability and may possibly be involved in decreasing the influx of metal ions into the cell cytoplasm to some extent. Further, decreases in cell permeability resulting from an increase in cell surface hydrophobicity, also increases the thermodynamic energy required to push molecules across the cell envelope into the cytoplasm, thus, making it an energetically expensive process (210).

Presence of metal ions of Cu\(^{2+}\) / Zn\(^{2+}\) during growth of HR, decreased the total lipid content of HR. Further the absence of Dehydrosqualene (in both Cu\(^{2+}\) / Zn\(^{2+}\)) and Sulphated diglycosyl archaeol in case of cells grown with Zn\(^{2+}\) suggest that HR may possibly be responding to the metal ion stress by alterations at the cell envelope level. In order to verify this, the chemical composition of isolated cell envelopes and plasma membranes of HR cells grown in NGSM with and without metal ions was investigated. Chemical analysis of cell envelopes and plasma membranes of HR cells grown in NGSM with and without metal ions indicated alterations in protein and lipid composition in both cases as seen in Fig. 41 & 43. A number of studies (335,336), indicate that microorganisms generally respond to environmental stress conditions, primarily by alterations at the cell surface level, since this is in direct contact with the outer environment.

Growth of HR on NGSM agar incorporated with copper (1 m\(^{-1}\) resulted in formation of pink colonies, which eventually turned cream and bluish on further incubation, indicating that metal ions pos
accumulated in the cells of HR, thus giving a bluish white tinge (Plate 4.3). In view of this, sorption of metal ions by resting cells of HR was studied. As depicted in Fig. 45, resting cells of HR grown in NGSM, on incubation with Cu$^{+2}$ ions, showed 60 PPM in the cells, after 16 h. Similarly, HR cells also showed an uptake of Zn$^{+2}$, up to 78 ppm in 16 h of incubation. Many microorganisms display metal resistance, mediated through plasmids (269). HR when screened for presence of plasmid by alkali lysis method, showed a single band, positioned close to the 23 Kbp band of λ DNA/Hind III digest. However, on screening for plasmid by the slot lysis method, recommended for megaplasmids generally seen in haloarchaea (119), the band of 23 Kbp was not detected. Instead, 2 other bands, were seen very close to the wells loaded with lysates of HR cells grown with and without metal ions (Fig. 46). The position of these bands very close to the well, even after electrophoresis for 15 h at 40mA, suggests that, they could be of megaplasmids, and the absence of the band seen in alkali lysis method, suggests that it could be a sheared part of the chromosome, possibly extracted during the alkali lysis. It is also possible that, the DNA may have got sheared giving a band on electrophoresis. However, the presence of these megaplasmids, although very widely reported in haloarchaea (119,120) may not be mediating the metal resistance of HR, as they are seen to be present in cells grown without metal ions also.

Halobacterium strain R$_1$ MTCC 3265 grows in mineral salts medium containing 20% NaCl and 0.2% glucose as sole source of carbon. The toxicity of Cu$^{+2}$ and Zn$^{+2}$ ions to HR increases with increase in their concentration. The MIC values of Cu$^{+2}$ and Zn$^{+2}$ to HR are 2 & 4 mM respectively. Continuous presence of metal ions of Cu$^{+2}$ / Zn$^{+2}$ at sub-lethal concentration of 1 mM, decreases the growth of HR at every consecutive growth cycle.
Presence of metal ions of $\text{Cu}^{2+}$ / $\text{Zn}^{2+}$ during growth alters the pigment profile between 540 and 680 nm; chemical composition of cell envelopes and plasma membranes; increases the cell surface hydrophobicity by 50 – 58% and abolishes the lipid moieties of Dehydrosqualene and Diglycosyl archaeol. Lysates of resting cells of HR incubated with metal ions showed an accumulation of 60 ppm ($\text{Cu}^{2+}$) and 75 ppm ($\text{Zn}^{2+}$). Plasmid screening by slot lysis method, showed the presence of two bands, which could be mega plasmid in nature, in HR grown with or without metal ions.

*Halobacterium* strain R₁ MTCC 3265 is tolerant to $\text{Cu}^{2+}$ and $\text{Zn}^{2+}$ and accumulates the two metal toxicants via alteration of cellular features.