ASM PAHO Microbial Susceptibility Testing Manual

Produced in conjunction with the Pan American Health Organization (PAHO) to provide laboratories and medical professionals with a comprehensive, self-instructional teaching manual on antimicrobial susceptibility testing. The manual was translated into Spanish and Portuguese by PAHO, reviewed, and printed in both languages. The CD-ROM contains the English and Spanish versions of the manual, as well as comprehensive reference material. Printed in both Spanish and English, the manual was designed for use by laboratories throughout the Americas. The purpose of this manual is to help laboratories in different hospitals and regions follow exactly the same procedures and quality control procedures, thereby ensuring consistent results throughout the region.

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5.1 Growth of arsenate resistant strains at various concentrations of sodium arsenate

The growth behaviour of six arsenate resistant strains viz. SI9, BL9, MPT4, Maj4, Man1 and Man2 was studied in presence of different concentrations of sodium arsenate (0-250mM). All the six isolates selected for growth studies exhibited sigmoid growth pattern; and in all the cases except for Vibrio sp.SI9, Vibrio campbellii sp. BL9 and Vibrio sp.. Maj4 which interestingly showed enhanced growth when arsenate was added to the medium, there was a reduction of growth in response to increasing arsenate concentration (Fig. 19.1-19.6). The reason for this growth enhancement probably lies in the fact that addition of arsenate causes the pH of the medium to increase (Anderson and Cook, 2004) and the strains which have pH optima above the neutral pH of the medium (pH 7.2) and are resistant to arsenic, show better growth in presence of arsenate. In prokaryotes (E.coli) there are two phosphate transporters, Pit and Pst, both catalyzing arsenate uptake but the Pit system appears to be predominant (Rosen, 2002). The bacteria have different mechanisms which enable them survive high arsenate concentrations. There could be three mechanisms of arsenate resistance in microbes:

a) Decreased uptake by the increased specificity for phosphate uptake.
b) Arsenate reduction and subsequent efflux of arsenite.
c) Sequestration by the phytochelatin like proteins present in the cell walls of few bacteria, mainly thermophilic archea.

Out of these resistance pathways arsenite efflux system is the most common and predominant detoxification mechanism working in most bacteria.
Fig. 19.1: Growth of *Vibrio* sp. SI9 in presence of sodium arsenate in MSM+ 0.4% glucose. (bars represent standard errors of means)

Fig. 19.2: Growth of *Vibrio* sp. BL9 in presence of sodium arsenate in MSM+ 0.4% glucose (bars represent standard errors of means)
Fig 19.3: Growth of *Aeromonas* sp. strain MPT4 in presence of sodium arsenate in MSM+0.4% glucose (bars represent standard errors of means)

Fig 19.4: Growth of *Vibrio* sp. Maj4 in presence of sodium arsenate in MSM + 0.4% glucose (bars represent standard errors of means)
Fig. 19.5: Growth of *Pseudomonas* sp. Man1 in presence of sodium arsenate in MSM + 0.4% glucose (bars represent standard errors of means)

Fig. 19.6: Growth of *Vibrio* sp. Man2 in presence of arsenate in MSM+0.4% glucose (bars represent standard errors of means)
5.2 Growth of arsenate resistant strains at various concentrations of arsenite

The growth behaviour of six arsenate resistant strains viz. SI9, BL9, MPT4, Maj4, Man1 and Man2 was studied in presence of different concentrations of sodium arsenite (0-7 mM). Strains SI9, MPT4 Man1 and Man2 showed slightly enhanced growth at a concentration of 1mM arsenite in the Mineral Salts Medium (MSM). A reduction of growth however was noticed at subsequently higher concentrations of sodium arsenite (Fig. 20.1-20.6). Arsenite in bacteria is transported into the cells by aqua-glyceroporins (glycerol transport proteins). In *E.coli* it is GlpF protein, which is a transmembrane integral protein, which was originally identified as the glycerol facilitator and is a member of the aqua-glyceroporin family (Stahlberg et al. 2000). The resistance to arsenite is mainly governed by the arsenite efflux pump comprising of ArsB protein to which sometimes an ATPase, ArsA is also attached causing enhanced rates of efflux (Ji and Silver 1992, Mukhopadhyay et al., 2002). Another mechanism of arsenite resistance is by means of arsenite oxidation through the activity of arsenite oxidase or peroxidation reactions with the membrane lipids (Jackson and Dugas, 2003).

5.3 Growth in presence of different carbon sources in Mineral medium + 1.5% NaCl

The growth behaviour of six selected strains SI9, BL9, MPT4, Maj4, Man1 and Man2 was studied in presence of four different carbon sources viz., glucose, lactose, sucrose and sorbitol (0.4% in each case) in Mineral medium + 1.5% NaCl (Fig. 21:i – vi). Glucose was found to be the best carbon source for all the strains except Man2 which showed a delayed but better growth in lactose (Fig.21:vi). The strains MPT4, Maj 4 and Man1 showed equally good growth in sucrose as well.
Fig. 20.1: Growth of *Vibrio* sp. SI9 in presence of sodium arsenite (0-2mM) in MSM + 0.4% glucose. (bars represent standard errors of means)

Fig. 20.2: Growth of *Vibrio* sp. BL9 in presence of sodium arsenite (0-3mM) in MSM + 0.4% glucose. (bars represent standard errors of means)
Fig. 20.3: Growth of *Aeromonas* sp. MPT4 in presence of sodium arsenite (1-6 mM) in MSM + 0.4% glucose. (bars represent standard errors of means)

Fig. 20.4: Growth of *Vibrio* sp. Maj4 in presence of sodium arsenite (0-3 mM) in MSM + 0.4% glucose. (bars represent standard errors of means)
Fig. 20.5: Growth of *Pseudomonas* sp. Maj4 in presence of sodium arsenite (0-3 mM) in MSM + 0.4% glucose. (bars represent standard errors of means)

Fig. 20.6: Growth of *Vibrio* sp. Man2 in presence of sodium arsenite (0-3 mM) in MSM + 0.4% glucose. (bars represent standard errors of means)
Marine vibrios grow best in presence of glucose or galactose as the sole source of carbon. These two sugars at concentration as low as 0.5 mg/ml elicited good growth of *Moritella marina* (formerly *Vibrio marinus*; Steven, 1990; Urakawa et al., 1998), on minimal medium plates (Rüger, 1988). Vibrios are well known for their capability of utilizing sorbitol as carbon source (Aeckersberg et al. 2001, Von Kruger et al. 1999, Collins and Thune 1996). Therefore, the growth of six selected arsenate resistant strains (four of which belong to the genus *Vibrio* and five fall under the family Vibrionaceae) was studied in presence of sorbitol as the carbon source. In the present experiments it was found that, although sorbitol can support the growth of all six strains, the growth was not as good as when glucose, galactose or sucrose were used in the medium as carbon source (Fig. 21:i–vi).

### 5.4 Designing of a phosphate limiting medium for arsenate uptake and biotransformation studies

The Sea Water Based Minimal Medium with limiting phosphate (SBMLP) was designed for arsenate uptake and biotransformation studies, which contained only 65μM phosphate and both the selected isolates Maj4 and SI9 were capable of growing well in it (Fig. 22.1,a–e & Fig. 22.2,a–e). Both the cultures showed satisfactory growth in SBMLP medium containing 25 and 50mM sodium arsenate also. It is interesting to mention that these two isolates chosen for arsenate uptake and biotransformation studies were able to grow without any phosphate in the medium PLM1 as well (Fig. 22.1a & 22.2a). Various types of phosphate limiting media have been designed and used by different researchers, which include 'Artificial sea water based minimal medium without any phosphate (Aeckersberg et al. 2001), Tris-glucose low phosphate (TGLP) medium (Von Kruger et al. 1999) and the Defined Minimal Medium (DMM Collins and Thune, 1996). The medium formulated by Aeckersberg et al. (2001) is designated as PLM1 in our experiments. The other phosphate limiting media viz. TGLP and DMM (with glucose/ sorbitol as carbon source) were also found to support normal
Fig. 21(i – vi): Effect of carbon source on growth of six selected Arsenate resistant strains SI9, BL9, MPT4, Maj4, Man1 and Man2.

Fig. 21(i):

Effect of carbon source on growth of *Vibrio* sp. SI9

![Graph showing growth of *Vibrio* sp. SI9](image)

- glucose
- lactose
- sucrose
- sorbitol

Time (h): 0, 4, 8, 12, 16, 20, 24

Abs 600 nm: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2

Fig. 21(ii):

Effect of carbon source on growth of *Vibrio* sp. BL9

![Graph showing growth of *Vibrio* sp. BL9](image)

- glucose
- lactose
- sucrose
- sorbitol

Time (h): 0, 4, 8, 12, 16, 20, 24

Abs 600 nm: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2
Fig. 21(iii):

Effect of carbon source on growth of *Aeromonas* sp. MPT4

![Chart showing growth of *Aeromonas* sp. MPT4 with different carbon sources.]

Fig. 21(iv):

Effect of carbon source on growth of *Vibrio* sp. Maj4

![Chart showing growth of *Vibrio* sp. Maj4 with different carbon sources.]

<table>
<thead>
<tr>
<th>Abs 600 nm</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>lactose</td>
<td>0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>sucrose</td>
<td>0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>sorbitol</td>
<td>0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Effect of carbon source on growth of *Pseudomonas* sp. Man1

![Graph showing growth of *Pseudomonas* sp. Man1](image)

Fig. 21(v):

Effect of carbon source on growth of *Vibrio* sp. Man2

![Graph showing growth of *Vibrio* sp. Man2](image)

Fig. 21(vi):
Fig. 22.1(a-e): Growth pattern of *Vibrio* sp. S19 in four different minimal media with limiting phosphate concentrations. (a) growth profile in PLM1, (b) growth profile in TGLP medium, (c) growth profile in DMM, (d) growth profile in SBMLP medium; & (e) growth profile in SBMLP+ 50mM sodium arsenate.
Growth of Vibrio sp. SI9 in SBMLP medium

0.3
0.25
- e
0.2 - CI
0.15 -
co 0.1 -
0.05 -
0
0 	 4 	 8 	 12 	 16 	 20 	 24 
time(h)

Growth of Vibrio sp. SI9 in SBMLP+50mM Arsenate

0.5
0.4 -
e 0.3 -
0.2 -
o 0.1 -
o
0 	 4 	 8 	 12 	 16 	 20 	 24 
time(h)
Fig. 22.2(a-e): Growth pattern of Vibrio sp. Maj4 in four different minimal media with limiting phosphate concentrations. (a) growth profile in PLM1, (b) growth profile in TGLP medium, (c) growth profile in DMM, (d) growth profile in SBMLP medium, and (e) growth profile in SBMLP+ 50mM sodium arsenate.
Growth of *Vibrio* sp Maj4 in SBMLP medium

(d)

Growth of *Vibrio* sp. Maj4 in SBMLP+50mM arsenate

(e)
growth of the isolates Maj4 and SI9 (Fig.21.1:b,c & Fig.21.2:b,c). The SBMLP was especially designed by us because of the fact that arsenate forms a precipitate in the media which contain Tris. HCl such as PLM1, TGLP and DMM. It was decided to use phosphate limiting medium for arsenate uptake and biotransformation experiments due to the fact that phosphate present in the medium interferes in the measurement of arsenate by spectrophotometric (Molybdenum blue) methods. Although there are indirect methods available for arsenate estimation which involve reduction of arsenate by L-Cysteine or other reducing agents, the use of phosphate limiting medium has an advantage of being direct, time saving and giving less error than the indirect methods.

5.5 Arsenate uptake by spectrophotometric method (Improved Molybdenum blue method)

Uptake of arsenate by the bacterial cells with respect to time was studied for the two best arsenate tolerating strains Vibrio sp. SI9 and Vibrio sp. Maj4. Arsenate uptake studies done in Mineral salts medium (which contains 50mM phosphate) as well as in phosphate limiting medium (SBMLP medium), both revealed that a substantial amount of arsenate is being depleted from the culture medium with respect to time. In Mineral salts medium (phosphate non-limiting) the isolate SI9 showed 56% and 66.6% uptake after 24h and 48h respectively (Table 18). Whereas, 46.6% and 50% uptake was noticed in case of the isolate Maj4 after 24 h and 48h of incubation. Significant uptake of arsenate was observed in case of SI9 (51.6%) and Maj4(68.5%) after 20h(max. growth) in SBMLP medium also(Table 19). There was no increase seen in the uptake levels after 20h in the phosphate limiting medium because the cultures lose their viability in nutrient deprived conditions. The spectrophotometric method based on the basic Molybdenum blue procedure of Johnson and Pilson (1972) has been
Table 18: Arsenate uptake by *Vibrio* sp. SI9 and *Vibrio* sp. Maj4 by spectrophotometric method (Improved Molybdenum blue method) in Mineral medium (MSM+0.4% glucose+1.5% NaCl) containing 50mM Arsenate.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>sample</th>
<th>$\text{Abs}_{815}$ (PO$_4^-$+AsO$_4^{3-}$)</th>
<th>$\text{Abs}_{875}$ (onlyPO$_4^-$)</th>
<th>Difference ($\text{Abs}<em>{815} - \text{Abs}</em>{875}$)</th>
<th>Residual arsenate in the medium</th>
<th>%Arsenate Utilised (uptake)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>control*</td>
<td>0.763 ± 0.0036</td>
<td>0.446 ± 0.0034</td>
<td>0.317 ± 0.0071</td>
<td>15µM</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>SI9 24h</td>
<td>0.573 ± 0.0030</td>
<td>0.435 ± 0.0031</td>
<td>0.138 ± 0.0061</td>
<td>6.5 µM</td>
<td>56.4 ± 0.85 %</td>
</tr>
<tr>
<td>3</td>
<td>SI9 48h</td>
<td>0.542 ± 0.0029</td>
<td>0.437 ± 0.0027</td>
<td>0.105 ± 0.0056</td>
<td>5µM</td>
<td>66.6 ± 0.78 %</td>
</tr>
<tr>
<td>4</td>
<td>Maj4 24h</td>
<td>0.643 ± 0.0029</td>
<td>0.474 ± 0.0040</td>
<td>0.169 ± 0.0069</td>
<td>8µM</td>
<td>46.6 ± 0.97%</td>
</tr>
<tr>
<td>5</td>
<td>Maj4 48h</td>
<td>0.568 ± 0.0046</td>
<td>0.412 ± 0.0011</td>
<td>0.156 ± 0.0057</td>
<td>7.5µM</td>
<td>50.0 ± 0.80 %</td>
</tr>
</tbody>
</table>

*control = 5µl of culture medium (MSM+ 1.5%NaCl+ 0.4%glucose+50mM sodium arsenate) in 10 ml of deionized water.

Note = Abs875 was taken after the sample was reduced with 5%Cysteine so as to eliminate any As (V) present.
### Table 19: Arsenate uptake in best selected Phosphate Limiting medium (SBMLP with 65μM Phosphate) at 28 ± 2°C

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment</th>
<th>Abs 815nm (mean± S.E.)</th>
<th>Percent uptake (mean± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 25mM As⁵⁺*</td>
<td>0.661 ± 0.0035</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>SI9+25mM As⁵⁺,20h</td>
<td>0.311 ± 0.0017</td>
<td>52.95 ± 0.48%</td>
</tr>
<tr>
<td>3</td>
<td>Maj4+25mM As⁵⁺,20h</td>
<td>0.203 ± 0.0025</td>
<td>69.30 ± 0.71%</td>
</tr>
<tr>
<td>4</td>
<td>Control 50mM As⁵⁺*</td>
<td>1.331 ± 0.0049</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>SI9+50mM As⁵⁺,20h</td>
<td>0.645 ± 0.00145</td>
<td>51.6 ± 0.29%</td>
</tr>
<tr>
<td>6</td>
<td>Maj4+50mM As⁵⁺,20h</td>
<td>0.419 ± 0.0017</td>
<td>68.55 ± 0.34%</td>
</tr>
</tbody>
</table>

*control = SBMLP medium with 25 or 50mM arsenate.
used by many workers including Anderson and Cook (2004) and Niggemyer et al. (2001). This method was further modified which included an extra reduction step with cysteine as reducing agent in order to eliminate interference caused by phosphate present in the culture medium (Carvalho et al., 1997). Vishnikin (2005) has devised a novel spectrophotometric method for determination of arsenate by using Polyoxometalates and micellar medium, in which 1% neonole (polyoxyethylene nonylphenole) was used as the reagent.

5.6 Determination of total arsenic in water samples and intracellular arsenic (uptake) by the resistant strains by HG-AAS

Total arsenic concentration was measured in the 9 water samples collected from different locations in Goa (Table 20). This was done because of the fact that out of 3700 sq. Km area of the state of Goa, 14% land area is under mining, 25% under forest cover and 36% for agriculture. Catchment area of Mandovi and Zuari is about 2500 Km² that is 68% of the total geographical area. Arsenic levels in the water samples were found to be within the safe limit of 50 ppb as prescribed in the W.H.O. guidelines. The water samples from Kakra, Majorda, MPT harbour, Dona Paula as well as all the well water samples analysed by HG-AAS were found to contain below 1 ppb of total arsenic. Water from Zuari and Mandovi estuaries contained 1.76 and 1.81 ppb, respectively (Table 20). The total arsenic values for Miramar water sample was 5.13 and that of Saint Inez sample 6.3 ppb, which are higher than other samples but still very well within the safe range of 50 ppb (Table 20). The reason for higher value obtained for Miramar beach water lies in the fact that it is connected to the Mandovi estuary at a close distance and encounters heavy load of tourists as compared to other sites. The higher value in case of St. Inez Nallah water may be attributed to industrial effluents and domestic waters falling into it. The rivers Mandovi and Zuari are
flowing through iron and mining areas and are used for iron and manganese ore transport to the MPT harbour. Two thirds of the Fe and Mn ores come from the mines located in the basins of these rivers. 90% of ores are transported in barges. Arsenic is present in the ores in the form of Fe.As.S. The Mn oxides enhance arsenite oxidation. The arsenate gets co precipitated with iron or gets adsorbed onto hydrous iron oxides. The iron ore of Mandovi/zuari catchment area has approximately 50.04 µg/g arsenic and partial dissolution can result in enrichment of estuarine water with arsenic. Both estuaries had the same arsenate levels in 1994 i.e. 0.11-0.24 µg/litre (Nair et al.1994). Total inorganic arsenic ranged from 3 to 150 µg/l in the coastal surface waters of Goa (Annual Report, NIO, India 1974, 1975). The results obtained by us indicate that the total inorganic arsenic levels have increased since 1994, although they are very much within the safety limits. The increase is most probably due to the increase in population and successively increasing load of tourists from all over the world (as industrial discharge of arsenic has been totally stopped by the law). Clesceri et al. (1998) have described the various methods of arsenic measurement including the silver diethyldithio cabamate method (detection limit 1-20µg). They have recommended ICP-ES for concentrations higher than 50 µg and ICP-MS method for lower concentrations if chloride does not interfere. Sehlin and Lindstrom (1992) have successfully used the ion chromatography technique for the detection of arsenate reduction. Andreae (1979) used a gas chromatograph along with a 63Ni electron capture detector mounted in parallel with flame ionization detector. Silver and Keach (1982) have used radioactive 74AsO4³⁻ to study arsenate uptake by E.coli cells containing cloned 4.2 Kb fragment of R773 with ars operon. Although all other techniques like HPLC,TLC, ion exchange chromatography, ICP-MS, ICP-ES etc. have been used by various authors (scientists) all over the world to detect arsenic in biological samples; still the hydride generation method has remained the most
popular method of choice and has emerged as a standard technique for total arsenic (Das et al. 1995) as well as speciation studies (Kashyap et al. 2006). The two isolates *Aeromonas* sp. MPT4 and *Pseudomonas* sp. Man1 were able to accumulate arsenic intracellularly (Table 21) whereas an efflux pump seems to be functioning in all other strains as evident from relatively constant levels of intracellular arsenic w.r.t. time (Table 21). There have been only scanty reports regarding bioaccumulation of arsenic by eubacteria. Only some thermophilic bacteria forming reddish brown biofilms from hot springs (Tazaki et al. 2003) have been known to have this property, where in it is found to accumulate intracellularly as Fe-As. The bacteria which are capable of bio-accumulating arsenic are of much importance for environmental bioremediation and waste water treatment. The genes responsible for bio-accumulation may be cloned into the genome of some easily culturable bacteria or even higher plants, which can serve as biosorbants for this highly recalcitrant oxyanion.

5.7 Detection of Arsenate biotransformation by thin layer chromatography
Two of the strains which showed enhanced growth in arsenate containing medium viz. *Vibrio* sp. S19 and *Vibrio* sp. Maj4 (Fig. 23) were selected for biotransformation studies. Arsenite spot could be detected in a 24 hour culture of *Vibrio proteolyticus* Maj4 grown in 100mM and 200mM Sodium arsenate in MSM+0.4% glucose (Fig. 24, Table 22). This clearly indicates that in this culture arsenite is the final product being formed. Ji and Silver (1992) have used the TLC of crude extract of cells grown in arsenic to demonstrate the phenomenon of arsenate reduction by *S. aureus* RN4220 cells containing plasmid pG109 (arsB and arsR deletion mutant, having intact arsC). They have used a 2-propanol/water (9:1, vol./vol.) as solvent system and the plates were developed by autoradiography. $^{73}$AsO$_4^{3-}$ was used in the growth medium during this uptake experiment. Mokashi and Paknikar (2002) have also used thin layer chromatography for biotransformation studies wherein, they have visualized
Table 21: Arsenic uptake by the two selected strains *Vibrio* sp. SI9 and *Vibrio* sp. Maj4

Hydride generation atomic absorption spectrometry (HG-AAS)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>culture</th>
<th>Intracellular arsenic ppb (µg/litre) 0 h</th>
<th>Intracellular arsenic ppb (µg/litre) 6 h</th>
<th>Intracellular arsenic ppb (µg/litre) 12 h</th>
<th>Intracellular arsenic ppb (µg/litre) 18 h</th>
<th>Intracellular arsenic ppb (µg/litre) 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a)SI9 in NB</td>
<td>5.4</td>
<td>131</td>
<td>167</td>
<td>196</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>b)SI9 in SBMLP</td>
<td>5.0</td>
<td>8.8</td>
<td>286</td>
<td>167</td>
<td>240</td>
</tr>
<tr>
<td>2</td>
<td>a)BL9 in NB</td>
<td>3.0</td>
<td>76.5</td>
<td>278</td>
<td>365</td>
<td>194.5</td>
</tr>
<tr>
<td></td>
<td>b)BL9 in SBMLP</td>
<td>7.0</td>
<td>105</td>
<td>336</td>
<td>463</td>
<td>365</td>
</tr>
<tr>
<td>3</td>
<td>a)MPT4 in NB</td>
<td>43</td>
<td>249</td>
<td>758</td>
<td>1203</td>
<td>1700</td>
</tr>
<tr>
<td></td>
<td>b)MPT4 in SBMLP</td>
<td>8.5</td>
<td>307</td>
<td>472</td>
<td>654</td>
<td>752</td>
</tr>
<tr>
<td>4</td>
<td>a)Maj4 in NB</td>
<td>10.5</td>
<td>281</td>
<td>267</td>
<td>298</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td>b) Maj4 in SBMLP</td>
<td>5.1</td>
<td>209</td>
<td>169</td>
<td>235</td>
<td>184</td>
</tr>
<tr>
<td>5</td>
<td>a)Man1 in NB</td>
<td>4.7</td>
<td>127</td>
<td>203</td>
<td>299</td>
<td>416</td>
</tr>
<tr>
<td></td>
<td>b)Man1 in SBMLP</td>
<td>6.3</td>
<td>89</td>
<td>486</td>
<td>885</td>
<td>1705</td>
</tr>
<tr>
<td>6</td>
<td>a) Man2 in NB</td>
<td>3.2</td>
<td>48</td>
<td>214</td>
<td>388</td>
<td>470</td>
</tr>
<tr>
<td></td>
<td>b) Man2 in SBMLP</td>
<td>4.9</td>
<td>38</td>
<td>178</td>
<td>307</td>
<td>361</td>
</tr>
</tbody>
</table>

NB = nutrient broth
SBMLP = sea water based minimal medium with limiting phosphate
Table 22: Rf values of arsenate and arsenite in different solvent systems as obtained by Paper chromatography and silica gel Thin layer chromatography

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solvent system</th>
<th>Mean Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>As3+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Iso-propanol:Water(9:1)</td>
<td>Paper chromatography: 0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica Gel TLC: 0.68</td>
</tr>
<tr>
<td>2</td>
<td>Iso-propanol:Water(7:3)</td>
<td>Paper chromatography: 0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica Gel TLC: 0.53</td>
</tr>
<tr>
<td>3</td>
<td>Iso-propanol:Water (4:1)</td>
<td>Paper chromatography: 0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica Gel TLC: 0.74</td>
</tr>
<tr>
<td>4</td>
<td>Iso-propanol-Water-Chloroform (7:3:4)</td>
<td>Paper chromatography: 0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica Gel TLC: 0.90</td>
</tr>
<tr>
<td>5</td>
<td>Methanol:Ammonia:Water(6:3:1)</td>
<td>Paper chromatography: 0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica Gel TLC: 0.91</td>
</tr>
<tr>
<td>6</td>
<td>Methanol:Ammonia:Water:Chloroform(6:3:1:4)</td>
<td>Paper chromatography: 0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica Gel TLC: 0.89</td>
</tr>
<tr>
<td>7</td>
<td>Ethyl acetate: Butanol: Acetic acid:Water(EBAW)-80:10:5:5</td>
<td>Paper chromatography: 0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Silica Gel TLC: 0.66</td>
</tr>
</tbody>
</table>
Fig 23: Effect of sodium arsenate on total protein of *Vibrio* spp. strains SI9 and Maj4 in MSM + 0.4% glucose + 1.5% NaCl

![Graph showing the effect of sodium arsenate on total protein of *Vibrio* spp. strains SI9 and Maj4](image)

**Effect of Sodium Arsenate on total protein**

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>SI9</th>
<th>Maj4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>100</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>150</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>200</td>
<td>160</td>
<td>160</td>
</tr>
</tbody>
</table>
Fig. 24: A Thin layer chromatogram showing Arsenate reduction (into arsenite) by *Vibrio* sp. Maj4

Spot 1: arsenate standard (1mg),
Spot 2: arsenite standard (1mg)
Spot 3: 0h culture of *Vibrio* sp. Maj4
Spot 4: 24h culture of *Vibrio* sp Maj4
Spot 5: 48h culture of *Vibrio* sp Maj4
formation of arsenate from arsenite by an As(III) oxidizing strain of *Microbacterium lacticum* isolated from arsenic contaminated tube well. There are two main approaches to the speciation of arsenic in environmental compounds. One is the rigorous procedure appropriate for the identification of compounds of previously unknown structure. Here arsenic species are separated from a large quantity of starting material, purified and isolated and their structures determined by X-ray crystallography, NMR spectroscopy, IR spectroscopy, Mass spectrometry, U.V.-Visible spectroscopy and elemental analysis. These methods afford an unequivocal identification but requires large amount of sample and much time (Morita and Edmonds, 1992). The other method is to combine a separation method with selective and sensitive detection methods. The thin layer chromatography method is a simple and economic method for studying arsenate reduction by bacteria and other microorganisms.

5.8 SDS- PAGE

The SDS-PAGE profile of total protein of the isolates did not reveal any induced proteins in presence of arsenate except for *Aeromonas* sp. MPT4 which showed two induced bands of 46 KDa and 28KDa (Fig. 25:GEL 1-6). These bands are possibly of some protein responsible for intracellular bioaccumulation of arsenic. As nothing is known about such proteins in bacteria it was not possible to identify these induced proteins. No induced band corresponding to arsenate reductase could be visualized by the normal SDS- PAGE technique, with Coomassie blue staining which may be due to its low concentration. The *ArsC* protein is an arsenate reductase that mediates reduction of arsenate prior to arsenite efflux. The size of *ArsC* is 12-15 KDa, which is a soluble enzyme couples the oxidation of thiols from glutathione/glutaredoxin or thioredoxin to the reduction of As(V) to As(III) (Ji and Silver 1992, PNAS). The *ars* operon as such is induced by As(III) and Sb(III)(Wu and Rosen, 1993). *ArsM* (arsenite S-adenosylmethionine methyltransferase) catalyses the formation of a number of methylated
Fig. 25(GEL 1-6): SDS-PAGE analysis of Arsenate induced proteins of the six selected arsenate resistant isolates after 24 hrs.

**GEL 1-3:** protein profile of arsenate resistant isolates in Nutrient Broth.

**GEL 4-6:** protein profile of arsenate resistant isolates in MSM+0.4% glucose + 1.5% NaCl.
GEL 3: Lane No. 1. BSA 500ng, 2. BSA 1000ng, 3. Maj4 control
4. Maj4 in 25mM sodium arsenate, 5. Maj4 in 50 mM sodium arsenate,
6. Man1 control, 7. Man1 in 25mM sodium arsenate, 8. Man1 in
50 mM sodium arsenate, 9. LMW Marker, 10. Gel loading buffer

GEL 4: Lane No. 1. LMW Marker, 2. BSA 500ng, 3. BSA 1000ng
4. S19 control, 5. S19 in 25mM sodium arsenate,
6. S19 in 50 mM sodium arsenate, 7. BL9 control, 8. BL9 in
25mM sodium arsenate, 9. BL9 in 50 mM sodium arsenate,
10. Gel loading buffer
GEL 5: Lane No.1, LMW Marker, 2. BSA 1000ng, 3. BSA 500ng, 4. Man2 control, 5. Man2 in 25mM sodium arsenate, 6. Man2 in 50 mM sodium arsenate, 7. MPT4 control, 8. MPT4 in 25mM sodium arsenate, 9. MPT4 in 50 mM sodium arsenate, 10. Gel loading buffer

GEL 6: Lane No.1. Gel loading buffer, 2. BSA 500ng, 3. BSA 1000ng, 4. Maj4 control, 5. Maj4 in 25mM sodium arsenate, 6. Maj4 in 50 mM sodium arsenate, 7. Man1 control, 8. Man1 in 25mM sodium arsenate, 9. Man1 in 50 mM sodium arsenate, 10. LMW Marker
intermediates from As(III) with trimethylarsine as the end product. This gene was found to be reported to be present in *Rhodopseudomonas palustris*. Its molecular weight is 29.6 KDa (283aa).