

**BIOREMEDIATION OF ARSENIC BY BACTERIA ISOLATED FROM ARSENIC CONTAMINATED MARINE ENVIRONMENT OF GOA HARBOUR OF INDIA****NEELAM SINGH**

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ABSTRACT

Biological characterization and bioremediation potential of an arsenic hyper-accumulating marine mesophilic bacterium, *Aeromonas punctata* strain MPT4 isolated from the Ferromanganese ore loading site of marmugao port trust harbour (N15°24' 28.3" and E 073° 48' 17.6"), Goa, India is presented in this study. The taxonomic placement of this isolate was done on the basis of biochemical tests (as per Bergey's Manual), FAME analysis and 16S rDNA sequencing. MIC of sodium arsenate and arsenite were determined in Mineral salts medium supplemented with 0.4% glucose and increasing concentrations of sodium arsenate and arsenite separately. Growth behaviour of this isolate reveals that it can tolerate well upto 150 mM sodium arsenate. Intracellular arsenic content of bacterial cells was estimated by HG-AAS. Significantly high levels of arsenate was found in the cell free extracts of sonicated cells, after washing the arsenate grown cells twice with 50mM Tris-Cl buffer. Interestingly, PCR analysis of genomic DNA and plasmid DNA using *arsC* specific primers revealed the absence of arsenate reductase gene. These findings clearly suggest that although *Aeromonas punctata* MPT4 is devoid of *arsC* gene, it hyperaccumulates arsenic (arsenate) and may be successfully used for bioremediation of arsenate contaminated marine environment.



KEYWORDS

Arsenic resistance, *Aeromonas*, bioaccumulations

INTRODUCTION

Arsenic is toxic to life forms at all concentrations and does not have any physiological role except in some bacteria which use it as an electron acceptor for anaerobic respiration (Laverman, et al.1995). Arsenic resistance in bacteria is a well studied phenomenon, although until recently (Anderson and Cook, 2004), the presence of arsenic resistance in the genus *Aeromonas* was not reported. These scientists from New Zealand, have reported it for the first time and the strain was PCR negative for the *ars* operon genes. Although the presence of Fe, Mn, Cu, Ni and Zinc accumulating bacteria have been reported from soil and rhizosphere, arsenic accumulating bacteria are lesser explored. Some engineered bacteria with arsenic hyper-accumulating genes have also been developed for this purpose (Sauge- Merle et al. 2003; Kostal et al. 2004). However there have been no reports of arsenic bioaccumulation in mesophilic bacteria and only some thermophilic bacteria forming reddish brown biofilms from hot springs composed of bacilliform and coccoid types of

bacteria have been known to have this property, wherein it is found to accumulate extracellularly in their cell walls as FeAs_2 (lollingite) (Tazaki et al. 2003).

In the present communication we have characterized an arsenate hyperaccumulating bacterium, *Aeromonas punctata*MPT4 which was isolated from subsurface waters (1m depth) of Fe-Mn ore loading sites of Marmugao Harbour Goa (Fig.1), with reference to growth in arsenate/arsenite containing media, arsenate bioaccumulation (HG-AAS), FAME analysis, 16s rDNA sequencing and taxonomic placement by NCBI blast.PCR analysis of genomic and plasmid DNA for the presence of *arsC* gene was also done. Scanning electron microscopy was also done to observe the morphological features of individual cells of the bacterial isolate. SDS-PAGE analysis of total proteins of arsenate bioaccumulating isolates was also performed to check the presence of arsenate induced proteins which may be playing a pivotal role in intracellular sequestration of arsenic(arsenate).

Figure 1
Growth of *Aeromonas* sp.MPT4 in presence of sodium arsenate in MSM+0.4% glucose

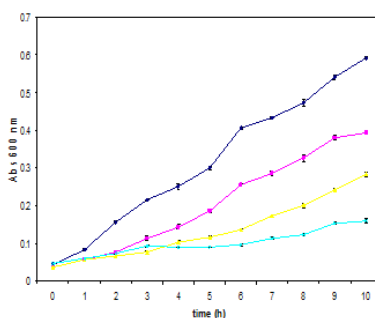


Fig1: Growth of *Aeromonas* sp.MPT4 in presence of sodium arsenate in MSM+0.4% glucose

—■— 0mM —■— 50mM —■— 150mM —■— 200mM

Figure 2
Growth of Aeromonas sp. MPT4 in presence of sodium arsenite in MSM + 0.4% glucose

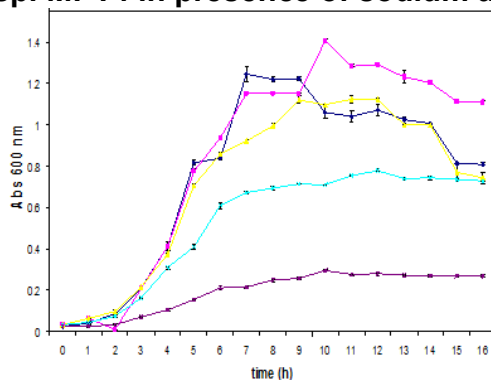
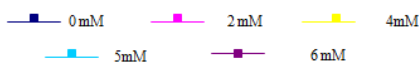


Fig2: Growth of Aeromonas sp. MPT4 in presence of sodium arsenite in MSM + 0.4% glucose



MATERIALS AND METHODS

Sterilization of media, solutions and apparatus:

All the media and solutions used were sterilized by autoclaving at 121°C temperature, 15 psi pressure for 20 minutes. The glassware and other apparatus were sterilized in an oven at 180 °C for an hour. After sterilization, the media and solutions were cooled to room temperature and then stored under refrigeration for their subsequent use. The sterilized glassware were stored separately in an oven at 60°C and cooled to room temperature before their subsequent use.

Isolation and Maintenance of the strain MPT4:

The *Aeromonas punctata* (strain MPT4) was screened and purified on nutrient agar prepared in ASW containing 10mM sodium arsenate and maintained on Mineral salts medium containing 50mM sodium arsenate and 0.4% glucose by subculturing once a week.

Determination of MIC for arsenate and arsenite:

The MIC was determined in the Mineral salts medium, as, the concentration of arsenate/arsenite at which the culture shows no visible growth (Abs₆₀₀ after 24 hours). Two percent inoculum from an overnight grown culture of the isolate was given in 10 ml of medium for each concentration in a 25 ml conical flask which was incubated for 24 hours at 28 ± 2 °C and 160 rpm.

Growth behaviour of the isolate in presence of arsenate and arsenite

The growth pattern of the strain was studied in Mineral Salts Medium with 0.4% glucose at different concentrations of sodium arsenate(0, 100, 150, 200 mM) and sodium arsenite(2,4,5 and 6mM) at 28 ± 2 °C and 160 rpm by recording absorbance at 600nm.

Fatty Acid Methyl Ester (FAME) Analysis:

The strain MPT4 was grown on tryptic soy agar(TSA) supplemented with 3%NaCl at 37°C for 24 hours. Whole cell fatty acids were extracted from the cells according to the MIDI protocol(Sasser,1990).GC analysis of fatty acids was performed on a Sherlock microbial identification system, Newark, USA fitted with a



cross linked methyl silicone fused capillary column(25m.0.2mm i.d.)and flame ionization detector(FID) and a sampler. Helium was used as a carrier gas. Samples were injected in a splitless mode at an oven temperature of 50°C. After 1 min the oven temperature was raised to 170°C at 30°C min⁻¹, then to 270°C at 2°C min⁻¹ and finally to 300 °C at 5°C min⁻¹.GC-MS analysis of the FAME was performed using a GCQ Plus GC/MS System(Thermoquest, USA)fitted with on column injection set at 45°C.Samples were injected using a sampler into a retention gap attached to an Ultra 2.50m, 0.32mm i.d. and 0.17 µm film thickness column using Helium as the carrier gas. The chromatograms and the mass spectra were applied using software ChemStation Version 4.02. Peaks are identified in comparison with known standards in the library that are included with the software ChemStation Version 4.02 and by taking into account the mass spectra. The fatty acids are designated taking into consideration the total number of carbon atoms, number of double bonds and the position of double bonds from the terminal(x) end of the molecule. The suffixes 'c' and 't' indicate cis and trans geometry.

16s rDNA sequence amplification and analysis:

16s rDNA amplification using eubacterial universal primers as follows:

27F : 5'CCA GAG TTT GAT CMT
GGC TCA- 3'

1525R: 5'TTC TGC AGT CTA GAA
GGA GGT GWT CCA GCC-3'.

(M=A/C, W=A/T)

The PCR amplified 16s rDNA fragment was sequenced [ABI PRISM 3730 DNA analyzer(48)] using the following 3 sequencing primers:

121F 5' GGC GGA CGG CTG AGT AAT 3'

343R 5' ACT GCT GCC TCC CGT A - 3'

704F 5'GTA GCG GTG AAA TGC GTA GA3'

The sequence was subjected to NCBI Blast in order to search homology with the other known

genera available in the database (Altschul, 1990).

Analysis of intracellular arsenic content (HG-AAS):

Intracellular arsenic was determined by using HG-AAS instrument (Perkin Elmer AAnalyst 700 AAS). Estimation of total cellular arsenic content in the sonicated samples was done as arsenite in a total volume of ten millilitres. To seven millilitre of sample, 1 ml of 5% Ascorbic acid+ 1ml Potassium Iodide (5%)+ 1 ml of conc. HCl was added and incubated for 45 min. at room temperature.All forms of organic as well as inorganic arsenic is reduced to As(III) by Ascorbic acid / KI treatment. The arsenite is further reduced to arsine, when it is passed through the glass cuvette(atomizer) heated by acetylene/air flame at 900° C. Arsenic atoms are released in the cuvette which absorb UV($\lambda_{193.7A}$) given out by the lamp. The Absorbance/transmittance is detected by the sensor which is proportional to the number of arsenic atoms present in the sample.

SDS-PAGE analysis of proteins to detect metallothionein like proteins:

The cells were disrupted gently by means of sonication (Sonicator LabsonicM) for 3 min in 3 cycles of 1 min each at 300 oscillations(30KHz), and supernatant collected after centrifugation at 12000xg; 4°C and total intracellular proteins in the cell free extract were analyzed on 12% polyacrylamide gel(Laemmli,1970). Protein staining was carried out using Coomassie Brilliant Blue R-250 stain. Gel was kept in this staining solution for 6 hours. Destaining was carried out in destaining sol. I (Methanol: acetic acid: water :: 5:1:4) for 1 hour and in destaining sol. II (Methanol: acetic acid: water :: 5:7:100) until clear bands appear.

PCR analysis of genomic and plasmid DNA to detect ars C gene:

An attempt was made to detect the presence of putative arsenate reductase gene (*arsC*) in the



arsenate resistant *Aeromonas punctata* strain MPT4 using four 4 sets of commonly used universal primers for *ars C* of gram negative bacteria. These are:

- i) *arsC1F/arsC1R* (Saltikov et al. 2002),
- ii) *amlt42F/amlt376R3* (Sun et al. 2004),
- iii) *smrc42F/smrc376R* (Sun et al. 2004)
- iv) *amlt42F/smrc376R* (Sun et al. 2004)

RESULTS AND DISCUSSION

Anderson and Cook (2004) reported arsenic resistance in the genus *Aeromonas* for the first time and showed that their strain *Aeromonas* sp. CA1 was able to transform arsenate into arsenite during growth in liquid broth cultures in rich medium; whereas, they were unable to demonstrate arsenate reducing activity in the crude extract, either by arsenomolybdenum blue method (Ji and Silver, 1992) or by NADPH oxidation method (Gladysheva et al. 1994, Renhui, 2003). It is interesting to note that they could not detect any amplification of the *arsB* or *arsC* gene in their isolate. Similar results were obtained in case of our strain of *Aeromonas* which is devoid of *arsC* gene which encodes arsenate reductase enzyme that is responsible for transformation of arsenate to arsenite. The presence of arsenite in the medium could be due to a subsidiary function of some other metal reductase such as sulphate reductase or selenate reductase.

Identification of the arsenate resistant (hypertolerant) bacterial isolate based on biochemical as well as molecular methods (16s ribosomal RNA gene sequence) revealed that this isolate (GenBankAcc NO. DQ979324) resembles 99% with *Aeromonas punctata* strain WAB1954 (GenBankAcc NO. AM 184293) and *Aeromonas caviae* (GenBankAcc NO. X 60409) of the NCBI database. FAME analysis revealed maximum similarity with *Aeromonas caviae* with a similarity index 0.671 (Fig 3) as the fatty acid profiles of both *A. punctata* and *A. caviae* are the same and hence only one of them has been included in the reference library of the supporting software. The culture showed negative response to Gram Stain and the scanning electron micrograph shows that the individual cells are straight rods with length 1.5-2.5 μm and 0.4 - 0.5 μm in diameter (Figs. 5-7).

The growth and MIC of arsenate were studied in Mineral salts medium +0.4% glucose.

Arsenate reductase gene (*ars C*) specific primers *arsC1 F/R*, *smrc 42F/R* and *amlt 42F/376R* were used for the PCR analysis of the genome and intracellular arsenic was estimated by HG-AAS. We got significantly high levels of arsenic in the sonicated cells, which were washed twice with 50mM Tris-Cl). These findings clearly suggest that *Aeromonas caviae* strain MPT4 can be successfully used for bioremediation of arsenic contaminated aquatic environment.

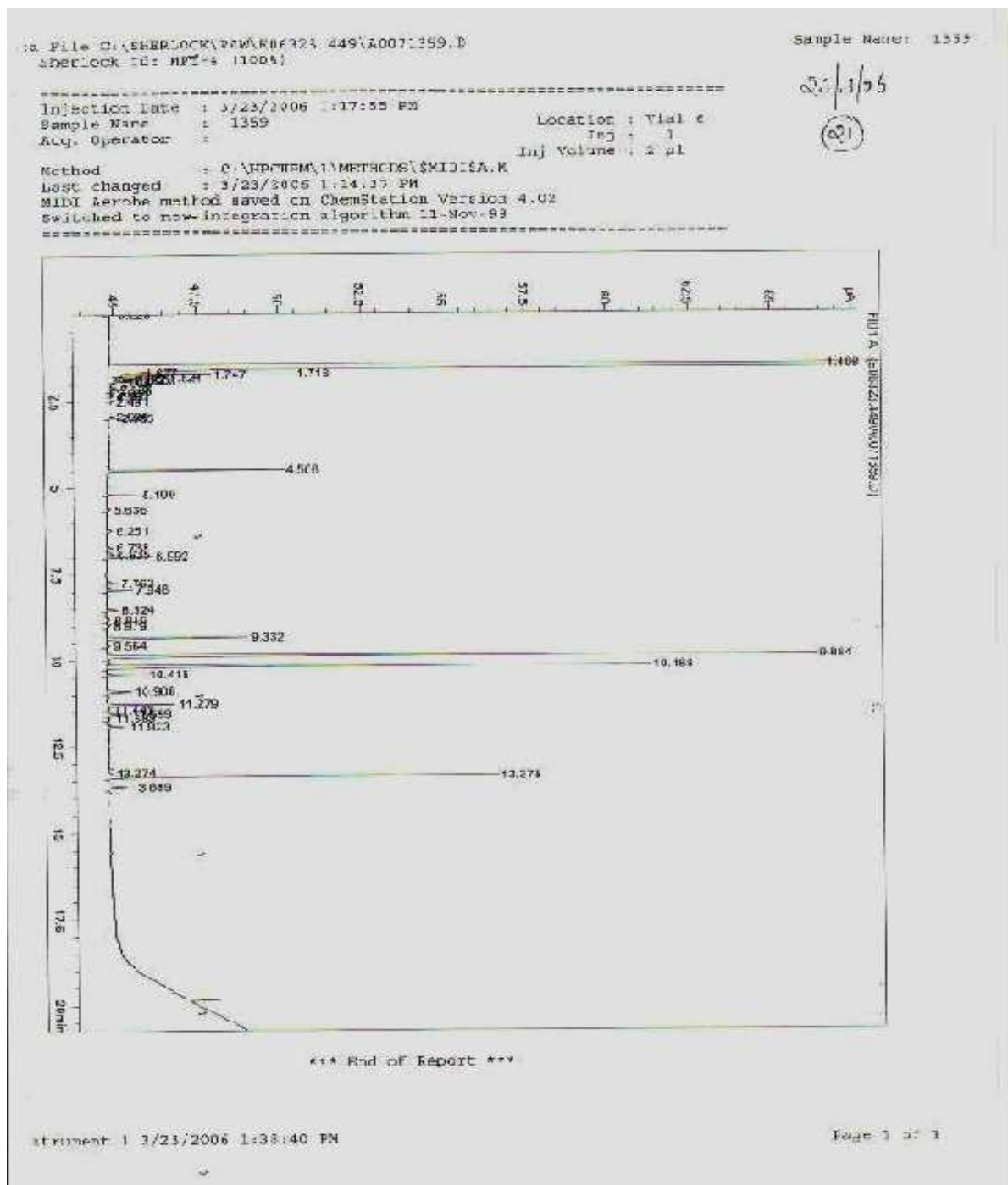
Table 1
Arsenic Uptake by cells of strain MPT4-Time course studies

Growth Media	Intracellular arsenic (ppb/mg protein)	Intracellular arsenic (ppb/mg protein)	Intracellular arsenic (ppb/mg protein)	Intracellular arsenic (ppb/mg protein)	Intracellular arsenic (ppb/mg protein)
	0 h	6 h	12 h	18 h	24 h
NB	0	230	296	986	1360
SBMLP	0	1686	1691	1952	2112

NB= Nutrient broth ; SBMLP= Sea water based minimal medium with limiting phosphate



Figure 3
Gas Chromatogram of Fatty acids of the strain





MPT4

E063234.49A [1359] MPT-4 (100%)

23/3/06
21

Page 1

Volume: DATA File: E063234.49A Seq Counter: 7 ID Number: 1359
Type: Samp Bottle: 6 Method: TSBA40
Created: 3/23/2006 1:14:36 PM
Created By: administrator
Sample ID: MPT-4 (100%)

Profile:

RT	Response	AsHt	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.025	481	0.021	---	4.013			< min rt	
1.498	4.636E+8	0.025	---	7.043	SOLVENT PEAK		< min rt	
1.677	489	0.015	---	7.412			< min rt	
1.718	12600	0.019	---	7.494			< min rt	
1.747	7640	0.024	---	7.555			< min rt	
1.821	3689	0.019	---	7.707			< min rt	
1.856	1314	0.018	---	7.779			< min rt	
1.882	980	0.018	---	7.832			< min rt	
1.908	1834	0.018	---	7.887			< min rt	
1.965	242	0.025	---	8.006			< min rt	
2.127	623	0.027	---	8.339			< min rt	
2.166	533	0.028	---	8.419			< min rt	
2.261	310	0.027	---	8.615			< min rt	
2.351	254	0.022	---	8.800			< min rt	
2.491	111	0.014	---	9.088			< min rt	
2.935	260	0.020	1.130	10.000	10:0	0.09	ECL deviates 0.000	Reference -0.011
2.985	771	0.025	---	10.077				
4.506	18050	0.027	1.050	12.001	12:0	5.70	ECL deviates 0.001	Reference -0.008
5.199	3487	0.028	1.032	12.614	13:0 ISO	1.08	ECL deviates 0.000	Reference -0.008
5.636	391	0.028	1.021	12.999	13:0	0.12	ECL deviates -0.001	Reference -0.009
6.251	875	0.034	1.010	13.455	12:0 3OH	0.27	ECL deviates 0.001	
6.738	867	0.034	---	13.813				
6.935	1513	0.045	0.998	13.959	unknown 13.957	0.45	ECL deviates 0.002	
6.992	6023	0.034	0.997	14.000	14:0	1.81	ECL deviates 0.000	Reference -0.008
7.760	1807	0.039	0.987	14.502	unknown 14.502	0.54	ECL deviates 0.000	
7.946	3235	0.032	0.985	14.623	15:0 ISO	0.96	ECL deviates 0.000	Reference -0.007
8.524	1778	0.038	0.978	15.000	15:0	0.52	ECL deviates 0.000	Reference -0.008
8.816	860	0.048	---	15.176				
8.979	539	0.026	---	15.274				
9.333	21166	0.038	0.970	15.486	Sum In Feature 2	6.17	ECL deviates -0.002	14:0 3OH/16:1 ISO 1
9.564	567	0.035	0.968	15.625	16:0 ISO	0.16	ECL deviates -0.002	Reference -0.010
9.884	107580	0.039	0.965	15.817	Sum In Feature 3	31.21	ECL deviates -0.005	16:1 w7e/15 iso 2OH
10.189	82372	0.038	0.962	16.000	16:0	23.83	ECL deviates 0.000	Reference -0.008
10.415	6148	0.042	0.960	16.131	15:0 ISO 3OH	1.77	ECL deviates -0.003	
10.908	3735	0.040	0.956	16.416	ISO 17:1 w9e	1.07	ECL deviates 0.000	
11.279	10526	0.040	0.953	16.630	17:0 ISO	3.02	ECL deviates 0.000	Reference -0.008
11.443	479	0.036	0.952	16.724	17:0 ANTEISO	0.14	ECL deviates 0.001	Reference -0.007
11.559	3420	0.045	0.951	16.791	17:1 w8e	0.98	ECL deviates -0.001	
11.683	764	0.038	0.950	16.862	17:1 w8e	0.22	ECL deviates 0.002	
11.923	2928	0.041	0.948	17.001	17:0	0.83	ECL deviates 0.001	Reference -0.007
13.274	561	0.037	0.938	17.765	18:1 w9e	0.16	ECL deviates -0.004	
13.375	64073	0.043	0.938	17.823	18:1 w7e	18.07	ECL deviates 0.000	
13.689	2967	0.042	0.935	18.000	18:0	0.83	ECL deviates 0.000	Reference -0.009
----	21166	---	---	---	Summed Feature 2	6.12	12:0 ALDIE 7	unknown 10.928
----	---	---	---	---	---	---	16:1 ISO 17:4:0 3OH	14:0 3OH/16:1 ISO 1
----	107580	---	---	---	Summed Feature 3	31.21	16:1 w7e/15 iso 2OH	15:0 ISO 2OH/16:1 w7e

ECL Deviation: 0.002 Reference ECL Shift: 0.008 Number Reference Peaks: 13
Total Response: 347854 Total Named: 344706
Percent Named: 99.09% Total Amount: 332598

Matches:
Library Sim Index Entry Name
TSBA40 4.10 0.671 Aeromonas-caviae

Sherlock Version 4.0B

Printed on 23-Mar-2006

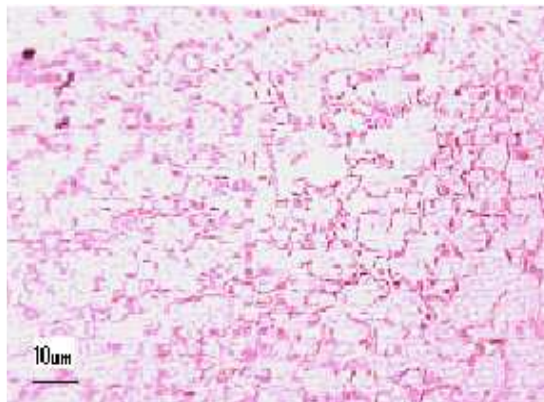


Figure 5
Light microscopy of Gram stained Cells of
***Aeromonas caviae* MPT4 under oil immersion (100X)**

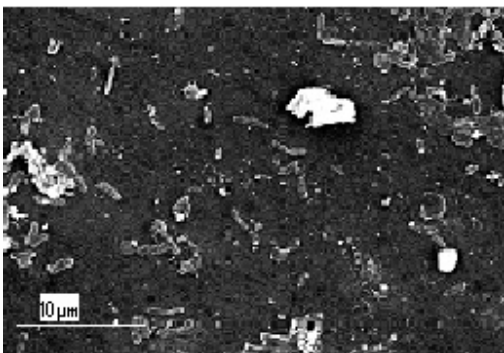


Figure 6
Scanning electron micrograph of
***caviae* sp. MPT4_(3,500X resolution)**

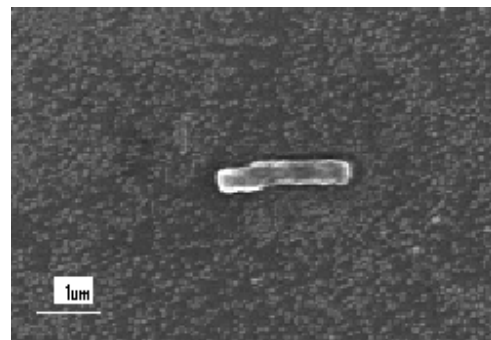


Figure 7
Scanning electron micrograph of *Aeromonas*
***caviae* sp.MPT4 (20000X Resolution)**

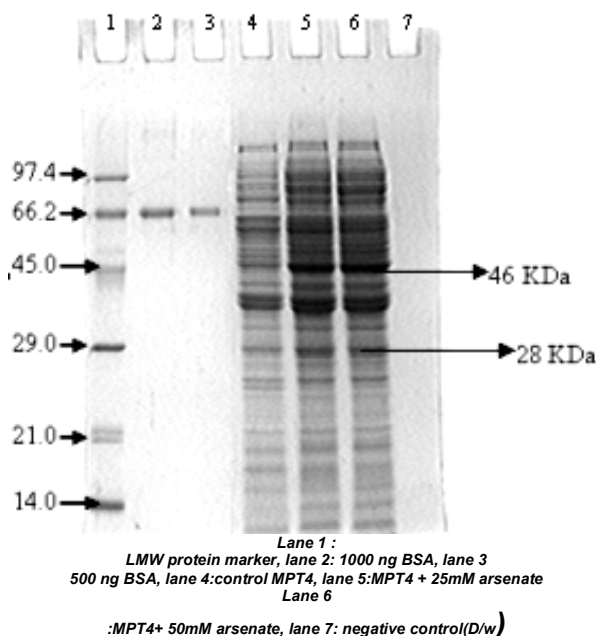
MIC of arsenate /arsenite and Growth behaviour of the strain at various levels of arsenate and arsenite:

The strain MPT4 showed no effect on its growth at 25 mM of arsenate (data not shown) but the growth was slightly reduced in case of 50mM and 100mM arsenate in Mineral Salts Medium containing 0.4% glucose as carbon source (Fig 4a). A significant reduction in growth was

observed in 150mM arsenate containing medium whereas little growth could be observed in the presence of arsenate at a concentration of 200 mM, being the MIC. In case of arsenite the isolate showed a better growth response at 2mM concentration (Fig 4b).

The time course study of arsenate uptake of *Aeromonas punctata* strain MPT4 clearly revealed that uptake of arsenic is 5-6 fold in nutrient broth whereas in SBMLP it was approx. 1.2 times

Figure 4
SDS-PAGE analysis of total cytoplasmic protein induced by Arsenate in *Aeromonas caviae* strain MPT4



Bioaccumulation of arsenic (HG-AAS analysis):

Significantly high levels of arsenic were detected in the supernatant fraction of sonicated cells, which were grown upto 24 hours in nutrient broth as well as sea water based minimal medium with limiting phosphate (SBMLP, with 6µM phosphate) separately and were washed twice with 50mM Tris-Cl buffer (pH 7.5). Interestingly, this isolate bioaccumulates 55% more arsenic i.e. 2112 ppb per mg total cytoplasmic protein in phosphate limiting Minimal medium (SBMLP) against in 1360 ppb in Nutrient broth after 24 hours of incubation at 28 ± 2 °C, 160 rpm. These findings clearly indicate that *Aeromonas caviae* strain MPT4 can be successfully used for bioremediation of arsenic contaminated aquatic environment.

SDS-PAGE analysis of Arsenate induced proteins:

Total protein analysis on of the isolate revealed three induced protein bands observed on 12% SDS PAGE when the strain MPT4 was grown in

presence of 25mM and 50 mM sodium arsenate. These polypeptides have got the size of 28KDa, 36KDa and 46 KDa respectively.

PCR analysis of genomic and plasmid DNA for *ars C* gene :

PCR mediated screening of arsenate reductase gene using 4 sets of commonly used universal primers for *arsC* gene in gram negative bacteria and genomic/plasmid DNA of *Aeromonas punctata* strain MPT4 as template clearly confirmed that this arsenate hyperaccumulating bacterial isolate does not possess the *arsC* gene (data not shown). Since these polypeptides are arsenate induced, they may possibly have some role in arsenic accumulation and detoxification.

CONCLUSION

The *Aeromonas punctata* strain MPT4 is a arsenate hyper-accumulating strain, resistant to



very high levels (150 mM) of arsenate and arsenite (6mM). This is the first report of arsenate bioaccumulation in a mesophilic bacterium, *Aeromonas punctata* strain MPT4. The results of the study contribute to a great extent towards an understanding of the mechanism of arsenic resistance in this bacterium and clearly indicates that this strain can be successfully employed for environmental bioremediation of arsenate contaminated marine sites.

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