Seminar attended
Workshops/ seminars attended and abstracts accepted

1. 10th International symposium on *Aeromonas* and *Plesiomonas*, May 2011, held at Galveston, Texas.

2. UGC sponsored national level symposium on “Pollution: Disbalancing the Global Environment: Threats and Prevention”, May 2011 held at Bolpur college, Burdwan University, India.

3. 37th Annual conference of Indian Immunology Society, February 2011, held at University of Jammu, India.


5. International conference on global climate change, 2010, in Centre for Environmental Studies, Visva-Bharati University, India.

6. Regional workshop on environmental engineering, 2008, in Centre for Environmental Studies, Visva-Bharati University, India.

7. 34th Annual Conference of the Indian Immunology Society, December 2007, in National AIDS Research Institute, Pune, India.

Publications
Effect of acute and chronic arsenic exposure on growth, structure and virulence of *Aeromonas hydrophila* isolated from fish

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**Article info**

**Abstract**

*Aeromonas hydrophila* being a ubiquitous bacterium is prone to arsenic exposure. The present study was designed to determine the role of arsenic on growth and virulence of *A. hydrophila*. Exposure to arsenic (1 mg L\(^{-1}\) and 2 mg L\(^{-1}\)) had no effect on growth but significantly inhibited the hemolytic and cytotoxic potential of exposed bacteria. Transmission electron microscopy revealed loss of membrane integrity and presence of condensed cytoplasm suggestive of acute stress in bacteria exposed to arsenic. Arsenic-adapted bacteria were developed by repeated sub-culturing in presence of arsenic. Arsenic-adaptation led to significant recovery in hemolytic and cytotoxic potential. The arsenic-adapted bacteria exhibited normal membrane integrity, decreased cytoplasmic condensation and possessed scattered polysome like structures in the cytoplasm. A positive correlation was observed between arsenic tolerance and resistance to several antimicrobials. Arsenic-adaptation failed to confer cross-protection to mercury and cadmium stress. SDS-PAGE analysis revealed the expression of two new proteins of approximately 85 kDa and 79 kDa respectively in arsenic-adapted bacteria. The expression of these proteins was confirmed by Western blotting. Arsenic-adaptation failed to confer cross-protection to mercury and cadmium stress. SDS-PAGE analysis revealed the expression of two new proteins of approximately 85 kDa and 79 kDa respectively in arsenic-adapted bacteria. The expression of these proteins was confirmed by Western blotting.

**Keywords:**

Arsenic

*Aeromonas hydrophila*

Pathogenicity

**1. Introduction**

Arsenic, a naturally occurring semi-metal and found in soil, air and water is a 'top priority global pollutant'. Environmental arsenic can exist in both organic and inorganic forms, the latter being more toxic to health. Inorganic arsenic is mostly present in the form of arsenite (As\(^{3+}\)) and arsenate (As\(^{5+}\)) of which the arsenite form is more toxic [1]. The effect of arsenic on higher animals is well documented and found to depend on both endogenous (membrane oxygen uptake and efflux pumps) and exogenous factors (mobility of arsenic forms) [2]. It has been reported that As\(^{5+}\) acts as an analogue of phosphate and disturbs critical cellular functions which includes uncoupling of ATP phosphorylation, while As\(^{3+}\) interferes with enzyme functions through covalent bond formation with sulfhydryl groups [1].

Our knowledge on the effect of arsenic on bacteria is poor. Although arsenic is detrimental to most organisms, bacteria are known to have evolved mechanisms to overcome the toxic effects of this pollutant; these include oxidation, reduction and methylation reactions which in turn can affect arsenic speciation in the environment [3,4]. Earlier workers have suggested the involvement of *ars* operon [5] and *As*\(^{\text{S}+}\) reductase complex (arr AB) [6] in the process. Some bacteria have specific efflux pumps to extrude arsenic from the cytoplasm thereby lowering the toxic effects of arsenic within a cell [1]. The absence or poor expression of aquaglyceroporins on the cell surface also interferes with the entrance of arsenic, thus enabling bacteria to survive in arsenic rich environments [6,7]. It has been observed that the gene(s) for resistance to arsenic in bacteria can be either chromosomal or plasmid encoded [1].

Information on the effects of environmental stress on bacterial life-cycle and virulence is important. Environmental stresses such as starvation, acid and heat shock have significant effect on the growth and physiology of bacteria [8–10]. Metal ions are widely distributed in nature and exposure to high concentrations could influence the growth and virulence of bacteria. *Vibrio parahaemolyticus* grown in the presence of cadmium was found to
withstand thermal and osmotic stress [11]. Mercury increases pigmentation in bacteria of the marine air-water interface [12]. In *Micrococcus luteus*, lead exposure was found to cause a moderate drop in lipid content of the cell accompanied by a noticeable decrease in cellular pigmentation [13]. It has also been confirmed that metal ions induce multiple antimicrobial resistance or Mar phenotype in several bacteria [14,15].

*Aeromonas hydrophila* is a Gram-negative facultative intracellular bacterium with wide range of host infectivity [16]. The effect of environmental stress on *Aeromonas* spp. is not well documented though few reports suggested the influence of environmental factors like temperature, salinity and pH on the pathogenicity of this bacterium [17–19]. Among the different metals ubiquitously distributed in nature arsenic appears to be the most common [1,2,5,7]. Consequently, the chances of bacteria getting exposed to this toxicant are higher than most other metals. To the best of our knowledge there is no information available on arsenic-microbe interactions affecting bacterial physiology. In the present study the effect of arsenic on growth and pathogenicity of *A. hydrophila* has been studied. *A. hydrophila* was selected as it is reported from arsenic prone areas [20,21], autochthonous to aquatic systems and ubiquitous at varying temperatures, salinity and nutritional conditions [22], pathogenic in nature and due to its well characterized virulence attributes and wide host range [16].

2. Results

2.1. Effect of arsenic on bacterial growth

*A. hydrophila* isolates were grown in presence of different concentrations of arsenic and growth monitored at different time intervals. It was observed that arsenic did not affect bacterial growth as both control and exposed wild-type *A. hydrophila* (Wt-Ah) isolates could attain log phase within 2 h, mid log phase between 4 and 6 h, late-log phase between 10 and 14 h and the stationary phase following 28 h of incubation at 30 °C (Fig. 1a and b). The growth curves of arsenic-adapted isolates (Ad-Ah) showed a similar trend as observed with control and exposed isolates (Supplementary Fig. 1). The *A. hydrophila* isolate possessed a 7.5 kb plasmid (Supplementary Fig. 2). To determine whether arsenic resistance trait was plasmid encoded the wild-type plasmid bearing (Wt-Ah) and plasmid cured (C-Ah) isolates were grown in presence of arsenic and growth monitored at regular intervals. It was observed that the C-Ah isolates grew efficiently in presence of arsenic (Fig. 1c and d). The transformed (T-Ah) isolates had the same growth characteristics as observed with wild-type control and exposed isolates (data not shown).

2.2. Effect of arsenic on virulence of *A. hydrophila*

2.2.1. Changes in hemolytic potential

Hemolytic potential is an important virulence attribute of pathogenic bacteria. To determine the effect of arsenic on hemolytic potential, *A. hydrophila* (Wt-Ah) isolates could attain log phase within 2 h, mid log phase between 4 and 6 h, late-log phase between 10 and 14 h and the stationary phase following 28 h of incubation at 30 °C (Fig. 1a and b). The growth curves of arsenic-adapted isolates (Ad-Ah) showed a similar trend as observed with control and exposed isolates (Supplementary Fig. 1). To determine whether arsenic resistance trait was plasmid encoded the wild-type plasmid bearing (Wt-Ah) and plasmid cured (C-Ah) isolates were grown in presence of arsenic and growth monitored at regular intervals. It was observed that the C-Ah isolates grew efficiently in presence of arsenic (Fig. 1c and d). The transformed (T-Ah) isolates had the same growth characteristics as observed with wild-type control and exposed isolates (data not shown).
Changes in cytotoxic potential

Arsenic induced alterations in bacterial cytotoxic potential were studied using head kidney macrophages (HKM) as target cells. It was observed that compared to control bacteria, the ability to induce macrophage cytotoxicity was significantly reduced \((p < 0.05)\) in the arsenic-exposed isolates with maximum reduction observed in bacteria exposed to 2 mg L\(^{-1}\) of arsenic \((p < 0.05)\) (Fig. 2b). The Ad-Ah isolates demonstrated a significant improvement in cytotoxic potential \((p < 0.05)\).

Changes in antimicrobial sensitivity and metal tolerance

The effect of arsenic on antimicrobial sensitivity was studied in \(A.\) hydrophila. It was observed that exposure to arsenic decreased the sensitivity to roxithromycin, co-trimoxazole, chloramphenicol and doxycycline suggesting a role of arsenic in modulating antimicrobial sensitivity in \(A.\) hydrophila (Table 1). Compared to arsenic-exposed isolates no significant difference in sensitivity to the different antimicrobials used was noted in the Ad-Ah isolates. To check whether arsenic could induce cross-protection to other metals, the \(A.\) hydrophila isolates were incubated in presence of MIC of mercury \((\text{Hg}, \ 12 \ \text{mg} \ \text{L}^{-1})\) and cadmium \((\text{Cd}, \ 350 \ \text{mg} \ \text{L}^{-1})\) and the changes in bacterial growth monitored. It was observed that arsenic could not confer cross-protection against mercury or cadmium in arsenic-exposed or Ad-Ah isolates.

2.3. Arsenic induced ultra-structural changes in \(A.\) hydrophila

Arsenic induced structural alterations in \(A.\) hydrophila were studied under TEM. It was observed that on exposure to arsenic the isolates underwent structural alterations characterized by loss of membrane integrity and cytoplasmic condensation which appeared to be more pronounced in bacteria exposed to 2 mg L\(^{-1}\) of arsenic. However, the Ad-Ah isolates appeared to have regained membrane integrity and had dispersed cytoplasm with scattered polysome like structures (Fig. 3).

2.4. Arsenic induced changes in \(A.\) hydrophila proteins

To determine the arsenic induced alterations in protein expression, \(A.\) hydrophila were grown in the presence of different concentrations of arsenic and the protein profile analyzed. No difference in the protein profile was observed in control and arsenic-exposed isolates. However, in the Ad-Ah isolates the expression of two new proteins of approximate molecular weights 85 kDa and 79 kDa were observed (Fig. 4).

3. Discussion

In the present study our objective was to determine the effect of arsenic on the growth and virulence of \(A.\) hydrophila. The concentrations of arsenic selected for this study have been reported in the Indo-Bangladesh region \([23]\) and also used for studies on bacteria \([24]\). When \(A.\) hydrophila was grown in the presence or absence of different concentrations of arsenic no difference in their growth profiles was observed. It is interesting to note that the \(A.\) hydrophila isolate used in the study was collected from arsenic free area and grew efficiently in the presence of arsenic suggesting that arsenic-resistant bacteria might be more widespread than generally thought. Although it is premature to conclude from this study the existence of early “bio-available” arsenic in this area or the bacteria had been pre-exposed to arsenic and migrated there, our observations support the notion that the genetic mechanisms for xenobiotic resistance are old and evolutionarily conserved \([6,7]\). We also observed no significant difference in the growth of arsenic-adapted and un-adapted bacteria which suggests arsenic resistance could be common in bacteria found in natural environments, regardless of the presence or absence of arsenic \([25]\).

It is well known that toxic metals select variants possessing genetic resistance determinants which confer the ability to tolerate higher levels of the toxic compounds \([1]\). It has also been suggested that in bacteria the genes conferring resistance to heavy metals are usually plasmid encoded \([7]\). The \(A.\) hydrophila isolate selected for our study possessed a plasmid and we were interested to determine whether arsenic resistance is plasmid encoded or chromosomal for the bacterium. We observed that compared to the wild-type plasmid bearing isolates the cured isolates grow slowly whether grown in medium supplemented with or without arsenic and reach stationary phase later. As the cured isolates could grow in exactly the same manner in presence or absence of arsenic our study suggests that plasmid has a general role in regulating bacterial growth not arsenic resistance because removal of the plasmid resulted in slow growth in \(A.\) hydrophila irrespective of whether arsenic is present or absent in the media. There are several studies which have suggested a general role of plasmid on the growth of several bacteria \([26–28]\). It was also noted that the transformed isolates also had same growth profile as observed with natural isolates when grown in presence of arsenic (data not shown). Thus, it is evident from our study that arsenic resistance trait is chromosomal in the isolate and supports earlier findings.
that have reported arsenic resistance may have chromosomal loci also [16].

The role of xenobiotics on bacterial virulence is not well studied. The present study was performed with the aim of studying the effect of arsenic on the virulence of *A. hydrophila*. Hemolysin production and cytotoxic potential are two well characterized virulence factors of *A. hydrophila* [28,29]. Our preliminary in vitro and in vivo studies had revealed that the *A. hydrophila* isolate induced hemolytic plaques on blood agar plates, caused the lysis of fish erythrocytes, was cytotoxic to CHO cells and HKM and when introduced into healthy fish led to the development of hemorrhagic ulcers and death (data not shown). This clearly indicated the isolate to be pathogenic and possessed hemolysin and cytotoxic traits. It was observed that arsenic significantly inhibited the hemolysin and cytotoxic potential of *A. hydrophila*. However, arsenic-adaptation helped the isolates in regaining hemolysin and cytotoxic potential significantly. Taken together our data suggests the presence of an inherent mechanism of adaptation in *A. hydrophila* which helps it to withstand the toxic effects of arsenic and regain its virulence potential. This is the first report on the role of arsenic in modulating bacterial virulence and this information is important since increased arsenic contamination can result in new selective pressures which might affect microbial-arsenic resistance and pathogenicity.

Earlier studies had reported that exposure to elevated concentrations of lead induced ultra-structural alterations and cytoplasmic leaking in *A. hydrophila* [30]. Although, *A. hydrophila* have been frequently isolated from arsenic contaminated areas [20,21] there is no information on the effect of arsenic on the structure of this bacterium. We observed that exposure to arsenic led to loss of membrane integrity and cytoplasmic condensation indicative of acute stress. On the other hand, the re-appearance of normal membrane integrity along with dispersed cytoplasm and scattered polysome like structures in arsenic-adapted bacteria indicate chronic exposure to arsenic induces structural and functional changes which helps the bacteria to withstand the deleterious effects of this xenobiotic. Interestingly de novo expression of two new proteins was also observed in arsenic-adapted isolates. Arsenic has been reported to induce the synthesis of stress proteins [1,31]. We are currently engaged in purifying the two proteins, sequence them and determine their role in the bacterial stress responsiveness to arsenic.

Resistance to metals and antimicrobials helps the bacteria to survive and spread under limiting conditions. Metal resistant bacteria have been reported for long and a relation between metal resistance and antimicrobial resistance appears to exist in bacteria as metal resistant isolates have been observed to be associated with single or multiple drug resistance [32]. Clear correlations between resistance to penicillin, erythromycin, tetracycline and tolerance to mercury, lead, cadmium and zinc have been reported in several bacteria [33,34]. It was also noted that vanadium induced multiple antimicrobial resistance or the Mar phenotype in the environmental isolates of *Escherichia hermannii* and *Enterobacter cloacae* [14]. Arsenic has also been reported to induce Mar phenotype in environmental isolates of *Versinia enterocolitica* [15]. Interestingly, there are no reports on the role of arsenic on antimicrobial or metal resistance in *A. hydrophila*. In the present study we report for the first time that arsenic increased the resistance to several structurally and functionally different antimicrobials in *A. hydrophila*. It has been suggested that arsenic can enhance the expression of cryptic efflux pumps and together with the reduced expression of outer

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**Table 1**

<table>
<thead>
<tr>
<th>Anti-microbials/Metals</th>
<th>CFU mL⁻¹ (18 h post incubation)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<tr>
<td></td>
<td>1 (exposed)</td>
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<td>Ro</td>
<td>75</td>
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<td>Co</td>
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<td>Do</td>
<td>45</td>
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<tr>
<td>Hg</td>
<td>12 × 10⁵</td>
</tr>
<tr>
<td>Cd</td>
<td>350 × 10⁵</td>
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</table>

Fig. 3. Arsenic induced ultra-structural changes in *A. hydrophila*, TEM figures of unexposed, 2 mg L⁻¹ arsenic-exposed and adapted (Ad-Ah) isolates. a) *A. hydrophila* grown in absence of arsenic; b) *A. hydrophila* grown in presence of 2 mg L⁻¹ arsenic; c) Ad-Ah grown in presence of 2 mg L⁻¹ arsenic. pm, plasma-membrane; c, cytoplasm; dpm, damaged plasma-membrane; cc, condensed cytoplasm; dc, dispersed cytoplasm; p, polysome like structure.
membrane proteins involved in the transport of antimicrobials could help in conferring antimicrobial resistance to bacteria [2,14,35]. Although from the present study it is not possible to define the exact mechanisms by which arsenic enhanced antimicrobial resistance in A. hydrophila our observations assume particular significance in studying microbe-antimicrobial relationship in arsenic prone areas. We further observed that exposure to arsenic failed to cross-protect the A. hydrophila isolates from mercury and cadmium stress. Thus, it appears that the mechanism of arsenic tolerance could be distinct from that against mercury and cadmium in A. hydrophila.

It can be concluded that arsenic can interfere with the virulence of bacteria. It suppresses several important virulence attributes but increases resistance to antimicrobials. However, the bacteria have also evolved mechanisms to withstand arsenic stress and regain the pathogenic potential. We believe that these observations would be useful for a better understanding of metal-microbe interactions and in designing antimicrobial strategies in arsenic prone areas.

4. Materials and methods

4.1. Animal care and maintenance

Clarias batrachus (50–70 g) with no history of infections were obtained from local breeders and maintained in 50 L glass tanks (five to six in each tank) under natural photoperiod [36]. The fish were kept for acclimatization for a period of 15 days before the start of the experimental work. During this period fish health was monitored both by appearance and pathological examinations. For pathological examinations 1–2 fish were sacrificed at regular intervals, the abdomen opened and checked for signs of infections and different organs plated on nutrient agar or potato-dextrose agar plates to determine the presence of bacterial and fungal growth respectively.

4.2. Preparation of arsenic stock

Arsenic stock solution (100 mM) was prepared by dissolving arsenic trioxide (As$_2$O$_3$, Sigma) in dilute acidic (HCl) distilled water and the pH was adjusted to 7.0 by adding 1 N NaOH [36]. The final solution was filtered and stored in room temperature and different working dilutions (1 mg L$^{-1}$, 2 mg L$^{-1}$) prepared in brain heart infusion broth (As-BHI).

4.3. Bacterial strains

Aeromonas hydrophila used in the study was a pathogenic isolate recovered from infected fish from a water body in Birbhum, West-Bengal which is reported to be arsenic free area in India (http://www.soesju.org/arsenic/wb.htm). In order to isolate the bacteria, fish were anesthetized with MS-222 (100 mg mL$^{-1}$, Sigma) and surface sterilized by dipping in 0.1% HgCl$_2$. The infected tissue was removed aseptically, homogenized in autoclaved saline (0.6%) and plated on nutrient agar (NA) plates supplemented with 100 µg mL$^{-1}$ of ampicillin. Following overnight incubation at 30 °C the isolated colonies were transferred to blood agar plates (HiMedia, India) containing 100 µg mL$^{-1}$ ampicillin and further incubated overnight at 30 °C. The single colonies appearing on the blood agar plates turned black following treatment with 1% sodium dimethyl–p-phenylenediamine monohydrochloride [37]. The bacteria were Gram-negative rods, oxidase and catalase positive and could grow on A-H medium [38] and Aeromonas selective medium (HiMedia, India). The identity of the isolates was confirmed using API 20E kit (bioMerieux, Inc) and other biochemical tests specific for A. hydrophila identification [39,40] and on agglutination with rabbit polyclonal anti-sera raised against reference A. hydrophila strain (strain 646) obtained from MTCC, Chandigarh, India. The isolate was grown on brain heart infusion broth (BHI) at 30 °C and maintained on nutrient agar (NA) slants at 4 °C.

4.4. Plasmid isolation and curing

A. hydrophila isolate used in the study is resistant to nalidixic acid. It was grown at 30 °C in the presence of 75 µg mL$^{-1}$ of nalidixic acid (Na, Himedia) and centrifuged at 10 000 × g for 10 min at 4 °C. The plasmid DNA was isolated by the alkaline lysis method [41], electrophoresed on 0.8% agarose gel in presence of molecular weight markers (λ DNA Hind-III/EcoRI, Gibco) and visualized after staining with 0.5% ethidium bromide (EtBr; Sigma) [42]. To cure the
plasmid, an aliquot from actively growing culture at 30 °C was transferred to fresh BHI maintained at 42 °C and left overnight without shaking [43]. The process was repeated twice, the bacterial culture diluted and plated on NA plates. The single colonies obtained were checked for the presence of the plasmid and screened for susceptibility to Na (20 μg mL−1) by replica plating [43]. The identity of Na sensitive colonies was checked and presence of plasmid studied by agarose gel electrophoresis. The plasmid cured isolates are henceforth termed as cured strains (C-Ah). The C-Ah isolates thus obtained were made competent by calcium chloride method [42] and transformation of the isolated plasmid into cured competent cells was carried out [44]. The identity of transformed bacteria (T-Ah) was confirmed by the same biochemical and serological techniques described above. The presence of plasmid DNA in the transformed bacteria was checked by agarose gel electrophoresis [28].

4.5. Bacterial growth

A. hydrophila were grown to late-log phase (12 h) at 30 °C in BHI, centrifuged at 10 000 × g for 15 min at 4 °C, the pellet washed thoroughly and re-suspended in phosphate buffered solution (PBS, pH 7.4). Equal number of wild-type bacteria (Wt-Ah) was added to BHI containing 1 mg L−1 and 2 mg L−1 of arsenic respectively (As-BHI) maintained at 30 °C and growth monitored at regular intervals of 600 nm. Arsenic-adapted bacteria (Ad-Ah) were developed by regularly passaging A. hydrophila in As-BHI media containing 1 mg L−1 and 2 mg L−1 of arsenic separately for more than 100 generations and their growth monitored as described above. In a parallel study the C-Ah and T-Ah isolates were grown in presence of different concentrations of arsenic and growth monitored at regular time intervals. The results represent mean ± S.E. of three independent observations.

4.6. Hemolysin assay

To determine the effect of arsenic on hemolytic potential of A. hydrophila, red blood cells (RBC) were collected from healthy C. batrachus in presence of EDTA. The assay mixture contained 3.7 mL PBS, 0.8 mL cell free supernatant obtained from the late-log phase (12 h) of exposed and Ad-Ah isolates respectively and 0.5 mL of fresh RBC [28]. As-BHI was used for studying the background hemolysis. The assay mixture was incubated at 30 °C for 5 h, debris removed by centrifugation at 400 × g at 4 °C and the absorbance read at 541 nm. One unit of hemolytic activity was defined as the activity in 0.8 mL filtrate to produce a supernatant having an absorbance of 1.0 at 541 nm [28]. The results represent mean ± S.E. of three independent observations.

4.7. Cytotoxicity assay

Cytotoxicity assays were done on head kidney macrophages (HKM) from C. batrachus. Briefly, head kidney leucocyte suspensions obtained from individual fish was individually enriched for phagocytes using a 34–51% percoll density gradient. Following centrifugation at 400 × g for 20 min at 4 °C, the phagocyte rich fraction appearing above the 34–51% interface was collected, washed and the cell number adjusted to 1 × 106 mL−1 in RPMI-1640 containing 10% fetal bovine serum (Gibco) supplemented with 1% penicillin-streptomycin (complete RPMI) and left overnight at 30 °C under 5% CO2 for adherence to sterile 90 mm petri-dishes. The non-adherent cells were removed carefully and the adherent macrophages obtained by incubation with 1% cell dissociation medium (Sigma) at 30 °C for 20 min. The purity of the cells was checked by staining with Wright-Giemsa Stain [36] and incubated separately in complete RPMI with live exposed and Ad-Ah isolates collected at late-log phase, at a multiplicity of infection (MOI) of 5 for 1 h at 30 °C. The cells were washed in RPMI-1640 containing 30 μg mL−1 chloramphenicol to remove the extra-cellular bacteria. The concentration of chloramphenicol used had no effect on HKM viability. The infected macrophages were distributed into each well (1 × 105 cells well−1) of 96 well flat bottom tissue culture plates (Nunc), incubated at 30 °C under 5% CO2 and their viability checked by trypan blue (0.5%) dye exclusion method 15 h post incubation and expressed in percent value. The results represent mean ± S.E. of three independent observations.

4.8. Antimicrobial sensitivity and metal resistance

The minimal inhibitory concentration (MIC) of the isolate for different antimicrobials as well as for mercury and cadmium was determined in 96-well microtiter plates by the twofold standard broth micro-dilution method in M-H broth (HiMedia) using an inocula of 1 × 105 cells mL−1 and the results interpreted following CLSI guidelines [45]. Live exposed and Ad-Ah isolates were grown in As-BHI containing MIC of different antimicrobials, mercury and cadmium and the changes in CFU were enumerated following overnight incubation at 30 °C. The results represent mean ± S.E. of three independent observations.

4.9. Transmission electron microscopy (TEM)

A. hydrophila were grown to late-log phase in presence of arsenic and fixed in 2.5% glutaraldehyde (Polaron Biorad) in 0.1 M phosphate buffer (pH 7.4) for 2 h. The fixed bacteria were further incubated for 1 h in 1% OsO4 (Sigma), dehydrated through graded series of ethanol and propylene oxide (Merck) and embedded in Epon 812 (TAAB), Ultra-thin sections (60 nm) were placed on nickel grids (Sigma) stained with uranyl acetate (BDH) and lead citrate (Polaron) and examined under Tecnai 12 Bio-Twin transmission electron microscope (FEI, The Netherlands) operating at 80 kV. The figure is representative of three replicate observations.

4.10. Electrophoretic studies

A. hydrophila isolates were grown in presence or absence of arsenic to late-log phase at 30 °C and harvested by centrifugation at 10 000 × g at 4 °C for 15 min. Bacteria were lysed in the presence of protease inhibitors (Sigma); the lysate centrifuged at 10 000 × g for 15 min at 4 °C, total protein content of the supernatant determined [46] and used for one-dimensional SDS-PAGE (10%) analysis along with molecular weight markers. The protein bands present in the gel were identified by silver staining and the molecular weights determined. The figure is representative of six replicate observations.

4.11. Statistical analysis

Results are expressed as mean ± S.E. calculated for each parameter considered in the present study. Pair wise comparison was also done between unexposed and arsenic-exposed group by employing paired t-test to determine the statistical significance between the groups. The value of p < 0.05 was considered statistically significant.

4.12. Policy and ethics

All experiments were carried out according to the procedures laid down by the Visva-Bharati University Animal Ethics Committee.
Conflict of interest statement
The authors do not have any conflict of interest in the present work. The funding agency had no involvement in the selection of site or sampling and choice of publication of the present work.

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Appendix. Supplementary information
Supplementary data related to this article can be found online at doi:10.1016/j.micpath.2010.10.005.

References
Role of virulence plasmid of *Aeromonas hydrophila* in the pathogenesis of ulcerative disease syndrome in *Clarias batrachus*

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Pathogenic *Aeromonas hydrophila* (strain VB21), a multiple-drug resistance strain contains a plasmid of about 21 kb. After curing of plasmid, the isolates became sensitive to antimicrobials, to which they were earlier resistant. The cured bacteria exhibited significant alterations in their surface structure, growth profile and virulence properties, and failed to cause ulcerative disease syndrome (UDS) when injected into the Indian catfish *Clarias batrachus*. Routine biochemical studies revealed that the plasmid curing did not alter the biochemical properties of the bacteria. After transformation of the plasmid into cured *A. hydrophila* the bacterium regained its virulence properties and induced all the characteristic symptoms of UDS when injected into fish. Thus, the plasmid plays a pivotal role in the phenotype, growth and virulence of *A. hydrophila* and pathogenesis of aeromonad UDS.

**Keywords:** Ulcerative disease syndrome, *Aeromonas hydrophila*, *Clarias batrachus*, Catfish, Plasmid, Transformation

*Aeromonas hydrophila* (Aeromonadaceae), a Gram-negative rod-shaped facultative intracellular bacterium with wide aquatic distribution is the causative agent of ulcerative disease syndrome (UDS) in fish\(^1\). It also infects amphibians, reptiles, birds and mammals\(^2\). The pathogenesis of *Aeromonas* is complex and multifactorial\(^3\). Important *A. hydrophila* virulence factors include haemolysins, protease, cholinesterases, enterotoxins, endotoxins and adhesins which together contribute to overall disease progress in fish\(^3,4\).

Plasmids are basically extra-chromosomal DNA material that encodes for several virulence determinants vital to the bacterial host\(^5\). Although plasmids are reported to be present in several fish pathogenic bacteria\(^6,7\), their role in the expression of virulence factors and also in the pathogenicity of *Aeromonas* is poorly understood. *Aeromonas* strains are known to harbor stable plasmids which play an important role in antimicrobial multiple-drug resistance (MDR)\(^7,10\), and virulence\(^11\). Recently, type III secretion system (TTSS) genes which influence pathogenicity of bacteria\(^12\) have been mapped in *A. salmonicida*\(^13\), and found located on a large thermodabile plasmid\(^14\). Loss of this plasmid results in the loss of its virulence potentials\(^14\).

*A. hydrophila* outbreak has a major impact in aquaculture and at present, no vaccine is commercially available to prevent such incidents\(^15\). Under these circumstances, studies on aeromonad plasmids have assumed significance, especially with the advent of MDR strains. In the present study, an attempt has been made to determine the role of plasmids in the virulence and pathogenicity of *A. hydrophila* in the Indian catfish *Clarias batrachus* in vivo.

**Materials and Methods**

*Clarias batrachus* (100-120 g) were maintained in 30 L tanks under laboratory conditions (5-6 fish per tank) with monitored water quality, dissolved oxygen content and pH, and fed on chopped chicken liver *ad-libitum*. The fish were acclimatized for 15 days prior to starting the experiments.

**Bacterial strain and culture conditions**

*A. hydrophila* strain (VB21) was isolated from a naturally infected *C. batrachus* collected from Santiniketan, West Bengal. Identification of bacteria was done using routine biochemical and serological
tests\textsuperscript{16} and from its ability to grow on \textit{Aeromonas} selective medium (HiMedia) or in \textit{A. hydrophila} medium (A-H)\textsuperscript{23}. For all experimental work, bacteria were grown to mid-log phase (11 h) in brain heart infusion broth containing 100 \(\mu\)g mL\(^{-1}\) ampicillin at \(pH\) 7.4 (BHI, HiMedia) overnight at 30\(^\circ\)C under shaking conditions and maintained in nutrient agar slants at 4\(^\circ\)C. The strain induced characteristic UDS lesions when injected in \textit{C. batrachus}.

**Antimicrobial sensitivity profile**

The antimicrobial sensitivity was determined by disc diffusion method based on NCCLS charts. The bacteria were plated on nutrient agar plates and incubated in the presence of different antimicrobials. Inhibition zone with diameter less than 13 mm was considered resistant (R), while zone diameter of 15-16 mm and a clear zone of 17 mm or more were considered as intermediate (I) and sensitive (S), respectively. The antimicrobials were used in test discs (HiMedia) and their concentrations were as follows: gatifloxacin (Gf, 5 \(\mu\)g), ampicillin (A, 10 \(\mu\)g), erythromycin (E, 15 \(\mu\)g), cefuroxime (Cu, 30 \(\mu\)g), chloramphenicol (C, 30 \(\mu\)g) and tetracycline (T, 30 \(\mu\)g).

**Determination of LD\(_{50}\) dose and development of disease model in \textit{C. batrachus}**

\textit{C. batrachus} (100 g body wt) with no history of \textit{A. hydrophila} infection or UDS were maintained under laboratory conditions. The strain was grown to mid-log phase and bacteria enumerated by correlating OD values (600 nm) of the growing culture at different time periods with the corresponding colony forming units (CFU) obtained by plate dilution method (OD 600 nm 1.17 = 3 \times 10^8 mL\(^{-1}\)). Different concentrations of the bacteria ranging from 1 \times 10^3–1 \times 10^{10} cells were injected in 100 \(\mu\)L saline (intramuscular, i.m.) into each group of fish (n = 7) and observed for changes in their behavioral patterns as well as development of hemorrhagic ulcers and tissue necrosis. The viability of the infected fish was checked for 14 days and the LD\(_{50}\) calculated\textsuperscript{18}.

**Plasmid isolation, curing and transformation**

Plasmid DNA isolated from wild-type \textit{A. hydrophila} (Wt-VB21) strain using Quiagen plasmid isolation kit (Quiagen Inc, USA) was purified by precipitation with polyethelene glycol and electrophoresed on 0.8% agarose gel in the presence of molecular weight markers (\(\lambda\) DNA Hind-III/EcoRI, Gibco). Curing of the plasmid was done with ethidium bromide (EtBr) treatment (30 \(\mu\)g mL\(^{-1}\), Sigma)\textsuperscript{16}. The loss of plasmid checked by agarose gel electrophoresis and the identity of cured bacteria (C-VB21) confirmed by routine biochemical tests. To reintroduce the plasmid, the cured isolates were made competent by calcium chloride method\textsuperscript{19} and plasmid DNA transformed into the competent cells\textsuperscript{20}. The identity of the transformed bacteria (T-VB21) was confirmed by agarose gel electrophoresis and routine biochemical tests like Gram staining, glucose fermentation, nitrate reduction, gelatinase and DNAse production, ability to hydrolyze aesculin and Voges-Proskauer (V-P) test. The transformation frequency was found to be 6.4 \times 10^{-7} cell\(^{-1}\).

**Scanning electron microscopy (SEM) studies**

Wild-type, cured and transformed isolates were harvested and fixed in 2.5% glutaraldehyde solution at 4\(^\circ\)C for 30 min. For scanning electron microscopy, 100 \(\mu\)L of each bacterial suspension containing 1 \times 10^8 bacteria was smeared on stub, coated with gold (IB-2 ion coater, Hitachi), air-dried and examined in a Hitachi S530 scanning electron microscope.

**Erythrocyte count and hemoglobin measurements**

Healthy fish were injected (i.m.) with 3 \times 10^6 wild-type, plasmid-cured and transformed isolates in 100 \(\mu\)L saline and the changes in hemoglobin content and total erythrocyte counts determined at different time intervals.

**Hemolytic potential**

Blood agar medium (1%) was prepared with freshly obtained de-fibrinated sheep blood and the isogenic isolates of \textit{A. hydrophila} strain VB21 plated on it separately. \(\beta\)-Hemolysin was observed as a transparent zone surrounding each bacterial colony following incubation at 30\(^\circ\)C for 18 h.

**Cytotoxicity assay**

Cytotoxicity assay was performed on Chinese hamster ovary (CHO) cells. In brief, 100 \(\mu\)L serial double dilutions (2-2408) of filter sterilized culture supernatants collected at late-log phase of growth from wild-type, cured and transformed isolates, were added to a confluent culture of CHO cells. The cells were incubated for 18 h at 37\(^\circ\)C, and the cytotoxicity was determined by observing changes in the cell morphology and trypan blue dye exclusion test.
Suckling mouse model

The oral LD$_{50}$ for wild-type, plasmid-cured and transformed isolates were determined in 4-6 days old suckling mice weighing 2.5-2.8 g$^{24}$. Suckling mice were administered with wild-type or transformed isolates (1 × LD$_{50}$), while for the cured isolates bacterial concentration corresponding to 10-fold the LD$_{50}$ calculated with the wild-type strain were introduced, and fluid accumulation (FA) in the gut studied. The FA ratio was expressed as the weight of the gut over the weight of remaining carcass. An arbitrary cut off value for positive effect was 96 ± 3 mg g$^{-1}$ and a FA ratio of 48 ± 2 mg g$^{-1}$ was considered as negative for fluid accumulation. Heat-killed $A. hydrophila$ served as negative control in this assay.

Statistical analysis

The paired differences of degree of pathogenicity between wild-type, plasmid-cured and transformed isolates of $A. hydrophila$ for each animal were compared by the paired ‘t’ test and Mann-Whitney U-test. The significance of differences between wild-type, plasmid-cured and transformed bacteria in all other experiments were determined by the Student’s t-test.

Results

Role of plasmid on growth and structure of $A. hydrophila$ and its characterization

Routine biochemical analysis demonstrated that plasmid curing did not alter the biochemical properties of $A. hydrophila$. However, the plasmid-cured (C-VB21) strains underwent change in their growth profile. The cured isolates exhibited a prolonged lag-phase, while the transformed isolates (T-VB21) had the same growth rate as observed in wild-type (Wt-VB21) (Table 1). Plasmid curing also resulted in significant changes in the surface structure of the bacteria. The Wt-VB21 and T-VB21 isolates appeared under SEM as typical smooth rod-shaped bacteria with blunt ends. The cured isolates had an elongated appearance with a rough surface coat bearing distinct wart-like structures (Fig. 1 a-c). The cured bacteria were also sensitive to antimicrobials to

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Mid-log phase (h)</th>
<th>Ulceration</th>
<th>Hemolytic Potential$^a$</th>
<th>Cytotoxicity (18 h)$^b$</th>
<th>LD$_{50}$ (Fish)</th>
<th>LD$_{50}$ (Suckling mice)</th>
<th>FA$^c$</th>
<th>P/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt-VB21</td>
<td>11</td>
<td>+++</td>
<td>+</td>
<td>62.20 %*</td>
<td>3.96 × 10$^7$</td>
<td>7.6 × 10$^7$</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>C-VB21</td>
<td>27</td>
<td>-</td>
<td>-</td>
<td>18.22 %</td>
<td>1.2 × 10$^9$**</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-VB21</td>
<td>12</td>
<td>+++</td>
<td>+</td>
<td>60.10 %*</td>
<td>3.1 × 10$^7$</td>
<td>7.6 × 10$^7$</td>
<td>P</td>
<td></td>
</tr>
</tbody>
</table>

Wt, wild-type, C, cured, T, transformed. + degree of positive response, - negative response. * p < 0.01; ** p <0.05
$^a$Beta-hemolytic potential was determined by the clear zone appearing around each bacterial colony spread on blood agar medium. + denotes beta-hemolytic positive, - denotes beta-hemolytic negative. $^b$% cytotoxicity of CHO cells were calculated following 18 h of incubation with $A. hydrophila$ isolates at an MOI of 50:1. $^c$Fluid accumulation (FA) in suckling mice gut was calculated 15-20 h post infection with $Aeromonas$ isolates. FA ratio in the range of 96 ± 3 mg g$^{-1}$ was considered positive (P) and 48 ± 2 mg g$^{-1}$ was considered negative (N). $^d$Suckling mice were orally administered with 1 × LD$_{50}$ wild or transformed bacteria and 10 × LD$_{50}$ cured bacteria to study FA (n=8)]

Fig. 1—SEM images of $A. hydrophila$ VB21 [(a): Wild-type aeromonads appear rod-shaped with blunt ends possessing a smooth phenotype (b): on plasmid removal, the cured bacteria became elongated with pointed ends and expressed rough warts-like surface coat; and (c): the transformed bacteria are rod-shaped with blunt ends re-expressing the smooth surface. Bars, 5 μm]
Table 2—Antimicrobial profiles of different wild-type (Wt-VB21) plasmid-cured (C-VB21) and transformed (T-VB21) *Aeromonas* strains

<table>
<thead>
<tr>
<th>Antibiotics used</th>
<th><em>A. hydrophila</em> strains</th>
<th>Gf</th>
<th>C</th>
<th>T</th>
<th>A</th>
<th>Cu</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt-VB21</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>C-VB21</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>T-VB21</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

Gf, gatifloxacin; C, chloramphenicol; T, tetracycline; A, ampicillin; Cu, cefuroxime; E, erythromycin; R, resistance; S, sensitive. Results were calculated following the manufacturers instructions after incubation at 30°C for 24 h. (n= 3; where n represents the number of times experiment was repeated)

which they were earlier resistant. When the plasmid was transformed into the cured bacteria, resistance to these antimicrobials was regained (Table 2). Characterization of the isolates using agarose gel electrophoresis demonstrated the presence of a plasmid of about 21 kb in the wild-type strain. The cured bacteria did not exhibit any plasmid band (Fig. 2).

**Role of plasmid in pathogenicity**

Healthy *C. batrachus* when injected with live Wt-VB21 developed characteristic UDS lesions. The severity of the lesions was directly proportional to the number of bacteria injected. When injected with high concentrations of Wt-VB21 (100 × LD<sub>50</sub>), the lesions appeared within 24 h and the animals died. However, fish were able to tolerate high number of C-VB21 (Table 1). Infection with the C-VB21 led to the formation of a small red area at the site within 12-16 h of the injection which remained restricted to the site of injection, and did not spread or develop into an ulcerative lesion. Infection with T-VB21 also resulted in similar lesions as observed with Wt-VB21, affecting the general health and viability.

Wt-VB21 strain produced hemolysin which was lost in the cured isolate with the removal of the plasmid (Table 1). Following transformation with the plasmid, bacteria regained the ability to produce hemolysin and induced fatal hemorrhagic lesions in healthy *C. batrachus*.

**Erythrocytes count and hemoglobin measurements**

Wt-VB21 infection lead to reduction of total erythrocytes count from second day onwards after infection compared to C-VB21 infected *C. batrachus* as well as control (Fig. 3a). Besides, total hemoglobin count of infected fish also reduced in fish infected with Wt-VB21, and after 4 days of infection hemoglobin content reduced to half of its normal value (Fig. 3b). T-VB21 isolates also showed same virulent profile like its wild-type isogenic form of *A. hydrophila*. 
Cytotoxicity assay

Infection with Wt-VB21 induced significant cytotoxicity in CHO cells (Table 1). When observed under microscope (X 40), the cells appeared to have lost their characteristic round shape and ability to adhere to the plastic surface of the culture flasks. On infection with C-VB21 isolates, the CHO cells did not undergo any change in their morphology and could adhere to the plastic surface and grow as confluent layers. Trypan blue assays did not document cell death on infection with C-VB21 isolates.

The role of plasmid in the virulence of *A. hydrophila* was also assessed by the suckling mice model (Table 1). Oral administration with $1 \times LD_{50}$ dose of Wt-VB21 resulted in the death of mice 15-20 h post-infection. The FA ratio of surviving mice was found to be in the range of 96 ± 3 mg g$^{-1}$. Administration of C-VB21 had very little effect on sucking mice. Even administering higher concentrations of C-VB21 ($10 \times LD_{50}$ of Wt-VB21) yielded a mean FA ratio within the range of 48 ± 2 mg g$^{-1}$. T-VB21 isolates of *A. hydrophila* had a $LD_{50}$ almost in Wt-VB21 range, and oral administration of T-VB21 isolates gave a similar FA ratio (96 ± 3 mg g$^{-1}$) as obtained with Wt-VB21 isolates. When the gut contents of surviving mice administered with Wt-VB21 or T-VB21 aeromonads were analyzed, extensive pathological lesions were noticed. Autopsy of the mice administered with C-VB21 isolates demonstrated no gross pathological alterations (data not given).

**Discussion**

*A. hydrophila* strain VB21 studied here possessed a plasmid of about 21 kb and was pathogenic as it caused UDS-like lesions, when introduced in Indian catfish *C. batrachus*. Plasmid curing changed the growth pattern of the bacteria, and the cured isolates took a longer time to enter into active growth phase as evidenced by the prolonged lag phase. Earlier studies demonstrated the role of plasmid on bacterial growth$^{21,22}$. In the present study, it was noted that plasmid-cured isolates underwent a transformation from smooth to rough phenotype. Study on *S. typhimurium* had shown that the plasmid-cured strains acquired a rough lipopolysaccharide, indicating the role of plasmid in encoding a complete smooth lipopolysaccharide$^{23}$. It was observed that with plasmid curing, the isolates became sensitive to antibiotics to which they were resistant earlier. It is interesting to note that a single high molecular weight plasmid is responsible for MDR in different strains of *Aeromonas*$^{7,8,24,25}$.

Development of fatal hemorrhagic ulcers was cause for concern in aeromonad UDS. The inability of C-VB21 strain to induce ulcers or affect the viability of fish even when injected at very high concentrations suggested the need of virulence plasmid in UDS pathogenesis. Moreover, injection of formalin-fixed or heat-killed bacteria did not lead to development of ulcers or cause fish death, also indicating that the bacteria could induce lesions only when they were viable. The present study also showed that plasmid curing did not alter the biochemical properties and sugar utilization profile of the cured isolates. From the curing experiments, it was also evident that EtBr treatment did not result in general loss of the plasmid, and bacteria that did not lose plasmid during curing attempts, retained their virulence proficiency when injected into healthy *C. batrachus*. These observations suggested that EtBr treatment did not induce any mutational change in *A. hydrophila* isolates and emphasized the importance of plasmid in UDS pathogenesis.

Several strains of *Aeromonas* release extracellular products (ECP) which are important for their pathogenicity$^{26,27}$. Hemolysins released by *A. hydrophila* could cause erythrocyte lysis and play important roles in pathogenesis$^{3}$, in addition to different enterotoxins released by the bacterium$^{28}$. It was noted that with the loss of the plasmid, the cured C-VB21 *A. hydrophila* strain lost the hemolytic potential and underwent a significant reduction in cytotoxicity.

To confirm further the role of plasmid in virulence of *A. hydrophila*, suckling mice were infected orally with live *A. hydrophila* isolates. It was observed that infection with C-VB21 significantly altered the $LD_{50}$ of the bacterial strain in the suckling mice model and did not lead to fluid accumulation in the gut or affect mice mortality. The accumulation of fluid in the intestine of mice infected with Wt-VB-21 was consistent with the earlier reports$^{28,29}$. It also indicated the presence of secretary toxins released by this bacterium$^{27}$ which were probably under the regulation of the 21kb plasmid.

It was observed that the virulence plasmid could be re-introduced into the cured bacteria and the transformed bacteria exhibited the wild-type virulence properties. The T-VB21 resembled the wild-types as typical smooth rod-shaped bacteria with blunt ends. They possessed a shortened lag phase, produced
hemolysins, cytotoxins, induced fatal hemorrhagic lesions characteristic of UDS and caused fluid accumulation in the gut of suckling mice.

Conclusion
The present study demonstrated that a plasmid of about 21 kb present in the pathogenic isolates of *A. hydrophila* is important for aeromonad UDS. Earlier, we reported the presence of similar plasmid bearing *A. hydrophila* from *Putius putius* 16. This indicates that the plasmid of about 21 kb is a common and stable plasmid of *A. hydrophila* important for the virulence of *Aeromonas* and the pathogenicity of aeromonad UDS as well. The study also indicates that the plasmid probably acts as a virulence marker for this bacterium. However, further studies are needed to ascertain the direct or indirect role of the plasmid in this regulatory mechanism. A detailed study of such virulent plasmids would help in understanding the pathogenesis of *A. hydrophila* and designing effective strategies to control its outbreak.

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