A rapid colony screening method for the detection of arsenate-reducing bacteria

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Abstract A rapid and simple method has been developed for the detection of arsenate reducing bacteria based on the presence of arsenite [As (III)], the end product of anaerobic arsenate [As (V)] respiration. Confirmation of As (III) product is made by the reduction of starch-iodine complex. The method can be used over a large pH range (5.5–9.0) and can easily be determined at arsenite concentration as low as 0.025 mM. Major advantages of this technique are that a large number of samples can be analyzed easily at a time.

Keywords Arsenate-reducing bacteria · Arsenite · Starch-iodine complex

Introduction

Arsenic is naturally occurring important global environmental toxicant that adversely affects human health. Millions of people in the world are suffering with skin lesions, cancers and other related diseases due to consumption of arsenic contaminated groundwater. Many people have died and hundreds of millions are at risk in many countries¹. The most abundant species are the inorganic As (III) and As (V). The biogeochemical cycle of this element strongly depends on microbial transformations that affect the mobility and distribution of arsenic species in the environment². Despite its toxicity, a number of microorganisms are capable of using either the oxidized form of inorganic arsenic As (V) or the reduced form As (III) in their metabolism⁵. Some bacteria use arsenite as the electron donor for chemoautotrophic growth while other bacteria use arsenate as the terminal electron acceptor in anaerobic respiration, thermodynamic considerations suggesting that dissimilatory reduction of arsenate could provide enough energy for microbial growth³. The bacteria that convert arsenate to arsenite are of environmental significance due to the formation of uncharged (H_3AsO_3) state which has high mobility than arsenate. Mobility and toxicity are the most important issues on both the regional and global scales. Arsenic mobility in natural environments is a major concern in arsenic rich areas. Dissimilatory arsenate reduction bacteria (DARB) may be involved in the solubilization, fate, and transport of arsenic by reducing arsenate to arsenite⁴.

Bacterial arsenate reduction represents a potential environmental implication to bioremediation and in the arsenic biogeochemical cycle. As few bacterial strains have been studied for their arsenic reduction ability⁵, therefore, it is difficult to make phylogenetically based gene probes for the identification of arsenate reducing bacteria.

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Traditionally, culture-based approaches are of interest for estimating population size of this group of bacteria in environmental samples. Earlier method for the most probable number estimation of arsenic reducing bacteria was based on arsenite-sulfide reaction, which is pH dependent and also has limitations at low-level detection⁶. Here, we report a rapid and simple microplate based colony screening method for the detection of arsenate reducing bacterial strains which can be used over a large pH range (5.5–9.0) and also respond at As (III) concentration as low as 0.025 mM.

Materials and Methods

The 56 arsenate resistant bacterial strains used in this study were isolated from arsenic contaminated area in West Bengal (Baliagram, Bhogobangola block 2, Murshidabad), India. Samples were serially diluted with sterile Milli-Q water and plated on yeast extract mannitol (YEM) agar medium (Mannitol, 10g; K_2HPO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.1 g; yeast extract, 0.5 g; water, 1000 ml, pH 7.0). Plates were incubated at $30 \pm 2^\circ C$ for 48 h. Pure cultures were obtained by successive isolation of colonies through repeated cross streaking. Stock of As (III) and As (V) prepared by dissolving sodium arsenite ($NaAsO_2$) and sodium arsenate (Na_2HAsO_4) in sterile deionized (MilliQ) water, stored at $4^\circ C$ in dark. A starch-iodine reagent [mixture of aqueous starch and Lugol's iodine solution (KI, 10g; I, 5g; water, 100ml)] was prepared freshly before use.

A preculture was prepared from each single colony in 20 ml YEM (without arsenate) broth and incubated at $30 \pm 2^\circ C$ for 24 h at 220 rpm. The preculture was centrifuged, washed twice with sterile Milli-Q water, and resuspended in 2 ml sterile water. A 20 μ l preculture suspensions were inoculated to 20 ml YEM broth supplemented with 2 mM arsenate and incubated under same condition. Strains were grown up to 0.5–0.6 O.D of cell suspension at 600 nm. Then cultures were centrifuged at 10,000 rpm for 5 min and supernatant was taken for further analysis.

One ml of the supernatant was added to the wells of a round-bottomed 36-well plate followed by addition of 30 μ l of starch-iodine complex. The plates were incubated in dark for 10 min.

Results and Discussion

The reaction is based on the specific detection of arsenite, the end product of anaerobic arsenate respiration. Starch-iodine complex is very useful for indicating redox titrations that involve iodine because of the distinct color change. When there is an excess oxidizing agent, the complex is

blue and in the presence of reducing agent, the I_3^- breaks up into iodine and iodide resulting in color disappearance⁷. The arsenite [As (III)] solution, a reducing agent⁸, reacts with starch-iodine complex, resulting in color ranging from blue-black to colorless. Individual effects of starch and iodine concentration were also studied and it was found that 10% lugol's iodine solution in 1% starch showed maximum stability (data not shown). Attempts were made to optimize the volume of starch-iodine complex to be added to arsenite solution and it was found that 3% freshly prepared complex solution gave best color indication (data not shown). Different concentrations of As (III) solution in YEM broth was placed in 36-microplate well, followed by addition to 3% freshly prepared complex solution and color changes were observed after incubation (Fig. 1). It was found that color change was depended upon the arsenite concentration in the medium and the color intensity was being distinguishable at concentration as low as 0.025 mM. Fig. 2 shows that the color intensity does not change significantly at a given pH range (5.5–9.0). Thus indicating it is independent of a broad range of pH (5.5–9.0).

The proposed screening method was used to test 56 strains isolated from the environmental samples and it was found that 23 strains was positive to arsenate reduction when all the strains were incubated with 2mM As (V). Among the positive strains, 14 strains showed completely colorless solution (Fig. 3, lane 2-lower three), *i.e.* amount of transformed As (III) from As (V) in the medium was

Fig. 1 Intensity of color variation in different concentrations of As (III) in YEM medium.

Fig. 2 Intensity of color variation in the range of pH (5.5–9.0), As (III) concentrations in the medium was 0.15 mM.

≥ 0.2 mM and in case of other positive strains (Fig. 3, lane 3-upper three), partial reduction of arsenate, i.e. arsenite in medium was < 0.20 mM. It was also observed that remaining 33 strains were resistant to arsenate but did not have detectable reduction capacity. These strains may be the non-transforming strains or they may be utilizing arsenate by any another metabolic process. Earlier also a number of non-transforming bacterial strains present in environmental

Fig. 3 Intensity of color variation for environmental sample (Lane 2–4), Lane 1-A, only YEM [without inoculum and As (V)]; and Lane 1-B, Bacterial culture supernatant in YEM medium [without As (V)]; Lane 1-C, YEM with 2mM As (V) [without inoculum].

sample belonging to β or γ -proteobacter groups have been reported⁹. Bacterial cultures without arsenate (Fig. 3, lane 1-B), only YEM, i.e. medium without inoculum and As (V) (Fig. 3, lane 1-A) and YEM with 2mM As (V) (Figure 3, lane 1-C) were used as control where the color remained unchanged. It indicates that As (V) itself or any other ions or any extracellular product present in the bacteria grown cultured medium could not interfere with the complex. Similar results were obtained by using chemically defined medium¹⁰ (CDM) instead of YEM. All the experiments were repeated in quadruplicate.

The method presented here is simple and rapid for the detection of arsenate reducing bacteria. The method is also cost effective and reliable which needs inexpensive starch-iodine complex, instead of high expensive and time-consuming HG-AAS, ICP-MS, or ICP-AES for the screening of large number of bacterial strains. This technique is easy to perform, and the reagents and equipment are readily available in most of the laboratories.

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