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Hydrogen formation by an arsenate-reducing *Pseudomonas putida*, isolated from arsenic-contaminated groundwater in West Bengal, India

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Abstract Anaerobic growth of a newly isolated *Pseudomonas putida* strain WB from an arsenic-contaminated soil in West Bengal, India on glucose, L-lactate, and acetate required the presence of arsenate, which was reduced to arsenite. During aerobic growth in the presence of arsenite arsenate was formed. Anaerobic growth of *P. putida* WB on glucose was made possible presumably by the non-energy-conserving arsenate reductase ArsC with energy derived only from substrate level phosphorylation. Two moles of acetate were generated intermediarily and the reducing equivalents of glycolysis and pyruvate decarboxylation served for arsenate reduction or were released as H_2. Anaerobic growth on acetate and lactate was apparently made possible by arsenate reductase *ArrA* coupled to respiratory electron chain energy conservation. In the presence of arsenate, both substrates were totally oxidized to CO_2 and H_2 with part of the H_2 serving for respiratory arsenate reduction to deliver energy for growth. The growth yield for anaerobic glucose degradation to acetate was $Y_{Glucose}$=20 g/mol, leading to an energy coefficient of Y_{ATP}=10 g/mol adenosine-5'-triphosphate (ATP), if the Emden–Meyerhof–Parnas pathway with generation of 2 mol ATP/mol glucose was used. During growth on lactate and acetate no substrate chain phosphorylation was possible. The energy gain by reduction of arsenate was $Y_{Arsenate}$= 6.9 g/mol, which would be little less than one ATP/mol of arsenate.

Keywords *Pseudomonas putida* · Arsenate · Hydrogen · Glucose · Acetate · Lactate

Introduction

Arsenic is a widespread metalloid compound on earth at an average concentration of 2 mg/kg soil. In some areas of the world, higher concentrations of arsenic in soil and sediments are found, for instance in Bangladesh and West Bengal, India, leading to groundwater concentrations of up to 1 mg/l (Chakraborti et al. 2002). Historically, weathering of arsenic-rich minerals in the Himalaya, transport of the particulate matter by the river Ganges and deposition of the arsenic-containing suspended material were considered to be responsible for the arsenic content of soil and sediments in the alluvial delta of West Bengal. Non-soluble arsenic compounds in soil may be mobilized and transferred as dissolved arsenic compounds into the aquifers (Harvey et al. 2005). Mobilization of arsenic, leading to the observed concentrations in groundwater, might be due to geochemical reactions, but also to microbial metabolism. A large number of bacteria (e.g. *Escherichia coli* and *Staphylococcus aureus*) have developed resistance-mechanisms to withstand toxicity of high concentrations of arsenic, but arsenic compounds in potable water are highly toxic for most prokaryotic and all eukaryotic organisms as well and lead to arsenicosis (Chakraborti et al. 2002). Arsenic-resistant bacteria characteristically express *arsC*-genes which reduce pentavalent arsenate to water-soluble trivalent

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arsenite, apparently without energy conservation (Rosen 1999). The arsenite is then exported by the microorganisms via an arsenite-specific transporter Arsb, which is located in the cytoplasmic membrane (Mukhopadhyay et al. 2002). On the other hand, there are also a number of bacteria, which are using arsenate for respiration (Oremland and Stolz 2005). These genera are characterized by dissimilatory respiratory arsenate reductase genes (*arrA*) and comprise a diverse phylogenetic group, including, e.g., *Bacillus*, *Shewanella*, and *Sulfurospirillum* species.

Pseudomonas putida is a Gram-negative, rod-shaped organism that is able to grow on a wide variety of organic substrates under aerobic or anoxic conditions. It is also known that *P. putida* has a wide spectrum of heavy metal and metalloid resistance genes, e.g., for copper, zinc, and nickel. Two open reading frames for arsenic resistance are encoded in *P. putida* KT2440 including the *arsC*-gene (Cánovas et al. 2003). In this study, a *P. putida* strain was isolated from arsenic-containing groundwater of West Bengal, India, and its arsenic metabolism during aerobic and anaerobic growth on several carbon sources was analyzed. This report shows for the first time the growth of *P. putida* under anaerobic conditions with arsenate as the only electron acceptor and the formation of hydrogen during complete mineralization of the carbon sources.

Materials and methods

Isolation of bacteria from arsenic-contaminated groundwater

Groundwater was pumped from a 19 ft deep household well in Sahispur (N 23° 04′15.5″, E 88°36′33.5″), district of Nadia and 100 ml of a representative sample was collected from the water stream in a sterilized plastic bottle after several minutes of pumping. The sample contained 285 μg/l total arsenic and a DOC of 1.5 mg/l. The bacterial population was 5×10^5/ml as determined by microscopical counting after ten-fold enrichment by centrifugation. For enrichment of anaerobic, arsenate-resistant bacteria, 1 ml of the water was inoculated into 20 ml R2A-medium (Reasoner and Geldreich 1985) in 100 ml serum bottles that contained 5 mmol/l arsenate. Serum bottles were closed with a rubber stopper that was fixed by an aluminum cap. The air atmosphere was exchanged for nitrogen. The bottles were incubated for 48 h at 27°C on a shaker at 160 rpm. After growth, 100 μl of the enrichment culture were streaked on R2A-agar plates that contained 5 mmol/l arsenate. The agar plates were transferred into an anaerobic jar and incubated at 27°C under a nitrogen atmosphere for 48 h. Several colonies were picked and re-streaked on R2A-agar plates which were subjected to the above mentioned incubation procedure for growth. After the second re-streaking single colonies were picked from the Petri dishes and transferred into serum bottles with 20 ml of R2A medium+5 mmol/l arsenate. After growth in liquid medium under the same conditions as above, cells from the best growing culture were either harvested for DNA extraction or 1 ml was inoculated into serum bottles with 20 ml of test medium that contained 1 g/l glucose, 0.25 g/l yeast extract, 0.3 g/l K_2HPO_4±0.695 g/l arsenate under a nitrogen atmosphere. Alternatively, glucose was omitted and 0.2 ml of a 1.5 M stock solution of sodium acetate or a 1 M stock solution of sodium L-lactate was added to 20 ml as the main carbon source to the media. No growth occurred in media without arsenate. For aerobic growth of the isolated culture the test medium was prepared in serum bottles, but instead of sodium-arsenate 0.695 g/l sodium arsenite was supplemented. For an OD of E_{578}=1.2, the cell dry weight was 2.2 mg/20 ml medium. The gas phase for aerobic cultures was synthetic air, which contained 21% oxygen, 79% nitrogen.

Identification of the bacterial isolate WB as a strain of *P. putida*

From the culture that grew best with arsenate as an electron acceptor in the absence of oxygen genomic DNA was isolated by the method of Murray and Thompson (1980). For identification of the strain universal eubacterial 16S rDNA sequencing primers 27F (5′-AGAGTTT GATCCTGGCT-CAG-3′) and 1492R (5′-GGTTACCTTGT TACGACTT-3′) (Lane 1991) were used. DNA amplification was performed in a total volume of 25 μl, containing 1 μl dissolved genomic DNA, 1.25 U *Taq*-polymerase, 10 pmol of each primer, 0.25 μl dNTP-mixture (2.5 mM each), 2.5 μl 10× polymerase chain reaction (PCR) buffer and 1.5 μl $MgCl_2$. 16S rDNA was amplified in a Biometra Thermocycler T Gradient using following conditions: 95°C for 5 min., followed by 35 cycles with 95°C for 30 s, 52°C for 1 min., 72°C for 2 min. and a final extension of 72°C for 5 min. Amplified DNA was isolated by gel-electrophoresis of aliquots of PCR mixtures (2 μl with 5 μl 6× loading-dye) using 1% agarose in 5×TBE-buffer and was sequenzed by MWG Biotech (Ebersberg, Germany). Sequences were compared with those in the NCBI database using the BLAST search program to find the most closely related strains. All further experiments were done with this isolate, identified below as *P. putida* WB (WB derived from "West Bengal") by its 16S rRNA gene (EMBL accession No. FN6886776).

To validate the classification as *P. putida* by the 16S rRNA gene strain WB was further characterized by microscopy, Gram staining and by testing for catalase and

oxidase. Biomass production during growth in the presence of arsenate and doubling times were also determined. The strain was deposited at DSMZ Braunschweig (DSM 23849).

Detection of the *arsC*- and *arrA*-gene

The *arsC*-gene of *P. putida* WB was analysed by PCR using primers *amlt-42-f* (3′-TCGCGTAATACGCTGGAGAT-5′) and *amltf-376-r* (3′-ACTTTCTCGCCGTCTTCCTT-5′) (Sun et al. 2004). Amplification reactions were performed in a total volume of 25 µl containing 2 µl dissolved genomic DNA, 1.25 U *Taq*-polymerase, 10 pmol of each primer, 0.25 µl dNTP-mixture (2.5 mM each), 2.5 µl 10× PCR-buffer and 1.5 µl $MgCL_2$. PCR conditions were as follows: 95°C for 3 min, followed by 40 cycles with 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The *arrA*-gene was also detected by PCR using primers *arrA for* (3′-TTATTCCAGG GAAGATG-5′) and *arrArev* (3′-TCTTTAAGCGGG GAATTC-5′) (Malasarn et al. 2004). PCR conditions for amplification were as follows: 95°C for 5 min, followed by 40 cycles with 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. Amplified DNA was verified by gel-electrophoresis of aliqouts of PCR-mixtures (2 µl with 5 µl 6× loading-dye) using 1% agarose in 5× TBE-buffer. As references strains *P. putida* DSM 291 and *Desulfosporosinus auripigmenti* DSM 13351 (obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig) were used.

Determination of glucose, acetate, lactate, arsenate, arsenite, hydrogen and cell mass

Glucose was determined by the colorimetric method of Miller (1959) D- and L-lactic acid were determined with the respective test kits of Boheringer (Mannheim). Acetate was determined by gas-chromatography (Model 437, Chrompack, equipped with a FID; Gallert and Winter 1997). Hydrogen and oxygen were also determined by gas-chromatography (Model CP9001, Chrompack, equipped with a TCD) according to Gallert and Winter (1997). Arsenate was determined by ion-exchange-chromatography (ICS90, Dionex) with a 4×250 mm IonPac A89-HC column (Dionex Austin, Texas USA). Arsenite in arsenate containing mixtures was also determined by ion-exchange-chromatography by substracting the arsenate amount after complete oxidation of arsenite with 1% H_2O_2 from the amount of arsenate before arsenite oxidation. Moles of hydrogen or oxygen in headspace gases were calculated with the ideal gas law. Cells from 20 ml cultures were pelleted by centrifugation and the pellet was washed twice with tap water before it was dried to constant weight at 105°C.

Results

Classification of isolate WB as *P. putida* WB by its 16S rRNA gene sequence and by other features

The 16S rRNA gene sequence of the DNA of isolate WB from groundwater taken in the district Nadia/West Bengal/India was detected with the universal primers 27F and 1492R (Lane 1991) of *Pseudomonas putida* KT2440 (Tumler and Fraser 2002) using the BLAST search program (Altschul et al. 1990). It was 100% identical with *P. putida* KT2440. The identity with several other *P. putida* strains from the NCBI data base was 99% (Table 1). Thus, isolate WB was a *P. putida* strain and is designated *P. putida* WB.

As expected, *P. putida* WB shared the main features of the genus *Pseudomonas*. It was a Gram-negative, motile, non-spore-forming rod-shaped bacterium, with a size of 1–1.5× 2–3 µm that reacted positive in oxidase- and catalase-tests. The ideal growth temperature was 27°C. It had a doubling time of 25 h with glucose as a substrate for aerobic growth. In anaerobic 20-ml cultures an optical density of E_{578}=1.2 represented 2.2 mg dry weight of *P. putida* WB cells.

Arsenate reduction and hydrogen production during anaerobic growth of *P. putida* WB and *P. putida* DSM 291 on glucose and acetate

P. putida WB grew exponentially on glucose under anaerobic conditions and formed almost 2 mol acetate per mol glucose, but only when arsenate was provided as an electron acceptor (Fig. 1a, b). Without arsenate *P. putida* WB did not grow at all. During glucose metabolism arsenate was reduced to arsenite and some hydrogen was released. During anaerobic growth to an optical density of $E_{578\ \eta m}$=1.2 (Fig. 1a at 100 h), 0.11 mmol glucose and 0.043 mmol arsenate (total amounts per 20-ml assay) were metabolized. An average of 0.2 mmol acetate was generated from 0.11 mmol glucose. All arsenate was reduced to arsenite and about 0.3 mmol hydrogen were liberated. The bacterial dry mass was 2.2 mg/0.11 mmol Glucose, equivalent to 20 g dry mass/mol glucose metabolized anaerobically in the presence of arsenate. The stoichiometry of glucose conversion to acetate in the presence of arsenate (Fig. 1b at 100 h) is given in Eq. 1. It is assumed that release of 1 mmol acetate from pyruvate decarboxylation gives 1 mmol CO_2. Biomass formation may have consumed about 10% of the substrate.

$$0.11C_6H_{12}O_6 + 0.043AsO_4^{3-} \rightarrow 0.2CH_3COOH + 0.2CO_2 + 0.043AsO_3^{3-} + 0.34H_2 + 3.7\text{mg cells} \quad (1)$$

Table 1 BLAST search for comparison of the *Pseudomonas putida* strain WB (accession No. FN686776) with other *P. putida* strains from NCBI gene bank

Relatedness of *P. putida* WB (accession no. FN686776) to	NCBI gene bank accession no.	Identity
P. putida KT2440 complete genome	AE015451.1	100%
P. putida partial 16S rRNA gene, strain WAB1869	AM184211.1	99%
P. putida strain CUG LPA11 16S ribosomal RNA gene, partial sequence	EU443615.1	99%
P. putida strain KNUC292 16S ribosomal RNA gene, partial sequence	EU239202.1	99%
P. putida strain D1 16S ribosomal RNA gene, partial sequence	EU878238.1	99%
P. putida strain BF-S5 16S ribosomal RNA gene, partial sequence	EU857418.1	99%
Pseudomonas sp. benan-1 16S ribosomal RNA gene, partial sequence	EU313808.1	99%

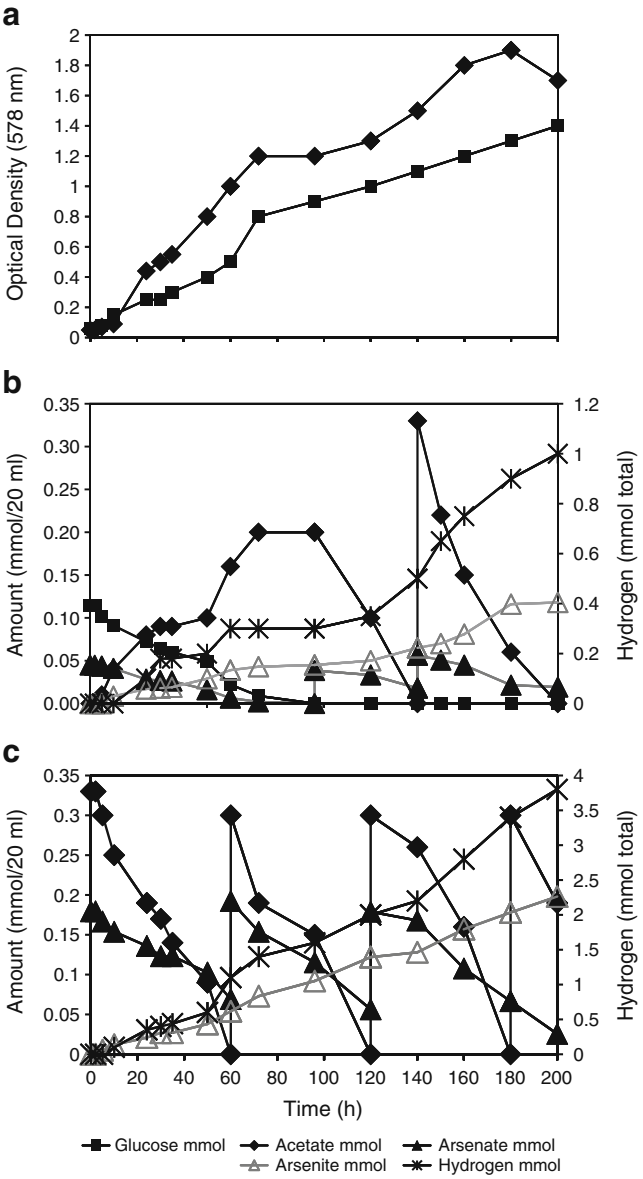

Fig. 1 **a**, **b**, **c** Anaerobic growth on glucose (*filled diamonds*) and acetate (*filled squares*) (**a**), as well as glucose (**b**) and acetate metabolism (**c**) of *Pseudomonas putida* WB in the presence of arsenate. Arsenate was oxidized to arsenite during glucose conversion to acetate, CO_2, and H_2. Acetate from glucose (**b**) or from external sources (**c**) could only be oxidized to CO_2 and H_2 when arsenate was present as an electron acceptor. CO_2 was not quantified. In the experiment of (**c**), acetate and arsenate were added at time zero and after 60 and 120 h, acetate once again after 180 h

After complete oxidation of glucose to acetate and reduction of all arsenate to arsenite growth of *P. putida* WB stopped. No growth at the expense of acetate occurred in the absence of arsenate (Fig. 1a, 70–100 h). However, growth continued with the acetate as carbon source if arsenate was replenished (Fig. 1a, 100 h onwards). During anaerobic metabolism of the acetate that was released during glucose degradation or was supplemented from an external source (Fig. 1b at 140 h) to CO_2 and reducing equivalents, the hydrogen served either for arsenate reduction or was released as H_2. The replenished 0.3 mol acetate at 140 h (Fig. 1b) were completely oxidized with the formation of 0.043 arsenite from arsenate and additionally 0.5 mmol H_2 were generated. Stoichiometrically there was a hydrogen deficiency from acetate oxidation and arsenate reduction, presumably caused by losses through the rubber stopper. Thus, the stoichiometry for anaerobic acetate oxidation by *P. putida* WB in the presence of arsenate was derived from a separate assay. Acetate was the sole growth substrate and much higher arsenate concentrations for hydrogen consumption were supplied. In addition, serum bottles were incubated bottom-up to avoid hydrogen losses (Fig. 1a, c). Acetate (0.3 mmol) was replenished three times after 60, 120, and 180 h, and arsenate was replenished two times after 60 and 120 h incubation time. Growth, acetate and arsenate reduction as well as arsenite and hydrogen formation were measured during the experiment. Concentrations were summed up and stoichiometries calculated for the whole experiment (Eq. 2; numbers are mmol total; 1 mmol acetate was assumed to give 2 mmol CO_2 since yeast extract could be used for biomass formation):

$$1.2CH_3COOH + 0.4AsO_4^{3-} \rightarrow 2.4CO_2 + 0.27AsO_3^{3-} + 4.3H_2 + 2.75\text{mg cells} \quad (2)$$

The hydrogen production rate apparently was not affected by accumulating hydrogen in the gas phase, indicating no influence of the H_2-partial pressure on hydrogen generation. After 240 h, 4.3 mmol of hydrogen were generated from a total of 1.2 mmol acetate while 0.4 mmol arsenate were apparently reduced to 0.27 mmol arsenite (the difference to arsenate may be due to incomplete oxidation during analysis). Thus, if 1.2 mmol acetate were anaerobically oxidized to 2.4 mmol CO_2 and theoretically 4.8 mmol H_2, and 0.4 mmol H_2 were required for reduction of 0.4 mmol arsenate to arsenite, 4.4 mmol H_2 should be generated. This was in good agreement with 4.3 mmol H_2 that were actually detected. Growth to a final OD of 1.5 was equivalent to 2.75 mg cells per 20 ml assay. Assuming a molar energy coefficient of Y_{ATP}=10 g/mol ATP, the 2.75 mg cell dry weight would be equivalent to 0.275 mmol ATP, that must have been generated from reduction of 0.4 mmol arsenate (arsenate added minus used) by arsenate reductase ArrA. Thus the energy conservation from reduction of 0. 4 mmol arsenate to arsenite was $Y_{Arsenate}$=6.9 g cells per mol arsenate that was respired.

The same experiments with glucose and acetate as growth substrates were preformed with the reference strain *P. putida* DSM 291 and similar stoichiometries were obtained (data not shown).

Respiration of arsenate with lactate as carbon source

To test anaerobic growth of *P. putida* WB with other carbon sources than glucose or acetate, 0.2 mmol of lactate was supplied as the main carbon source in the test medium together with 0.043 mmol of arsenate, which was replenished after 60 h (Fig. 2a, b). *P. putida* WB grew exponentially to an optical density of 1.2 (Fig. 2a) while the lactate was decarboxylated stoichiometrically to acetate. After 140 h lactate was completely oxidized and totally 0.086 mmol arsenate were reduced to arsenite (Fig. 2b). Acetate utilization apparently started already when little lactate was still left, but growth and acetate utilization stopped after 140 h when arsenate was used up (Fig. 2b). At that time 0.4 mmol hydrogen were produced. The stoichiometry for lactate utilization and arsenate reduction from Fig. 2b can be formulated as follows (Eq. 3):

$$0.2CH_3CH_2COOH + 0.086AsO_4^{3-} \rightarrow 0.05CH_3COOH + 0.086AsO_3^{3-} + 0.4H_2 + 2.2\text{mg cells} \quad (3)$$

In a control experiment *P. putida* DSM 291 was supplied with 0.2 mmol lactate and 0.86 mmol arsenate. The organisms grew exponentially and oxidized the lactate to intermediarily 0.3 mmol acetate which disappeared after lactate depletion. Overall, 0.045 mmol arsenite and 0.4 mmol hydrogen were formed (data not shown).

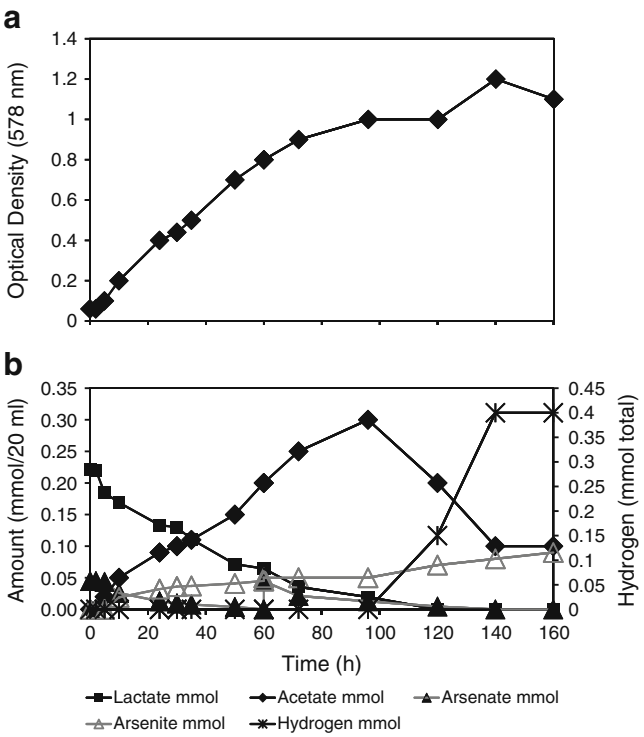

Fig. 2 **a**, **b** Growth (**a**) and L-lactate metabolism of *Pseudomonas putida* WB (**b**) during anaerobic arsenate respiration. Arsenate was replenished after 60 h. The L-lactate was stoichiometrically decarboxylated to acetate, and then the acetate oxidized under hydrogen formation, as long as arsenate was available

Aerobic growth with glucose as the only carbon source in the presence of arsenite

To test the influence of arsenite on aerobic growth of *P. putida* WB 0.079 mmol sodium arsenite was added to the medium. There was an exponential growth for 96 h to a final optical density of 1.6, which was equivalent to 3.3 mg cells in the 20 ml assay (Fig. 3a). Totally, 0.48 mmol of glucose was respired with 3.25 mmol oxygen and 0.22 mmol arsenite were oxidized to 0.22 mmol arsenate (Fig. 3b). The oxygen supply for each portion was calculated for glucose respiration and arsenite oxidation. In a control experiment without glucose, only 0.03 mmol arsenite were oxidized after 96 h, excluding a rapid chemical oxidation. With the data of Fig. 3b the following stoichiometry could be derived (CO_2 estimated from glucose respiration; Eq. 4).

$$0.48C_6H_{12}O_6 + 0.22AsO_3^{3-} + 3.25O_2 \rightarrow 0.22AsO_4^{3-} + 2.88CO_2 \quad (4)$$

P. putida DSM 291 reacted similarly (data not shown). As in the experiment with the *P. putida* WB, arsenite was completely oxidized to arsenate after 96 h. For complete oxidation of 0.48 mmol glucose and 0.22 mmol arsenite theoretically 3 mmol oxygen were required.

Identification of the *arsC*- and the *arrA*-gene

To demonstrate the genetic capacity of *P. putida* WB for detoxification and/or respiration of arsenate by arsenate reductases *arsC*- and *arrA*-genes were isolated. Both genes were detected in *P. putida* WB. *arcC* contained 350 bp and *arrA* 620 bp. As positive controls *D. auripigmenti*, a sulfur-reducing bacterium that is known to be able to reduce arsenate and *P. putida* DSM 291 were used. Both control strains and the isolated *P. putida* strain WB showed the bands for the *arsC*- and *arrA*-genes (Fig. 4).

Discussion

This study reports the isolation of a *P. putida,* strain WB from arsenic-contaminated groundwater in West Bengal and its ability to generate hydrogen during anaerobic mineralization of different carbon sources with arsenate as an electron acceptor. The arsenic concentration in West

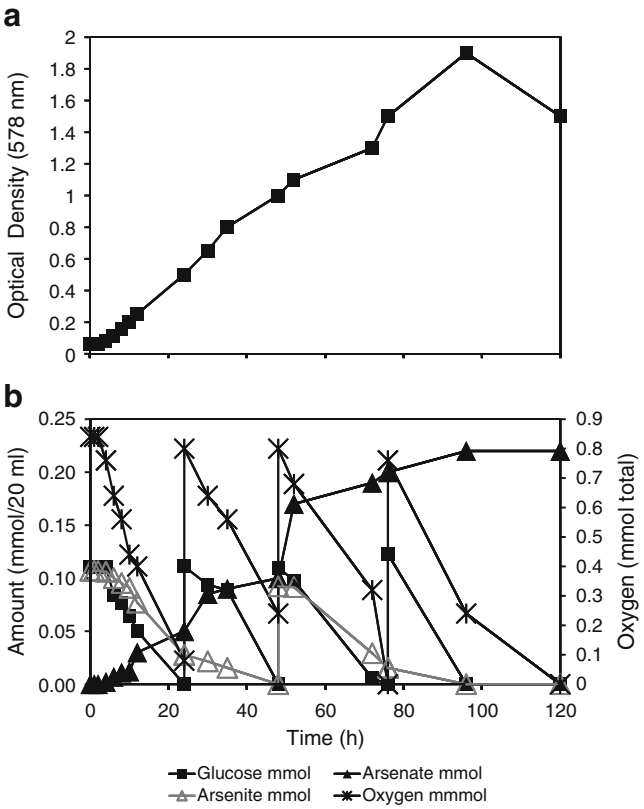

Fig. 3 **a**, **b** Aerobic growth (**a**) and glucose metabolism of *Pseudomonas putida* WB in the presence of arsenite (**a**)

Bengal can be as high as 1 mg/l (Chakraborti et al. 2002), whereas in the location, where the groundwater samples were taken, about 300 µg/l arsenic was found. A number of studies in this area on geochemical and microbial mobilization of arsenic in soil, contaminating the groundwater are available in the literature (e.g. Nickson et al. 2000; Héry et al. 2010). A new bacterial species that was isolated from an aquifer in West Bengal, *Deinococcus indicus,* was resistant against arsenic and was able to grow

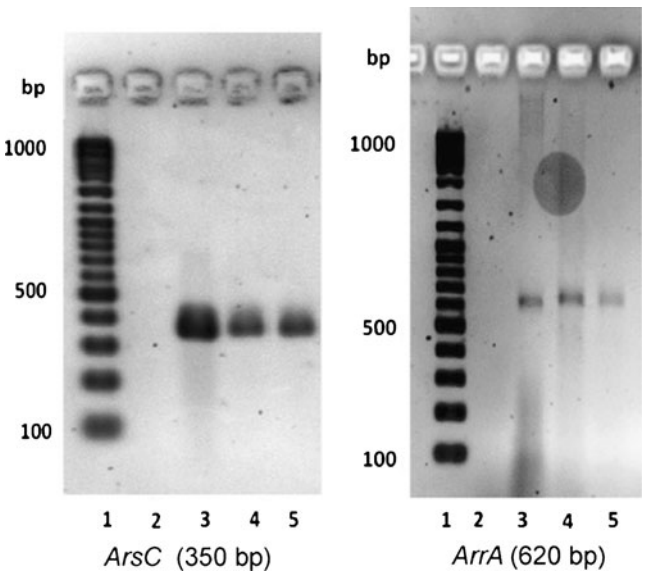

Fig. 4 Agarose gels of the PCR products of arsC- and arrA-genes of *Pseudomonas putida* WB and reference strains. *Lane 1*, DNA standard; *lane 2*, sterile deionized water; *lane 3*, *Desulfosporinus auripigmenti*; *lane 4*, *P. putida* DSM291; *lane 5*, *P. putida* WB

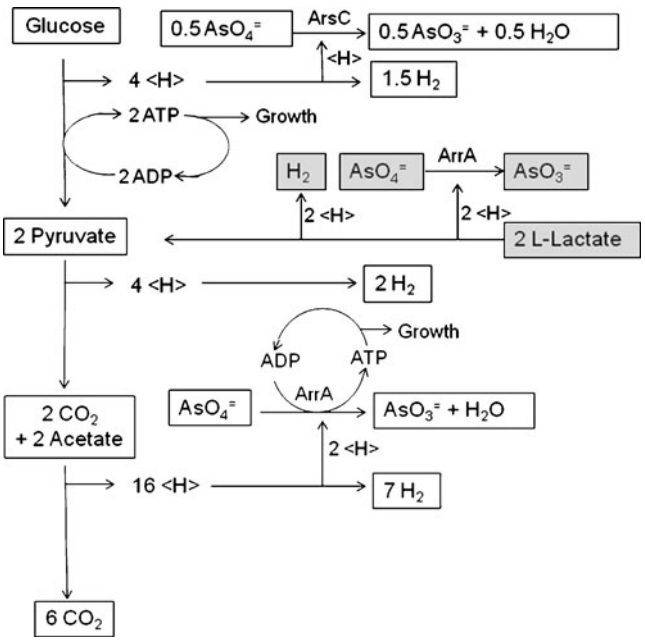

Fig. 5 Scheme of anaerobic respiration of glucose, acetate or lactate by *Pseudomonas putida* WB in the presence of arsenate. Lactate seems to be fed in via pyruvate

aerobically on different carbon sources such as lactose or arabinose in the presence of 10 mmol/l arsenate (Suresh et al. 2004). However, no accurate turnover of carbon sources and arsenic was determined. There are a number of other studies describing isolates from different arsenic-containing environments that were able to respire arsenate (Huber et al. 2000; Lear et al. 2007; Bachate et al. 2009; Zhou et al. 2010). Newamn et al. (1997) described a sulfur-reducing bacterium, *D. auripigmenti* that reduced 5 mmol/l of arsenate in 144 h completely to arsenite while growing on 5 mmol/l of lactate, but no growth on acetate or only acetate oxidation was observed. In our study *P. putida* WB reduced 4.5 mmol/l of arsenate during anaerobic growth on 8.5 mmol/l lactate. In contrast to Newman's study we added arsenate twice, 2.25 mmol/l each (0.045 mmol total/20 ml assay). The arsenate was reduced completely in 120 h. Srivastava et al. (2009) isolated a *Pseudomonas* sp. DRBSI that was closely related to *Pseudomonas stutzeri* which was able to reduce up to 100 mmol/l of arsenate under aerobic conditions in NB-medium. No hydrogen formation was observed, as expected for aerobic conditions. In contrast *P. putida* WB from our study does not reduce arsenate under aerobic conditions, but it oxidizes arsenite to arsenate. Santini et al. (2002) isolated a strictly anaerobic *Bacillus*-like strain JMM-4 from arsenic-contaminated mud from a gold-mine in Australia that was able to grow anaerobically on arsenate with lactate as the sole carbon source. This bacterium reduced 5 mmol/l of arsenate completely to arsenite in parallel to the oxidation of 5 mmol/l of lactate. The formation of hydrogen was not investigated. In contrast to *D. auripigmenti* (Newman et al. 1997) the JMM-4 *Bacillus*-like strain could use acetate as a sole carbon source. Another well-known arsenate-reducing bacterium, *Chrysiogenes arsenatis* was also able to grow on acetate as electron donor with arsenate as the terminal electron acceptor. This species reduced 3.9 mmol/l of arsenate while oxidizing 1 mmol/l of acetate (Macy et al. 1996). The formation of hydrogen was again not investigated. Jones et al. (2000) enriched a strain of *Clostridium intestinales*, an arsenate-reducer from an agricultural soil that contained naturally elevated concentrations of arsenic (840 μmol/kg) and investigated the reduction of arsenate under anaerobic conditions in serum bottles with 50 ml nutrient solution at 25°C and under shaking. As a carbon-source ^{14}C-spiked glucose was used (5 mmol/l) and 0.6–5 mmol/l of As(V) was the sole electron acceptor. Hydrogen production after complete consumption of glucose was equimolar to the released carbon dioxide. Since butyrate was the main fermentation product the authors concluded that their strain was using the butyric acid pathway, generating 1 mol of butyrate, 2 mol of CO_2 and 2 mol of H_2 per mol glucose. Traces of acetic acid, formic acid, butanol and sec-butyl butyrate were also detected. The formation of hydrogen in these experiments seemed not to be dependent on arsenate reduction, thus *C. intestinales* did not grow by arsenate respiration.

P. putida WB in contrast had an essential requirement for arsenate to be able to grow at all on glucose, lactate or acetate in the absence of oxygen. Two arsenate reductases were apparently expressed by *P. putida* WB (Fig. 4), the non-respirative ArsC for detoxification (Rosen 2002) and ArrA for respirative arsenate reduction (Malasarn et al. 2004). Anaerobic glucose degradation to acetate did not require energy from arsenate reduction since 2 mol of ATP were available per mol glucose by substrate level phosphorylation during glycolysis via the EMP-pathway. This was apparently the case since 2.2 mg of cell mass (derived from the OD measurement) was formed from 1.1 mmol of glucose, resulting in $Y_{Glucose}$=20 g/mol. With a Y_{ATP}=10 g/mol this would indicate a specific energy gain of 2 mol ATP. The reducing equivalents generated by glycolysis were partially used for arsenate reduction and partially released as hydrogen. No glucose utilization was possible in the absence of arsenate.

Two moles acetate were generated from 1 mol glucose and remained in the medium when arsenate was used up, and growth stopped. Only when arsenate was replenished (Fig. 1b at 90 h), growth continued and acetate was oxidized under formation of arsenite and hydrogen. The energy for growth on acetate must have come from respiratory arsenate reduction, since acetate oxidation per se would not allow energy conservation. If 1 mol ATP could be generated by reduction of 1 mol arsenate, then the Y_{ATP} was 6.9 g/mol. In Fig. 5 the reactions during anaerobic growth of *P. putida* WB on glucose, acetate and lactate are summarized. Based on the obtained reaction stoichiometries with the respective substrates and on the growth response, the involvement of ArsC and ArrA was included. Under anaerobic conditions in the absence of oxygen glucose could be converted to acetate with an energy gain from substrate level phosphorylation during glycolysis only in the presence of arsenate when at least some of the hydrogen was removed by non-respiratory ArsC for arsenate reduction. Anaerobic acetate oxidation was coupled to respiratory arsenate reduction and proceded to completion only if enough arsenate was present. Lactate was sequentially oxidized via pyruvate and acetate and was associated with growth of *P. putida*. Lactate oxidation to pyruvate and the subsequent decarboxylation of pyruvate to acetate as well as the anaerobic oxidation of acetate both seemed to allow growth by arsenate respiration with ArrA.

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