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ORIGINAL PAPER

An arsenate-reducing and alkane-metabolizing novel bacterium, *Rhizobium arsenicireducens* sp. nov., isolated from arsenic-rich groundwater

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Abstract A novel arsenic (As)-resistant, arsenate-respiring, alkane-metabolizing bacterium KAs 5-22^T, isolated from As-rich groundwater of West Bengal was characterized by physiological and genomic properties. Cells of strain KAs 5-22^T were Gram-stain-negative, rod-shaped, motile, and facultative anaerobic. Growth occurred at optimum of pH 6.0–7.0, temperature 30 °C. 16S rRNA gene affiliated the strain KAs 5-22^T to the genus *Rhizobium* showing maximum similarity (98.4 %) with the type strain of *Rhizobium naphthalenivorans* TSY03b^T followed by (98.0 % similarity) *Rhizobium selenitireducens* B1^T. The genomic G + C content was 59.4 mol%, and DNA–DNA relatedness with its closest phylogenetic neighbors was 50.2 %. Chemotaxonomy indicated UQ-10 as the major quinone; phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol as major polar lipids; $C_{16:0}$, $C_{17:0}$, 2-OH $C_{10:0}$, 3-OH $C_{16:0}$, and unresolved $C_{18:1}$ ω7C/ω9C as predominant fatty acids. The cells were found to reduce O_2, As^{5+}, NO_3^-, SO_4^{2-} and Fe^{3+} as alternate electron acceptors. The strain's ability to metabolize dodecane or other alkanes as sole carbon source using As^{5+} as terminal electron acceptor was supported by the presence of genes encoding benzyl succinate synthase (*bss*A like) and molybdopterin-binding site (*mop*B) of As^{5+} respiratory reductase (*arr*A). Differential phenotypic, chemotaxonomic, genotypic as well as physiological properties revealed that the strain KAs 5-22^T is separated from its nearest recognized *Rhizobium* species. On the basis of the data presented, strain KAs 5-22^T is considered to represent a novel species of the genus *Rhizobium*, for which the name *Rhizobium arsenicireducens* sp. nov. is proposed as type strain (=LMG 28795^T=MTCC 12115^T).

Keywords Arsenic · Groundwater · *Rhizobium arsenicireducens* · Arsenate reduction · Hydrocarbon utilization

Introduction

Geogenic arsenic (As) in alluvial groundwater of West Bengal, Bangladesh and several other parts of Southeast Asia has created severe problems in drinking water resources, affecting millions of people. Geomicrobiological activities that form the integral components of As biogeochemical cycle in subsurface environment have been attributed to the prevalence of toxic As level in groundwater (Malasarn et al. 2008; Blum et al. 2009; Ohtsuka et al. 2013; Zhu et al. 2014). Bacterial transformation of sediment bound As (As^{5+}) and/or Fe (Fe^{3+}) of host minerals [Fe (Mn/Al)-oxides/hydroxides, phyllosilicates, and arseno-pyrites] is considered as the most feasible mechanism for As mobilization in sub-oxic alluvial groundwater (Paul et al. 2015). Some inhabitant bacteria use the sediment associated As^{5+} as a terminal electron acceptor during

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their anaerobic metabolism reducing it to more soluble As^{3+} species and are often categorized as dissimilatory As^{5+}-reducing bacteria (DARB) (Newman et al. 1998; Saltikov and Newman 2003; Kudo et al. 2013; Osborne et al. 2015). Although, the anaerobic reduction of sediment bound As^{5+} by DARB has been a subject of considerable interest to decipher the role of such processes in As biogeochemistry and in As release within the alluvial environment, until today thorough characterization of bacterial strains isolated from As-rich alluvial groundwater and capable of such transformation remains mostly elusive.

Alluvial aquifer of West Bengal is characterized to be of low nutrient content. Presence of alkane hydrocarbons in As-rich aquifer of West Bengal and organisms capable of utilizing many of them as sole carbon source and respiring As^{5+} have been recently reported (Ghosh et al. 2015; Paul et al. 2015). Under anaerobic condition, very low concentrations of metabolizable substrates (mostly in the form of aromatics, long-chain alkanes, hopanes and steranes as well as geochemically driven simpler organic molecules) have been found to be the key factors driving the dissimilatory (respiratory) As^{5+} reduction (Lear et al. 2007; Paul et al. 2015). With respect to taxonomic and physiological characterization of DARB, up to now 21 cultivable representatives have been studied (Saltikov et al. 2003; Saltikov and Newman 2003; Kudo et al. 2013; Osborne et al. 2015). Interestingly, except the strain WB-3 *Desulfuromonas*/*Pelobacter* sp. affiliated to the class *Gammaproteobacteria* (Osborne et al. 2015), no other strain was isolated from As-rich alluvial environment. No pure culture has been isolated from As-contaminated groundwater and studied for its taxonomic characterization, overall physiology and As biotransformation ability (respiratory function) under anaerobic condition. Although many studies have shown the abundance of members of the class *Alphaproteobacteria* (mostly *Rhizobium* species) within As-rich aquifer and investigated their capability of using As^{5+} anaerobically (Lear et al. 2007; Fan et al. 2008; Kudo et al. 2013; Sarkar et al. 2014; Drewniak et al. 2015; Paul et al. 2015), none of them have been taxonomically described. The present study was carried out to highlight the taxonomic description of an As^{5+}-respiring strain KAs 5-22^T isolated from As-contaminated groundwater of India.

Taxonomic description of the genus *Rhizobium* denotes its affiliation to the family *Rhizobiaceae,* order *Rhizobiales,* class *Alphaproteobacteria*, and phylum *Proteobacteria*. Since its first description by Frank (1889), 94 validly named species (LPSN, http://www.bacterio.net/) were affiliated to the genus *Rhizobium*. Members of the genus *Rhizobium* are characterized as Gram-stain-negative, non-spore forming and chemo-organotrophic rod-shaped bacteria with $C_{18:1}$ ω7c as the major fatty acid and DNA G + C content between 57 and 66 mol% (Tighe et al. 2000; Young et al. 2001). Members are well distributed in soil habitat and endowed with immense environmental as well as agricultural significance for their ability to fix nitrogen (N_2) in legume crops. They have been recognized for their ability to form root nodules on legumes and fix nitrogen (Viteri and Schmidt 1987; Young et al. 2001). Although most of the members have been isolated from nodules of leguminous plants (Mnasri et al. 2007), in recent years, an increasing number of new members have been isolated from diverse non-legume niches including sand dunes, effluent treatment plant, activated sludge, bioreactor, pesticide-contaminated sites, freshwater river, and sea water (Kaur et al. 2011; Ramana et al. 2013; Liu et al. 2015; Sheu et al. 2015). Among the recently described members, presence of diverse metabolic functions like naphthalene degradation (*R. naphthalenivorans*; Kaiya et al. 2012), selenite reduction (*R. selenitireducens*; Hunter et al. 2007), utilization of hydrocarbons for exopolysaccharides production (*R. alamii*; Berge et al. 2009), degradation of aniline (*R. borbori*; Zhang et al. 2011), polycyclic aromatic hydrocarbon (*R. petrolearium*; Zhang et al. 2012), triazophos (*R. flavum* Gu et al. 2014), etc. has been reported. During our earlier survey on cultivable microbial diversity of highly As-contaminated groundwater of West Bengal, one such *Rhizobium* strain, designated as KAs 5-22, was isolated (Sarkar et al. 2013). Polyphasic taxonomic approach including phenotypic, chemotaxonomic, phylogenetic, genotypic, as well as in vitro physiological tests and functional gene-based analysis was carried out to investigate the species novelty and metabolic potential of the strain.

Materials and methods

Bacterial strains and growth conditions

Strain KAs 5-22^T (MTCC 12115^T, BCCM 28795^T) was previously isolated from high As content groundwater (N 22°47.750 and E 88°44.121), of West Bengal, India, in the month of August, 2009 (Sarkar et al. 2013). Unless otherwise indicated, the strain was maintained and routinely subcultured on Luria Bertani (LB) agar or minimal salt medium (MSM) (Kazy et al. 1999). Reference strains of *Rhizobium* (*R. daejeonense* DSM 17795^T, *R. aggregatum* DSM 1111^T, *R. selenitireducens* LMG 1111^T, *R. radiobacter* IAM 12048^T, *R. rossetiformans* MTCC 9454^T, *R. naphthalenivorans* KCTC 23252^T), *Escherichia coli* K-12, and *Shewanella* sp. ANA-3 were obtained, grown in appropriate media and used in different experiments.

Phylogenetic and molecular analysis

Genomic and plasmid DNA were extracted according to the standard methods (Sambrook and Russel 2001).

Amplification of genes encoding 16S rRNA, *arr*A, *bss*A like, *nif*H and *nod*A was performed by using either universal specific or degenerate primers (Table S1). PCR amplicons were gel purified, cloned in pTZ57R/T vector (InsTA clone kit, Thermo scientific), and sequenced. For sequencing the nearly complete stretch of 16S rRNA gene, internal primers were used, and individual sequences were assembled, edited (BioEdit version 7.1.11), and subjected to similarity search in NCBI Genbank, Ribosomal Database Project (RDP), and EzTaxon-e server. Phylogenetic dendrograms were constructed using neighbor-joining (NJ) and maximum-likelihood (ML) methods with bootstrap analyses based on 1000 replications. Nucleotide sequences obtained for the coding genes were translated using ExPASy tools, where the appropriate reading frame was selected. The amino acid sequences were searched for protein similarity in non-redundant protein database (BLASTP), and functional domain similarity through conserved domain database (CDD). The NJ tree was constructed by using deduced amino acid sequences of the genes with respective similar sequences.

DNA G + C content and DNA–DNA hybridisation

Molar G + C content of the strain KAs 5-22^T was determined using the thermal denaturation method (De Ley et al. 1970) where *Escherichia coli* K-12 NCIM 2563 was used as an internal standard. DNA–DNA hybridization was carried out between strain KAs 5-22^T and its closest neighbors using the SyBr green binding fluorimetry-based method (Gonzalez and Saiz-Jimenez 2005). Optimum renaturation temperature ($\boldsymbol{T_{OR}}$) was calculated from the melting curve analysis. Hybridization was performed in 2X sodium chloride sodium citrate (SSC) buffer incorporating 0.1X SyBr dye following the real-time PCR program as described by Gonzalez and Saiz-Jimenez (2005). For each cycle, the relative fluorescence unit (RFU) (with an interval of 0.2 s) was plotted and ΔT_m as well as binding percentage (B_d%) was calculated following the equation as described by De Ley et al. (1970). The difference in ΔT_m values between homologous and hybrid DNA of 5 °C or higher was considered the cutoff value to discriminate between bacterial species (Wayne et al. 1987; Rosselló-Mora and Amann 2001). All experiments were conducted in triplicate, and the mean was indicated as sample values.

Morphological, biochemical, and physiological characterization

Cell morphology was studied using bright-field optical microscopy, fluorescence microscopy, and electron microscopy. For all microscopy, mid log phase cells were harvested, washed with normal saline (0.85 % w/v), fixed [formaldehyde or glutaraldehyde (0.2 %, v/v) in 0.1 mM phosphate buffer saline (PBS), at 4 °C, as appropriate, for 12 h], dehydrated (for SEM), stained appropriately, and viewed. Fluorescence microscopy was performed by staining cells with acridine orange (AO) [100 µg/mL (v/v)] and incubating in the dark for 20 min at 4 °C. For SEM, the cell suspension was fixed with glutaraldehyde; fixed cells were serially dehydrated with increasing concentration of ethanol (30–100 %) (v/v), placed on poly-L-lysine-coated cover glass, coated with gold, and viewed under SEM (SEM, JEOL JSM5800) using a Cu grid. Gram reaction was studied by using Gram's staining kit (Hi-Media). Motility was tested by the flagella staining protocol of Kodaka et al. (1982). Strain KAs 5-22^T along with all the taxonomically related type strains was tested for catalase, oxidase, and other general biochemical properties (viz. nitrate reduction, utilization of gelatin, esculin, citrate, urea, etc.) using either appropriate kits (Hi-Media or Bio-Merieux) as per the manufacturer's instructions, or following the standard procedures (Cowan and Steel 1965; Kelly and Fulton 1953; Smibert and Krieg 1994). Assimilation of carbon, nitrogen sources, and enzyme production was examined by API 20NE kit (Bio-Merieux) and Biolog GEN-III microplate following manufacturer's instructions. Temperature, pH, and salt (NaCl %) tolerance was monitored by allowing cell growth in MSM medium. Anaerobic growth was performed by growing cells on LB agar which was purged with a mixture of $CO_2/N_2/H_2$ [(90:5:5, (%)] and amended with L-Cys-HCl (0.05 %, v/v) and Na_2S (2 %, v/v of 70 mM stock solution) using an anaerobic work station (Coy Laboratories, USA). Inoculated plates were incubated in anaerobic gas jar with anaero gas-pak system. Antibiotic susceptibility was tested on Mueller-Hinton agar (Hi-Media) using disk diffusion method. The antibiotics disks used were as follows: ceftriaxone (30 µg), cefixime (5 µg), amikacin (30 µg), cefotaxime (30 µg), chloramphenicol (30 µg), ofloxacin (5 µg), polymyxin-B (300 units), tetracycline (30 µg), ciprofloxacin (5 µg), and erythromycin (15 µg). Arsenic resistance was tested following bacterial growth in LB broth amended with graded concentrations of As^{3+} ($NaAsO_2$; 0.1–30 mM) and As^{5+} (Na_2HAsO_4; 1–300 mM). The lowest concentration of As species, sufficient to completely inhibit bacterial growth, was considered as minimum inhibitory concentration (MIC).

Chemotaxonomic characterization

Cellular fatty acid methyl esters (FAMEs) and isoprenoid quinones were analyzed using cells from the mid log phase of growth. Cells mass collected through centrifugation was subjected to saponification followed by methylation and esterification of fatty acids (Miller 1982; Kuykendall et al. 1988). FAMEs were separated using a gas chromatograph (GC,

CLARUS 500, PerkinElmer) fitted with Omega-Wax capillary column (30 mm × 0.25 mm, d_f 0.25 µ) and detected by flame ionization detector (FID). FAMEs were identified by comparing with the bacterial fatty acid mixture standard (Sigma). Isoprenoid quinones were extracted from cells and analyzed by reverse phase high-performance liquid chromatography (HPLC) using Sorbax C18 reverse phase column (Agilent) (Komagata and Suzuki 1987). A mixture of methanol: isopropanol (2:1, v/v), using a flow rate of 1 mL min^{-1}, was used for elution of quinones which were subsequently identified by liquid chromatography–mass spectrometry (LC–MS) using positive ionization mode (Hiraishi et al. 1996). Polar lipids were extracted and analyzed by two-dimensional (2-D) thin-layer chromatography (TLC) (Komagata and Suzuki 1987).

Test for use of different electron and carbon donors, electron acceptors and As respiratory growth

Utilization of different electron and carbon donors was tested under both aerobic and anaerobic conditions using MSM supplemented with different sugars (at a concentration of 30 mM) and hydrocarbons (at a concentration of 100 mgL^{-1}). Bacterial growth was monitored by measuring the culture turbidity at 600 nm with a UV–Vis spectrophotometer (Cary 50, Varian). Anaerobic utilization of substrates was tested with As^{5+} (5 mM) as terminal electron acceptor. Utilization of different terminal electron acceptors (TEAs) was assessed by adding a range of test compounds (As^{5+}, Fe^{3+}, NO_3^-, etc.) into MSM with either glucose (30 mM) or lactate (30 mM) or nonadecane (100 mgL^{-1}) as sole carbon–electron source. Anaerobic growth was performed in serum vials (50 ml) containing MSM added with L-Cys-HCl (0.5 g L^{-1}) as reducing agent and resazurin (0.01 %, w/v) as redox indicator. Vials were crimp sealed keeping 30 % (v/v) head space filled with filtered N_2 and autoclaved. All anaerobic experiments were set up within anaerobic work station (Coy laboratory, USA) purged with N_2. Concentrations of TEAs during growth were measured by standard spectrophotometric methods. Concentrations of glucose and nonadecane were estimated by a modified phenol sulfuric acid method (Albalasmeh et al. 2013) and GC-FID, respectively. Concentration of As was measured by inductively coupled plasma–mass spectrometer (ICP-MS) (Varian 810 ICP-MS System). All experiments were done in triplicate, and mean was quoted as sample values.

Results and discussion

Phylogenetic and molecular analysis

Comparison of the nearly complete 16S rRNA gene sequence (1440 nucleotides; Genbank accession number JX173993) indicated the taxonomic affiliation of strain KAs 5-22^T to the genus *Rhizobium*, with highest sequence similarity to *R. naphthalenivorans* TSY03b^T (98.4 %) and *R. selenitireducens* B1^T (98.0 %) strains while similarities with all other species of *Rhizobium* were in the range of 93.0–97.5 %. The NJ phylogenetic analysis showed that the strain KAs 5-22^T formed a coherent cluster with *R. naphthalenivorans* TSY03b^T and *R. selenitireducens* B1^T by a bootstrap value of 97.0 % (Fig. 1) and confirmed the affiliation of strain KAs 5-22^T within the genus *Rhizobium*. Similar tree topology was also inferred through ML method suggesting robustness of the phylogenetic tree (Supplementary Fig. S1).

The genomic G + C content of the strain KAs 5-22^T was found to be 59.4 ± 1.0 %, a value that fits well within the 57–63 % GC content range reported for the members of *Rhizobium* genus and also with that of its closest taxonomic neighbors *R. naphthalenivorans* TSY03b^T and *R. selenitireducens* B1^T (Table 1). As reported by Gonzalez and Saiz-Jimenez (2005), members of the genus *Rhizobium* have G + C mol% that may vary up to 10 %, whereas members of the same species have G + C mol% values that are within 3–5 % of each other. DNA–DNA hybridization analysis of strain KAs 5-3^T with two close taxonomic neighbors indicated 50.2 ± 1.5 % (ΔT_m of 6.6 °C) of relatedness with both *R. selenitireducens* B1^T and *R. naphthalenivorans* TSY 03b^T. In relation to other related type members, DNA level relatedness of 48.5 ± 1.0 % (ΔT_m of 6.8 °C), 47.0 ± 1.2 % (ΔT_m; 6.8 °C), and 42.0 % (ΔT_m 7.2 °C) was observed with *R. daejeonense* L61^T and *R. aggregatum* IFAM 003^T and *R. rossetiformans* W3^T, respectively. Since the difference in ΔT_m between strain KAs 5-22^T and closest type strains is higher than 5 °C (maximum of 7.2 °C and minimum of 6.6 °C) and hybridization data indicated <70 % similarity at DNA level, the strain KAs 5-22^T may be considered as a novel species (Wayne et al. 1987; Stackebrandt and Goebel 1994). Thus, the phylogenetic analysis and DNA–DNA relatedness (% hybridization) clearly indicated that strain KAs 5-22^T belongs to the genus *Rhizobium* and represents a distinct species.

Morphological, biochemical, and physiological characterization

All phenotypic properties of strain KAs 5-22^T and its taxonomic neighbors are presented in Table 1. Colonies of strain KAs 5-22^T were observed to be circular, convex, translucent, and entire with gram stain negative, rod-shaped (1.2–1.5 µm × 0.6–0.8 µm dimensions), motile cells capable of facultative anaerobic growth (Supplementary Fig. S2). Positive responses of strain KAs 5-22^T toward nitrate, citrate, β-galactosidase, N-acetyl glucosamine, L-ornithine, reduction of tetrazolium salts and inability to use glucose

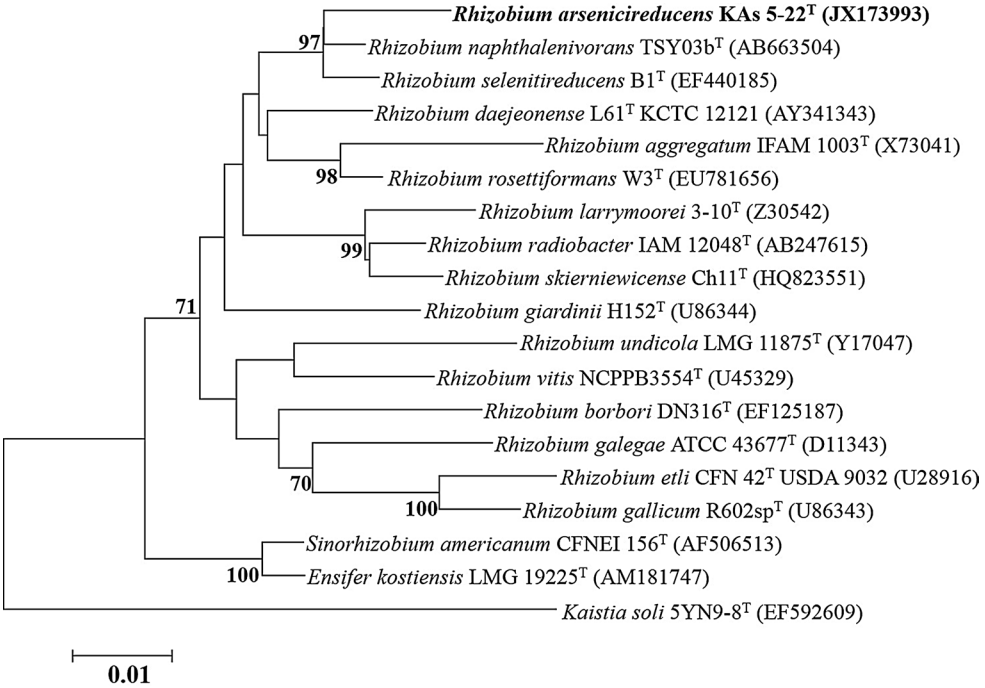

Fig. 1 Neighbor-joining tree based on 16S rRNA gene sequences showing phylogenetic relationship between strain KAs 5-22^T and its closely related phylogenetic neighbors, constructed through MEGA version 5.0. Numbers at nodes represent bootstrap values obtained with 1000 replications. The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. *Bar* 0.01 indicates 1 % nucleotide substitution

(fermentative), arabinose, mannose, manitol, gluconate, caprate, adipate, PAC are in line with similar results for *R. naphthalenivorans* TSY03b^T and *R. selenitireducens* B1^T. The strain showed its ability to utilize various D-sugars, deoxy sugars, amino sugars, sugar acids, and sugar phosphates, but inability to use di- or oligo-sugars and sugar alcohols (except glycerol) (data not shown). Along with the motile nature of the strain KAs 5-22^T, its ability to grow at 28–30 °C, pH 6–8 and 1–3 % NaCl; use of glucose, NAG, with few organic acids and inability to use cellulose is in good agreement with the common traits of *Rhizobium* members (Young et al. 2001). Ability to utilize L-proline, serine, and reduction of methylene blue could be attributed to non-nodulating nature of the strain (Kaur et al. 2011). Strain KAs 5-22^T also showed high resistance toward As, compared to its reference strains, among which none of them could be able to tolerate As concentrations higher than 0.5 mM for As^{3+} and 2 mM for As^{5+} (Table 1).

Chemotaxonomic characteristics

The predominant quinone of the strain KAs 5-22^T was found to be UQ-10, along with minor percentage of UQ-8 (Table 2), which was consistent with other members of the genus *Rhizobium* (Tighe et al. 2000). UQ-10 was earlier reported to be the major quinone of *R. naphthalenivorans* TSY03b^T (Kaiya et al. 2012). Among the predominant fatty acids (>5 % of the total fatty acid content) of KAs 5-22^T, $C_{10:0}$ 2-OH (11.3 %) and $C_{16:0}$ 3-OH (10.25 %), were not detected in any of the closest relatives tested, while $C_{16:0}$ (5.8 %) and $C_{17:0}$ (6.56 %) were found to be present in others, but at relatively lower proportions. Unresolved $C_{18:1}$ ω7C/ω9C found to be present at higher proportion (26.8 %) seems to be signature for *Rhizobium* members. Polar lipid profile of strain KAs 5-22^T showed a similar composition to that of all the strains compared, particularly with respect to presence of PE, PG, and DPG, while absence of PL1, PL2 and GL1 was observed in both the closest taxonomic neighbors *R. naphthalenivorans* TSY03b^T and *R. selenitireducens* B1^T (Table 2).

Use of different e-donors, e-acceptors and As respiratory growth

The strain KAs 5-22^T was found to be capable of using various sugar, sugar acids and hydrocarbons including both long-chain alkanes and poly aromatics (Table 3). Carbon or electron preference of the closest taxonomic type strains with that of the strain KAs 5-22^T was compared (Table 3). A good agreement on both utilization and non-utilization of several of the test substrates is found between strain KAs 5-22^T and its closest neighbors *R. naphthalenivorans* TSY03b^T and *R. selenitireducens* B1^T. With respect to metabolism of hydrocarbons, observed performance of strain KAs 5-22^T is in line with *R. naphthalenivorans* TSY03b^T. Notably, with respect to utilization of sugars except lactate and citrate none of the other carbon sources are utilized by the two closest relatives under anaerobic condition. Similar to strain KAs 5-22^T, *R. naphthalenivorans* TSY03b^T showed its ability to utilize pentadecane and nonadecane under anaerobic condition.

Table 1 Differential phenotypic properties of the strain KAs 5-22^T with its closely related type strains

Properties	Bacterial strains					
	1	2	3	4	5	6
Habitat	CGW	BR	SS	CBR	LW	HDS
Gram	−ve, rod	−ve, rod	−ve, rod	−ve, rod	−ve, rod	−ve, rod
Motility	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Temp opt. (°C)	28–30	28–32	28–30	28–30	28–30	25–40
(10 °C)	+	−	−	+	+	−
(42 °C)	+	−	−	+	+	−
pH	6–8	6–8	6–9	7–8	5–9	6–10
NaCl (%)	0.5–3	0.5–2	0.5–3	0.5–3	0.5–3	0.5–3
Nitrate	+	+	−	−	−	+
Citrate	+	−	+	−	−	+
Urea	+	−	−	+	+	−
ESC	−	+	−	+	+	+
GEL	−	−	+	−	−	−
PNPG	+	+	+	+	+	+
Glucose (f)	−	−	−	+	+	+
C source						
Glucose	+	+	−	+	+	+
ARA	−	−	+	+	ND	ND
MNE	−	−	−	+	ND	ND
MAN	−	−	−	+	+	+
NAG	+	−	+	+	ND	ND
MAL	−	+	−	+	+	+
GNT	−	−	−	−	ND	ND
CAP	−	−	−	+	ND	ND
ADI	−	−	−	−	ND	ND
MLT	−	−	+	−	ND	ND
PAC	−	−	−	−	ND	ND
N source						
L-Ornithine	+	+	ND	−	+	+
L-Proline	−	+	ND	+	−	+
L-Serine	−	+	ND	+	−	+
D-Serine	+	−	ND	ND	ND	ND
Reduction of tetrazolium dye	+	+	−	ND	ND	ND
*nif*H gene	−	−	−	−	+	+
As resistance [mM] (III/V)	10/100	0.2/1	0.2/2	0.2/1.5	0.2/2	0.5/1.5
DNA G + C (%)	59.4	60.8	60.3–60.9	60.1–60.9	62.7	62.3

Strains 1, KAs 5-22^T (isolated from arsenic-contaminated groundwater, this study); 2, *R. selenitireducens* LMG 24075^T (bioreactor, this study, except for G + C mol% taken from Hunter et al. 2007); 3, *R. naphthalenivorans* TSY 03b^T(sediment slurry from anaerobic microcosm, this study, except for G + C mol% taken from Kaiya et al. 2012); 4, *R. daejeonense* DSM 17795^T (cyanide bioreactor, this study, except for G + C mol% taken from Quan et al. 2005); 5, *R. aggregatum* DSM 1111^T (lake water, Kaur et al. 2011); 6, *R. rosettiformans* CCM 7583^T (hexachlorocyclohexane dumpsite, Kaur et al. 2011); + Positive, − Negative, *ND* no data available, *GLC* Glucose, *URE* urease, *GEL* gelatin, *PNPG* β-galactosidase, *ARA* arabinose, *MNE* mannose, *MAN* mannitol, *NAG* N-acetyl glucosamine, *MAL* malate, *GNT* gluconate, *CAP* caprate, *ADI*- adipate, *MLT* malate, *CIT* citrate, *PAC* phenyl acetate. All data are from this study unless otherwise indicated

Table 2 Differential chemotaxonomic properties of the strain KAs 5-22^T and its taxonomically related type strains

Fatty acids	Bacterial strains					
Saturated	1	2	3	4	5	6
16:0	5.8	2.6	2.0	2.2	2.7	3.2
17:0	6.5	1.2	–	–	–	1.0
18:0	1.4	2.9	10.0	2.1	2.5	3.8
19:0 cyclo ω8C	–	1.5	5.0	16.8	–	–
Unsaturated						
18:1	–	–	–	1.3	–	–
20:1	4.8	–	1.5	–	–	–
Hydroxyl						
10:0 2-OH	11.3	–	–	–	–	–
16:0 3-OH	10.2	–	–	–	–	–
18:0 3-OH	–	2.6	2.5	3.1	2.4	5.1
Unresolved						
12:0 unknown, 16:1 iso I/I 3-OH	3.6	5.8	3.5	12.4	5.5	8.7
18:1 ω7C and/or ω9C	26.8	60.5	38.5	51.5	70.8	65.4
Major quinone	UQ-10	ND	UQ-10	ND	ND	ND
Polar lipids						
PE	+	+	+	+	ND	+
PG	+	+	+	+	ND	+
DPG	+	+	+	+	ND	+
CL	+	+	−	+	ND	+
PL1	+	−	−	+	ND	−
PL2	+	+	−	+	ND	−
GL1	+	+	−	+	ND	−
AGL	+	−	−	+	ND	−

Strains 1, KAs 5-22 (this study); 2, *Rhizobium selenitireducens* LMG 24075^T (this study); 3, *Rhizobium naphthalenivorans* TSY 03b^T (this study); 4, *Rhizobium daejeonense* DSM 17795^T (data taken from Quan et al. 2005); 5, *Rhizobium aggregatum* DSM 1111^T(data taken from Kaur et al. 2011); 6, *Rhizobium rosettiformans* CCM 7583^T (data taken from Kaur et al. 2011); + detected; − not detected, *ND* not determined, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *DPG* diphosphatidylglycerol, *CL* choline-containing lipid, *PL* phospholipid, *GL* glycolipid, *AGL* aminoglycolipid; For fatty acids, all values are percentage of total fatty acids, fatty acid with value <1 % are considered as negligible and stated as negative, all data are from this study unless otherwise indicated. For polar lipids, data are taken from the published literatures. Figure showing polar lipid profile of strain KAs 5-22T is presented as Supplementary Fig. S3

Anaerobically, strain KAs 5-22^T was found to use As^{5+}, Fe^{3+}, SO_4^{2-}, NO_3^- and NO_2^- as TEAs with an order of preference (As^{5+}> NO_3^- > Fe^{3+}> SO_4^{2-} > NO_2^-). As^{5+} respiratory growth was further characterized with As^{5+} as the sole TEA and glucose or nonadecane as C and energy source, and it was found that anaerobic growth rate was low compared to that of aerobic growth. Anaerobically, strain KAs 5-22^T showed a faster growth rate and higher cell yield for glucose [final cell yield (CFU/ml) of 1×10^9 (nonadecane) and 1×10^{12} (glucose)] (Fig. 2). Within 60 h, ~95 % of the added As^{5+} was reduced to As^{3+} with glucose, while at ~80 h, complete reduction was achieved with both the substrates. Addition of nitro-phenol (as respiratory inhibitor) confirmed its As^{5+} respiratory metabolism.

Hydrocarbon metabolizing ability of KAs 5-22^T, reducing As^{5+} is in line with its natural habitat, i.e., alluvial aquifer enriched with geogenic As (mainly as As^{5+} sorbed on Fe/Mn(Al) oxides-hydroxide minerals) that has been found to contain low concentration of alkane and other hydrocarbons (Ravenscroft et al. 2001; McArthur et al. 2004; Rowland et al. 2006; Ghosh et al. 2015). Alkane metabolism by bacterial strains isolated from As-rich groundwater of West Bengal has been recently reported (Paul et al. 2014, 2015).

Functional gene-based analysis

Genetic potential of strain KAs 5-22^T to metabolize hydrocarbons and respire with As^{5+} was evaluated by analyzing genes encoding benzyl succinate synthase (*bss*A-like gene)

Table 3 Comparison on electron donor/carbon substrates utilization profile of the strain KAs 5-22^T and other closely related type strains

Electron/carbon substrates	Bacterial strains (growth in the presence of O_2/As^{5+} as electron acceptors)						
	1	2	3	4	5	6	7
Glucose	+/+	+/−	+/−	−/−	−/−	−/−	+/−
Lactate	+/+	+/+	+/+	+/−	+/−	−/−	+/+
Citrate	+/−	+/+	+/−	+/−	+/−	−/−	+/−
Glycerol	+/−	+/−	+/−	+/−	+/−	+/−	+/−
Acetate	+/−	+/−	−/−	−/−	−/−	−/−	+/−
Pyruvate	+/−	−/−	+/−	−/−	−/−	−/−	−/−
Fructose	+/−	−/−	+/−	+/−	+/−	−/−	−/−
Mannitol	−/−	+/−	+/−	+/−	+/−	+/−	+/−
Arabinose	−/−	+/−	+/−	−/−	−/−	−/−	+/−
Fumarate	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Succinate	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Formate	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Ethanol	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Sucrose	−/−	−/−	−/−	+/−	+/−	−/−	−/−
Ascorbate	−/−	−/−	−/−	−/−	+/−	−/−	−/−
Inositol	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Bicarbonate	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Cyclohexane	+/−	+/−	+/−	−/−	−/−	+/−	+/−
Dodecane	+/w	−/−	−/w	−/−	−/−	+/−	−/−
Pentadecane	+/+	−/−	−/+	−/−	−/−	−/−	−/−
Nonadecane	+/+	−/−	−/+	−/−	−/−	−/−	−/−
Naphthalene	+/−	−/−	+/−	−/−	−/−	−/−	−/−
Phenanthrene	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Pyrene	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Anthracene	+/−	−/−	+/−	−/−	−/−	−/−	−/−

Strains 1, KAs 5-22^T; 2, *R. selenitireducens* LMG 24075^T; 3, *R. naphthalenivorans* TSY 03b^T; 4, *R. daejeonense* DSM 17795^T; 5, *R. aggregatum* DSM 1111^T; 6, *R. rosettiformans* CCM 7583^T; 7, R. radiobacter 12048^T; + Positive, − Negative; growth OD (600 nm) > 0.1 considered as positive; growth OD (600 nm) <0.5 considered as negative, *w* weak growth (0.1 > OD < 0.5) after 48 h of growth. All data are from this study, unless otherwise indicated

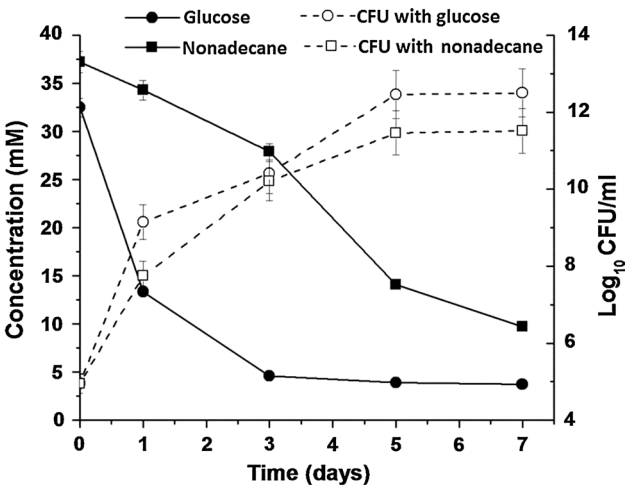

Fig. 2 Growth (as $\log_{10}$ CFU/ml) kinetics of KAs 5-22^T and utilization of substrates under dissimilatory As^{5+}-reducing condition

and As^{5+} respiratory reductase (*arr*A). Anaerobic oxidation of hydrocarbons proceeds through fumarate addition (as a primary reaction step) (catalyzed by benzyl succinate synthase family of proteins), followed by β-oxidation pathway (Aitken et al. 2013). Amplification of 450 bp of putative *bss*A-like gene (accession KX011179) and its phylogenetic analysis with similar genes (belonging to pyruvate formate lyase superfamily [like *bss*A, *nms*A and *ass*A]) confirmed its affiliation (Supplementary Fig. S3). Similarly, the strain's ability to use As^{5+} as respiratory substrate was complemented by detection of *arr*A gene stretch (560 bp; accession KR340465). Similarity search showed highest identity (90 %) of this sequence with molybdopterin-binding superfamily (*mop*B) of membrane bound respiratory reductases. NJ tree of deduced amino acid sequence further confirmed its evolutionary closeness with the molybdopterin-binding protein of DMSO transmembrane reductase

superfamily (Supplementary Fig. S4). Amplification of genes for nitrogen fixation (*nifH*) and nodulation (*nod*A) was further attempted. Presence of *nif*H gene could be detected only from the plasmid, while *nod*A could not be amplified (both genomic and plasmid DNA), indicating the strain's nitrogen fixing non-nodulating behavior.

In conclusion, strain KAs 5-22^T showed a close taxonomic resemblance with members of the genus *Rhizobium*, thereby providing evidence on its affiliation to the same genus. The observed distinctiveness of strain KAs 5-22^T in terms of taxonomic and physiological characters clearly indicated its uniqueness, which might be attributed to strategies for competitive survival of the organism (strain KAs 5-22^T) in As-rich oligotrophic aquifer environment. Based on the results obtained, it is proposed that strain KAs 5-22^T represents a novel species of the genus *Rhizobium*, with the name *Rhizobium arsenicireducens*.

Description of *Rhizobium arsenicireducens* sp. novel

Rhizobium arsenicireducens (ar.se.ni.ci.re.du'cens. L. n. arsenicon, arsenic; L. part. adj. reducens, N.L. part. adj. arsenicireducens, arsenic-reducing, referring to the ability of the organism to reduce arsenic during its growth).

Cells are rod-shaped, gram-stain-negative, motile, facultative anaerobic with catalase and oxidase positive activity. On agar plate, colonies are small, circular with entire margins, pale creamy, and approximately 1–2 mm in diameter. Optimum growth occurs at pH 6.0–8.0 with 1 % NaCl at 28–30 °C. It shows positive reaction for citrate, N-acetyl glucosamine, glucose, urea, D-galactose, D-fructose, L-fucose, D-glucose-6-phosphate, D-fructose-6-phosphate, dextrin, trehalose, inosine, glycerol, L-rhamnose, L-galactonic acid, L-gluconic acid, L-glucuronic acid, L-lactic acid, L-malic acid, lactate, methyl pyruvate, α-ketoglutarate, α-hydroxy butyrate, acetic acid, citric acid, bromo-succinic acid, acetoacetic acid, propionic acid, N-acetyl mannosamine, and N-acetyl neuraminic acid. It can also use L-alanine, L-aspartate, D-serine but not L-glutamate, D-aspartate, L-serine, L-arginine, glycyl proline, L-histidine, pyroglutamate and amino butyric acid as preferred N source. It shows resistance to erythromycin, troleandomycin, lincomycin, aztreonam and can metabolize tetrazolium dyes. The major cellular fatty acids are $C_{16:0}$, $C_{17:0}$, 2-OH $C_{10:0}$, 3-OH $C_{16:0,}$ and unresolved $C_{18:1}$ ω7C and/or ω9C, and the major quinone is UQ-10. The DNA G + C content is 59.4 mol%. The polar lipids include phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, unidentified aminolipid, glycolipid, and one choline-containing lipid. Presence of genetic determinants encoding *mop*B of *arr*A, PFL domain of *bss*A-like gene, and *nif*H along with apparent absence of *nod*A gene confirmed further the physiological novelty of the strain. Based on biochemical, chemotaxonomic, genotypic and physiological properties, the strain is proposed to be a novel representative species of the genus *Rhizobium,* for which the name *Rhizobium arsenicireducens* sp. nov. is proposed.

The type strain KAs 5-22^T (=BCCM/LMG 28795^T=MTCC 12115^T) is isolated as an As^{5+}-reducing and highly As-resistant bacterium, from As-contaminated groundwater of North 24 Parganas, West Bengal, India.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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