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Isolation and characterization of an arsenic-resistant bacterium from a bore-well in West Bengal, India

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Abstract - An arsenic-resistant bacterium, strain KRPC10Y^T, was isolated from arsenic-infested bore-well of West Bengal, India. The bacterium was resistant to exceeding concentrations of arsenate (30 mM) and arsenite (20 mM). The bacterium was Gram-positive, rod-shaped, motile and yellowish to orange-pigmented. The major fatty acids were anteiso-C15:0, iso-C15:0. The DNA G+C content was 49 mol %. Based on its phenotypic, chemotaxonomic and phylogenetic characteristics, it was identified as a member of the genus *Planococcus* and is the first known *Planococcus* resistant to arsenic. KRPC10Y^T was positive for indole, catalase, tolerated up to 12.0% NaCl and exhibited phenotypic differences with other type strains of genus *Planococcus*. Strain KRPC10Y^T thus could be a novel species of the genus *Planococcus*. The type strain is KRPC10Y^T (= MTCC 7758^T, = JCM 13947^T).

Key words: arsenic-resistance, bacteria, fatty-acids, Gram-positive, *Planococcus*.

INTRODUCTION

Arsenic is a toxic, naturally occurring element that is sometimes found at high concentrations in well water. Arsenic contamination of water has been reported in various parts of the world, including India, where arsenic has contaminated the groundwater in the entire Bengal delta, which covers the Indian state of West Bengal and parts of Bangladesh (Bhattacharya *et al*., 2002, 2006). The source of arsenic in this site has been attributed to both the oxidation of pyrite/arsenopyrite in the aquifer (Bhattacharya *et al*., 2002, 2006) and the reduction of ferrous-coated sand grains (Nickson *et al*., 1998).

According to a recent research report published in *Nature* (Islam *et al*., 2004), bacteria are responsible for the release of arsenic into water from surrounding earth. The microbes gain energy by changing the chemistry of minerals containing both iron and arsenic, and release the arsenic into the water as a by-product of the reaction. Without such bacterial activity, the arsenic would remain in an insoluble form, and thus be unable to contaminate the water. The presence of arsenic in the environment may thus lead to the enrichment of arsenic-resistant bacteria. Diverse microbial flora that is resistant to arsenic, including *Deinococcus indicus* (Suresh *et al*., 2004), *Pseudomonas fluorescens* (de Vicente *et al*., 1990; Prithivirajsingh *et al*., 2001), *Bacillus subtilis* (Sato and Kobayashi, 1998), *Thermus aquaticus* and *Thermus thermophilus* (Gihring *et al*., 2001), *Yersinia enterocolitica* and *Yersinia intermedia* (Bansal *et al*., 2000), *Streptomyces noursei* (Friedrich *et al*., 1984) and *Desulfitobacterium* sp. (Niggemyer *et al*., 2001) has already been reported from various habitats.

The goal of this research study was to isolate bacteria from arsenic-contaminated environments that contain mechanisms of resisting arsenic and begin characterization and identification of the isolate. We hypothesize that the isolated bacteria highly resistant to arsenic could represent good candidates for bioremediation processes of native polluted sediments. In the present study, we report the isolation of an arsenic-resistant novel species that was isolated from arsenic contaminated bore-well of village Kamdevkathi, North 24 Parganas, West Bengal, India; North 24 Parganas represent one of the most severely arsenic-effected districts of West Bengal.

The isolated bacterial strain, KRPC10Y^T, is resistant to both arsenite and arsenate. Based on its phenotypic characteristics, chemotaxonomic properties and 16S rRNA gene sequence, the strain was identified as representing a novel species of the genus *Planococcus*, for which the name *Planococcus bengal* sp. nov. is proposed.

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MATERIALS AND METHODS

Strain origin and estimation of arsenic content in sample water. Water sample was collected from an arsenic-contaminated bore well, supplying drinking water to village Kamdevkathi, North 24 Parganas, West Bengal, India. The arsenic content of the sample was analyzed at the School of Environmental Studies, Jadavpur University, Kolkata. Flow injection-hydride generation-atomic absorption spectrometry (FI-HG-AAS) was used for the estimation of arsenic in the collected sample. A Perkin-Elmer Model-3100 (Boston, MA, USA) spectrometer equipped with a Hewlett-Packard (Houston, TX, USA) Vectra computer with GEM software, Perkin-Elmer EDL System-2, arsenic lamp (lamp current 400 mA) was utilized for the purpose.

Media and culture conditions. To check for the presence of bacteria in the collected arsenic-contaminated water sample, the dilution plating technique on Luria-Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.4, 1.5% Bactoagar, all w/v) (Difco, BD, USA) was used. After incubating the plates at 37 °C for 2 days, six different colony morphotypes were visualized. Arsenic sensitivity of the six colony morphotypes was tested thereafter. Cultures were grown in either LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.4, all w/v) or MOPS minimal media (containing either 5 mM high phosphate or 0.2 mM low phosphate) (Neidhardt *et al*., 1974) and M9 minimal media (5X M9 salt, 1M $MgSO_4$, 20% glucose, 0.1 M $CaCl_2$) containing either sodium arsenate (Na_2HAsO_4) or arsenite ($NaAsO_2$) to determine the tolerance of the culture to arsenate and arsenite. The bacterial strain KRPC10Y^T representing one of the morphotypes showing high resistance to arsenic was thus isolated and further characterized. LB agar without arsenic was primarily used for growth, maintenance and biochemical tests of the strain. The optimum pH and temperature for growth were 7.4 and 37 °C, respectively. The pure culture was maintained on LB slants at room temperature or preserved as suspension in 20% glycerol (v/v) at –70 °C.

Morphology, motility and physiological tests. Strain KRPC10Y^T was observed under a phase-contrast microscope (DIPLAN; Leitz) to ascertain motility.

For the various physiological tests listed in Table 1 and referred in the description of the species, the cultures were grown at 37 °C in LB broth and the tests were performed as described by Lanyi (1987) and Smibert (1994). Gram reaction was determined using the HiMedia Gram Staining kit according to the manufacturer's instructions. To check for the level of resistance to arsenic, strain KRPC10Y^T was grown at 37 °C in LB broth containing either 500 mM, 1.25 mM, 2.5 mM, 5 mM, 10 mM, 20 mM, 30 mM and 40 mM arsenate (Na_2HAsO_4) or arsenite ($NaAsO_2$). To check for arsenic resistance of bacteria at low phosphate level the strain was grown in low phosphate MOPS minimal media (0.2 mM phosphate) in presence or absence of arsenate (30 mM) and arsenite (20 mM). Growth was monitored spectrophotometrically (at OD_{600}) for a period of 72 h (Hitachi U-1100 spectrophotometer, Tokyo, Japan). For carbon utilization studies, KRPC10Y^T was grown in bacterial minimal medium [1.05% (w/v) K_2HPO_4, 0.45% (w/v) KH_2PO_4, 0.1% (w/v) $(NH_4)_2SO_4$, 1.5% (w/v) agar] supplemented with 0.5% (w/v) filter sterilized various carbon compounds like glucose, fructose, sucrose, galactose, maltose, lactose, glycerol, lactic acid, glutamic acid, cellulose or glycogen as the sole source of carbon.

The sensitivity of strain KRPC10Y^T to various antibiotics was checked in LB agar. LB broth was used to check the NaCl (%) tolerance tests with different concentration (0 to 15%) of NaCl. Similarly growth condition at different pH (pH 4 – pH 8) was performed.

DNA extraction and Mol % G + C determination. DNA was isolated by the method of Sambrook (1989). Briefly, several colonies were picked from the agar plate and transferred to a 1.5 ml microcentrifuge tube. The biomass was resuspended in 0.1 ml sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). The cells were pelleted (13400 *x g*, 5 min, Biofuge, Heraeus Insruments, Germany), washed three times in 1 ml sterile TE buffer, and resuspended in 0.1 ml TE. TE buffer containing 3% (w/v) SDS (0.2 ml) was added to the cells and mixed by a vortex mixer for 3 min. The cell lysate was extracted three times with 0.6 ml TE buffered phenol and three times with chloroform. The aqueous phase was transferred to a fresh microcentrifuge tube and two volumes of ice-cold absolute ethanol were added. The DNA was precipitated overnight at -20 °C and pelleted by centrifugation (13400 *x g*, 10 min). The DNA was redissolved in 50 ml sterile TE buffer and used directly in PCR reactions. DNA preparations typically contained 50-200 ng DNA per ml. Determination of the mol% G+C content of the DNA was determined from the melting point (*T*m) curves obtained using a Hitachi Spectrophotometer as described by Shivaji *et al*. (1989). The equation of Schildkraut (1965) was used to calculate the G+C content (mol %) of the DNA.

TABLE 1 - Differential phenotypic properties of strain KRPC10Y^T and *Planococcus* species

Characteristics	*Planococcus bengal* JCM 13947^T	*Planococcus maritimus* JCM 11543^T	*Planococcus citreus* DSM 20549^T
Growth in NaCl:			
10%	+	+	+
12%	+	+	+
15%	–	+	+
Max. Temperature for growth (°C)	40	41	ND
Acid production from:			
D-Glucose	+	+	+
D-Mannitol	+	+	–
Melibiose	–	–	–
DNA G+C content (Mol %)	49	48	48-51

Strain/species: column 1: KRPC10Y^T, column 2: *Planococcus maritimus* JCM 11543^T (Yoon *et al*., 2003), column 3: *Planococcus citreus* DSM 20549^T (Hao & Komagata, 1985). All strains/species are positive for Gram stain, motility, catalase and growth in the absence of NaCl and are negative for oxidase, urease, nitrate reduction and acid production from D-cellobiose and lactose.
+: Positive, –: negative, ND: Not determined.

Fatty acid analysis. Cells were grown in LB broth at 37°C to the early stationary phase, harvested by centrifugation (7000 *x g*, 10 min) and washed twice with sterilized 0.1 M phosphate buffer saline (PBS, pH 6.8). Cellular fatty acid methyl esters were obtained using the method described by Sato (1988) and were analyzed as described by Reddy *et al*. (2002). The peaks were identified by GLC-MS (GC-17A Shimadzu) using DB-5 column (30 mm x 0.25 mm x 0.25 mm) and a temperature program of 160 °C for 2 min, 3 °C/min, 220 °C for 15 min.

Amplification and sequencing of 16S rRNA genes. The chromosomal DNA of strain KRPC10Y^T was isolated as described above and DNA concentrations were quantified by UV spectrophotometry at 260 nm (Hitachi U-1100 spectrophotometer, Tokyo, Japan). The bacterial 16SrRNA gene was amplified with universal 16S rDNA primers, forward 5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCA-3′, and reverse 5′-CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3′ (Weisburg *et al*., 1991). The initial denaturation step consisted of heating the reaction mixture at 95 °C for 3 min, and this was followed by an annealing step (45 °C, 1 min) and an extension step (72 °C, 2 min). The thermal profile consisted of 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 1 min and extension at 72 °C for 2 min. A final extension was carried out at 72 °C for 5 min. The amplification reaction produced DNA molecules of about 1.5 kb. The amplified DNA fragment was separated on a 1% agarose gel, eluted from the gel and purified using the QIA quick gel extraction kit (Qiagen). The purified PCR product was sequenced using an ABI PRISM model Avant 3700 automatic DNA sequencer and the Big Dye Terminator cycle sequencing kit (Applied Biosystems, USA). To have an almost complete bacterial 16S rRNA gene sequence, a pair of appropriate nested primers was also used for the sequencing reaction.

16SrRNA gene sequence accession number. The nucleotide sequence realized for this study has been deposited in EMBL Database and appears under the accession number DQ375559.

BLAST analysis and molecular phylogenetic tree. Sequence similarity with other type strains of the genus *Exiguobacterium* was assessed using BlastN (http://www.ncbi.nlm.nih.gov/blast) with the 16S rRNA gene sequence of KRPC10Y^T as query in the non- redundant database of NCBI. From pairwise alignments, sequence identities were recorded for 16S rRNA gene sequence of the reported type strains with KRPC10Y^T. Phylogenetic analysis was performed using MEGA version 3.0 (Kumar *et al*., 2004). For the construction of phylogenetic tree 16S rRNA gene sequence of the strain KRPC10Y^T was aligned by multiple sequence alignment using ClustalW 1.6 algorithm, with type strains of the genera, *Planococcus,* retrieved from the GenBank database. To convert sequence similarities into evolutionary distances both Jukes-Cantor model, which is based on simple probabilistic approach, and Kimura 2-parameter model, which takes into account both transition and transversion, were used, considering gamma correction with a gamma value of 0.25. To determine the evolutionary position of the strain KRPC10Y^T, phylogenetic trees were constructed using the Neighbor Joining and UPGMA method with the 16S rRNA gene sequence of KRPC10Y^T along with the species of the closely related genera. For the estimation of the sampling error and to evaluate the robustness of the inferred trees bootstrapping was performed with 1000 replicates and 70000 random seeds. To assess the stability amongst the clades of the phylogenetic tree, the original tree was compared with the consensus bootstrap tree.

RESULTS AND DISCUSSION

Morphology and physiological characterization

Cells were rod-shaped and occurred singly, in pairs or in groups of three and were motile as observed through phase contrast microscopy. They were Gram-positive and strictly aerobic. Colonies were smooth, glistening, low-convex, circular, uniform-edged, punctiform and yellow to orange in colour and 1-2 mm in diameter after 3 days cultivation at 30 °C on Nutrient agar. Pigment was insoluble in water but soluble in methanol. Temperature is another important environmental factor, which affects bacterial growth (Herbert and Bhakoo, 1979). Bacteria were psychrophilic and exhibited optimum growth at 30 °C, but growth occurred at 4 to 40 °C but not at above 41 °C. The optimum temperature range for the growth of KRPC10Y^T in the present study support the results observed by Bouchard *et al*. (1996), Christiansen *et al*. (1996) and Niggemeyer *et al*. (2001). Optimal pH for growth is 6-8. Growth occurred weakly at pH 5 but not at pH 4.5.

Bacteria grew in the presence of 0-12% (w/v) NaCl. The strain was also able to survive in low phosphate MOPS medium supplemented with arsenate (30 mM) and arsenite (20 mM). The strain was positive for catalase, indole, amylase and protease, but negative for oxidase, benzidine, methyl red, indole and H_2S production, and nitrate to nitrite reduction. It hydrolysed starch and gelatin but not urea, Tween 20 or aesculin. It was resistant to kanamycin and ampicillin, but sensitive to penicillin, gentamicin, chloramphenicol, tetracycline, neomycin and streptomycin. It could utilize glucose, fructose, glycerol and glutamic acid, but it couldn't utilize sucrose, lactose, lactic acid, galactose, maltose, cellulose or glycogen, when these were provided as the only carbon source. It produced acid from glucose and fructose but not from lactose, or cellobiose. Other characteristics are listed in Table 1.

Arsenic resistance of strain KRPC10Y^T

Strain KRPC10Y^T was isolated from water, infested with enormously high arsenic concentrations, of an arsenic-contaminated bore well, supplying drinking water to a village located in West Bengal, India. When the water sample was subjected to arsenic content analysis by AAS, it indicated the presence of exceedingly high concentration of dissolved arsenic. The arsenic content was approximately 210 µg/l which is quite high when compared to the World Health Organization (WHO) recommended permissible value of arsenic in drinking water i.e., 10 µg/l (WHO, 1996). To check for the level of resistance to arsenic, strain KRPC10Y^T was grown in gradually increasing concentrations of both arsenate (Na_2HAsO_4) and arsenite ($NaAsO_2$). When the growth was monitored spectrophotometrically it was found that the bacterium grew in the presence of extremely high concentrations of arsenate (30 mM) and arsenite (20 mM). To our knowledge, bacterial resistance to such high concentrations of arsenic has not been reported so far (Table 2). Jackson *et al*. (2005) isolated numbers of culturable arsenate (V) resistant bacteria from which some were capable to tolerate very high level of arsenate; in addition, Zelibor *et al*. (1987) isolated As (V) bacteria as well in water samples, however, they did not test for As (III) resistance.

Arsenic compounds are highly toxic for most microorganisms, yet certain microorganisms have evolved a variety of mechanisms to cope with toxicity of arsenic. Microorganisms inhabiting these arsenic-polluted environments encounter selective pressure to develop metal resistance mechanisms including minimizing the amount of arsenic that enters the cell (Dopson *et al*., 2003); Therefore, KRPC10Y^T might also have evolved such survival strategies that are subjected to further investigations,

TABLE 2 - Arsenic resistance as observed in different bacterial strains

Bacterial strains	Resistance to arsenate (Na_2HAsO_4)	Resistance to arsenite ($NaAsO_2$)
Bacillus arsenicus (JCM 12167^T)[a]	20.0 mM	0.5 mM
Deinococcus indicus (DSM15307^T)[b]	10.0 mM	0.2 mM
Bacillus indicus (DSM 15820^T)[b]	20.0 mM	3.0 mM
Bacillus subtilis (NCDO 1769^T)[c]	4.0 mM	0.5 mM
Strain KRPC10Y^T (JCM 13947^T)[d]	30.0 mM	20.0 mM

[a] (Shivaji *et al*,. 2005), [b] (Suresh *et al*., 2004), [c] (Sato and Kobayashi, 1998), [d] This study.

TABLE 3 - Cellular fatty acid profiles of strains KRPC10Y^T and the type strains of genus *Planococcus*

Fatty acid	Strain							
	1	2	3	4	5	6	7	8
Straight chain fatty acids								
C15:0	1.6	4.53	1.73	–	5.49	3·1	6.7	7.2
C16:0	1.5	1.1	1.1	2.2	–	0·8	–	1.4
C17:0	2.9	–	–	–	2.07	2·5	2.3	–
Branched chain fatty acids								
iso C14:0	2.2	6.18	14.56	5.33	4.67	13·1	8.8	13.1
iso C15:0	16.8	4.53	5.31	5.76	2.85	9·5	–	5.1
anteiso- C15:0	61.8	49.46	30.44	53.51	49.83	30.6	52.1	40.9
iso C16:0	5.5	6.41	18.19	7.06	5.65	18.5	8.1	7.2
iso C17:0	2.5	–	3.50	–	2.84	3.1	–	1.7
anteiso - C17:0	–	5.40	3.93	4.04	4.55	4.4	5.2	2.6
iso C18:0	–	–	2.26	–	–	2.1	–	–
Unsaturated fatty acids								
$C_{16:1}\omega 7c$	5.3	12.51	15.94	10.05	8.53	8.9	9.8	11.4
$C_{16:1}\omega 11c$	–	–			1.74	0.7	2.5	2.5

Strain 1: KRPC10Y^T, strain 2: *Planococcus mcmeekinii* ATCC 700539^T, strain 3: *Planomicrobium okeanokoites* ATCC 33414^T, strain 4: *Planococcus psychrophilus* CMS 53or^T, strain 5: *Planococcus stackebrandtii* K 22-033^T (Mayilraj *et al*., 2005), strain 6: *Planococcus maritimus* JCM 11543^T(Yoon *et al*., 2003), strain 7: *Planococcus citreus* DSM 20549^T, strain 8: *Planococcus kocurii* NCIMB 629^T (X62173) (Hao & Komagata, 1985). Values are percentages of total fatty acids. – Not detected.

to resist and thrive in high concentrations of both arsenate and arsenite.

Cellular fatty acids

The main cellular fatty acids in strain KRPC10Y^T were branched. The cellular fatty acid pattern of strain KRPC10Y^T was similar to those of members of the genus *Planococcus* (Table 3) (Yoon *et al*., 2003), but significant differences were also observed at the levels of the fatty acids, Anteiso-C15: 0, Iso-C15: 0, Iso-C16: 0, C16: 1ω7C and Iso-C14: 0 further differentiating strain KRPC10Y^T from other type strains of genus *Planococcus* (Table 3). Anteiso-C15: 0 was the major fatty acid constituting approximately 62% of the cellular fatty acids in strain KRPC10Y^T.

DNA composition and 16S rRNA analysis

The DNA G+C content of strain KRPC10Y^T was 49 mol %. Phylogenetic analyses based on 16S rRNA gene sequence showed that strain KRPC10Y^T fall within the evolutionary radiation enclosed by the genus *Planococcus* and is most closely related to *Planococcus citreus* ATCC14404^T. Strain KRPC10Y^{T}exhibited a maximum similarity of 94% with *Planococcus citreus* ATCC14404^T following BLAST analysis. About 90-93% sequence identity was observed with other *Planococcus* species following BLAST analysis. From the phylogenetic tree, it is evident that the strain KRPC10Y^T forms a separate clade with *Planococcus citreus* ATCC14404^T (Fig. 1) with a high bootstrap value of 90%. High degree of similarity of the original tree with the bootstrap consensus tree indicates the validity of the phylogenetic tree. Phylogenetic trees constructed by UPGMA, using the evolutionary distances computed with Jukes-Cantor and Kimura 2-parameter models also yielded similar stable groupings. A representative phylogenetic tree along with the bootstrap values expressed as percentage of 1000 replications constructed based on Neighbor-Joining method using Kimura 2-parameter model is shown in Fig. 1. Therefore, it appears that, based on the more than 3% difference at the 16S rRNA gene sequence level with the closest related *Planococcus* species, strain KRPC10Y^T probably represents a novel species according to accepted criteria (Goebel, 1994).

Our study significantly contributes to the understanding of the microbial diversity of bacteria under extreme arsenic concentrations in the Bengal Delta plain that remain largely uncharacterized despite its great theoretical interest and importance. Moreover, given the harmful consequences of arsenic exposure on human health, the bio remediation of arsenic from these contaminated sites with the use of arsenic resistant microorganisms can be a potential remedial tool. The successful exploitation of these bacterial strains with proper biotechnology will therefore be highly beneficial. Future research should therefore be concentrated and channelized for a deeper understanding about these bacterial strains and on identifying the molecular mechanism of *Planococcus* mediated arsenic resistance to improve arsenic bioremediation processes.

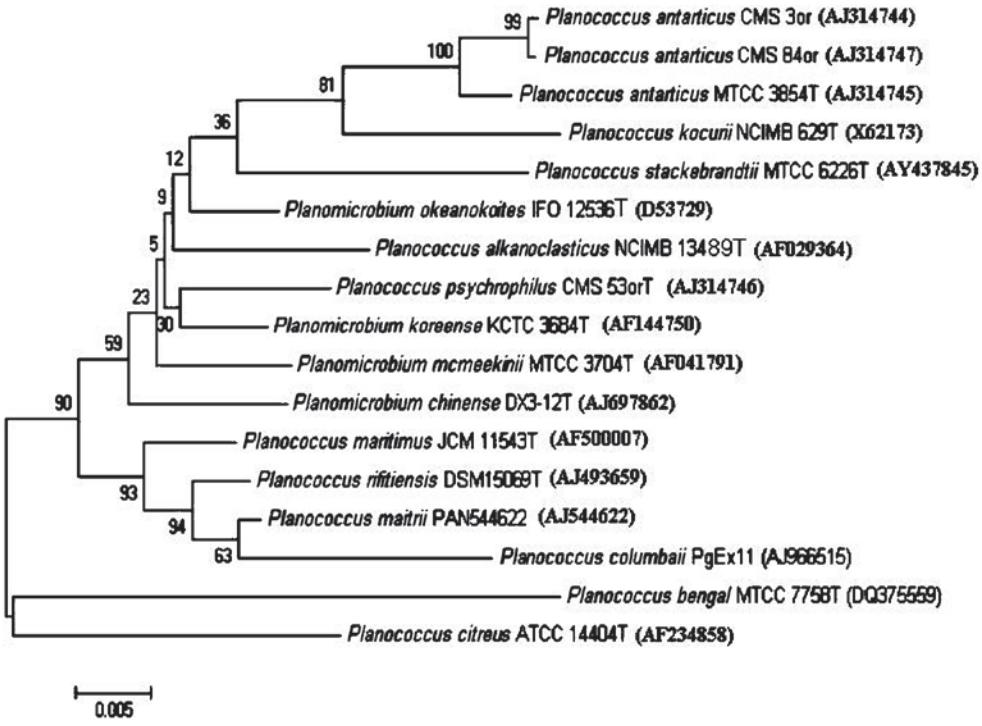

FIG. 1 - Neighbour-joining tree based on 16S rDNA sequences showing the positions of strain KRPC10Y^T, *Planococcus* species, and *Planomicrobium* species. Bootstrap values (expressed as percentages of 1000 replications) are shown at the nodes.

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