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TAXONOMIC DESCRIPTION
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Rhodohalobacter barkolensis sp. nov., isolated from a saline lake and emended description of the genus *Rhodohalobacter*

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Abstract

A Gram-stain-negative, non-motile, aerobic, rod-shaped bacterium, designated 15182^T, was isolated from a saline lake in China. The novel strain 15182^T was able to grow at 10–40 °C (optimum, 37 °C), pH 7.0–8.0 (optimum, 7.5) and with 0.5–4 % NaCl (optimum, 2–3 %, w/v). The phylogenetic analysis based on 16S rRNA gene sequences revealed that strain 15182^T was most closely related to the genus *Rhodohalobacter* by sharing the highest sequence similarity of 97.0 % with *Rhodohalobacter halophilus* JZ3C29^T. Chemotaxonomic analysis showed that the sole respiratory quinone was menaquinone 7, the major fatty acids included $C_{16:0}$ N alcohol and $C_{16:1}\omega 11c$. The major polar lipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, four uncharacterized glycolipids, one uncharacterized phospholipid and two uncharacterized lipids. The genomic DNA G+C content of the strain 15182^T was 42.4 mol%. The average nucleotide identity value between 15182^T and *R. halophilus* JZ3C29^T was 75.4 %, and the *in silico* DNA–DNA hybridization value of the two strains was 19.1 %. On the basis of its phenotypic, chemotaxonomic, genotypic and genomic characteristics presented in this study, strain 15182^T is suggested to represent a novel species in the genus *Rhodohalobacter*, for which the name *Rhodohalobacter barkolensis* sp. nov. is proposed. The type strain is 15182^T (=KCTC 62172^T=MCCC 1K03442^T). An emended description of the genus *Rhodohalobacter* is also presented.

The genus *Rhodohalobacter*, belonging to the family *Balneolaceae*, was first proposed by Xia *et al.* [1]. It was well-established based on the phylogenetic, chemotaxonomic, phenotypic, physiological and biochemical characteristics [1]. Those characteristics, such as low 16S rRNA gene sequence similarity, fatty acid component and acid production from specific substrates, could clearly divide the genus *Rhodohalobacter* from other genera of family *Balneolaceae*. The type species, *Rhodohalobacter halophilus*, was isolated from a saltern located in Feicheng, PR China [1]. At the time of writing, the genus *Rhodohalobacter* contained only one species, *R. halophilus*.

During the survey of the archaea and bacteria diversity of a saline lake, a novel aerobic, red, non-motile, Gram-stain-negative bacterial strain, designated 15182^T, was isolated on marine agar 2216 (MA). The lake, named Barkol, is located in Xinjiang province, China (43° 37′ 9.68″ N, 92° 46′ 20.72″ E). The pH of the lake water is 7.0 and the salinity is 22.6 % (w/v). *R. halophilus* JZ3C29^T was used as a reference strain. The isolate represents a novel species of the genus *Rhodohalobacter* based on the phenotypic, chemotaxonomic, phylogenetic and genomic data presented in this study.

The novel isolate was obtained by the following procedure. The lake water was diluted and spread onto MA using a ten-fold dilution series method. Obvious colonies formed after 7 days incubation at 30 °C. Distinctive colonies were picked out and purified by repeated restreaking. Purity was confirmed by the uniformity of cell morphology. The isolate was routinely cultured in marine broth 2216 (MB) medium and maintained at −80 °C with 20 % (v/v) glycerol.

Cell morphology and motility were determined by using optical microscope (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) [2]. Cells grown on plates were suspended and stained with uranyl acetate and then fixed on the copper mesh before observed with transmission electron microscopy. Growth at various NaCl concentrations (0, 0.5 and 1.0–15.0 %, at increments of 1 %, w/v) was investigated in modified MB medium without Na^+

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Keywords: *Rhodohalobacter*; phylogenetic analysis; whole genome sequencing.
Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 15182^T is MF618255. The GenBank accession number for the whole genome sequence of strain 15182^T is PISP00000000.
Two supplementary figures are available with the online version of this article.

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ions. The temperature range for growth was tested in MB medium by incubating cells at various temperatures (4, 10, 20, 25, 30, 37, 40, 45, 50 and 55 °C). The pH range for growth (from pH 5.5 to 10.0, at intervals of 0.5 pH units) was determined in MB medium with the addition of 25 mM buffering agents, including MES (pH 5.5–6.5), PIPES (pH 6.5–7.5), Tricine buffer (pH7.5–8.5) and CAPSO (pH 9.0–10.0).

The stimulation of growth by a single carbon source was performed by using the GN2 MicroPlate (Biolog) according to the manufacturer's instructions and the description of Park *et al.* [3], with modified MB medium. The medium contained 25 mM PIPES, 0.1 % NH_4Cl, 0.001 % (w/v) yeast extract and removal of trypticase peptone. Before using this medium, we tested different concentrations of yeast extract and trypticase peptone to confirm the basal medium for the experiment of utilization of carbon sources. We found that yeast extract rather than trypticase peptone was essential for growth. API ZYM, 20NE and 50CH kits (bioMérieux) were used according to the manufacturer's instructions. Catalase and oxidase activities, PHB production, H_2S production from thiosulfate and L-cysteine, and hydrolysis of starch, casein, L-tyrosine and cellulose were tested according to Zhu *et al.* [4]. Hydrolysis of Tweens 20, 40 and 80 were examined as described by Sun *et al.* [5]. The indole production test was assayed according to Zhang *et al.* [6]. Hydrolysis of algin was tested on modified MA with 2 % (w/v) sodium alginate as described by Cowan and Steel [7]. Nitrate reduction and hydrolysis of aesculin and gelatin were performed using the methods described by Mata *et al.* [8]. Antibiotic susceptibility tests were determined on MA plates for 7 days at 37 °C using antibiotic discs containing the following (μg per disc, unless indicated): amikacin (30), amoxicillin (10), bacitracin (0.04 IU), cephalothin (30), chloramphenicol (30), clindamycin (2), doxycycline (30), erythromycin (15), gentamicin (10, 2), kanamycin (30), mefoxin (30), nalidixic acid (30), norfloxacin (10), novobiocin (30), nystatin (100), ofloxacin (5), penicillin G (10 IU), rifampicin (5), streptomycin (10), tetracycline (30) and vancomycin (30). The strains were considered susceptible, intermediate and resistant respectively when the diameter of the inhibition zone was >5 mm, 2~5 mm and <2 mm according to Nokhal and Schlegel [9]. Anaerobic growth was determined in an anaerobic jar (MGC) with AnaeroPack (MGC) using modified MA, to which 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite and 20 mM sodium nitrate were respectively added as electron acceptors at 37 °C for 30 days [10].

Cells of strain 15182^T and *R. halophilus* JZ3C29^T, which were grown on MB medium for 5 days at 37 °C, were used for polar lipid and isoprenoid quinone analysis. Polar lipids were analysed by two-dimensional thin-layer chromatography (TLC) with silica gel 60 F254 plates (Merck) as described previously [11]. Isoprenoid quinones were extracted and purified by TLC, and then identified via a high-performance liquid chromatography-mass spectrometry system (Agilent) [12]. For the preparation of cellular fatty acid methyl esters (FAMEs), the two strains were harvested and freeze-dried at the exponential stage of growth according to Kuykendall *et al.* [13]. Identification and quantification of the FAMEs were performed by using the Sherlock Microbial Identification System (MIDI) with the standard MIS Library Generation software (Microbial ID).

We used a quick bacteria genomic DNA extraction kit (DongSheng Biotech) to obtain a high-quality PCR template. An almost-complete 16S rRNA gene sequence of the isolate was obtained by PCR using the primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′)/1492R (5′-GGTTACCTTGTTACGACTT-3′) and the PCR products were cloned into pMD19-T vector (Takara) for sequencing [14]. The sequence was compared with the closely related organisms provided by the EZTAXON service [15]. Multiple sequences were aligned with CLUSTAL_W version 1.8 [16]. Phylogenetic trees were reconstructed using the neighbour-joining [17], maximum-likelihood [18] and maximum-parsimony [19] methods with the MEGA 7.0 program package [20]. Evolutionary distances were calculated according to Kimura's two-parameter model [21] for the neighbour-joining method. The DNA G+C content was determined by reversed-phase high-performance liquid chromatography as described by Mesbah and Whitman [22].

The genome of strain 15182^T was sequenced at the Analysis and Test Centre of the College of Life Sciences of Zhejiang University using the Illumina HiSeq platform. The sequencing generated approximate 1.96 Gb of clean data (approximate 544-fold genome coverage). The *de novo* assembly of the reads was performed using CLC Genomics Workbanch 6.0 (CLC bio). The quality of microbial genomes was assessed using the bioinformatics tool CheckM 1.0.8 [23]. The open reading frames (ORFs) were predicted and annotated by using Glimmer version 3.0 [24] and the Rapid Annotation using Subsystem Technology (RAST) online server [25]. The genome sequence of *R. halophilus* JZ3C29^T (MDWE01000000) was retrieved from the GenBank database. The average nucleotide identity (ANI) was calculated using the OrthoANIu algorithm of the Chun lab's online Average Nucleotide Identity calculator [26]. The *in silico* DNA–DNA hybridization (DDH) value was calculated by using the Genome-to-Genome Distance Calculator [27].

Cells of strain 15182^T were rod-shaped, non-motile and had no flagella. Cells ranged in size from 0.3 to 0.7 µm wide and 2.5 to 20.6 µm long (Fig. S1, available in the online version of this article). The morphological characteristics between 15182^T and *R. halophilus* JZ3C29^T were similar, which was consistent with the result of the phylogenetic analysis. Strain 15182^T grew at 10–40 °C (optimum 37 °C), pH 7.0–8.0 (optimum 7.5) and with 0.5–4 % NaCl (optimum 2–3 %, w/v). In contrast to strain 15182^T, *R. halophilus* JZ3C29^T could adapt to wider and higher NaCl concentrations (Table 1). The NaCl ranges for growth of strain 15182^T and *R. halophilus* JZ3C29^T are 0.5–4 % (w/v) and 2–16 % (w/v),

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Table 1. Differential phenotypic and genotypic characteristics of the strain 15182^T and *R. halophilus* JZ3C29^T

Stains: 1, strain 15182^T; 2, *R. halophilus* JZ3C29^T. +, Positive; −, negative.

Characteristic	1	2
Growth in NaCl (%, w/v):		
Range	0.5–4	2–16*
Optimum	2–3	8–10*
Growth temperature (°C):		
Range	10–40	20–50*
Optimum	37	40*
Growth pH:		
Range	7.0–8.0	7.0–9.0*
Optimum	7.5	7.5–8.5*
Hydrolysis of:		
Aesculin	+	−
Enzyme activities:		
Lipase (C14)	−	+
α-Glucosidase	−	+
Acid production from:		
D-Mannose	−	+
Potassium-2- ketogluconate	+	−
Stimulation of:		
Adonitol, D-alanine, α-ketoglutaric acid, L-proline, L-serine, xylitol, 2-aminoethanol, 2,3-butanediol	+	−
D-Arabitol, D-fructose, D-galacturonic acid, gentiobiose, D-glucose-6-phosphate, L-histidine, D-mannose, melibiose	−	+
Susceptibility to:		
Doxycycline (30 µg)	−	+
Ofloxacin (5 µg)	−	+
Streptomycin (10 µg)	+	−
Polar lipids†	DPG, PG, PE, PL1, GL1, GL2, GL3, GL4, L1, L2	DPG, PG, PE, PL1, PL2, GL1, GL2, GL3, GL4, L2
DNA G+C content (mol%)	42.4	44.4*

*Data was cited from Xia *et al.* [1].

†DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; GL1–GL4, four uncharacterized glycolipids; PL1, PL2, two uncharacterized phospholipids; L1, L2, two uncharacterized lipids.

and the optima are 2–3 % (w/v) and 8–10 % (w/v) respectively. The annotation results of the two genomes indicated that *R. halophilus* JZ3C29^T harbours genes related to choline and betaine uptake and betaine biosynthesis system such as *betA* and *betT* genes. However, those genes were not detected in the genome of strain 15182^T. Glycine betaine (*N,N,N*-trimethylglycine) has been shown to be a very efficient osmolyte found in a wide range of bacterial and plant species, where it is accumulated at high cytoplasmic concentrations in response to osmotic stress. The lacking of betaine uptake and biosynthesis-related genes may be the main reason for the narrow and low NaCl tolerance of strain 15182^T. Strain 15182^T was susceptible to amoxicillin (10), cephalothin (30), chloramphenicol (30), clindamycin (2), erythromycin (15), mefoxin (30), norfloxacin (10), novobiocin (30), penicillin G (10 IU), rifampicin (5), streptomycin (10) and vancomycin (30), but resistant to amikacin (30), bacitracin (0.04 IU), doxycycline (30), gentamicin (10 , 2), kanamycin (30), nalidixic acid (30), nystatin (100), ofloxacin (5) and tetracycline (30). Detailed results of physiological and biochemical tests are given in species description. The differentiating characteristics between strain 15182^T and its reference strain are summarized in Table 1.

The polar lipids profile of strain 15182^T included diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, four glycolipids (GL1–GL4), one uncharacterized phospholipid (PL1) and two uncharacterized lipids (L1, L2), which was in accordance with reference strain *R. halophilus* JZ3C29^T in this study, except for the two minor components (L1, PL2) (Fig. S2). Fatty acid analysis revealed that $C_{16:0}$ N alcohol and $C_{16:1}\ \omega 11c$ were the major fatty acids in strain 15182^T as well as in the reference strain (Table 2). Menaquinone 7 (MK-7) was detected as the sole respiratory quinone of the strain 15182^T which was in accordance with the genus description.

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Table 2. Fatty acid composition (%) of strain 15182^T and strain *R. halophilus* JZ3C29^T

Strains: 1, 15182^T; 2, *R. halophilus* JZ3C29^T. Values are percentages of the total fatty acids. Data of the two strains was taken from this study. Fatty acids representing less than 0.5 % in both strains are omitted. –, Not detected. Major components (≥10 %) are highlighted in bold.

Fatty acid	1	2
Saturated:		
$C_{16:0}$ N alcohol	**20.3**	**23.0**
Unsaturated:		
$C_{15:1}\omega 8c$	9.3	5.6
$C_{16:1}\omega 11c$	**51.6**	**60.7**
$C_{18:1}\omega 9c$	2.2	1.8
$C_{18:1}\omega 5c$	0.6	–
$C_{17:1}\omega 7c$	9.5	3.6
Summed feature 3*	3.5	3.2
Summed feature 4*	2.4	1.3

*Summed feature 3 contained $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$. Summed feature 4 contained $C_{17:1}$ iso I/anteiso B.

An almost-complete 16S rRNA gene sequence (1501 nt) of strain 15182^T was obtained. Similarity analysis based on it and other representative bacteria with validly published names revealed that the strain belonged to the genus *Rhodohalobacter* and the most closely related strain was *R. halophilus* JZ3C29^T (97.0 %). The similarity values between the strain 15182^T and all other validly published strains were below 92 %. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain 15182^T clustered with *R. halophilus* JZ3C29^T in a separated branch in the neighbour-joining, maximum-likelihood and maximum-parsimony trees with high bootstrap values (Fig. 1). The DNA G+C nucleotide content of the bacterium calculated from the draft genome sequence was 42.4 %, which was slightly different from the estimated DNA G+C content (43.8 mol %) obtained by HPLC. The ANI value between strain 15182^T and *R. halophilus* JZ3C29^T was 75.4 %. The *in silico* DDH value (the recommended results from formula 2) indicated that strain 15182^T and *R. halophilus* JZ3C29^T shared 19.1 % DNA relatedness. The ANI value and *in silico* DDH value were significantly lower than the threshold value (ANI, 94–96 % and *in silico* DDH 70 %) for species demarcation [28, 29].

In conclusion, strain 15182^T exhibits many typical characteristics of the genus *Rhodohalobacter*, such as being catalase-positive and oxidase-negative, NaCl requirement for growth, having MK-7 as the sole respiratory quinone, and having diphosphatidylglycerol, phosphatidylethanolamine, unidentified glycolipids (GL1–GL4) and an unidentified phospholipid (PL1) as the major polar lipids. However, strain 15182^T could also be distinguished from the type strain *R. halophilus* JZ3C29^T by several different characteristics, i.e. (1) narrow range and low optimum NaCl concentration for growth, (2) strictly aerobic condition for growth, (3) unique carbon source utilization pattern, (4) hydrolysis of aesculin, (5) lack of lipase (C14) and α-glucosidase activity. Additionally, the relatively low ANI and *in silico* DDH values between strain 15182^T and *R. halophilus* JZ3C29^T clearly indicated that strain 15182^T should represent a novel species of genus *Rhodohalobacter*.

Based on of the polyphasic taxonomic characterization presented in this study, strain 15182^T is proposed to represent a novel species of genus *Rhodohalobacter,* with the name *Rhodohalobacter barkolensis* sp. nov.

EMENDED DESCRIPTION OF THE GENUS *RHODOHALOBACTER* XIA *ET AL*. 2017

Cells are facultatively anaerobic or strictly aerobic. The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, unidentified glycolipids, unidentified phospholipids and unidentified lipids. The rest of the genus description is identical to that given by Xia *et al.* [1].

DESCRIPTION OF *RHODOHALOBACTER BARKOLENSIS* SP. NOV.

Rhodohalobacter barkolensis (bar.kol.en′sis. N.L. masc. adj. *barkolensis* referring to the Barkol lake in China, from which the type strain was isolated).

Cells are Gram-stain-negative, rod-shaped, non-motile and have no flagella. Cells are approximately 0.3–0.7 µm wide and 2.5–20.6 µm long. After incubation for 5 days on MA plates, colonies are 1–2 mm in diameter, convex, smooth and circular, viscid and reddish. The pH and temperature ranges for growth are pH 7.0–8.0 and 10–40 °C (optima at pH 7.5 and 37 °C). The NaCl concentration range for growth is 0.5–4 % (w/v) and optimal growth occurs at 2–3 % (w/v). No growth is observed in anaerobic conditions by anaerobic respiration with $S_2O_3^{2-}$, SO_3^{2-}, SO_4^{2-}, NO_2^- or NO_3^- as electron acceptors. Positive in tests for catalase activity, and hydrolysis of aesculin, algin and starch. Negative for oxidase activity, nitrate reduction, indole production, H_2S production (from thiosulfate or L-cysteine), hydrolysis of casein, gelatin, tyrosine, CM-cellulose, Tween 20, Tween 40 and Tween 80. Acid is produced from mannitol, D-glucose, aesculin, maltose, starch, glycogen, potassium-2-ketogluconate and potassium-5-ketogluconate. The following carbon sources stimulated growth: dextrin, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, raffinose, m-inositol, lactose, D-sorbitol, sucrose, trehalose, turanose, xylitol, α-ketoglutaric acid, α-ketovaleric acid, D,L-lactic acid, D-saccharic acid, D-alanine, L-alanine, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, 2-aminoethanol, 2,3-butanediol, glycerol and D,L,α-glycerol phosphate. The following enzymic activities are present: alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, chymotrypsin and

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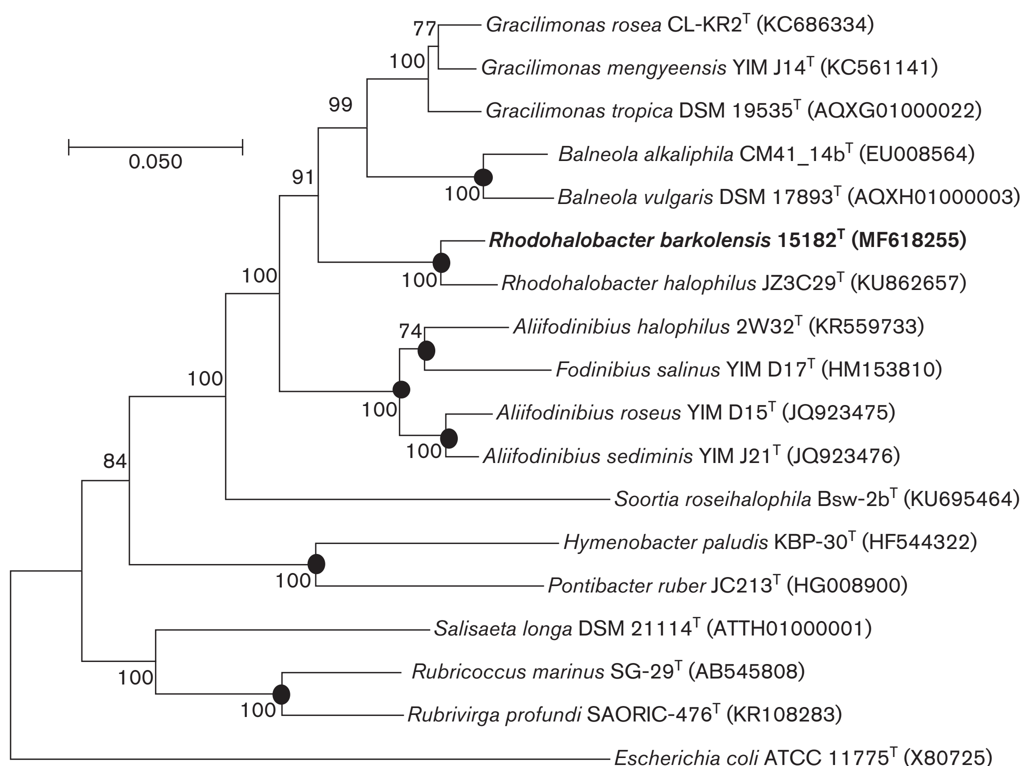

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of strain 15182^T and related species. Bootstrap values based on 1000 replicates are listed as percentages at branching points. Only bootstrap values above 70 % are shown. Filled circles indicate that the corresponding nodes were also recovered in both maximum-likelihood and maximum-parsimony trees. *Escherichia coli* ATCC 11775^T (GenBank accession no. X80725) was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

naphthol-AS-BI-phosphohydrolase. The respiratory quinone is menaquinone 7 (MK-7). Major fatty acids are $C_{16:0}$ N alcohol and $C_{16:1}\omega 11c$. The major polar lipids include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, four uncharacterized glycolipids, one uncharacterized phospholipid and two uncharacterized lipids.

The type strain, 15182^T (=MCCC 1K03442^T=KCTC 62172^T), was isolated from a saline lake in Xinjiang, China. The DNA G+C content is 42.4 mol% (by genome).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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