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Reclassification of *Halomonas caseinilytica* Wu et al. 2008 as a later synonym of *Halomonas sinaiensis* Romano et al. 2007, and emendation of the species description

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Abstract The taxonomic relationship between *Halomonas sinaiensis* DSM 18067^T and *Halomonas caseinilytica* JCM 14802^T has not been established, despite the high similarity (99.6 %) of their 16S rRNA gene sequences. To clarify their taxonomic positions, a polyphasic approach was applied to both type strains. Genomic relatedness analyses between *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T resulted in an average nucleotide identity of 99.5 % and an estimated DNA–DNA hybridization of 96.1 % by the genome-to-genome distance calculator, indicating that they belong to a single species. Phenotypic and chemotaxonomic characteristics showed no pronounced differences between the two type strains. Based on the results of this polyphasic study, it is proposed that *H. caseinilytica* JCM 14802^T is a later heterotypic synonym of *H. sinaiensis* DSM 18067^T. An emended description for the species *H. sinaiensis* is given.

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Introduction

Halomonas sinaiensis was described by Romano et al. (2007) for a novel halophilic bacterium isolated from sand and water samples from a salt lake in Egypt. *Halomonas caseinilytica* was described by Wu et al. (2008) for a novel halophilic bacterium isolated from a soil sample from a saline lake in China. Since the publication of these studies, 27 *Halomonas* species have been proposed (List of prokaryotic names with standing in nomenclature; http://www.bacterio.net/halomonas.html). However, the taxonomic relationship between *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T has not yet been determined, despite the type strains of both species sharing a high 16S rRNA gene sequence similarity (99.6 %). Such a high level of 16S rRNA gene sequence similarity requires an investigation of genomic DNA–DNA relatedness for separating species (Rosselló-Mora and Amann 2001; Stackebrandt and Ebers 2006).

Based on the original descriptions of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T (Romano et al. 2007; Wu et al. 2008), some phenotypic features seemed to be different between the two

species, such as colony colour, oxygen requirement for growth, minimum temperature for growth, NaCl range for growth, hydrolysis of casein and gelatin, and acid production from certain carbohydrates. However, the previous studies of Romano et al. (2007) and Wu et al. (2008) employed different test media and incubation conditions for the phenotypic assays (e.g. casamino acids were supplied as organic substances in the former study, while glucose and peptone were added in the latter study; the assays were performed under different salinity conditions). As phenotypic analyses are strongly recommended to be performed under identical conditions for comparison of closely related taxa (Tindall et al. 2010), phenotypic characteristics of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T have to be re-examined under identical test conditions to confirm these phenotypic differences.

In the present study, *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T were compared using a polyphasic analysis to clarify the precise taxonomic relationship of the two species.

Materials and methods

Strains and culture conditions

The type strains of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and the Japan Collection of Microorganisms (JCM), respectively. Unless otherwise specified, all characteristics of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T were determined for cultures grown aerobically at 35 °C for 3 days on medium 1 (Romano et al. 2007) with slight modifications of the concentrations of NaCl (2 %, w/v) and KCl (0.026 %, w/v) (hereafter, MM1), supplemented with 1.5 % Bacto agar (Difco).

Whole genome sequencing and analysis of genomic relatedness

For genomic comparison, whole genome sequencing was carried out for *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T. Genomic DNAs of both type strains were extracted using a commercial kit (DNeasy Blood & Tissue kit; Qiagen). Genome sequencing was performed using an Illumina MiSeq with a paired end library (300 bp × 2) at ChunLab Inc. (Seoul, Korea). Sequence reads were assembled using the CLC Genomics Workbench version 8.0 (CLC Bio, Aarhus, Denmark). The draft genomes of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T were deposited at DDBJ/EMBL/GenBank under accession numbers NZ_BDEO00000000 and NZ_BDEP00000000, respectively. The degree of genome-based relatedness was estimated by both an average nucleotide identity (ANI) value, following the BLAST-based ANI calculation method described by Goris et al. (2007), and the genome-to-genome distance calculation (GGDC) method described by Auch et al. (2010). The DNA G+C content was calculated from a draft genome of each strain.

Phylogenetic analyses of housekeeping gene sequences

Three housekeeping genes including 16S rRNA, 23S rRNA and *gyrB* genes were analysed to resolve phylogenetic relationships of *H. sinaiensis*, *H. caseinilytica* and related *Halomonas* species (Arahal et al. 2002; Okamoto et al. 2004). Nearly complete 16S rRNA gene sequences of *H. sinaiensis* DSM 18067^T (GenBank accession no. AM238662) and *H. caseinilytica* JCM 14802^T (FR749914) were aligned with those of closely related taxa retrieved from GenBank using the RDP aligner (Cole et al. 2014) based on secondary structures. The 23S rRNA and *gyrB* genes of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T were retrieved from the annotated draft genomes using the Prokka program (Seemann 2014) and deposited at DDBJ/EMBL/GenBank under accession numbers LC155959, LC155961, LC155960 and LC155962, respectively. Each gene was aligned with those of closely related taxa (de la Haba et al. 2012) using the ClustalX program (Thompson et al. 1997). Phylogenetic analysis was performed using the program MEGA 6.0 (Tamura et al. 2013). Phylogenetic trees were inferred using the neighbour-joining (NJ) (Saitou and Nei 1987), maximum-parsimony (MP) (Fitch 1971) and maximum-likelihood (ML) (Felsenstein 1981) methods using bootstrap analyses based on 1000 replications. NJ analysis was performed using the Kimura two-parameter model (Kimura 1980) with the gamma-distributed option. MP analysis was made with the tree inference option of tree-bisection-reconnection (TBR). For ML analysis, the general time

reversible (GTR) (Tavaré 1986) model with the gamma distribution was selected based on the result of the best-fit model.

Cellular fatty acids

The fatty acid methyl esters derivatised from whole cells of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T grown on marine agar (MA; Difco) at 35 °C for 3 days were analysed with gas chromatography (Agilent technologies 7890B) according to the instructions of the Microbial Identification System (MIDI; version 6.2), with reference to the TSBA6 database. Under the employed culture conditions, both type strains appear to be in exponential phase (data now shown).

Phenotypic tests

To compare phenotypic characteristics of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T, the following experiments were performed in duplicate for both type strains at the same time and repeated on different days. Anaerobic growth of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T was tested on MA in the presence of nitrate (Romano et al. 2007) using a GasPak Anaerobic system (BBL) at 25 °C for 3 weeks, along with a strictly aerobic bacterium (*Rhodococcus aerolatus* PAMC 27367^T; Hwang et al. 2015) and a strictly anaerobic bacterium (*Clostridium* sp. PAMC 80033) included as experimental controls. The temperature range for growth was examined by the ability to form colonies on solid medium 1 (Romano et al. 2007) incubated at 4 and 10–65 °C (in increments of 5 °C). The pH range (pH 4.0–11.0 at intervals of 0.5 pH unit) for growth was determined by assessing turbidity measured as OD_{600} in pH-buffered MM1 broth using citric acid-phosphate buffer for pH 4.0–5.0, MES for pH 5.5–6.5, MOPS for pH 7.0–7.5, AMPD for pH 8.0–9.5 and CAPS for pH 10.0–11.0, each at a final concentration of 50 mM, at 35 °C for up to 2 weeks. The NaCl requirement for optimal growth was determined using synthetic ZoBell marine broth with various NaCl concentrations (0, 2 and 5–35 % at intervals of 5 %, w/v).

Decomposition of casein and hypoxanthine was determined according to the protocols described by Smibert and Krieg (1994). Degradation of Tweens 40, 60 and 80 was investigated as described by Hansen and Sørheim (1991). Acid production from carbohydrates was tested as described by Lemos et al. (1985). In addition, other enzyme activities were assayed in duplicate using the API 20NE and API ZYM kits (bioMérieux) according to the manufacturer's instructions except that the cell suspension was prepared as described by Hwang et al. (2009). Carbon utilisation was tested using medium 2 (Romano et al. 2007) with slight modifications of the concentrations of NaCl (2 %, w/v), KCl (0.026 %, w/v) and tested compounds (0.4 %, w/v). Carbon utilisation was scored as negative when growth was equal to, or less than, that in the negative control with no carbon source. Growth was measured by monitoring changes in the OD_{600} every 6 days for 24 days incubation at 35 °C. Sensitivities to antibiotics were assessed using disc-diffusion methodology (Bauer et al. 1966) with 6 mm diameter discs containing the following (μg per disc); gentamycin (10), kanamycin (30), nalidixic acid (30), penicillin G (10), rifampicin (30) and tetracycline (30).

Results and discussion

The draft genomes of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T are comprised of 3,477,089 bp (25 contigs, N50 = 293,132 bp) and 3,571,641 bp (38 contigs, N50 = 241,229 bp), respectively. Genomic relatedness analyses between both type strains gave an ANI value of 99.5 ± 0.7 % and in silico DNA–DNA hybridization value of 96.1 ± 1.7 % by GGDC. The observed ANI value is notably higher than the ANI cut-off values (95–96 %) proposed for delineating bacterial species (Goris et al. 2007; Richter and Rosselló-Móra 2009). In addition, the in silico DNA–DNA hybridization value also showed that *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T belong to single genomic species (Richter and Rosselló-Móra 2009). The DNA G+C content calculated from the draft genome sequences of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T was 63.6 and 63.4 mol%, respectively, which are similar to those reported previously (Table 1).

A pairwise comparison of the nearly complete 16S rRNA gene sequences of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T showed a high similarity of 99.6 %. For the complete 16S rRNA gene sequences from each genome sequence, both type

Table 1 Phenotypic properties of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T

Characteristic	1	2
Isolation source	(Salt lake, Egypt)	(Salt lake, China)
Colony color	White[d] (white[d])	White[d] (light yellow)
Anaerobic growth	+ (+)	+ (–)
Growth conditions		
Temperature range (°C)	15–55 (25–50)	15–50 (4–48)
Optimum temperature (°C)	35 (35)	35 (30)
NaCl range (%, w/v)	0–25 (0–30)	0–25 (0.5–15)
Optimum NaCl (%, w/v)	2–10 (5–15)	2–10 (3–5)
pH range	5.5–8.5 (6–9)	5.5–9.0 (5–9)
Optimum pH	7.0–7.5 (7)	7.0–7.5 (7–8)
Decomposition of		
Casein, gelatin	+ (–)	+ (+)
Esculin	+ (ND)	+ (+)
Hypoxanthine	+ (ND)	+ (ND)
Tween 80	– (ND)	– (–)
Acid production from		
L-Arabinose	+ (–)	+ (+)
D-Lactose, D-maltose, sucrose, D-trehalose	– (–)	– (+)
L-Rhamnose	+ (ND)	+ (+)
Utilization of sole carbon		
D-Mannitol, L-ornithine, D-raffinose	+ (ND)	+ (+)
D-Galactose, D-mannose	– (ND)	– (+)
D-Xylose	– (ND)	– (ND)
Susceptibility to		
Gentamycin	S (ND)	S (ND)
Kanamycin, tetracycline	S (R)	S (S)
Nalidixic acid, rifampicin	S (ND)	S (S)
Penicillin G	R (S)	R (R)
DNA G+C content (mol%)	63.6[c] (64.7[a])	63.4[c] (63.0[b])

Strains 1, *H. sinaiensis* DSM 18067^T; 2, *H. caseinilytica* JCM 14802^T. Data were obtained in the present study. Data in parentheses are taken from the previous studies of Romano et al. (2007) for *H. sinaiensis* DSM 18067^T and Wu et al. (2008) for *H. caseinilytica* JCM 14802^T. + positive, – negative, *R* resistant, *S* sensitive, *ND* not determined

[a] HPLC analysis

[b] Thermal denaturation analysis

[c] Whole genome analysis

[d] Grown on solid medium 1 (Romano et al. 2007)

strains differed by only six bases among 1534 locations, resulting in the same similarity value. An almost complete 23S rRNA gene sequence (size range of 3412–3422 bp) was found in each genome sequence, and these share a high similarity of 99.5 %. A complete sequence of *gyrB* gene with a size of 2421 bp was retrieved from each genome sequence and they have a high similarity of 99.6 %.

Phylogenetic analyses of the 16S rRNA, 23S rRNA and *gyrB* gene sequences of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T consistently revealed that they constitute a distinct subclade within a robust clade encompassing *Halomonas elongata* DSM 2581^T, *Halomonas eurihalina* ATCC 49336^T, *Halomonas almeriensis* M8^T and *Halomonas halmophila* ATCC 19717^T (Fig. 1). The level of divergence in the 3 housekeeping gene sequences (0.4–0.5 %) between *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T appears to be too low to distinguish two species within the robust clade, of which the overall mean sequence divergence are 1.7 ± 0.1, 2.0 ± 1.1 and 16.3 ± 5.9 % for 16S rRNA, 23S rRNA and *gyrB* genes, respectively (Fig. 1).

The fatty acid profile of *H. sinaiensis* DSM 18067^T was very similar to that of *H. caseinilytica* JCM 14802^T under identical cultivation conditions (Table 2). The dominant fatty acids (>10 %) of both type strains were identified as $C_{19:0}$ cyclo ω8*c* (36.6–42.4 %), $C_{16:0}$ (26.7–27.9 %) and $C_{18:1}$

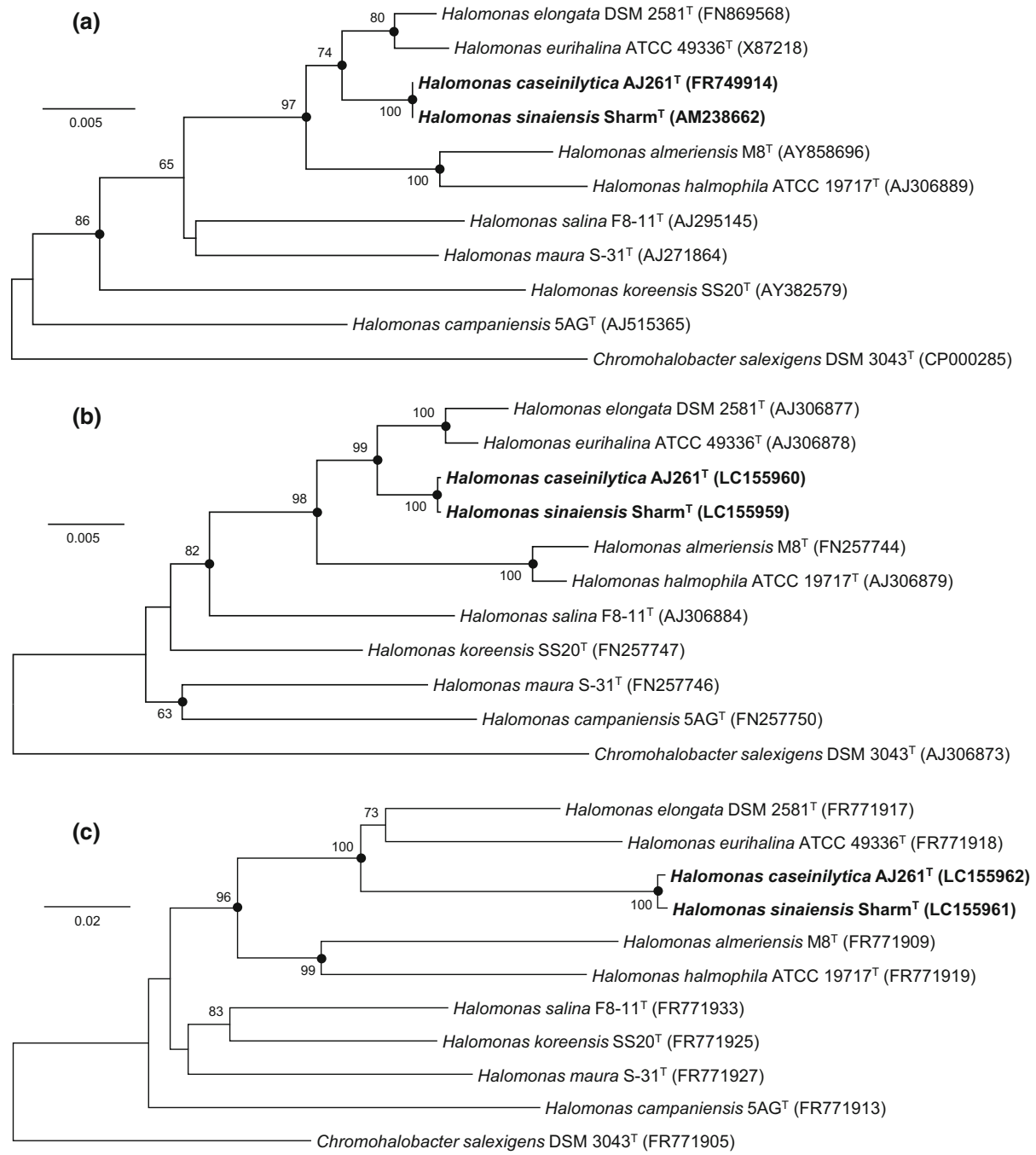

Fig. 1 Neighbour-joining trees showing the phylogenetic positions of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T and related species on the basis of **a** 16S rRNA gene, **b** 23S rRNA gene and **c** *gyrB* gene sequences. Only bootstrap values above 60 % are shown (1000 resamplings) at the branching points. *Solid circles* indicate that the corresponding nodes were also obtained in the maximum-parsimony and the maximum-likelihood trees. The corresponding gene of *Chromohalobacter salexigens* DSM 3043^T was used as an outgroup in each phylogenetic tree

Table 2 Cellular fatty acid contents of *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T

Fatty acids	1	2
Saturated		
$C_{10:0}$	2.1	2.1
$C_{12:0}$	3.1	3.3
$C_{14:0}$	Tr	Tr
$C_{16:0}$	26.7	27.9
$C_{17:0}$	Tr	Tr
$C_{18:0}$	1.0	1.1
Unsaturated		
$C_{18:1}$ ω7*c* 11-methyl	1.1	Tr
$C_{20:2}$ ω6,9*c*	Tr	Tr
Hydroxy		
$C_{12:0}$ 3-OH	5.7	6.3
$C_{17:0}$ cyclo	2.3	3.0
$C_{19:0}$ cyclo ω8*c*	42.4	36.6
Summed feature[a]		
3	1.0	1.4
8	13.0	15.7

Strains 1, *H. sinaiensis* DSM 18067^T; 2, *H. caseinilytica* JCM 14802^T. Fatty acids were analysed for cells grown on marine agar at 35 °C for 3 days. Values are percentages of total fatty acids. *Tr* trace amount (<1 %)

[a] Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 3 comprises $C_{16:1}$ ω6*c* and/or $C_{16:1}$ ω7*c*; summed feature 8 comprises $C_{18:1}$ ω6*c* and/or $C_{18:1}$ ω7*c*

ω6*c* and/or $C_{18:1}$ ω7*c* (13.0–15.7 %; Table 2), which were also found in abundant amounts as previously reported (Romano et al. 2007; Wu et al. 2008). The fatty acid profiles in this study do not support the conclusion that *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T are separate species.

Most phenotypic characteristics showed the same results between *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T (Table 1; Fig. 2). Colony colour; anaerobic growth; lower limit of temperature (15 °C), NaCl concentrations (0–25 %, w/v) and lower limit of pH (5.5) for growth; decomposition of casein and gelatin; acid production from L-arabinose, D-lactose, D-maltose, sucrose and D-trehalose; and susceptibility to kanamycin, penicillin G and tetracycline were all found to be identical for both type strains (Table 1), whereas those features had been shown to be different in the original studies for *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T (Romano et al. 2007; Wu et al. 2008). Furthermore, decomposition of esculin, hypoxanthine and Tween 80; acid production from L-rhamnose; utilisation of D-galactose, D-mannitol, D-mannose, L-ornithine, D-raffinose and D-xylose as sole carbon; and susceptibility to gentamycin, nalidixic acid and rifampicin all showed congruent results between both type strains (Table 1), characteristics which had previously been uncharacterised for either type strains (Romano et al. 2007; Wu et al. 2008). Although a few phenotypic characteristics are different between both type strains (i.e. upper limits of temperature and pH for growth; Table 1), *H. sinaiensis* DSM 18067^T and *H. caseinilytica* JCM 14802^T share common properties in most phenotypic features when tested under identical conditions.

Based on the genomic DNA–DNA relatedness, phylogenetic, chemotaxonomic and phenotypic features obtained in the present study, we propose to reclassify *H. caseinilytica* Wu et al. (2008) as a later heterotypic synonym of *H. sinaiensis* Romano et al. (2007).

Emended description of *H. sinaiensis* Romano et al. (2007)

The characteristics of this species are as described by Romano et al. (2007) and Wu et al. (2008), with the following amendments. Facultative anaerobe. Colony colour is white or light yellow depending on the medium. Grows at 15–55 °C (optimum, 35 °C) and at pH 5.0–9.0 (optimum, pH 7.0–7.5). Growth occurs at NaCl concentrations of 0–25 % (w/v) (optimum, 2–10 %). Positive for decompositions of casein, gelatin and hypoxanthine. Acid is produced from L-arabinose but not from D-lactose, D-maltose, sucrose and D-trehalose. D-Galactose, D-mannose and D-xylose are not utilised as sole carbon source. According to the API ZYM test, positive for acid and alkaline phosphatases and naphthol-AS-BI-phosphohydrolase, but negative for *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α-fucosidase, α- and β-galactosidases, α- and β-glucosidases, β-glucuronidase, leucine arylamidase, lipase (C14), α-mannosidase, trypsin and valine arylamidase. According to the API 20NE test, positive for esculin hydrolysis, β-galactosidase (PNPG), gelatinase, glucose fermentation and nitrate

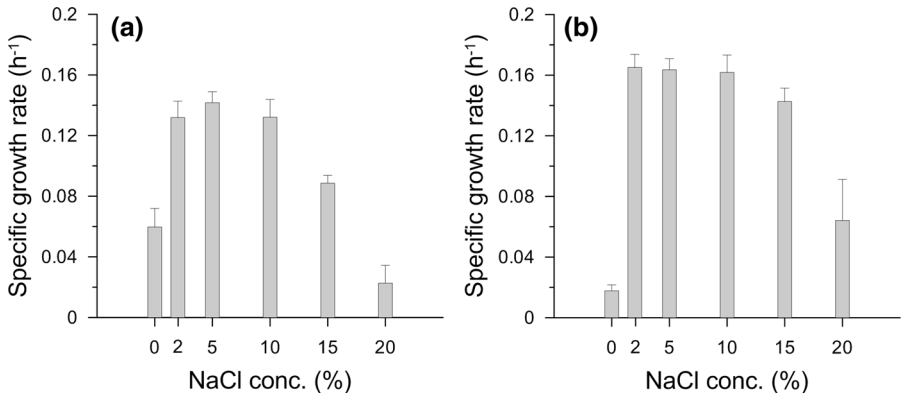

Fig. 2 Specific growth rates of **a** *H. sinaiensis* DSM 18067^T and **b** *H. caseinilytica* JCM 14802^T along the NaCl concentrations (%; w/v). The NaCl requirement for optimal growth was determined using synthetic ZoBell marine broth using a shaking incubator at 35 °C. Turbidity of OD_{600} was measured every 3 h for 24 h. *Error bar* represents SD of triplicate samples at each NaCl concentration

reductase, but negative for arginine dihydrolase, indole production and urease. The DNA G+C content is 63.0–64.7 mol%.

The type strain is Sharm^T (= DSM 18067^T - = ATCC BAA-1308^T). A second strain is AJ261 (= CGMCC 1.6773 = JCM 14802).

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