Alishewanella tabrizica sp. nov., isolated from Qurugöl Lake

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A novel Gram-negative, aerobic, motile and rod-shaped bacterium was isolated from Qurugöl Lake located in a mountainous region near Tabriz city in the north-west of Iran. Growth occurred at pH 6–10 (optimum, pH 7 ± 0.5) and at 10–40 °C (optimum, 30 °C). Strain RCRI4^T was able to grow in the absence and presence of NaCl to 3 % (w/v). The major fatty acids were $C_{17:0}$, $C_{16:1}\omega7c/C_{15}$ iso3-OH, $C_{17:1}\omega8c$ and $C_{16:0}$. The G+C content of genomic DNA was 45.3 mol%. Based on the 16S rRNA and *gyrB* gene sequences, phylogenetic analyses indicated that strain RCRI4^T associated with the genus *Alishewanella*, and closely related type strains include *Alishewanella agri* BLO6^T (97.8 %), *Alishewanella aestuarii* B11^T (97.7 %), *Rheinheimera perlucida* BA131^T (97.5 %), *Alishewanella fetalis* CCUG 30811^T (97.3 %) and *Alishewanella jeotgali* MS1^T (97.1 %). The level of DNA–DNA relatedness between strain RCRI4^T and phylogenetically the closest related strains, *A. agri* BLO6^T and *R. perlucida* BA131^T, was 9 and 14 %, respectively. On the basis of phenotypic, chemotaxonomic and phylogenetic results, it is suggested that strain RCRI4^T represents a novel species of the genus *Alishewanella*, for which the name *Alishewanella tabrizica* sp. nov. is proposed. The type strain is RCRI4^T (=LMG 26473^T=JCM 17275^T=KCTC 23723^T).

The genus *Alishewanella*, belonging to the family *Alteromonadaceae*, was first proposed by Vogel *et al.* (2000). *Alishewanella fetalis*, the first species within the genus, was isolated from an autopsy of a human fetus in Sweden. A second species of *Alishewanella* was isolated from the tidal flat sediment in Yeoso, South Korea, and named *Alishewanella aestuarii* (Roh *et al.*, 2009). Most recently, *Alishewanella jeotgali* MS1^T was reported from a traditional fermented food in Korea (Kim *et al.*, 2009) and *Alishewanella agri* BL06^T was identified from landfill soil from iron manufacture in Pohang, South Korea, by Kim *et al.* (2010).

During a survey of the microbial biodiversity in Qurugöl Lake, a fresh water lake near Tabriz city – the centre of East Azerbaijan State, which is a mountainous region in the north-west of Iran – a Gram-negative, rod-shaped and motile bacterium was isolated. The isolated strain, RCRI4^T (Rice and Citrus Research Institute), was subjected to a polyphasic taxonomic investigation. Based on the results of a polyphasic taxonomic study, this strain is considered to represent a novel species of the genus *Alishewanella*.

Strain RCRI4^T was isolated from a water sample of Qurugöl Lake on marine agar medium containing (per l): peptone, 5.0 g; yeast extract, 1.0 g; Fe^{3+}-citrate, 0.1 g; NaCl, 19.45 g; $MgCl_2$ (dried), 8.8 g; Na_2SO_4, 3.24 g; $CaCl_2$, 1.8 g; KCl, 0.55 g; $NaHCO_3$, 0.16 g; KBr, 0.08 g; $SrCl_2$, 34.0 mg; H_3BO_3, 22.0 mg; sodium silicate, 4.0 mg; NaF, 2.4 mg; $(NH_4)NO_3$, 1.6 mg; Na_2HPO_4, 8.0 mg; agar, 15.0 g. pH was adjusted to 7.6 ± 0.2. Gram staining was performed as described by Gerhardt *et al.* (1994) and the Gram reaction was tested by using the KOH lysis test (Gregersen, 1978). Cell morphology and motility were observed under a light microscope (ECLIPSE 80*i*, Nikon), and a transmission electron microscope image was

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and partial *gyrB* gene sequences of strain RCRI4^T are GQ505294 and JN106465, respectively.

Four supplementary figures and a supplementary table are available with the online version of this paper.

taken by the Korean Collection for Type Culture, after the cells had been incubated for 1 day at 30 °C.

Growth was tested at different NaCl concentrations in lab-made marine broth medium (the same as marine agar but with 5.9 g $MgCl_2$) with no supplement or supplemented with 0.5–10 % (w/v) NaCl at intervals of 0.5 % (Yoon *et al.*, 2007).

The cells were incubated in an anaerobic chamber for 48 h in order to determine the extent of growth under anaerobic conditions. Cell growth was investigated at −5, 0, 5, 10, 15, 20, 25, 30, 37, 40, 45 and 50 °C. In order to determine the optimal growth temperature, the spectrophotometric absorbance of the samples was measured at 600 nm after 48 h. The pH tolerance was determined in marine broth with the pH set from 4 to 12 (using increments of 1 pH unit).

Growth on MacConkey agar, nutrient agar, blood agar (Tryptic soy agar containing 5 % sheep blood), brain heart infusion (BHI) and LB medium was tested. These media were purchased from Scharlau Chemie (Spain). Growth was also studied on sea water, agar containing (per l): beef extract, 10.0 g; peptone, 10.0 g; agar, 20.0 g; tap water, 250 ml; artificial sea water, 750 ml. Artificial sea water consists of NaCl, 28.13 g; KCl, 0.77 g; $CaCl_2.2H_2O$, 1.6 g; $MgCl_2.6H_2O$, 4.80 g; $NaHCO_3$, 0.11 g; $MgSO_4.7H_2O$, 3.5 g.

Biochemical tests consisted of determination of oxidase and catalase activity, gelatin liquefaction, ability to hydrolyse starch, Tween 20, Tween 80 and casein; production of indole, H_2S, nitrate reduction and motility tests were carried out according to MacFaddin (2000). Motility was determined by using the hanging-drop technique (Skerman, 1967).

The ability to use various carbon sources, the production of acid from sugars and the physiological profile of the RCRI4^T strain were performed using the API 20E, API 20 NE and API 50 CH B/L kits. API ZYM and ID 32E were used to identify the enzyme activities of the strain RCRI4^T according to the manufacturer's instructions (bioMérieux).

Fatty acid analysis was performed by BCCM/LMG. In order to determine fatty acid analysis, the cells were grown on marine agar (Difco) at 28 °C for 24 h in aerobic conditions. This test was also performed using the cells grown for 48 h on Columbia Blood Agar=LMG medium. For fatty acid extraction, the procedure was performed according to the recommendations of the commercial identification system MIDI (Microbial Identification System). The cells were harvested from the overlap between the second and third quadrants. For the identification of the obtained fatty acids, the TSBA 50 (Rev 5.0) MIDI database was used.

For genotypic characterization, DNA was isolated from the strain RCRI4^T according to the method described by Corbin *et al.* (2001) with some modifications. For phylogenetic analysis based on the 16S rRNA gene sequence, the gene was amplified using PCR in the presence of forward 16F27 (5′-AGAGTTTGATCTGGCTCAG-3′), reverse 16R1488 (5′-TACCTTGTTAGGACTTCACC-3′) and internal r530 (5′-GGTCATTAAGGCTAATTGCG-3′) primers at least twice (Karlson *et al.*, 1993).

The amplified fragment was purified using the High-Pure PCR Product Purification kit (Roche) and then sequenced by utilizing forward, reverse and internal primers by Macrogen (Korea). The 16S rRNA gene reads were assembled using Chromas pro software and aligned using the multiple sequence alignment program CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). Phylogenetic trees were constructed using neighbour-joining, maximum-parsimony and maximum-likelihood methods in MEGA version 5 (Tamura *et al.*, 2011).

gyrB was amplified by PCR in the presence of forward UP-1S (5′-GAAGTCATCATGACCGTTCTGCA-3′) and reverse UP-2Sr (5′-AGCAGGGTACGGATGTGCGAGCC-3′) primers (Yamamoto & Harayama, 1995). The amplified fragment also was sequenced by utilizing forward, reverse and 500R (5′-CTTGATAGGAATCGTTCCACTGC-3′) primers by Macrogen (Korea). Phylogenetic analysis was performed using the same method for 16S rRNA gene sequence by using the neighbour-joining method with 1000 random replicates.

Genomic DNA G+C content was verified by BCCM/LMG. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). For determination of G+C content, the DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase. The resulting deoxyribonucleosides were investigated by using HPLC (Shimadzu), under chromatography conditions [temperature 45 °C, 10 µl sample, solvent 0.3 M $(NH_4)H_2PO_4$/acetonitrile, 40 : 1 (v/v), pH 4.4, 1.3 ml min^{-1}] adapted from Tamaoka & Komagata (1984). The G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) according to the method of Mesbah *et al.* (1989).

DNA–DNA hybridization between strain RCRI4^T and reference strains, *Alishewanella agri* BL06^T and *Rheinheimera perlucida* BA131^T, was performed by BCCM/LMG. DNA–DNA hybridizations were performed in the presence of 50 % formamide at 45 °C between strain RCRI4^T and *A. agri* BL06^T and at 42 °C between strain RCRI4^T and *R. perlucida* BA131^T, according to a modification of the method described by Ezaki *et al.* (1989). The DNA–DNA relatedness percentage reported is the mean of six–seven hybridizations.

Strain RCRI4^T is a rod-shaped motile bacterium with a polar flagellum (Fig. 1). The cells grow without NaCl and also in the presence of 0.5, 1, 1.5, 2 and 3 % NaCl (optimum 0–0.5 %). Strain RCRI4^T was able to grow at 10–40 °C with a pH range of 6–10, optimum growth at 30 °C and pH of 7±0.5 in lab made marine broth with 0.5 % NaCl. The other phenotypic characteristics of strain

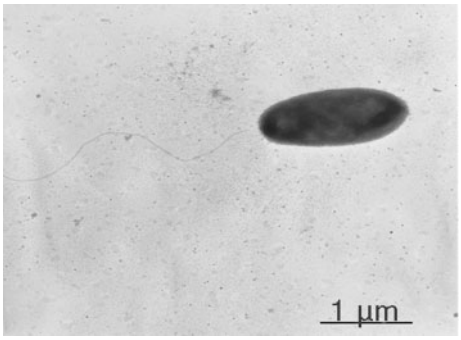

Fig. 1. Morphological characteristics of strain RCR14^T as visualized by TEM.

RCRI4^T are summarized in the species description in Table 1.

Analysis of the 16S rRNA gene sequences revealed that strain RCRI4^T belongs to the genus *Alishewanella* (Fig. 2). Similarities between strain RCRI4^T and closely related type strains, achieved using EzTaxon (Chun *et al.*, 2007), were 97.8 % with *A. agri* BLO6^T, 97.7 % with *Alishewanella aestuarii* B11^T, 97.5 % with *R. perlucida* BA131^T, 97.3 % with *Alishewanella fetalis* CCUG 30811^T and 97.1 % with *Alishewanella jeotgali* MS1^T. According to the phylogenetic tree derived from the maximum-likelihood, strain RCRI4^T is monophyletic with other species of *Alishewanella* (Fig. 2). Based on the neighbour-joining and maximum-parsimony trees, strain RCRI4^T is paraphyletic with the rest of *Alishewanella* species since *R. perlucida* BA131^T is also included (Figs S1 and S2, available in IJSEM Online). Furthermore, members of the genus *Alishewanella* have a short hypervariable region in the 16S rRNA gene sequence from 66 to 103 (*Escherichia coli* numberings), which is shorter than in other bacteria, as described by Vogel *et al.* (2000). Strain RCRI4^T contains a short hypervariable region that is as long as other *Alishewanella* species and consequently has high affinity with other *Alishewanella* species in this region. Furthermore, the 16s rRNA gene sequence in RCRI4^T is shorter (12 bases) than the closest species of *Rheinheimera*, *R. perlucida* BA131^T (Fig. S3). All these results support the prediction that strain RCRI4^T belongs to the genus *Alishewanella*. In addition, DNA–DNA hybridization between strain RCRI4^T and *R. perlucida* BA131^T was 14 %. DNA–DNA hybridization experiments between the genomic DNA of strain RCRI4^T and its close relative *A. agri* BL06^T was 9 % (Table S1). This finding strongly suggests that the strain RCRI4^T is a novel species due to less than 70 % relatedness with closely related species in the genus *Alishewanella* (Stackebrandt &

Table 1. Comparison of the characteristics of *Alishewanella tabrizica* RCRI4^T and its closely related species including all *Alishewanella* species and *Rheinheimera perlucida*

Taxa: 1, strain RCRI4^T; 2, *A. aestuarii* B11^T; 3, *A. agri* BL06 ^{T}; 4, *A. fetalis* CCUG 30811^T; 5, *A. jeotgali* MS1^T (Kim *et al.*, 2010); 6, *R. perlucida* BA131^T (Brettar *et al.*, 2006). (+), Very weak; ND, not detected; +, positive; −, negative.

Characteristic	1	2	3	4	5	6
Motility	+	+	−	−	+	+
Growth at:						
4 °C	−	−	−	−	+	+
10 °C	(+)	−	+	−	+	+
Growth in						
0 % NaCl	+	+	+	−	−	+
6 % NaCl	−	−	+	+	−	+
8 % NaCl	−	−	−	+	−	+
NaCl concentration range (%)	0–3	0–5	0–6	0–15	1–2	0–8
Hydrolysis of aesculin	+	−	+	+	+	ND
Assimilation of:						
D-Glucose	−	−	+	−	+	−
Malate	+	−	−	+	−	ND
Glycerol	−	−	−	+	−	−
D-Fructose	+	+	−	+	−	−
Inositol	−	−	−	+	−	ND
D-Mannitol	−	+	−	+	−	ND
Aesculin	+	−	+	−	+	ND
Trehalose	+	−	−	−	+	−
Raffinose	−	+	−	−	−	ND
Glycogen	−	−	−	−	+	+
DNA G+C content (mol%)	45.3	49.5	54.8	51.0	53.6	48.9
Isolation source	Lake water	Tidal flat sediment	Soil	Human foetus	Fermented food	Seawater

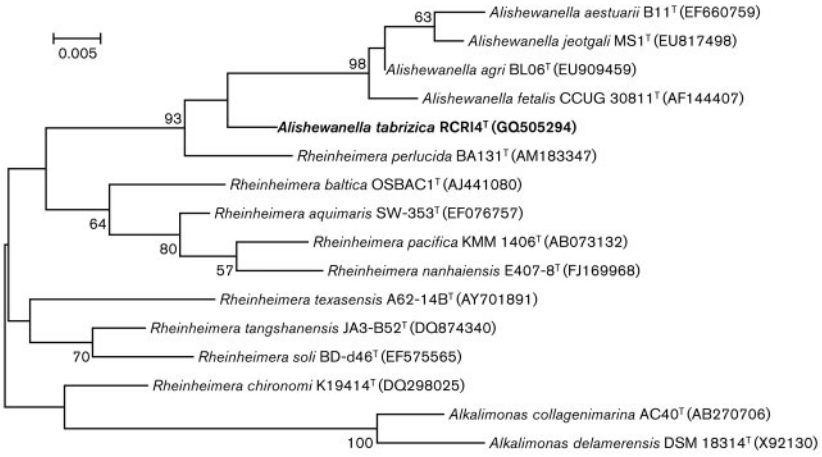

Fig. 2. Phylogenetic relationships of the members of *Alishewanella tabrizica* RCRI4^T based on the partial sequence of the 16S rRNA gene. The sequence alignment was performed using the CLUSTAL_X program and the tree was generated using the maximum-likelihood method in MEGA5 software. Numbers at nodes refer to bootstrap values; only values $\geqslant$50 % are shown. Bar, expected changes per nucleotide.

Goebel, 1994; Wayne *et al.*, 1987). The *gyrB* sequence was compared by nBlast. RCRI4^T *gyrB* showed similarities higher than 75 % only with *Alishewanella* species, but its similarities with close genera were lower than 75 %. The phylogenetic tree based on the *gyrB* gene sequence is shown in Fig. S4.

Phenotypic characteristics of strain RCRI4^T groups this strain in the genus *Alishewanella* due to the following features. (i) The major fatty acid composition of strain RCRI4^T is $C_{17:0}$ (15.02 %), $C_{16:1}\omega7c/C_{15}$ iso 3-OH (15.20 %) and $C_{17:1}\omega8c$ (13.90 %), which is similar to that of the members of the genus *Alishewanella* (Brettar *et al.*, 2002), (ii) *Alishewanella* species, including the proposed species (strain RCRI4^T) in the present study, are Gram-negative rods. They have oxidase and catalase activities and are able to hydrolyse gelatin. They do not produce indole or arginine dihydrolase and they can reduce nitrate to nitrite and nitrogen (Kim *et al.*, 2009; Vogel *et al.*, 2000). Strain RCRI4^T is a Gram-negative, rod and facultatively anaerobic. (iii) Members of the genus *Rheinheimera* grow at temperatures from 4 to 30 °C with optimum growth at 20–25 °C (Brettar *et al.*, 2002) but members of the genus *Alishewanella*, except for *Alishewanella jeotgali* MS1^T, cannot grow at 4 °C and the optimum temperature for them is 30–37 °C (Kim *et al.*, 2010). Strain RCRI4^T cannot grow at 4 °C and the optimum temperature for growth of strain RCRI4^T is 30 °C.

The results of all of these findings, including 16S rRNA gene sequence, DNA–DNA hybridization, morphophysiological and biological tests, justify a distinction for strain RCRI4^T on species level in the genus *Alishewanella*. Therefore, strain RCRI4^T (=LMG 26473^T=JCM 17275^T=KCTC 23723^T) is suggested to represent a novel species named *Alishewanella tabrizica* RCRI4^T.

Description of *Alishewanella tabrizica* sp. nov.

Alishewanella tabrizica (tab.ri.zi.ka N.L. fem. adj. *tabrizica* pertaining to Tabriz, a city in the north-west of Iran, the centre of East Azerbaijan province, where the type strain was isolated and characterized).

The isolated bacterium is a Gram-negative, rod-shaped, motile bacterium with a polar flagellum of 1.5–2.0 × 0.9 μm. The cells produce colonies with pallid colour on marine agar medium. The shape of the colonies is circular, the angle of the colonies is convex and their edges are entire.

The cells do not require NaCl for growth and tolerate NaCl up to 3 % with optimum growth in 0–0.5 % NaCl. The cells are able to grow at 10–40 °C, in a pH range of 6–10 with the most efficient growth at 30 °C and pH 7$\pm$0.5 (Table 1). The strain RCRI4^T can grow on MacConkey agar, nutrient agar, blood agar, BHI and LB media but not sea water agar. The biochemical experiments indicate that strain RCRI4^T is capable of producing oxidase, catalase, amylase, urease, tyrosinase, aesculinase and gelatinase but is not able to produce indole, ornithine, arginine and lysine decarboxylase and phenylalanine deaminase, according to lab-made tests. It is positive for reduction of nitrate to nitrite.

According to the API 50CH test, acid is produced from D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, methyl-α-D-glucopyranoside, aesculin, D-cellobiose, maltose, sucrose, trehalose, gentiobiose, turanose and potassium 5-ketogluconate, but is not produced from glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl-β-D-xylopyranoside, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, inulin, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate. In API 20NE, maltose and malic acid are assimilated, but L-arabinose, D-mannose, D-mannitol, potassium gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid are not assimilated. Moreover, the strain RCRI4^T has enzyme activities for lipase, β-glucosidase, *N*-acetyl-β-glucosaminidase, β-galactosidase, α-glucosidase, α-galactosidase, α-maltosidase and L-aspartic acid arylamidase activities, but does not have

Table 2. Fatty acid content (%) of *Alishewanella tabrizica* RCRI4^T and close relatives

Taxa: 1, strain RCRI 4^T; 2, *A. aestuarii* B11^T; 3, *A. agri* BL06^T; 4, *A. fetalis* CCUG 30811^T; 5, *A. jeotgali* MS1^T(Kim *et al.*, 2010); 6, *R. perlucida* BA131^T(Brettar *et al.*, 2006). tr, Trace (less than 1.0 %). Cells were grown on Columbia blood agar for 2 days and/or marine agar for 1 day. Growth conditions: A, marine agar; B, blood agar. $C_{15:0}$ $\omega 8c$ is only reported in *A. agri* by Kim *et al.* (2010). Numbers in bold type refer to dominant fatty acid fractions.

Fatty acid	1A	1B	2B	3B	4B	5B	6B
$C_{10:0}$	0.17						
$C_{10:0}$ 3-OH	0.22	0.32					
$C_{11:0}$	0.29	0.78					0.51
$C_{11:0}$ 3-OH	2.61	5.74	1.7	3.3	2.3	2.3	1.78
$C_{12:0}$	1.11	0.99					1.28
$C_{12:0}$ 3-OH	3.58	4.81	3.7	2.4	4.0	3.3	3.27
$C_{12:0}$ iso 3-OH	0.51	0.61					
$C_{13:0}$	0.38	0.69					0.54
$C_{13:0}$ 3-OH							1.32
$C_{14:0}$	0.75	0.51					1.13
$C_{14:0}$ iso 3-OH	0.55	0.78					
$C_{15:0}$			1.9	2.2	1.2	1.4	
$C_{15:1}\omega$ 6*c*	1.16	0.77					0.57
$C_{15:1}\omega$ 8*c*	3.33	2.56				1.6	3.04
$C_{16:0}$	**8.88**	**9.45**	**14.3**	**10.1**	**11.4**	**13.4**	**18.24**
$C_{16:1}$ $\omega 7c$							**23.61**
$C_{16:0}$ iso	2.05	1.57	tr	2.6	1.5	2.4	1.23
$C_{17:0}$	**7.0**	**15.02**	**8.7**	**10.2**	**8.9**	**8.9**	**7.88**
$C_{17:0}$ iso		0.55					0.47
$C_{17:0}$ anteiso	0.83	0.94		1.0	tr	tr	0.81
$C_{17:1}\omega 6c$	1.27	0.88	tr	1.8	1.6	1.9	0.88
$C_{17:1}\omega 8c$	**17.83**	**13.90**	**16.0**	**20.2**	**18.4**	**19.8**	**18.3**
$C_{18:0}$	0.53	1.33					0.73
$C_{18:0}$iso	0.60	0.44	tr	1.1	1.8	2.0	0.29
$C_{18:1}\omega 7c$	**10.18**	**6.80**	**18.0**	**12.7**	**23.4**	**21.7**	**9.39**
$C_{18:1}\omega 7c$ 11-methyl		0.28					0.51
$C_{18:1}\omega 9c$		0.91	2.0	1.1	1.4	1.3	
Sum in features 1*	2.61	6.85	1.9	2.6	2.4	1.8	
Sum in features 2†	3.13	4.45	3.2	2.1	3.2	1.9	
Sum in features 3‡	**27.98**	**15.20**	**15.0**	**15.1**	**13.4**	**16.5**	
Unknown 11.799	1.83	2.86					

*Sum in features 1 comprises $C_{13:0}$ 3-OH/$C_{15:1}$ H, or both.
†Sum in features 2 comprises $C_{14:0}$ 3-OH/$C_{16:1}$ iso.
‡Sum in features 3 comprises $C_{16:1}\omega 7c$ /C_{15} iso 3-OH.

enzyme activity for β-glucoronidase and malonate. Sodium pyruvate acidification is negative.

The major fatty acids are $C_{17:0}$, $C_{16:1}\omega$7c and/or C_{15} iso 3-OH, $C_{17:1}\omega$8c, and $C_{16:0}$ (Table 2). The G+C content of genomic DNA is 45.3 mol%.

The type strain is RCRI4^T (=LMG 26473^T=JCM 17275^T=KCTC 23723^T), isolated from Qurugöl Lake, which is located in Azerbaijan, a mountainous region in the north-west of Iran.

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