Rheinheimera longhuensis sp. nov., isolated from a slightly alkaline lake, and emended description of genus *Rheinheimera* Brettar et al. 2002

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The bacterial strain LH2-2^T was isolated from freshwater of Longhu Lake, a slightly alkaline lake (pH 8.8) in north-east China. Cells of strain LH2-2^T were Gram-staining-negative, non-spore-forming rods, 0.3–0.5 µm wide and 2.0–4.0 µm long. Cells were motile by means of a single polar flagellum. The strain was strictly aerobic and heterotrophic and oxidase- and catalase-positive. Growth occurred at 0–36 °C (optimum, 26–34 °C), pH 6.5–11 (optimum, pH 8.0–8.6) and in the presence of 0–2 % (w/v) NaCl (optimum, 1 %). Strain LH2-2^T contained Q-8 as the major respiratory quinone. The major fatty acids were summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 21.9 %), C_{17:1ω8c} (18.9 %), C_{18:1ω7c} (16.4 %) and C_{16:0} (12.7 %) after growth on marine agar 2216. The DNA G+C content was 47 mol% (*T_m*). The 16S rRNA gene and a conserved portion of the *gyrB* gene were sequenced and used for phylogenetic analyses. Phylogenetic trees based on 16S rRNA gene and *gyrB* sequences showed that strain LH2-2^T was associated with the genus *Rheinheimera* and closely related to the type strains of *Rheinheimera* species, and showed the highest 16S rRNA gene sequence similarity to *Rheinheimera pacifica* KMM 1406^T (97.4 %), *R. aquimaris* SW-353^T (97.1 %) and *R. chironomi* K19414^T (96.5 %). The DNA–DNA relatedness of strain LH2-2^T to *R. pacifica* NBRC 103167^T, *R. aquimaris* JCM 14331^T and *R. chironomi* LMG 23818^T was 39, 31 and 23 %, respectively. Based on these results, it is concluded that strain LH2-2^T represents a novel species of the genus *Rheinheimera*, for which the name *Rheinheimera longhuensis* sp. nov. is proposed. The type strain is LH2-2^T (=CGMCC 1.7003^T =NBRC 105632^T). An emended description of the genus *Rheinheimera* is also provided.

The genus *Rheinheimera*, a branch of the gammaproteobacteria, was created by Brettar et al. (2002) and its description has subsequently been emended by Merchant et al. (2007), Chen et al. (2010) and Li et al. (2011). At the time of writing, the genus *Rheinheimera* comprises ten recognized species: *Rheinheimera baltica* (the type species; Brettar et al., 2002), *R. pacifica* (Romanenko et al., 2003), *R. perlucida* (Brettar et al., 2006), *R. aquimaris* (Yoon et al., 2007), *R. texasensis* (Merchant et al., 2007), *R. chironomi* (Halpern et al., 2007), *R. soli* (Ryu et al., 2008), *R. tangshanensis* (Zhang et al., 2008), *R. aquatica* (Chen et al.,

2010) and *R. nanhaiensis* (Li et al., 2011). Members of *Rheinheimera* have mostly been isolated from aquatic environments (except for *R. soli*, *R. nanhaiensis* and *R. tangshanensis*, which were isolated from soil, marine sediments and plant roots, respectively), including seawater (*R. baltica*, *R. pacifica*, *R. perlucida* and *R. aquimaris*) and freshwater (*R. texasensis*, *R. aquatica* and *R. chironomi*; the latter was isolated from a chironomid egg mass sampled from river water). Species of *Rheinheimera* are able to grow on and to degrade rapidly the more easily degradable organic fraction of marine organic matter (Bianchi & Bianchi, 1995; Pinhassi & Berman, 2003; Poretsky et al., 2005), suggesting that they may play important roles in aquatic environments in biogeochemical cycling of elements such as carbon. In this communication, a freshwater bacterial isolate capable of growth at 0 °C, strain LH2-2^T, was investigated for its taxonomic position by a polyphasic approach.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LH2-2^T is EU183319.

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

Strain LH2-2^T was isolated from surface water of a slightly alkaline lake, Longhu Lake (46° 41' 47.0" N 124° 26' 11.1" E; maximum depth 3 m, 12 °C, chemical oxygen demand 80 mg l⁻¹, pH 8.8) in Daqing, Heilongjiang Province, China, during the study of the bacterial diversity of the freshwater in this lake in 2008. For isolation, serially tenfold-diluted water samples were spread onto low-organic Luria–Bertani (LOLB) agar (containing 1.0 g tryptone, 0.5 g yeast extract and 10.0 g NaCl l⁻¹, pH 8.0) and incubated at 30 °C for 2 days. Strain LH2-2^T was obtained after several streakings and transfers on LOLB agar plates, and it could also grow well on normal Luria–Bertani agar and marine 2216 agar (MA; Difco); the strain grew slowly on R2A agar.

All experiments were performed in parallel on strain LH2-2^T and type strains of species of the genera *Rheinheimera* and *Alishewanella*, except for morphological studies and DNA G + C content analyses.

Routine cultivation was conducted at 30 °C with MA (pH 8.0) unless otherwise indicated. Gram staining was performed according to Gerhardt *et al.* (1994). Endospore formation was tested by malachite green staining (Dong & Cai, 2001). Flagellation was examined by transmission electron microscope (H-600; Hitachi) after negative staining with 1% (w/v) phosphotungstic acid. Morphological observations were carried out using a scanning electron microscope (FEI Quanta 2000). The growth temperature range was determined with a TN3F temperature-gradient incubator (Advantec) and results were recorded after 2 and 7 days. The pH range for growth (pH 5.0–11.5) was tested in marine 2216 broth (MB; Difco) (inoculum size 0.01%) with the pH adjusted by addition of 5 M NaOH or HCl and adjusted again with sterile NaOH or HCl after autoclaving.

Tolerance of NaCl was examined in modified MB (laboratory prepared) with final NaCl concentrations of 0–2.5% (w/v) (at intervals of 0.5%). Physiological and biochemical tests, including catalase and oxidase activity, hydrolysis of casein, starch and Tweens 20 and 80, indole production and nitrate reduction, were carried out according to Dong & Cai (2001). Oxygen requirement was determined by incubation in an Oxoid AnaeroGen system. Anaerobic respiration tests were performed as described by Brettar *et al.* (2002) in MB using thiosulfate, sulfite, nitrate and ferric iron as electron acceptors. Formation of H₂S from thiosulfate was tested with lead acetate paper. Some carbon source utilization tests were performed according to the methods of Dong & Cai (2001); further carbon source utilization tests were performed using API 50CH test strips (bioMérieux) according to the manufacturer's instructions. Strain LH2-2^T was also characterized by using API 20NE and API ZYM systems (bioMérieux) following the manufacturer's instructions. Susceptibility to antibiotics was determined by the agar diffusion method using filter-paper discs (Beijing Pharmaceutical Company) containing various antibiotics as specified in the species description.

Strain LH2-2^T was heterotrophic, aerobic and oxidase- and catalase-positive. No growth occurred under anoxic

conditions, even in the presence of the tested electron acceptors. Cells were Gram-staining-negative, non-spore-forming rods, 0.3–0.5 µm wide and 2.0–4.0 µm long, and motile by means of a single polar flagellum (Fig. S1, available in IJSEM Online). Colonies were 1–2 mm in diameter, smooth, non-pigmented, transparent, raised and circular with entire margins after cultivation on MA (pH 8.0) for 24 h. Strain LH2-2^T grew at 0–36 °C, pH 6.5–11 and in the presence of 0–2% NaCl (w/v), with optimal growth at 26–34 °C, pH 8.0–8.6 and 1.0% NaCl (w/v). No growth was observed at –1 or 37 °C or at pH 6.0 or 11.5. More physiological and biochemical properties of strain LH2-2^T are provided in Table 1 as well as in the species description.

Biomass for chemotaxonomic analyses was cultivated on MA at 30 °C for 24 h. Cellular fatty acids were analysed using the standard MIDI Sherlock Microbial Identification System (version 6.0), and peaks were identified on an Agilent 6890N Network GC system using the peak-naming table TSBA6. Isoprenoid quinones were extracted and purified according to the method of Collins (1985) and analysed by HPLC (Wu *et al.*, 1989), with a previously characterized mixture of various menaquinones and ubiquinones (Hu *et al.*, 2004) as a reference. Polar lipids were extracted using a chloroform/methanol system and identified using two-dimensional TLC, as described by Kates (1986). Merck silica-gel 60 F₂₅₄ aluminium-backed thin-layer plates were used in TLC analysis. The major fatty acids of strain LH2-2^T were summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 21.9%), C_{17:1}ω8c (18.9%), C_{18:1}ω7c (16.4%) and C_{16:0} (12.7%). Strain LH2-2^T had a fatty acid profile similar to those of the type strains of the most closely related *Rheinheimera* and *Alishewanella* species under the same cultivation conditions (Table S1). All strains had summed feature 3 and C_{16:0} as dominant fatty acids. The major isoprenoid quinone of strain LH2-2^T was Q-8 (>95%). The results shown in Fig. S2 indicate that all tested members of the genera *Rheinheimera* and *Alishewanella* exhibited quite similar polar lipid profiles. All tested strains contained phosphatidylglycerol and phosphatidylethanolamine as the major polar lipids, and some unknown phospholipids and aminolipids as minor polar lipids. In comparison with its closest relative, *R. pacifica* NBRC 103167^T, strain LH2-2^T contained one more unknown aminolipid (AL3) and lacked one unknown phospholipid (PL3), in addition to phosphatidylglycerol and phosphatidylethanolamine (Fig. S2).

The nearly complete 16S rRNA gene of strain LH2-2^T (1488 nt) was amplified and sequenced as described by Zhang *et al.* (2003). The 16S rRNA gene sequence was then compared with available 16S rRNA gene sequences in GenBank databases using the BLAST program (Altschul *et al.*, 1990) at NCBI (<http://www.ncbi.nlm.nih.gov>). Alignments of 16S rRNA gene sequences were performed with the CLUSTAL_X program (version 1.64b; Thompson *et al.*, 1997) and then phylogenetic analyses were carried out using three treeing algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971)

and maximum-likelihood (Felsenstein, 1981) methods. The neighbour-joining tree reconstructed with Kimura's two-parameter model and the pairwise-deletion option in MEGA version 3.1 (Kumar *et al.*, 2004) is shown in Fig. 1. Two major clusters, corresponding to the genera *Rheinheimera* and *Alishewanella*, can be observed. Strain LH2-2^T belonged to the *Rheinheimera* cluster (95.3–97.4 % sequence similarity), with the highest sequence similarity to *R. pacifica* KMM 1406^T (97.4 %) and *R. aquimaris* SW-353^T (97.1 %). Species belonging to the genus *Alishewanella* formed a distinct cluster and were closely related to the *Rheinheimera* species. Strain LH2-2^T also showed high sequence similarity to members of the genus *Alishewanella* (96.3–97.1 %), with the highest sequence similarity to *Alishewanella aestuarii* B11^T (97.1 %). The maximum-parsimony (Fig. S3) and maximum-likelihood (Fig. S4) trees showed almost the same topologies as the neighbour-joining tree.

The *gyrB* gene (encoding gyrase B) has also been used in phylogenetic analyses of related bacteria, such as the genus *Alishewanella* (Fonnesbech Vogel *et al.*, 2000) and *Pseudomonas putida* strains (Yamamoto & Harayama, 1995). In this study, a conserved fragment of *gyrB* (approx. 1200 nt) from strain LH2-2^T, *R. tangshanensis* CGMCC 1.6362^T, *R. aquimaris* JCM 14331^T, *R. perlucida* LMG 23581^T, *R. chironomi* LMG 23818^T and *R. pacifica* NBRC 103167^T was amplified and sequenced as described by Fonnesbech Vogel *et al.* (2000) and Yamamoto & Harayama (1995). An unrooted neighbour-joining tree based on *gyrB* sequences (Fig. S5) indicated that members of the genus *Alishewanella* formed a distinct cluster in the tree, with high sequence similarities (>91.4 %) to each other. However, members of the genus *Rheinheimera* formed two distinct clusters, and LH2-2^T displayed quite low *gyrB* sequence similarities to members of the genera *Rheinheimera* (73.9–77.7 %) and *Alishewanella* (76.2–78.7 %). These results further suggested that LH2-2^T is a member of the genus *Rheinheimera*.

DNA base composition was determined by thermal denaturation (Marmur & Doty, 1962), with DNA from *Escherichia coli* K-12 as a reference. The DNA G+C content of strain LH2-2^T was 47 mol% (*T_m*), within the range of values reported for the genus *Rheinheimera* (47.0–50.5 mol%).

Strain LH2-2^T showed some important properties that are in agreement with those reported for the genus *Rheinheimera* but different from the properties of members of the genus *Alishewanella*, such as being strictly aerobic (in particular, no growth under anoxic conditions even in the presence of electron acceptors), flagellated and not requiring NaCl for growth, being able to assimilate *N*-acetylglucosamine and malate and showing *N*-acetyl-β-glucosaminidase activity. However, strain LH2-2^T also showed a range of phenotypic characteristics that differentiated it from currently described *Rheinheimera* species (Table 1), such as the ability to grow at 0 °C, nitrate reduction, assimilation of various carbon resources and

production of different enzymes. To distinguish strain LH2-2^T further from closely related *Rheinheimera* species, DNA–DNA hybridization was performed by using the initial renaturation rate method of De Ley *et al.* (1970) and modified by Huß *et al.* (1983). Results showed that the DNA–DNA relatedness of strain LH2-2^T to *R. pacifica* NBRC 103167^T, *R. aquimaris* JCM 14331^T and *R. chironomi* LMG 23818^T was 39, 31 and 23 %, respectively.

Combining the above results of phenotypic, chemotaxonomic and genotypic studies, it is concluded that strain LH2-2^T represents a novel species of the genus *Rheinheimera*, for which the name *Rheinheimera longhuensis* sp. nov. is proposed. An emended description of the genus *Rheinheimera* is also provided.

Emended description of the genus *Rheinheimera* Brettar *et al.* 2002

The description is as given by Brettar *et al.* (2002) and emended by Merchant *et al.* (2007), Chen *et al.* (2010) and Li *et al.* (2011) with the following additional changes. Growth occurs at 0–43 °C. The major polar lipids are phosphatidylglycerol and phosphatidylethanolamine.

Description of *Rheinheimera longhuensis* sp. nov.

Rheinheimera longhuensis (long.hu.en'sis. N.L. fem. adj. *longhuensis* pertaining to Longhu Lake, from which the organism was first isolated).

Heterotrophic, aerobic, oxidase- and catalase-positive. Cells are Gram-staining negative, non-spore-forming rods, 0.3–0.5 µm wide and 2.0–4.0 µm long, motile by means of a single polar flagellum. Colonies are 1–2 mm in diameter, smooth, non-pigmented, transparent, raised and circular with entire margins after cultivation on MA (pH 8.0) for 24 h. Grows at 0–36 °C (optimum, 26–34 °C) and pH 6.5–11 (optimum, pH 8.0–8.6) and in the presence of 0–2 % (w/v) NaCl (optimum, 1 %). Positive for hydrolysis of starch, casein, Tweens 80 and 20 and DNA. Utilizes methionine, L-glutamic acid, valine, asparagine and DL-lysine. Negative for H₂S production. According to API 20NE and API 50CH tests, positive for nitrate reduction, hydrolysis of aesculin and gelatin and assimilation of D-glucose, maltose, malate, trehalose, D-fructose, *N*-acetylglucosamine, aesculin, cellobiose, sucrose, starch and glycogen; all other test results are negative. In API ZYM analysis, the following enzyme activities are detected: acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosaminidase and cystine arylamidase (weakly positive). Major cellular fatty acids when grown on MA are summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{17:1}ω8c, C_{18:1}ω7c and C_{16:0}. The major quinone is Q-8. The DNA G+C content of the type strain is 47 mol% (*T_m*). The polar lipids consist of phosphatidylglycerol, phosphatidylethanolamine, two unknown phospholipids

Table 1. Properties of strain LH2-2^T and members of the genera *Rheinheimera* and *Alishewanella*

Strains: 1, LH2-2^T (data from this study); 2, *R. pacifica* KMM 1406^T; 3, *R. aquimaris* SW-353^T; 4, *R. chironomi* K19414^T; 5, *R. tangshanensis* JA3-B52^T; 6, *R. perlucida* BA131^T; 7, *R. baltica* OSBAC1^T; 8, *R. texasensis* A62-14B^T; 9, *R. soli* BD-d46^T; 10, *R. aquatica* GR5^T; 11, *R. nanhaiensis* E407-8^T; 12, *A. fetalis* CCUG 30811^T; 13, *A. aestuarii* B11^T; 14, *A. jeotgali* MS1^T; 15, *A. agri* BL06^T. Data for reference strains are from Romanenko *et al.* (2003), Yoon *et al.* (2007), Halpern *et al.* (2007), Zhang *et al.* (2008), Brettar *et al.* (2002, 2006), Merchant *et al.* (2007), Ryu *et al.* (2008), Chen *et al.* (2010), Li *et al.* (2011), Fonnesbech Vogel *et al.* (2000), Roh *et al.* (2009) and Kim *et al.* (2009, 2010) unless indicated. All strains are positive for catalase and oxidase activities, hydrolysis of gelatin, starch and Tween 80 and API ZYM tests for alkaline phosphatase and leucine arylamidase; all strains are negative for hydrolysis of L-arginine, indole production, fermentation of D-glucose, assimilation of mannose, gluconate, caprate, adipate and phenylacetate and API ZYM tests for lipase (C14), β -galactosidase, β -glucuronidase, α -mannosidase and α -fucosidase. +, Positive; –, negative; w, weakly positive; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Source of isolation	Lake water	Deep seawater	Seawater	Chironomid egg mass	Rice roots	Surface seawater	Surface seawater	Lake water	Soil	Freshwater pond	Marine sediments	Human fetus	Tidal flat sediment	Fermented food	Landfill soil
Pigmentation	None	None	Yellowish white	None	None	None	Blue	None	None	Greenish yellow	Slightly yellow	None	None	Ivory	None
Cell width (µm)	0.3–0.5	0.6–0.8	0.3–0.7	0.3–0.7	0.4–1.0	0.6–1.2	0.5–1.5	0.7–0.8	0.9–1.5	0.5–1.0	0.3–0.5	0.5–1.0	NR	1	0.5
Cell length (µm)	2.0–4.0	1.8–2.0	0.3–5.0	1.0–2.4	1.3–2.5	0.9–2.4	0.9–2.5	1.25–2.5	2.0–3.5	1.5–2.0	1.0–2.0	2	NR	2–6	1–2
Flagella*	S, P	M, P and L	S, P	S, P	S, P or L	S, P	S, P	S, P or M, P and L	S, P	S, P	S, P	None	S, P	S, P	None
Oxygen requirement†	A	A	A	A	A	FAN	A	FAN	FAN	A	FAN	FAN	NR	FAN	A
Growth temperature (°C)															
Range	0–36	4–37	4–43	4–40	10–37	4–37	4–30	25–37	15–35	10–40	10–48	25–42	18–44	4–40	10–44
Optimum	26–34	25–30	30–37	30–37	30	20–30	20–25	30–37	25–30	35	37	37	37	NR	NR
Growth in NaCl (% w/v)															
Range	0–2	0–8	0–8	0–2	0–3	0–8	0–6	0–1	0–4	0–2.0	0–8	1–8	0–5	1–2	0–6
Optimum	1	0.5–2.5	1–3	0.5–1	1	1–3	1–3	0	0–1	0.5–1.0	0.5–2.5	NR	3	NR	NR
pH for growth															
Range	6.5–11	5.5–10.5	5.5–10.5	6.5–10.0	6.0–8.5	5.7–10	5.7–10	6.5–9.6	6.5–8.0	7.0–8.0	5.5–10.0	NR	NR	6.5–9.0	5.5–12
Optimum	8.0–8.6	8.0–8.5	7.0–8.0	7.5–8.0	7.0	7.0	7.0	7.5–8.0	7.0–7.5	8.0	7.5–8.5	NR	NR	NR	NR
Nitrate reduction	+	–‡	+‡	+‡	–‡	+	–	+	+	+	+	+‡	+‡	+‡	+‡
Hydrolysis of aesculin	+	+	+	–	+	–	+	+	+	+	+	–	–	+	+
Assimilation of:															
D-Glucose	+	+‡	–‡	+‡	+‡	–	+	+	+	+	+	–‡	–‡	+‡	+‡
L-Arabinose	–	+‡	–‡	–‡	+‡	–	–	+	+	–	–	–‡	–‡	–‡	–‡
Mannitol	–	–‡	–‡	–‡	–‡	–	–	–	–	–	–	+‡	–‡	–‡	–‡
Maltose	+	+‡	+‡	+‡	+‡	–	+	–	+	+	+	+‡	+‡	+‡	+‡
Malate	+	–‡	–‡	+‡	+‡	–	–	–	–	–	–	–‡	–‡	–‡	–‡
Citrate	–	+‡	–‡	–‡	–‡	–	–	–	–	–	–	–‡	–‡	–‡	–‡
N-Acetylglucosamine	+	+‡	+‡	+‡	+‡	+	+	+	+	+	+	–‡	–‡	–‡	–‡

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
API ZYM test results															
Esterase (C4)	+	+	+	+	+	+	—	+	+	—	+	+	+	+	+
Esterase lipase (C8)	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+
Valine arylamidase	w	—	—	w	—	—	—	—	—	—	+	+	—	—	—
Cystine arylamidase	w	—	—	w	—	—	—	+	—	—	—	+	w	—	w
Trypsin	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+
α -Chymotrypsin	+	+	—	+	—	+	—	+	+	+	+	+	+	+	+
Acid phosphatase	+	+	+	+	+	+	—	+	w	+	+	+	w	w	w
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+
α -Galactosidase	—	—	—	—	—	—	—	—	w	—	—	—	—	—	—
α -Glucosidase	—	—	—	—	—	—	—	—	w	—	—	—	—	—	—
β -Glucosidase	—	—	—	—	—	—	+	—	—	—	—	—	—	+	—
<i>N</i> -Acetyl- β -glucosaminidase	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—
DNA G+C content (mol%)	47	49.6	50.5	49.9	47.0	48.9	48.9	48.2	49.2	51.9	51.0	51	49.5	53.6	54.8

*L, Lateral; M, multiple; P, polar; S, single.

†A, Aerobic; FAN, facultatively anaerobic.

‡Results from this study.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain LH2-2^T and members of related species. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at branch points. *Serratia entomophila* DSM 12358^T was used as an outgroup. Bar, evolutionary distance (K_{nucl}) of 0.01.

and three unknown aminolipids. Susceptible (diameter of inhibition zone >20 mm) to (μg per disc unless indicated) gentamicin (10), kanamycin (30), chloramphenicol (30), ampicillin (10), tetracycline (30), neomycin (30) and carbenicillin (100). Weakly resistant (diameter of inhibition zone 10–20 mm) to streptomycin (10), polymyxin (300) and novobiocin (5). Resistant (diameter of inhibition zone <10 mm) to bacitracin (1 U) and vancomycin (30).

The type strain is LH2-2^T (=CGMCC 1.7003^T =NBRC 105632^T), isolated from surface water of Longhu Lake in north-east China.

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