International Journal of Systematic and Evolutionary Microbiology (2005), **55**, 165–170 DOI 10.1099/ijs.0.63315-0

Pontibacillus chungwhensis gen. nov., sp. nov., a moderately halophilic Gram-positive bacterium from a solar saltern in Korea

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Three moderately halophilic, spore-forming strains, designated BH030062^T, BH030049 and BH030080, were isolated from a solar saltern in Korea. Phylogenetic analyses and comparative 16S rRNA gene sequence studies revealed that the isolates represent a novel distinct monophyletic lineage within the phyletic group classically defined as the genus *Bacillus* and are most closely related to members of the genera *Gracilibacillus* (93·7–95·1 % similarity), *Virgibacillus* (93·5–94·8 %), *Halobacillus* (94·8–95·9 %), *Filobacillus* (94·4–94·8 %) and *Lentibacillus* (93·3–93·7 %). Strain BH030062^T was strictly aerobic, rod-shaped, Gram-positive and motile by means of peritrichous flagella. It grew in the presence of 1–15 % (w/v) NaCl and at temperatures of 15–45 °C. The cell wall peptidoglycan contained A1γ-*meso*-diaminopimelic acid as the diagnostic diamino acid. The predominant cellular fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. DNA G+C content was about 41 mol% and the major isoprenoid quinone was MK-7. On the basis of their physiological and molecular properties, the isolates represent a new genus, *Pontibacillus* gen. nov., and novel species, *Pontibacillus chungwhensis* sp. nov. The type strain is BH030062^T (=KCTC 3890^T=DSM 16287^T).

Moderately halophilic bacteria that grow optimally in media containing 1–15 % (w/v) NaCl are widely distributed in different marine environments. They are of interest because this group of bacteria has great biotechnological potential for the production of compatible solutes or hydrolytic enzymes (Margesin & Schinner, 2001). Taxonomically, they include both Gram-positive and Gram-negative microorganisms. Aerobic spore-forming moderately halophilic Gram-positive rods are also taxonomically diverse and have been isolated from marine environments and related habitats. They were originally assigned to the genus *Bacillus*, but molecular and chemical analyses have shown that they form several phylogenetically distinct lineages within the group classically defined as the genus *Bacillus* (Ash *et al.*, 1991; Stackebrandt & Liesack, 1993; Nielsen *et al.*, 1994). The distinct lineages have been described as the genera *Halobacillus* (Spring *et al.*, 1996), *Gracilibacillus* (Wainø *et al.*, 1999), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Filobacillus* (Schlesner *et al.*, 2001) and *Lentibacillus* (Yoon *et al.*, 2002). Recently, some members of the genera *Salibacillus* and *Bacillus* have been transferred to the genera *Virgibacillus* and *Gracilibacillus* (Wainø *et al.*, 1999; Heyrman *et al.*, 2003). Some members of the genera *Filobacillus* and *Halobacillus* have shown Gram-negative or Gram-variable reactions, although phylogenetically they belong to the Gram-positive bacteria (Schlesner *et al.*, 2001; Yoon *et al.*, 2003). In this study, three moderately halophilic Gram-positive bacteria were isolated from the Chungwha solar saltern in Korea. Using a polyphasic approach, the strains were classified in a new genus, for which the name *Pontibacillus* gen. nov. is proposed.

The three strains (BH030062^T, BH030049 and BH030080) were isolated from a solar saltern of the Tae-An area on the Yellow Sea in Korea. The strains were isolated from soil samples that were diluted serially, spread on marine agar 2216 (MA; Difco) with the addition of 5 % (w/v) NaCl [final concentration: 6·94 % (w/v) NaCl] and incubated for 2 days at 35 °C. Requirement of and tolerance to NaCl were determined in nutrient broth (NB; Difco; 3·0 g beef extract l^{-1} and 5·0 g peptone l^{-1}) supplemented with modified artificial sea water (ASW) containing (l^{-1}): 0–30 % (w/v) NaCl, 5·94 g $MgSO_4.7H_2O$, 4·53 g $MgCl_2.6H_2O$, 0·64 g KCl and 1·3 g $CaCl_2$. The isolates were routinely grown aerobically on MA for 2 days at 35 °C,

Published online ahead of print on 30 July 2004 as DOI 10.1099/ijs.0.63315-0.

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BH030062^T, BH030049 and BH030080 are AY553296, AY603361 and AY603362, respectively.

A transmission electron micrograph showing the general morphology of strain BH030062^T is available as supplementary material in IJSEM Online.

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except where otherwise indicated. Anaerobic growth was determined by incubation in an anaerobic chamber at 35 °C on MA. Growth was tested at different temperatures (4–55 °C) on MA and at different pH values (pH 5·0–10·0) in NB supplemented with ASW containing 2·36 % (w/v) NaCl.

Cell morphology was studied using light microscopy and transmission electron microscopy. Motility was observed at 12 and 36 h in wet mounts with a light microscope (Nikon E600). The flagellum type was examined by transmission electron microscopy using cells from the exponential growth phase. Cells were mounted on Formvar-coated copper grids and negatively stained with 1 % potassium phosphotungstate (pH 7·0). Grids were examined in a JEOL JEM-1010 transmission electron microscope operated at 60 kV. Endospores were stained according to the method of Schaeffer-Fulton (Smibert & Krieg, 1981).

Gram staining was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Catalase activity was determined by production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck). Hydrolysis of aesculin, casein, starch, Tween 80, urea, hypoxanthine, tyrosine, gelatin and xanthine was determined on MA according to previously described methods (Cowan & Steel, 1965; Lanyi, 1987; Smibert & Krieg, 1994). Nitrate reduction was performed according to the method of Lanyi (1987). Acid production from carbohydrates was tested as described by Leifson (1963); all suspension media were supplemented with ASW containing 2·36 % (w/v) NaCl.

For quantitative analysis of whole cell fatty acids, strains BH030062^T, BH030049 and BH030080 were cultivated on either MA or MA plus 3 % (w/v) NaCl for 2 days at 35 °C. Fatty acid methyl esters were prepared from 40 mg wet cells and analysis was carried out using GC/MS chromatography according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Preparation of cell walls from the test strains and analysis of peptidoglycan structures were carried out using methods described by Schleifer & Kandler (1972), with the modification that TLC on cellulose sheets was performed instead of paper chromatography. Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using HPLC fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM). Methanol/2-propanol (2 : 1, v/v) was used as the mobile phase and isoprenoid quinones were detected at 270 nm. The DNA G+C contents of strains BH030062^T, BH030049 and BH030080 were determined by reversed-phase HPLC using the method of Tamaoka & Komagata (1984). DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness of the three isolates. The isolates were grown aerobically on MA for 2 days at 35 °C and chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996). Randomly primed DNA labelling with digoxigenin (DIG)-dUTP and detection of hybrids by enzyme immunoassay on nylon membranes were performed using the DIG High Prime DNA Labelling and Detection Starter kit II (Roche Applied Science) according to the manufacturer's instructions.

PCR amplification, sequencing and assembling of the 16S rRNA gene were carried out as described previously (Jeon *et al.*, 2004). The resultant sequences were compared with 16S rRNA gene sequences available from GenBank using the BLAST program to determine the approximate phylogenetic affiliation and aligned with members of the group classically defined as the genus *Bacillus* using the software CLUSTAL W (Thompson *et al.*, 1994). Sequence similarity values were computed using Similarity Matrix version 1.1 (Ribosomal Database Project II; http://rdp.cme.msu.edu/html/analyses.html; Cole *et al.*, 2003). Gaps at the 5′ and 3′ ends of the alignment were omitted from further analyses. The phylogenetic trees were constructed using three different methods: neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms available in the PHYLIP software, version 3.6 (Felsenstein, 2002). Evolutionary distance matrices were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed with the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE in the PHYLIP package.

Colonies of strain BH030062^T were yellow, low convex, smooth and circular/slightly irregular after 2 days incubation at 35 °C on MA. The strain grew in media containing 1–15 % (w/v) NaCl; optimum growth occurred on media containing 2–5 % (w/v) NaCl. It did not grow without NaCl or in the presence of more than 15 % (w/v) NaCl. It grew in nutrient agar (Difco) supplemented with ASW, but not in nutrient agar with just NaCl, indicating that the strain required salts other than NaCl for growth. Strain BH030062^T grew at pH 6·5–9·5 in NB containing 3 % (w/v) NaCl; optimal growth occurred at pH 7·5–8·5. Growth was observed at temperatures between 15 and 45 °C, with optimum growth temperatures of 35–40 °C.

Strain BH030062^T was Gram-positive and strictly aerobic. Cells were rod-shaped with a width of 0·6–0·9 μm and length of 2·3–3·0 μm and motile by means of peritrichous flagella after 2 days incubation at 35 °C on MA (see the transmission electron micrograph available as supplementary material in IJSEM Online). Spherical endospores were formed at terminal positions in swollen sporangia, enabling this strain to be distinguished from some other closely related Gram-positive bacteria (Table 1).

Strain BH030062^T was catalase-positive and oxidase-negative. It did not reduce nitrate to nitrite. Strain BH030062^T hydrolysed casein, starch and Tween 80, but not aesculin, L-tyrosine, hypoxanthine, xanthine, gelatin or urea. The strain produced acid from D-glucose, D-ribose, maltose, glycerol and D-trehalose, but not from D-xylose,

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Table 1. Characteristics of strain BH030062^T and some related species

Strains: 1, BH030062^T; 2, *Gracilibacillus halotolerans* DSM 11805^T; 3, *Virgibacillus salexigens* ATCC 700290^T; 4, *Halobacillus salinus* KCCM 41590^T; 5, *Filobacillus milosensis* DSM 13259^T; 6, *Lentibacillus salicampi* KCCM 41560^T. Data from Schlesner *et al.* (2001), Wainø *et al.* (1999), Garabito *et al.* (1997), Yoon *et al.* (2002, 2003) and this study. +, Positive; −, negative; V, variable; NA, not available. Cells of all strains are rod-shaped.

Characteristic	1	2	3	4	5	6
Size (μm)	0·6–0·9 × 2·3–3·0	0·4–0·6 × 2·0–5·0	0·3–0·6 × 1·5–3·5	0·7–1·1 × 1·5–4·0	0·3–0·5 × 3·0–7·0	0·4–0·7 × 2·0–4·0
Pigmentation	Yellow	Creamy white	None	Pale orange–yellow	White	Cream
Flagellation	Peritrichous	Peritrichous	NA	Peritrichous	One flagellum	One flagellum
Gram reaction	+	+	+	+(V)*	−	+/−†
Spore shape‡	S	E	E	E	S	S/O
Spore position§	T	C/T	C/ST	C/ST	T	T
NaCl range (%, w/v)	1–15	0–20	10–20	0–23	2–23	2–23
Catalase	+	+	+	+	+	+
Oxidase	−	+	+	+	−	+
Nitrate reduction	−	+	−	NA	−	NA
Hydrolysis of:						
Aesculin	−	+	+	+	−	−
Casein	+	−	+	+	−	+
Gelatin	−	+	+	+	−	NA
Starch	+	+	−	−	−	−
Tween 80	+	+	−	+	NA	+
Urea	−	+	NA	−	NA	−
Acid production from:						
D-Glucose	+	+	+	+	−	NA
D-Fructose	−	NA	+	+	−	−
D-Mannitol	−	+	−	+	−	−
D-Ribose	+	NA	NA	−	−	−
D-Xylose	−	+	−	−	−	−
Maltose	+	NA	+	+	−	−
D-Trehalose	+	NA	−	+	−	−
Major fatty acids	iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$	anteiso-$C_{15:0}$, $C_{16:0}$, anteiso-$C_{17:0}$	NA	iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$	NA	anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$
Cell wall type	*m*-DAP (A1γ)	*m*-DAP (A1γ)	*m*-DAP	L-Orn–D-Asp (A4β)	L-Orn–D-Glu (A4β)	*m*-DAP
Major quinone	MK-7	MK-7	MK-7	MK-7	NA	MK-7
G+C content (mol%)	41	38	39·5	45	35	44

*Gram-positive but variable in older cultures.
†Gram-variable.
‡Spore shape: E, ellipsoidal; S, spherical; O, oval.
§Spore position: C, central; T, terminal; ST, subterminal.

L-arabinose, L-rhamnose, α-D-lactose, adonitol, D-raffinose, D-mannitol, D-fructose, arbutin, D-salicin, D-melibiose or D-mannose. Typical phenotypic and chemotaxonomic properties of strain BH030062^T are summarized and compared with those of phylogenetically related type strains of the group classically defined as the genus *Bacillus* in Table 1.

Analysis of the cell wall peptidoglycan showed that strain BH030062^T possessed A1γ-*meso*-diaminopimelic acid (DAP) as the diagnostic diamino acid, in common with the great majority of endospore-forming Gram-positive bacilli. The major isoprenoid quinone was menaquinone-7 (MK-7). The cellular fatty acid profile of strain BH030062^T was characterized by anteiso-$C_{15:0}$ (~31·1 %), iso-$C_{15:0}$ (~28·5 %) and iso-$C_{16:0}$ (~10·2 %) as the major fatty acids on MA. Strains BH030062^T, BH030049 and BH030080 had very similar fatty acid profiles on both MA and MA supplemented with 3 % (w/v) NaCl (Table 2), indicating that they may possibly belong to the same species. The genomic DNA G+C contents of strains BH030062^T, BH030049 and BH030080 were about 41·0, 40·8 and

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Table 2. Fatty acid profiles (%) of strains BH030062^T, BH030049 and BH030080 on MA and MA supplemented with 3 % (w/v) NaCl

Data are expressed as percentages of total fatty acids. Fatty acids representing less than 0·5 % in all data are not shown.

Fatty acid	BH030062^T		BH030049		BH030080	
	MA	MA + 3 % NaCl	MA	MA + 3 % NaCl	MA	MA + 3 % NaCl
Straight-chain fatty acids						
$C_{15:0}$	0·72	0·80	0·73	0·61	0·60	0·93
$C_{16:0}$	1·03	2·34	1·47	1·59	0·44	1·42
Branched fatty acids						
iso-$C_{14:0}$	7·30	7·82	12·66	8·24	12·03	14·66
iso-$C_{15:0}$	28·45	23·31	23·24	21·41	30·75	24·36
anteiso-$C_{15:0}$	31·14	29·72	25·95	35·72	43·30	32·81
iso-$C_{16:0}$	10·17	9·47	18·41	9·04	4·26	14·40
iso-$C_{17:0}$	1·67	1·64	1·36	1·25	0·26	0·83
anteiso-$C_{17:0}$	8·11	7·76	6·36	8·45	2·12	5·75
Unsaturated fatty acids						
$C_{16:1}\omega 7c$ alcohol	7·30	11·69	7·24	8·99	2·67	3·13
$C_{16:1}\omega 11c$	0·81	1·63	0·77	1·73	0·36	0·52
Summed feature*						
4	1·37	1·81	0·75	1·81	0·39	ND

ND, Not detected.

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

41·5 %, respectively. The major fatty acid profiles, the major lipoquinone and the DNA G+C content are typical of the group classically defined as the genus *Bacillus* (Table 1). DNA–DNA hybridization was assessed to evaluate the genomic DNA relatedness among strains BH030062^T, BH030049 and BH030080. The values obtained from DNA–DNA hybridization experiments of strain pairs BH030062^T/BH030049, BH030062^T/BH030080 and BH030049/BH030080 were about 97, 92 and 94 %, respectively. It was concluded that the DNA–DNA relatedness values were sufficiently high for these three strains to be classified as a single species (Stackebrandt *et al.*, 2002).

Almost-complete 16S rRNA gene sequences of strains BH030062^T, BH030049 and BH030080 were obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. Phylogenetic analysis indicated that the three isolates clustered as a group within the Gram-positive bacteria with low G+C content showing halophilic or halotolerant properties. A tree constructed by neighbour-joining analysis clearly showed that the three isolates formed a distinct monophyletic clade with a 79·0 % bootstrap value within the group classically defined as the genus *Bacillus* (Fig. 1). The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to those of the tree constructed by neighbour-joining analysis (data not shown). The 16S rRNA gene sequence of strain BH030062^T was highly similar to the sequences of BH030049 and BH030080 (99·9 and 99·3 % similarity, respectively). The 16S rRNA gene sequence similarity values of the three strains to sequences of closely related type strains of Gram-positive bacteria were as follows: *Gracilibacillus* (93·7–95·1 %), *Virgibacillus* (93·5–94·8 %), *Halobacillus* (94·8–95·9 %), *Filobacillus* (94·4–94·8 %) and *Lentibacillus* (93·3–93·7 %).

Therefore, on the basis of 16S rRNA gene sequences and phylogenetic analyses, it is clear that strains BH030062^T, BH030049 and BH030080 should be assigned to a novel genus within the phyletic group classically defined as the genus *Bacillus* as representatives of a novel species; the name *Pontibacillus chungwhensis* gen. nov., sp. nov. is proposed; the type strain is BH030062^T (=KCTC 3890^T=DSM 16287^T).

Description of *Pontibacillus* gen. nov.

Pontibacillus (Pon.ti.ba.cil′lus. L. n. *pontus* the sea; L. dim. n. *bacillus* small rod; N.L. masc. n. *Pontibacillus* bacillus pertaining to the sea).

Cells are Gram-positive, spore-forming rods. Catalase-positive and oxidase-negative. Urease-negative. Spherical endospores are formed terminally in swollen sporangia. Strictly aerobic and moderately halophilic. Cells are motile by means of peritrichous flagella. Cell wall peptidoglycan contains A1γ type *meso*-DAP. Major isoprenoid quinone is MK-7. DNA G+C content is 40·8–41·5 mol% (HPLC

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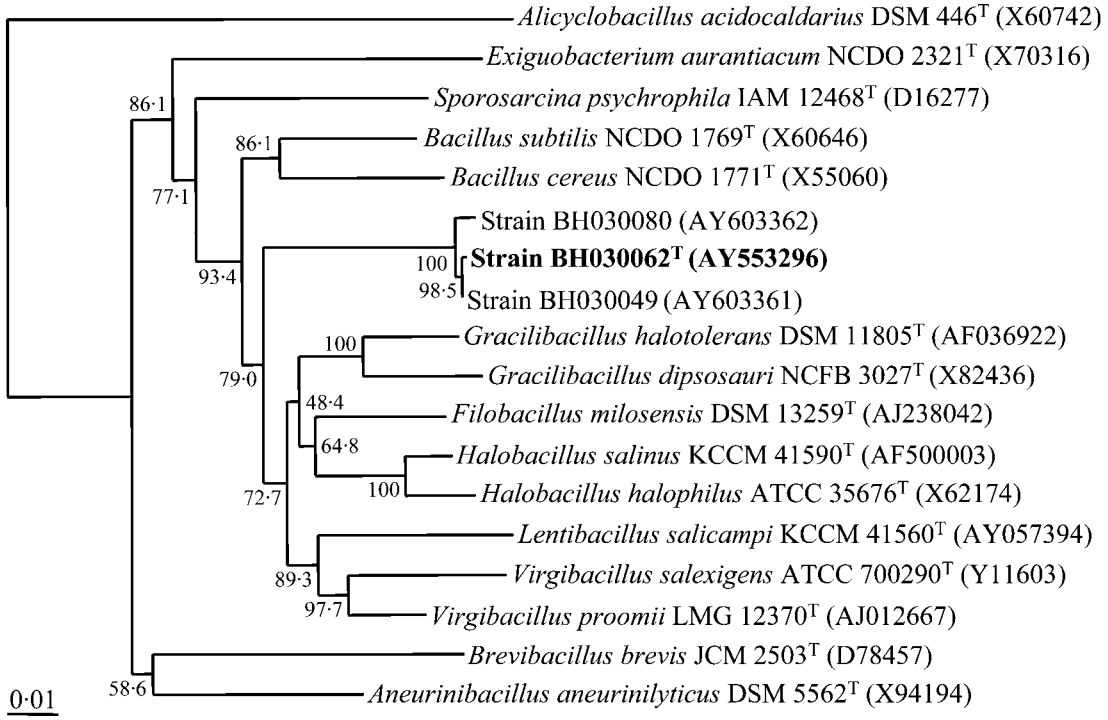

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequences of strain BH030062^T and other related taxa belonging to the Gram-positive bacteria with low G+C content. Bootstrap values are shown as percentages of 1000 replicates. *Alicyclobacillus acidocaldarius* DSM 446^T was used as an outgroup. Bar, 0·01 change per nucleotide position.

method). Predominant cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$ on MA. Phylogenetically, the genus belongs to the family *Bacillaceae*.

The type species is *Pontibacillus chungwhensis*.

Description of *Pontibacillus chungwhensis* sp. nov.

Pontibacillus chungwhensis (chung.when′sis. N.L. masc. adj. *chungwhensis* belonging to Chungwha, where the organism was isolated).

Cells are approximately 0·6–0·9 μm wide and 2·3–3·0 μm long. Strictly aerobic rods. Colonies are yellow, low convex, smooth and circular/slightly irregular on MA. Grows at 15–45 °C (optimum: 30–35 °C), pH 6·5–9·5 (optimum: pH 7·5–8·5) and 1–15 % (w/v) NaCl (optimum: 2–5 %, w/v). No growth occurs without NaCl or in the presence of more than 15 % (w/v) NaCl. Nitrate is not reduced to nitrite. Casein, starch and Tween 80 are hydrolysed. Does not hydrolyse aesculin, L-tyrosine, hypoxanthine, xanthine, gelatin or urea. Acids are produced from D-glucose, D-ribose, maltose, glycerol and D-trehalose, but not from D-xylose, L-arabinose, L-rhamnose, α-D-lactose, adonitol, D-raffinose, D-mannitol, D-fructose, arbutin, D-salicin, D-melibiose or D-mannose. Cellular fatty acid profiles on MA and MA plus 3 % (w/v) NaCl are given in Table 2.

The type strain is BH030062^T (=KCTC 3890^T=DSM 16287^T), isolated from a solar saltern on the Yellow Sea in Korea. DNA G+C content of the type strain is 41·0 mol% (HPLC method).

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (Grant MG02-0101-002-1-0-0) and KRIBB Research Initiative Program, Republic of Korea.

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