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Flavobacterium jocheonensis sp. nov., Isolated from Marine Green Alga *Ulva pertusa*

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A bacterial strain, labeled UR11^T, was isolated from green alga *Ulva pertusa* collected from Jeju Island, Korea. UR11^T was identified as a gram-negative, rod-shaped, motile by gliding and aerobic bacterial strain with yellow colonies on R2A plates. The strain UR11^T grew over at a temperature range of 10°C to 30°C (optimally at 25°C), a pH range of 6.0–11 (optimally at pH 7.0) and a Nacl range of 0.5–5% Nacl (w/v). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain UR11^T was a member of the genus *Flavobacterium*. Strain UR11^T shared close similarity with *F. jejuensis* EC11^T (98.0%) *F. jumunjinense* HME7102^T (96.1%), *F. haoranii* LQY-7^T (95.3%), *F. dongtanense* LW30^T (95.1%), and *F. ahnfeltiae* 10Alg 130^T (94.9%). The major fatty acids (>5%) were iso-$C_{15:0}$ (33.9%), iso-$C_{15:1}$ G (12.4%), iso-$C_{17:0}$ 3-OH (9.0%), iso-$C_{16:0}$ (7.0%) and iso-$C_{15:0}$ 3-OH (6.3%). The major polar lipids were phosphatidylethanolamine, seven unknown aminolipids, two unknown aminopolarlipids and two unknown lipids. DNA-DNA hybridization value was 58% at strain UR11^T with *F. jejuensis* EC11^T. Based on phenotypic, chemotaxonomic and phylogenetic evidence, strain UR11^T represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium jocheonensis* sp. nov. is proposed. The type strain is *Flavobacterium jocheonensis* is UR11^T (=KCTC 52377^T =JCM 31512^T).

Keywords: Green alga, marine bacteria, *Ulva pertusa*, *Flavobacterium*, 16S rRNA gene

Introduction

Marine algae provide surfaces for consistent colonization by microbial communities and microorganisms that inhabit these surfaces and have a positive effect by interacting with the algae. [1–3] For example, the microbes that inhabit algae help the host grow normally while also helping to release, settle and grow the spores of algae [4, 5].

The genus *Flavobacterium*, type genus of the family *Flavobacteriaceae* and member of the phylum *Bacteroidetes*, was first proposed by Bergey *et al*. (1923) with emended description by Bernardet *et al*. (1996) Kang *et al*., and Dong *et al*. (2013). At the time of writing, this genus consists of 211 species with validly published names (http://www.bacterio.net/flavobacterium.html). The members of *Flavobacterium* have been isolated from a wide range of habitats including sea water [10, 11], freshwater [12, 13], and soil [14–17] as well as marine algae [18, 19]. They are gram-negative, aerobic, with gliding motility, yellow-pigmented, rod-shaped and contain menaquinone 6 (MK-6) as the major respiratory quinone. [20–22]. Members of *Flavobacterium* show a genomic DNA G+C content in the range of 30–52 mol%.

In this paper, we report the isolation and characterization of the genus *Flavobacterium* associated with marine green alga *Ulva pertusa* for which the name *Flavobacterium jocheonensis* sp. nov. is proposed.

Materials and Methods

Bacterial strains

Strain UR11^T was isolated from the green alga *Ulva pertusa* collected on Jeju Island (Korea), by a standard dilution plating method. The sample of alga was serially diluted (10-fold dilutions) using sterile 0.85% (w/v) NaCl solution and 0.1ml homogenates of each dilution were spread onto R2A agar plates (Difco, USA) and incubated for 7 days at 25°C. The novel isolate

UR11^T was routinely cultivated on R2A agar at 25°C and preserved as R2A broth (Difco) supplemented with glycerol suspension (20%, v/v, glycerol in water) at −80°C. *Flavobacterium jejuensis* KCTC 42149^T, *Flavobacterium jumunjinense* KCTC 23618^T, *Flavobacterium haoranii* KCTC 23008^T, *Flavobacterium dongtanense* KACC 15621^T and *Flavobacterium ahnfeltiae* KCTC 32467^T were obtained from Korean Collection for Type Cultures (KCTC) and Korean Agricultural Culture Collection (KACC) and used as reference strains.

Morphology and Physiological Characteristics

Cell morphology of strain UR11^T was examined by light microscopy (Nikon, Japan) and scanning electron microscopy (SUPRA 55VP, ZEISS) with cells grown aerobically for 3 days at 25°C on R2A agar. Gram staining was performed using the Gram Stain Kit (BBL, Difco, USA), according to the manufacturer's instructions. Gliding motility was investigated on R2A broth with 0.5% agar, by the method of Bowman (2000). Anaerobic growth was determined in an anaerobic jar with the AnaeroPack (Oxoid, UK) on R2A agar at 25°C for 4 weeks. Growth was conducted at varied temperatures (5°C, 10°C 15°C, 20°C, 25°C, 30°C, 35°C, 37°C, 40°C, and 45°C) and pH 5.0–11.0 (intervals of 1.0 pH unit) in R2A broth at pH 5.0–11.0 (intervals of 1.0 pH unit) for 2 weeks at 25°C. Salt tolerance was tested on R2A agar containing various concentrations of NaCl (0–7%, w/v) at 1% intervals. Catalase activity was tested by observing bubble production using a catalase reagent (BioMérieux, UK). Oxidase activity was determined by oxidase reagent (BioMérieux). The hydrolysis of DNA (1%), starch (3%), cellulose (1%), Tween 20, 40, 60, and 80 (1%) and casein (1%) were tested after containing on R2A agar. Furthermore, these biochemical tests were determined using the API 20NE and API ZYM tests (BioMérieux), according to the manufacturer's instructions.

Phylogenetic Analysis

Genomic DNA of strain UR11^T was extracted and purified as described by Wilson (1987). Amplification of the 16S rRNA was carried out by PCR using universal 27F and 1522R primers [25]. The PCR product was then cloned using the TOPO Cloning Kit (Invitrogen, USA) and sequenced by Genotech (Korea). The complete 16S rRNA gene sequence (1,510 bp) was assembled with SeqMan software (DNASTAR). Similarity searches were achieved using the BLAST program (http://blast.ncbi.nlm, nih.gov/Blast.cgi) and EzTaxon-e server [http://eztaxon-e.ezbiocloud.net/; 26, 27]. Multiple sequence alignments were performed using the CLUSTAL X program, version 1.83 [28] and gaps were edited by the BioEdit program [29]. Phylogenetic analysis was performed using the software package MEGA version 6.0 [30]. Phylogenetic trees were constructed using the neighbor-joining [31], maximum-parsimony [32], and maximum-likelihood [33] methods. Bootstrap values were estimated based on 1000 replications [34]. DNA-DNA hybridization (DDH) was performed between UR11^T and *Flavobacterium jejuensis* EC11^T using the microplate method [35].

Chemotaxonomy

For analysis of cellular fatty acids, cells of strain UR11^T and four reference strains were grown at 25°C on R2A agar for 3 days. Fatty acids were extracted, methylated as described by the standard protocol of the Sherlock Microbial Identification System (MIDI; version 4.5), analyzed by gas chromatography (GC 7890A; Agilent, USA), and then identified using the TSBA 5.0 library [36]. Polar lipids from strain UR11^T were extracted as described by Minnikin *et al*. (1984) and analyzed by 2-dimensional thin-layer chromatography (TLC) [37]. The polar lipid pattern was identified by comparing results following staining with molybdophosphoric acid, ninhydrin, Zinzadze and α-naphthol. For determining the G+C DNA content, genomic DNA was extracted according to standard procedures described by Wilson (1987) from cells that had been cultured on R2A agar for 3 days at 25°C and analyzed by the Korean Culture Center of Microorganisms (KCCM, Korea).

Results

Morphology and physiological characteristics

Strain UR11^T cells were aerobic, gram-negative rods (0.16–0.23 μm × 1.43–1.62 μm), and gliding motility was observed. The colonies were yellow, circular, smooth and 1–2 mm in diameter after growth for 3 days at 25°C on R2A agar. Growth occurred at 5–35°C (optimum 25°C) at pH 6.0–10.0 (optimum pH, 7.0) but not at pH 5.0 and 11.0. and in the range of 0–5% NaCl (w/v), with optimal growth occurring in the absence of NaCl. The morphological, physiological and biochemical characteristics are explained below in the species description, and comparison with reference strains is presented in Table 1.

Phylogenetic Analysis

The 16S rRNA gene sequence of strain UR11^T showed the highest similarity to *F. jejuensis* EC11^T (98.0%), *F. jumunjinense* HME7102^T (96.1%), *F. haoranii* LQY-7^T (95.3%), *F. dongtanense* LW30^T (95.1%), and *F. ahnfeltiae* 10Alg 130^T (94.9%). Phylogenetic analysis based on 16s rRNA gene sequences using the neighbor-joining algorithm showed that strain UR11^T formed a phyletic lineage distinct from other members of the genus *Flavobacterium* (Fig. 1). Strain UR11^T of DNA-DNA hybridization (DDH) value was 58% that showed less than 70% DDH comparing *Flavobacterium jejuensis* EC11^T.

Chemotaxonomy

The major fatty acids (>5% of the total fatty acids) of strain UR11^T were iso-$C_{15:0}$ (33.9%), iso-$C_{15:1}$ G (12.4%), iso-$C_{17:0}$ 3-OH (9.0%), iso-$C_{16:0}$ (7.0%) and iso-$C_{15:0}$ 3-OH (6.3%) (Table 2). The polar lipid profile consisted of phosphatidyl-

Table 1. Biochemical characteristics of strain UR11^T and related type strains.

Characteristic	1	2	3	4	5	6
Temp. range for growth (°C)	5–35	5–30	5–30	15–35	5–35	5–30
Nitrate reduction	-	+	-	-	-	+
Catalase	+	+	+	-	+	+
Oxidase	+	+	+	-	-	-
Hydrolysis of :						
DNA	+	+	-	-	-	-
CAS (casein)	+	+	+	+	-	+
Tween 80	-	-	+	+	+	+
Assimilation of:						
Esculin degradation	+	+	+	+	-	+
D-Glucose	+	+	+	+	-	-
L-Arabinose	-	-	-	-	+	-
D-mannose	+	+	+	+	-	+
D-Mannitol	-	-	-	-	-	+
N-acetyl-*D*-glucosamine	+	-	-	-	-	+
D-maltose	+	+	+	+	-	+
Gluconate	-	-	-	-	-	+
Adipate	+	-	+	+	-	+
Malate	+	-	-	-	-	-
Citrate	+	-	-	+	-	-
Enzyme activities:						
α-chymotrypsin	+	+	-	+	+	-
β-glucuronidase	+	-	-	+	-	-
α-glucosidase	+	+	-	+	-	-
β-glucosidase	+	+	-	+	-	-
N-acetyl-β-glucosamidase	+	-	-	-	-	-
G+C mol%	32.6	28.1[a]	36.5[b]	34[c]	30[d]	34.3[e]

Strain: 1, UR11^T 2, *F. Jejuensis* KCTC42149^T (this study) 3, *F. jumunjinense* KCTC23618^T (this study) 4, *F. haoranii* KCTC23008^T (this study) 5, *F. dongtanense* KACC15621^T (this study) 6, *F. ahnfeltiae* KCTC32467^T (this study). All data were obtained in this study as +, Positive; -, negative. All strains are gram-negative, motile by gliding. All strains were positive for Gelatin, Alkaline phosphatase, Esterase (C4), Esterase Lipase (C8), Leucinearylamidase, Valinearylamidase, Crystinearylamidase, Trypsin, Acidphospatase and Naphtol-AS-BI-phosphohydrolase. All strains were negative for Indole production, Glucose fermentation, Arginine dihydrolase, Urease, Caprate, Phenylacetate, Lipase (C14), α-galactosidase, β-galactosidase, α-mannosidase and α-fucosidase.
Data from [a]Park *et al*., (2015); [b]Joung *et al*., (2013); [c]Zhang et al., (2010); [d]Xiao *et al*., (2011); [e]Nedashkovskaya *et al*., (2014).

Table 2. Cellular fatty acid composition of strain UR11T and type strains.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{14:0}$	0.6	TR	0.6	TR	1.2	0.6
$C_{16:0}$	0.6	TR	0.8	1.0	3.7	1.2
Hydroxylated						
$C_{15:0}$ 3-OH	1.1	-	-	1.0	0.8	0.8
$C_{16:0}$ 3-OH	0.9	TR	0.6	0.8	1.1	1.2
$C_{17:0}$ 3-OH	0.6	-	-	-	-	TR
iso-$C_{14:0}$ 3-OH	0.6	TR	TR	0.6	-	TR
iso-$C_{15:0}$ 3-OH	6.3	7.6	8.8	4.5	15.3	11.6
iso-$C_{16:0}$ 3-OH	3.9	2.6	1.0	4.0	TR	1.2
iso-$C_{17:0}$ 3-OH	9.0	11.2	10.8	8.6	TR	10.9
Branched						
iso-$C_{13:0}$	1.0	0.9	1.0	0.9	TR	1.5
iso-$C_{14:0}$	3.2	-	-	-	-	0.8
iso-$C_{15:0}$	33.9	41.4	39.9	32.2	TR	44.9
iso-$C_{15:1}$ G	12.4	9.8	13.9	20.3	8.9	10.0
iso-$C_{16:0}$	7.0	3.8	2.2	5.3	1.4	1.3
iso-$C_{16:1}$ H	2.4	2.4	1.3	-	-	-
iso-$C_{17:1}$ ω9*c*	1.2	2.4	4.4	1.3	3.0	3.7
anteiso-$C_{15:0}$	1.5	1.2	1.8	8.0	1.6	3.3
Unsaturated						
$C_{15:1}$ ω6*c*	4.1	3.3	3.3	1.0	TR	0.9
$C_{17:1}$ ω6*c*	1.1	0.9	0.8	0.2	-	-
Summed features						
3	TR	TR	2.9	1.0	TR	1.1

Strain: 1, UR11T 2, *F. Jejuensis* KCTC42149^T 3, *F. jumunjinense* KCTC23618^T 4, *F. haoranii* KCTC23008^T 5, *F. dongtanense* KACC15621^T 6, *F. ahnfeltiae* KCTC32467^T. All data incurred in this study are recorded as -, not detected; TR, trace amount (<0.5%). All strains were incubated in R2A agar plate at 25°C for 3 days. Fatty acids that account <0.5% of the total fatty acids in all strains were deleted.
*Summed features were represented in case of two fatty acids that cannot be separated by MIDI system. Summed features 3, iso-C15:0 2-OH/ $C_{16:1}$ ω7*c*.

ethanolamine (PE), seven unknown aminolipids (AL1-7), two unknown aminopolarlipids (APL1 and APL2) and two unknown lipids (L1 and L2; Fig. 2). The polar lipid profile of strain UR11^T was very similar to those of the reference strains *F. jejuensis* EC11^T, *F. jumunjinense* HME7102^T, *F. haoranii* LQY-7^T, *F. dongtanense* LW30^T and *F. ahnfeltiae* 10Alg 130^T but could be differentiated by the presence or absence of several other polar lipids. The major respiratory quinone of the UR11^T strain was menaquinone-6 (MK6). The G+C content of the genomic DNA was found to be 32.6 mol, as shown in Table 1.

Discussion

Flavobacterium jocheonensis (jo.cheon.en'sis.N.L. fem. adj. *jocheonensis* refers to Jocheon in Jeju Island, where the type

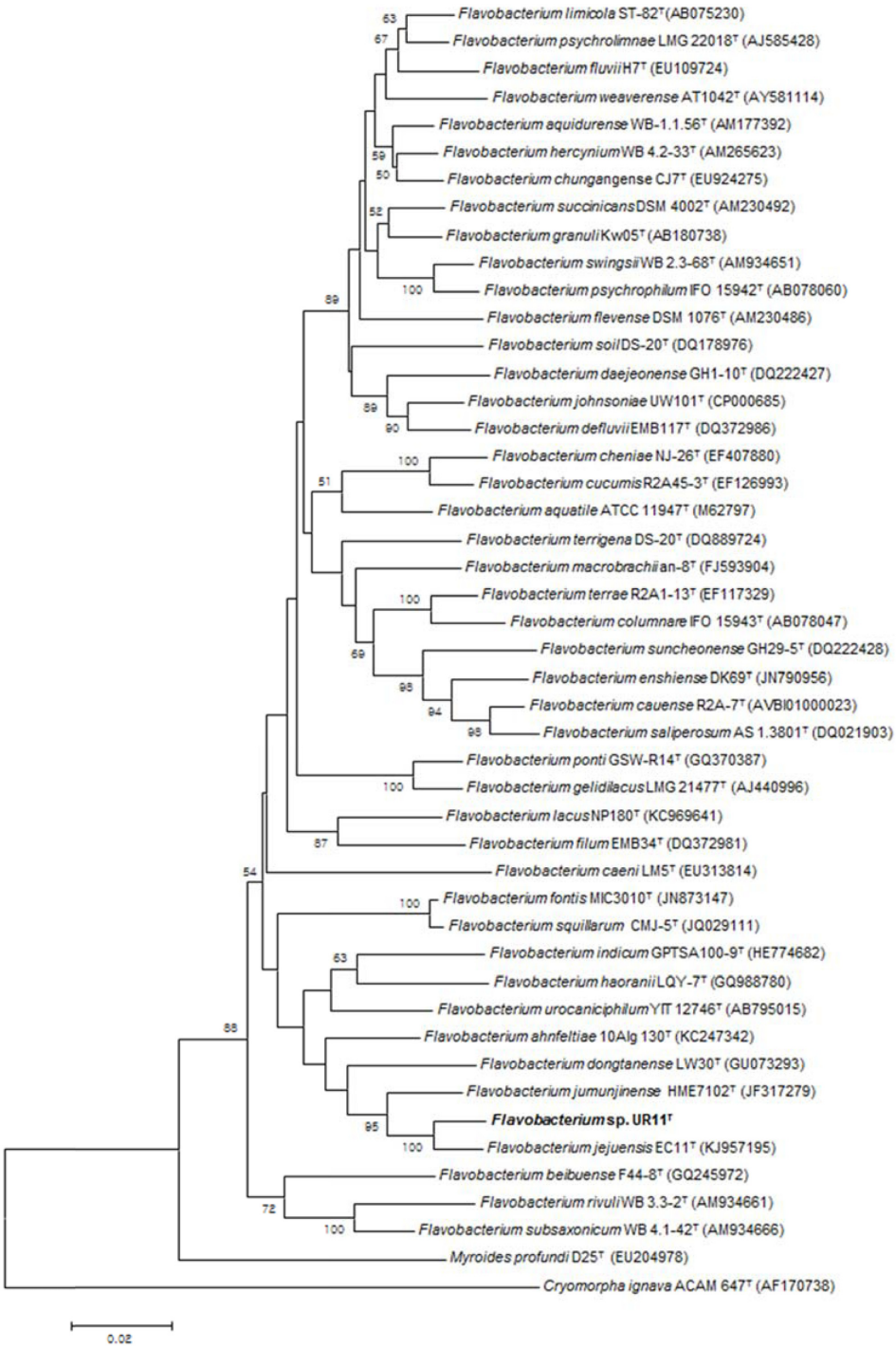

Fig. 1. Phylogenetic tree of the 16S rRNA gene sequences of strain UR11^T and other related taxa.
GenBank accession numbers are placed in parentheses. Boostrap values (>50%) are based on 1,000 replications. Bar 0.02 nucleotide substitutions per nucleotide position.

strain was isolated).

Strain UR11^T are gram-negative, motile by gliding, aerobic and rod-shaped, approximately 0.16-0.23 μm wide and 1.43–1.62 μm long. Colonies were yellow, circular and smooth after 3 days of incubation at 25°C on R2A agar. Growth occurs at 5–35°C, but not at 37°C, 40°C, and 45°C

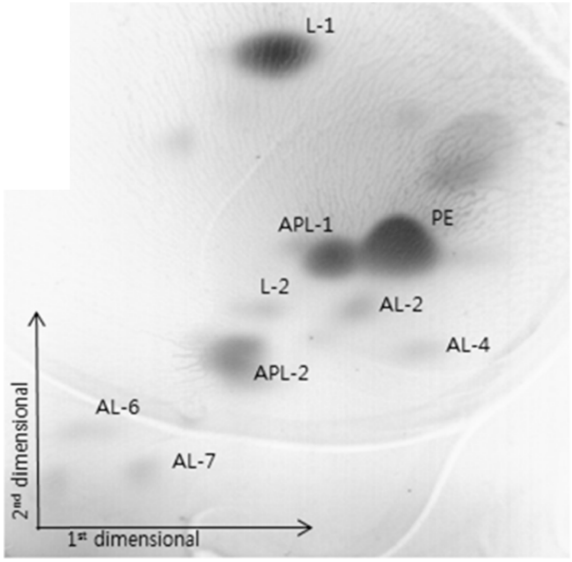

Fig. 2. Two dimensional thin-layer chromatogram of total polar lipids of strain UR11^T.
Total polar lipids were identified by spraying with molybdophosphoric acid reagent. PE, phosphatidylethanolamine; AL1-7, unknown aminolipids; APL1-2, unkown aminopolarlipids; L 1–2, unknown lipid.

(optimum temperature, 25°C) and pH 6.0–10.0 (optimum pH, 7.0), but not at pH 5.0 and 11.0. Growth was observed at 0–5% NaCl (w/v). Oxidase and catalase activities are positive. Strain UR11 hydrolyzes aesculin, gelatin, casein, DNA, Tween 40 and 60, but does not hydrolyze starch, cellulose, Tween 20 and 80. The strain tested negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, β-galactosidase, L-arabinose, D-mannitol, gluconate, caprate and phenylacetate but positive for D-glucose, D-mannose, *N*-acetyl-*D*-glucosamine, D-maltose, adipate, malate and citrate. In ZYM testing, alkaline phosphatase, esterase(C4), esterase lipase(C8), leucine arylamidase, valine arylamidase, crystine arylamidase, trypsin, α-chymotrypsin, acid phos- phatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-glucosidase, β-glusidase and *N*-acetyl-β-glucosamidase are present, but lipase(C14), α-galactosidase, β-galactosidase, α-mannosidase and α-fucosidase are not present. The main fatty acids (>5%) of strain UR11^T were iso-$C_{15:0}$ (33.9%), iso-$C_{15:1}$ G (12.4%), iso-$C_{17:0}$ 3-OH (9.0%), iso-$C_{16:0}$ (7.0%) and iso-$C_{15:0}$ 3-OH (6.3%). The major fatty acid composition of strain UR11^T was similar to that of *F. jejuensis* EC11^T, with minor differences in their respective proportions. The polar lipids profile consisted of phosphatidylethanolamine, seven unknown aminolipids, two unknown aminopolarlipids and two unknown lipids. They all contained phosphatidylethanolamine (PE). PE is present in many species of the genus *Flavobacterium* [38]. Menaquinone-6 is the predominant quinone. The major respiratory quinone of the UR11^T strain was menaquinone-6 (MK6), which is also characteristic for members of the genus *Flavobacterium*. The G+C content is 32.6 mol. A DDH experiment was performed with *F. jejuensis* EC11^T as closest phylogenetic neighbor. DNA-DNA hybridization (DDH) between the strain UR11^T and *Flavobacterium jejuensis* EC11^T showed 58% relatedness. The DNA-DNA hybridization value of less than 70% shows that the new isolates belong to a novel species. The results demonstrate that strain UR11^T is a novel species of the genus *Flavobacterium* [39, 40].

The type strain, UR11^T (=KCTC 52377^T = JCM 31512^T), was isolated from green alga *Ulva pertusa* in Jeju Island, Republic of Korea. The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain UR11^T is KX24431.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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