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ORIGINAL PAPER

Lysobacter oculi sp. nov., isolated from human Meibomian gland secretions

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Abstract A Gram-stain negative, rod-shaped bacterial, catalase and oxidase positive strain (83-4^T) that formed yellow colonies was isolated from human Meibomian gland secretions. Strain 83-4^T belongs to the genus *Lysobacter* according to phylogenetic analysis based on 16S rRNA gene sequences. The DNA G+C content was 67.1 mol%. The circular genome was 2.6 Mb, which contained 2431 protein-coding sequences, 75 pseudogenes, 46 tRNAs, 3 rRNAs and 4 ncRNAs. A bacteriocin cluster and aryl polyene cluster were also found in the genome. The average nucleotide identity value was 79.6% between isolate 83-4^T and the closely related type strain *Lysobacter* tolerans UM1^T. The estimated DNA–DNA hybridization value between strain 83-4^T and *L. tolerans* UM1^T was 41.6%. Diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol were the major polar lipids. *Iso*-$C_{15:0}$, *iso*-$C_{11:0}$ 3-OH, *iso*-$C_{11:0}$ and summed feature 9 (*iso*-$C_{17:1}\omega 9c$) were the major fatty acids. Ubiquinone (Q-8) was the only respiratory quinone. Therefore, based on the data of phylogenetic analysis, chemotaxonomical and biochemical analyses, it is concluded that strain 83-4^T represents a novel species of the genus *Lysobacter* with the name of *Lysobacter oculi* sp. nov. The type strain is 83-4^T (= CGMCC 1.13464^T = NRBC 113451^T).

Keywords *Lysobacter* · Novel species · 16S rRNA gene · Biochemical analyses

Hua Bai and Huibin Lv have contributed equally to this work.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 83-4^T is MG822667. The GenBank accession number for the whole-genome sequence of strain 83-4^T is CP029556.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10482-019-01289-1) contains supplementary material, which is available to authorized users.

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Introduction

The genus *Lysobacter* belonging to the family *Lysobacteraceae* within the order *Lysobacterales* of *Gammaproteobacteria* was first proposed by Christensen and Cook (1978). Currently, the genus contains 48 species with validly published names (http://www.bacterio.net/lysobacter.html). Species of the genus have been isolated from various environmental sources, including soil, sludge and aqueous habitats. Typical chemotaxonomic features of the genus *Lysobacter* include: Q-8 as the major respiratory quinone; *iso*-branched fatty acids are major fatty acids; diphosphatidylglycerol (DPG), phosphatidylethano lamine (PE) and phosphatidylglycerol (PG) as the major polar lipids and high G+C contents ranging between 61.7 and 70.7 mol% (Christensen and Cook 1978; Luo et al. 2012; Weon et al. 2006). Members of the genus *Lysobacter* could be used as biocontrol agents because of their ability to lyse a variety of microorganisms (Ahmed et al. 2003; Christensen and Cook 1978; Hashizume et al. 2004; Ryazanova et al. 2005), which indicates the importance of isolating new isolates for future agricultural applications.

In this study, we characterized a novel bacterium of the genus *Lysobacter*, designated 83-4^T, which was isolated from the Meibomian gland secretions during an investigation of the composition of the ocular microbiome in patients with Meibomian gland dysfunction.

Materials and methods

This study was approved by the Human Research and Ethics Committee of Peking University Third Hospital under the number D2018086. Signed informed consent was obtained from each participant.

Strain isolation and cultivation of strains

Meibomian gland secretions were taken by the ophthalmologist with sterile transport swabs in a sterile environment. Sterile swabs which were exposed in the air of the operation room for 10 s each time were used as blank controls. The samples were plated on blood agar plate (10 g/L tryptone, 3 g/L beef powder, 5 g/L NaCl, 50 mL/L defibrinated sheep blood and 15 g/L agar) at 37 °C for 72 h. Following the incubation, a yellow colored colony was isolated and purified. The purified strain was routinely grown on tryptic soy broth (TSB) medium and maintained as cell suspensions in 20% glycerol (v/v) at − 80 °C.

Growth conditions, phenotypic, biochemical and antibiotic resistance analyses

To determine the temperature range for growth, the strain 83-4^T and reference strain *L. tolerans* UM1^T (Margesin et al. 2018) (obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ) were cultured on tryptic soy agar (TSA) and kept at 4, 15, 20, 30, 37, 42 and 45 °C for 48 h. Growth at pH 4–12 (at intervals of 1 pH unit) was assessed using appropriate buffers. 0.1 M citric acid/0.1 M sodium citrate buffer was used for pH 4.0–5.0; 0.1 M KH_2PO_4/0.1 M NaOH buffer was used for pH 6.0–8.0; 0.1 M $NaHCO_3$/0.1 M Na_2CO_3 was used for pH 9.0–10.0; 0.05 M Na_2HPO_4/0.1 M NaOH was used for pH 11.0 and 0.2 M KCl/0.2 M NaOH was used for pH 12.0. NaCl tolerance was determined by growing strain 83-4^T and *L. tolerans* UM1^T on TSA for 48 h at different NaCl concentrations (0–10%, at an interval of 0.5%). 1% (w/v) N,N,N9,N9,-tetramethyl 1,4-phenylenediamine reagent (bioMérieux) was used to determine the oxidase activity. Catalase activity was tested by bubble production after the application of 3% (v/v) hydrogen peroxide solution. Other physiological and biochemical properties were examined with API kits (API 20NE and API ZYM system) according to the manufacturer's instructions (bioMérieux). The morphological study of bacterial cells was carried out using a transmission electron microscope JEM-1400 and Gram stain test was performed as described by Magee et al. (1975).

Antibiotic sensitivity tests were determined by the agar diffusion method (Bauer et al. 1966) using the filter-paper discs (diameter, 5 mm) on TSA with varying amounts of antibiotics. The antibiotics tested were ampicillin (100 mg L^{-1}), spectinomycin (100 mg L^{-1}), streptomycin (10 mg L^{-1}), tetracycline (10 mg L^{-1}), gentamicin (50 mg L^{-1}), erythromycin (20 mg L^{-1}), kanamycin (50 mg L^{-1}) and chloramphenicol (34 mg L^{-1}).

Chemotaxonomic characteristics analysis

For fatty acid methyl esters and quinones analysis, the strain 83-4^T and the closely related type strain, *L. tolerans* UM1^T, were cultured on TSA at 30 °C for 36 h. Fatty acid methyl esters were analyzed using the standard (MIDI) Sherlock Microbial Identification System (version 6.0) and peaks were identified on an Agilent 6890 N Network GC system using the TSBA6 peak-naming table.

Quinones were extracted and purified from 200 mg dry cell mass according to the method of Collins (1985) and analyzed by HPLC (Wu et al. 1989). Polar lipids were extracted by using the chloroform/methanol system described by Minnikin et al. (1984). Two-dimensional thin-layer chromatography (TLC) was used to identify polar lipids. TLC was carried out on the Merck silica gel 60 F_{254} aluminium-backed thin-layer plates using chloroform/methanol/water (65:25:4, v/v) for the first dimension and chloroform/methanol/acetic acid/water (80:12:15:4, v/v) for the second dimension. The identification of individual lipid spots was performed by spraying with 5% molybdophosphoric acid followed by heating at 100 °C for 5–8 min.

16S rRNA sequence and analyses

The 16S rRNA gene was amplified by using colony PCR with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR products were cloned into pMD19-T vector and sequenced by Beijing Genomics Institute (BGI, Beijing, China). 16S rRNA gene sequence similarities were analyzed using the BLASTN program (Altschul et al. 1990) and the EzBioCloud server (Yoon et al. 2017a). For phylogenetic analysis, the 16S rRNA sequence of strain 83-4^T was aligned with the sequences of the closely related taxa using CLUSTAL_W (Thompson et al. 1994). Three methods, including neighbor-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) were selected to reconstruct the phylogenetic tree using MEGA version 7.0 (Kumar et al. 2016). Maximum likelihood tree was calculated using Kimura 2-parameter model (K2 + G + I) (Kimura 1980). Evolutionary distances in the neighbor-joining tree were calculated using Kimura's 2-parameter model (Kimura 1980). The topology of the tree was evaluated with 1000 bootstrap replications (Felsenstein 1985).

Genome sequencing and analyses

Strain 83-4^T was cultured in tryptic soy broth at 30 °C for 36 h. Whole genome sequencing analysis was performed by GENEWIZ. Genomic DNA was extracted using a TIANamp Genomic DNA Kit (TIANGEN). Qubit dsDNA HSAssay Kit and Nanodrop 2000 (Thermo Fisher Scientific) was used for estimating the DNA concentration of the solution. For the whole genome assembly, the genomic DNA was randomly fragmented to ~ 10 Kb by sonication (Covaris g-TUBE) followed by preparing SMRTbell templates using DNA Template Prep Kit. Single-molecule real-time (SMRT) sequencing was performed on a PacBio Sequal platform. Additionally, next generation sequencing libraries were constructed following the manufacturer's protocol (VAHTS Universal DNA Library Prep Kit for Illumina) and sequenced on an Illumina HiSeq platform.

The complete genome sequence data was deposited in the GenBank/EMBL/DDBJ. The average nucleotide identity (ANI) value between strain 83-4^T and *L. tolerans* UM1^T, was calculated using the ANI calculator (https://www.ezbiocloud.net/tools/ani) (Yoon et al. 2017b). The estimated DNA–DNA hybridization (DDH) value was calculated using the Genome-to-Genome Distance Calculator 2.1 available at the DSMZ server (http://ggdc.dsmz.de/distcalc2.php) (Meier-Kolthoff et al. 2013). The genome sequence of *L. tolerans* UM1^T (accession number: GCF_900155935.1) was obtained from the NCBI database. The DNA G+C content of strain 83-4^T was determined according to the genomic DNA sequence.

Results and discussion

According to the EzBioCloud server analysis, the 16S rRNA gene sequence of strain 83-4^T showed maximum similarity of 97.4% with that of *Lysobacter tolerans* UM1^T. Furthermore, the 16S rRNA gene of the strain 83-4^T shared 95.8–96.6% similarities to several type strains in the genus *Luteimonas*. Among the genus, the closely related *Luteimonas rhizosphaerae* shared a similarity of 96.6% with strain

83-4^T. The relationships between 83-4^T and members of the genus *Lysobacter* and *Luteimonas* were supported by the phylogenetic trees topology. The phylogenetic tree illustrated that the strain 83-4^T is closely related to *L. tolerans* UM1^T (Fig. 1, Supplementary Figs. S1 and S2). The phylogenetic analysis indicated that strain 83-4^T groups within the genus *Lysobacter*.

For genome sequencing by PacBio Sequal platform, the total number of the reads was 816,606, which yielded 2,273,102,668 bp of sequence information. The Illumina HiSeq platform provided 21,067,660 reads, yielding a 3,139,252,569 bp of sequence information. The PacBio reads were assembled by the Hierarchical Genome Assembly Process version 4 (HGAP4) (Chin et al. 2013). Illumina reads were used to improve the genome accuracy. The complete circular genome size is 2,591,885 bp. The G+C content is 67.1 mol%. The genome is annotated applying the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al. 2016), which predicted 2431 protein-coding sequences, 75 pseudogenes, 46 tRNAs, 3 rRNAs and 4 ncRNAs. The analysis of secondary metabolite producers antiSMASH (version 5.0.0beta1-ca06418) indicated that a bacteriocin cluster and aryl polyene cluster were in the genome (Blin et al. 2017). The ANI value between strain 83-4^T and *L. tolerans* UM1^T was 79.6%, which is lower than the standard criteria for classifying strains as different species (95–96% identity) (Richter and Rossello-Mora 2009). The estimated DDH value between strain 83-4^T and *L. tolerans* UM1^T was 41.6%, which is well below the 70% threshold for prokaryotic species delineation by Wayne et al. (1987). Therefore, the strain 83-4^T represents a novel species belonging to the genus *Lysobacter*.

Strain 83-4^T formed circular, yellow and smooth colonies on the TSA after incubation at 30 °C for 72 h. The morphological study of bacterial cells was carried out by transmission electron microscopy, which indicated that strain 83-4^T was non-flagellated and rod-shaped (2.7 × 1.3 μm) (Supplementary Fig. S3). The Gram stain test was performed in standard protocol and strain 83-4^T was found to be Gram-stain negative. According to the antibiotic sensitivity tests, *L. tolerans* UM1^T was sensitive to chloramphenicol, while *L. oculi* 83-4^T was resistant to all the antibiotics in the test. The temperature range for growth of strain 83-4^T was 15–37 °C and optimal growth occurred at 30 °C. The pH range for growth of strain 83-4^T was pH 6–9 (optimum pH 7). The optimum NaCl concentration for growth of strain 83-4^T was 0–2%. The tests for oxidase and catalase activities were positive. In the API 20NE test, the strain was negative for assimilation of glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, capric acid, adipic acid, malic acid, citric acid and phenylacetic acid. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase were positive in the API ZYM system. The detailed physiological and biochemical characteristics of strain 83-4^T and the reference strain are given in Table 1.

As shown in Table 2, the predominant cellular fatty acids of the strain 83-4^T were *iso*-$C_{15:0}$ (22.4%), *iso*-$C_{11:0}$ 3-OH (15.1%), *iso*-$C_{11:0}$ (11.5%) and summed feature 9 (*iso*-$C_{17:1}\omega 9c$; 27.6%), while those of *L. tolerans* UM1^T were *iso*-$C_{17:0}$ (28.5%), $C_{11:0}$ 2-OH (12.2%), *iso*-$C_{16:0}$ (7.5%) and summed feature 9 (*iso*-$C_{17:1}\omega 9c$; 43.2%) Ubiquinone-8 was the only quinone detected in the strain 83-4^T and *L. tolerans* UM1^T. The major polar lipids present in the strain 83-4^T were DPG, PG and PE (Supplementary Fig. S4).

The chemotaxonomic features of strain 83-4^T including the presence of Q-8 as quinone, DPG, PG and PE as major polar lipids and *iso*-$C_{15:0}$, *iso*-$C_{11:0}$ 3-OH, *iso*-$C_{11:0}$ and *iso*-$C_{17:1}\omega 9c$ as major cellular fatty acids, which are consistent with the characteristics of genus *Lysobacter* (Christensen and Cook 1978; Luo et al. 2012; Weon et al. 2006). Besides the consistent characteristics with *L. tolerans* UM1^T, such as temperature range and NaCl tolerance for growth, colony color, oxidase and catalase activity, there are also different characteristics between 83-4^T and the related type strain *L. tolerans* UM1^T. For example, the pH range for growth of strain83-4^T was low compared to that of *L. tolerans* UM1^T. Strain 83-4^T was found to be negative in the enzyme assay for trypsin and positive in the enzyme assay for acid phosphatase. Strain *L. tolerans* UM1^T, by contrast, was found to be positive for the trypsin assay and weakly positive for the acid phosphatase assay. Strain 83-4^T was resistant to chloramphenicol while *L. tolerans* UM1^T was sensitive to chloramphenicol. Strain 83-4^T could hydrolyze the gelatin as distinct from strain *L. tolerans* UM1^T. Cell size of the proposed new strain (2.7 × 1.3 μm) was much larger than the type strain (1.1 × 0.4 μm) (Table 1). Strain 83-4^T and *L.*

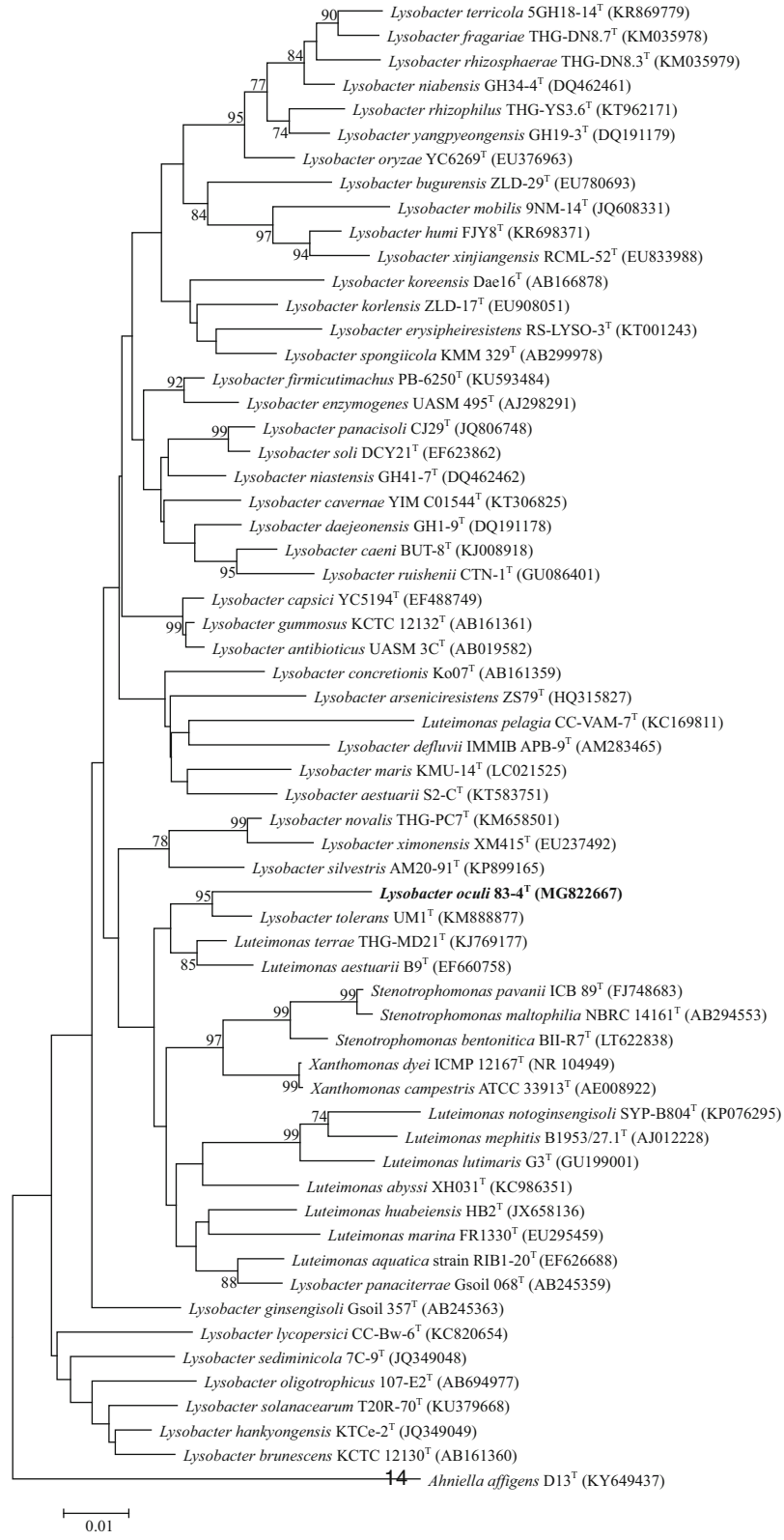

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain 83-4^T to the closely related strains. GenBank accession numbers are given in parentheses. The 16S rRNA gene sequence of *Ahniella affigens* D13^T (KY649437) was used as an outgroup. Bootstrap values (based on 1000 replications, only values above 70%) are shown at branch points. Bar, 0.01 substitutions per nucleotide position

Table 1 Differential morphological, physiological and biochemical characteristics of strains 83-4^T and *L. tolerans* UM1^T

Characteristic	1	2
Habitat	Human meibomian gland secretions	Soil
Cell size (μm)	2.7 × 1.3	1.1 × 0.4
pH for growth		
Range	6.0–9.0	6.0–10.0
Optimum	7.0	8.0
Enzymatic activities		
Trypsin	−	+
Acid phosphatase	+	w
Antibiotic susceptibility		
Chloramphenicol	R	S
Hydrolysis of gelatin	+	−
DNA G+C content (mol %)	67.1	64.3

Strains: 1, *Lysobacter oculi* 83-4^T; 2, *L. tolerans* UM1^T; + positive, − negative, *W* weakly positive, *S* sensitive, *R* resistant. All data were from this study except for DNA G+C content and cell size of the reference strain (Rani et al. 2016)

Table 2 Cellular fatty acid contents (%) of strain 83-4^T and *L. tolerans* UM1^T

Fatty acid	*L. oculi*	*L. tolerans* UM1^T
Straight chain		
$C_{14:0}$	0.5	–
$C_{16:0}$	2.4	2.7
Branched		
iso-$C_{11:0}$	11.5	–
iso-$C_{14:0}$	1.3	1.1
iso-$C_{15:0}$	22.4	–
iso-$C_{15:1}$ F	3.2	–
iso-$C_{15:1}$ G	–	2.9
iso-$C_{16:0}$	4.8	7.5
iso-$C_{17:0}$	4.2	28.5
anteiso-$C_{15:0}$	1.0	–
Hydroxy		
$C_{11:0}$ 2-OH	TR	12.2
iso-$C_{11:0}$ 3-OH	15.1	
Summed feature[a]		
3	2.2	TR
9	27.6	43.2

All data are from this study. – not detected, *TR* trace (< 0.5%). Fatty acids amounting to less than 0.5% of the total fatty acids in all strains are not mentioned in this table

[a]Summed features are groups of two or three fatty acids that could not be separated by GLC with the Microbial Identification System (MIDI). Summed feature 3 contained $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 9 contained $C_{16:0}$ 10-methyl and/or *iso*-$C_{17:1}\omega9c$

tolerans UM1^T shared a DNA relatedness value of 41.6% clearly distinguishing them as different species. In addition, the major cellular fatty acids of strain 83-4^T were different to those of the reference strain (Table 2). Based on the distinct biochemical, physiological and chemotaxonomic analyses, strain 83-4^T should be classified as a novel species of *Lysobacter*, here designated as *Lysobacter oculi* sp. nov.

Description of *Lysobacter oculi* sp. nov.

Lysobacter oculi (*o'cu.li*. L. gen. n. *oculi*, of the eye).

Gram-stain negative, non-motile, rod-shaped bacterium with a 2.7 × 1.3 μm cell size. Colonies are circular, smooth and yellow. Growth is observed at 15–37 °C (optimum 30 °C), at pH 6–9 (optimum pH 7) and in the presence of 0–2% NaCl. Oxidase and catalase positive. Cells are able to hydrolyze gelatin but not aesculin and urea. Tests for nitrate reduction, glucose fermentation and indole production are negative. The major polar lipids are DPG, PG and PE. Predominant fatty acids are *iso*-$C_{15:0}$, *iso*-$C_{11:0}$ 3-OH, *iso*-$C_{11:0}$ and summed feature 9. The G+C content of the type strain is 67.1 mol% and the size of the complete genome of the type strain is 2.6 Mb. Ubiquinone (Q-8) was identified as the only respiratory quinone.

The GenBank accession number for the 16S rRNA gene and genome sequence of strain 83-4^T is

MG822667 and CP029556, respectively. The type strain is 83-4^T (= CGMCC 1.13464^T = NRBC 113451^T), isolated from human Meibomian gland secretions at Peking University Third Hospital in Beijing, China.

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Conflict of interest The authors declare that there are no conflicts of interest.

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