Thiovirga sulfuroxydans gen. nov., sp. nov., a chemolithoautotrophic sulfur-oxidizing bacterium isolated from a microaerobic waste-water biofilm

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A novel mesophilic, chemolithoautotrophic, sulfur-oxidizing bacterium, designated strain SO07^T, was isolated from a microaerobic waste-water biofilm. Chemolithoautotrophic growth was observed with elemental sulfur, sulfide and thiosulfate as sole electron donors and oxygen as electron acceptor. Anaerobic and heterotrophic growth were not observed. Nitrate was not used as a terminal electron acceptor. The optimum pH and temperature for growth were pH 7·5 and 30 °C, respectively. The major isoprenoid quinone was Q-8. The DNA G+C content of strain SO07^T was 47·1 mol%. Phylogenetic analysis of 16S rRNA gene sequences demonstrated that strain SO07^T formed a monophyletic group in the *γ-Proteobacteria* with only 89 % similarity to members of the genus *Halothiobacillus*, its nearest phylogenetic neighbours. In addition, the isolate differed from members of the genus *Halothiobacillus* in its requirement for and tolerance of NaCl; strain SO07^T was unable to grow in NaCl concentrations of more than 180 mM. On the basis of phylogenetic, chemotaxonomic and physiological data, it is proposed that isolate SO07^T (=JCM 12417^T=ATCC BAA-1033^T) represents the type strain of a novel species in a new genus, *Thiovirga sulfuroxydans* gen. nov., sp. nov.

During investigations into the sulfur cycle in a microaerobic waste-water biofilm, a survey combining the molecular techniques of 16S rRNA gene cloning followed by fluorescence *in situ* hybridization revealed the presence of a novel sulfur-oxidizing bacterium (approximately 10^8 cells cm^{-3}), designated strain SO07^T, which was present at the oxic biofilm strata where high concentrations of elemental sulfur (S^0) accumulate. The use of S^0 as an electron donor was an effective measure to establish an enrichment culture of strain SO07^T and for further purification (Ito *et al.*, 2004). The 16S rRNA genes of large numbers of uncultured bacterial clones that are closely related to strain SO07^T have been found in GenBank (Benson *et al.*, 2003), suggesting the widespread distribution and ecological importance of these types of bacteria in the environment. Analysis of phylogenetic and physiological characteristics showed that strain SO07^T represents the type strain of the type species of a new genus; the name *Thiovirga sulfuroxydans* gen. nov., sp. nov. is proposed.

The waste-water biofilm sample was collected from a sewer line that transports primary settling tank effluent at the Soseigawa municipal waste-water treatment plant, Sapporo, Japan. Enrichment and isolation were performed using a slightly modified version of medium used for neutrophilic *Thiobacillus* species (Kuenen *et al.*, 1991), designated SOB medium in this study. The composition of SOB medium (in g l^{-1}) was KH_2PO_4 (0·5), K_2HPO_4 (0·5), NH_4Cl (0·5), $MgSO_4.7H_2O$ (0·1), $CaCl_2$ (0·05) and $NaHCO_3$ (1·0), plus 1 ml trace element solution l^{-1} (Kuenen *et al.*, 1991). The procedures for enrichment and isolation have been described previously (Ito *et al.*, 2004). Cells grown in liquid SOB medium with thiosulfate (6·5 mM) as a sole electron donor were used for studies on phenotypic properties and chemotaxonomic traits unless otherwise specified. Aerobic

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Abbreviation: EDS, energy-dispersive X-ray spectroscopy.

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growth was examined in SOB medium supplemented with sulfide (13 mM), thiosulfate (6·5 mM), elemental sulfur (416 mg l^{-1}), methanol (1 mM), formate (1 mM), acetate (1 mM) or propionate (1 mM) as electron donor. Anaerobic growth was examined in SOB medium containing the same electron donors as in the aerobic growth test and with nitrate (0·74 mM) as electron acceptor; the headspace gas contained only N_2 gas (99·99 %, v/v). Fermentative growth was tested in SOB medium containing the same electron donors as in the aerobic growth test under anoxic conditions without nitrate. Mixotrophic growth was tested in SOB medium containing thiosulfate (6·5 mM) together with one of formate (13 mM), acetate (13 mM) or propionate (13 mM). Substrate utilization was evaluated by measuring each substrate concentration and by DAPI direct counting as described by Hobbie *et al.* (1977). The concentrations of sulfate, thiosulfate, nitrite, nitrate, formate, acetate and propionate in the culture solutions were determined with an ion chromatograph equipped with either an IonPac AS9-HCC column for sulfate, thiosulfate, nitrite and nitrate or an ICE-AS1 column for formate, acetate and propionate (model DX-100; Nippon Dionex). The concentration of total dissolved sulfide (H_2S, HS^- and S^{2-}) was determined by the methylene blue method (Cline, 1969). The temperature range for growth was examined in SOB medium containing thiosulfate (6·5 mM) at 5, 10, 15, 20, 25, 30, 34, 37, 42, 47 and 55 °C. The pH range for growth (4·0–10·0) was tested in SOB medium containing thiosulfate at 30 °C. The NaCl requirement and tolerance of the isolate were examined in SOB medium containing various NaCl concentrations (0·03, 0·08, 0·13, 0·18, 0·23, 1·0, 2·0, 3·0 and 4·0 M). Growth of strain SO07^T was monitored by measuring the optical density at 540 nm. All the growth tests described above were conducted at 30 °C and pH 7·0 unless otherwise specified.

For morphological observations, cells grown on solid SOB medium containing 1·5 % agar at 25 °C for 1 day were negatively stained with 1 % (w/v) phosphotungstic acid and then observed under a Hitachi model H-800 transmission electron microscope at an acceleration voltage of 75 kV. For ultrastructure analysis, cells grown on SOB agar medium were immersed in 2 % (v/v) glutaraldehyde in 0·1 M phosphate buffer for 2 h; cells were then washed twice for 10 min and then for 1 h in 0·1 M phosphate buffer. Cells were fixed in 1 % OsO_4 in 0·1 M phosphate buffer for 2 h and then washed again in the same way. The fixed cells were dehydrated in a graduated acetone series (70–100 %) and subsequently embedded into the resin. Thin sections were cut with a Reichert model ultracut S ultramicrotome, placed on copper grids and stained with 10 % (w/v) uranyl acetate and 1 % (w/v) lead citrate. The stained thin sections were examined by transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (TEM-EDS) with a JEOL model JEM-2000ES at an acceleration voltage of 200 kV. For scanning electron microscope observations, cells incubated in liquid SOB medium containing thiosulfate at 25 °C for 1 day were fixed with 2 % (v/v) glutaraldehyde in 0·1 M phosphate buffer for 2 h. Further preparation steps were conducted according to Yumoto *et al.* (2001). The cultures were mounted on aluminium stubs and observed under a Hitachi model S-4000 scanning electron microscope at an acceleration voltage of 3·5 kV.

Analyses of whole-cell fatty acids and isoprenoid quinones were performed as described previously (Yumoto *et al.*, 2001). Genomic DNA for the analysis of G+C content was prepared as described by Marmur (1961). The G+C content (mol%) of the genomic DNA was determined by HPLC according to the method of Tamaoka & Komagata (1984). The levels of DNA relatedness were determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microplates. The DNA–DNA hybridization study was conducted only against *Halothiobacillus neapolitanus* (strain JCM 3861), the type species of the genus *Halothiobacillus* and most closely related species to strain SO07^T, because 16S rRNA gene similarities between strain SO07^T and all members of the genus *Halothiobacillus* are low (less than 89 %).

PCR amplification, purification of PCR products and 16S rRNA gene sequencing were carried out as described previously (Ito *et al.*, 2004). Phylogenetic inferences were made with the 16S rRNA gene sequence database associated with the ARB software package (Ludwig *et al.*, 2004). Phylogenetic trees were then constructed by using the ARB neighbour-joining and maximum-parsimony algorithms. Bootstrap analysis was performed to establish a confidence level for nodes. 16S rRNA gene sequence similarity values were calculated by using the program Similarity_Matrix in the Ribosomal Database Project II (Cole *et al.*, 2003).

Chemolithoautotrophic growth of strain SO07^T was observed on sulfide, thiosulfate and elemental sulfur. Growth on thiosulfate reduced the pH to a minimum of 6·0. The pH range for growth of strain SO07^T was 6·0–9·0, with optimal growth at pH 7·5. The temperature range for growth was 15–42 °C, with an optimum growth temperature of 30–34 °C. This strain grew at NaCl concentrations of 30–180 mM. No growth was observed in 230 mM NaCl. Heterotrophic growth was not observed when tested with acetate, formate, propionate or methanol. Anaerobic growth did not occur in the presence of any organic or inorganic substrates when nitrate was used as an electron acceptor. No fermentative growth was observed on methanol, formate, acetate or propionate. Mixotrophic tests on thiosulfate with formate, acetate or propionate revealed growth inhibition.

Colonies of strain SO07^T grown on solid SOB medium containing thiosulfate were 0·5–1·0 mm in diameter, white-coloured and lens-shaped. The colour and morphology of the colonies did not change during incubation for 5 days. The isolate was Gram-negative, catalase-positive and oxidase-positive. Cells were rod-shaped (0·5–0·8 × 1·0–2·0 μm), non-spore-forming and

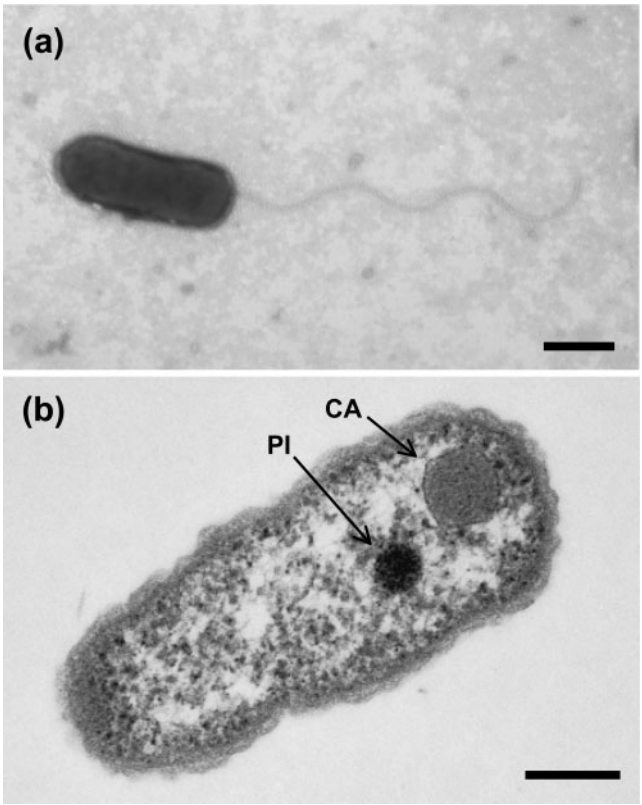

Fig. 1. Transmission electron micrographs of cells of strain SO07^T. (a) Negatively stained cell showing the single polar flagellum (bar, 0·4 μm). (b) An ultrathin section of a cell stained with uranyl acetate and lead citrate (bar, 0·2 μm). Cells in (a) and (b) were grown on thiosulfate and sulfide, respectively. CA, carboxysome; PI, polyphosphate-like inclusion. The carboxysome was enveloped by a thin membrane-like structure.

motile by means of a single polar flagellum (Fig. 1). Structure of the cell wall was a typical Gram-negative-stained type (Fig. 1b). Scanning electron microscope observations revealed that the cells had large surface areas with cobble-like structures on their surfaces (Fig. 2). Carboxysome-like inclusions were observed in the cells (Fig. 1b). The number of carboxysome-like inclusions in exponential-growth-phase cells incubated with thiosulfate averaged about 6 ± 3 ($n=20$) per cell, whereas that with sulfide was 2 ± 1 ($n=20$) per cell. It has been reported that the number of carboxysomes per cell in *Thiomonas intermedia* (formerly *Thiobacillus intermedius*) is proportional to the specific activity of ribulose-1,5-bisphosphate carboxylase (Purohit *et al.*, 1976). The higher number of carboxysomes in cells incubated with thiosulfate may explain the higher maximum specific growth rate on thiosulfate of 0·41 h^{-1}, compared with 0·30 h^{-1} on sulfide. Storage of polyphosphate-like inclusions was observed when strain SO07^T was grown with sulfide as electron donor (Fig. 1b). The polyphosphate-like inclusions contained phosphorous, which was determined by TEM-EDS analysis.

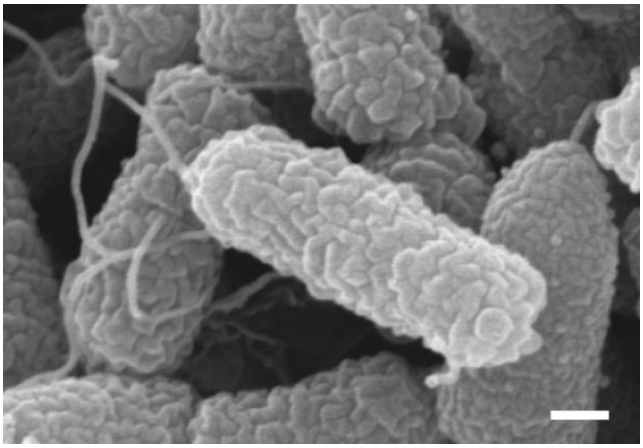

Fig. 2. Scanning electron micrograph of cells of strain SO07^T grown on SOB medium containing thiosulfate. Bar, 0·2 μm.

Analysis of quinone compounds revealed Q-8 to be the major isoprenoid quinone. Predominant cellular fatty acids of strain SO07^T were $C_{12:0}$ (2 %), $C_{16:0}$ (19 %), $C_{18:0}$ (16 %), $C_{16:1}$ (30 %) and $C_{18:1}$ (31 %). The DNA G+C content of strain SO07^T was 47·1 mol%. The DNA–DNA relatedness between strain SO07^T and *H. neapolitanus* JCM 3861 was less than 2 %.

Comparison of 16S rRNA gene sequences revealed that strain SO07^T formed a monophyletic group within the *γ-Proteobacteria*, as supported by high bootstrap values (Fig. 3), and can clearly be distinguished from members of the genus *Halothiobacillus* (less than 89 % sequence similarity between them). A slightly higher level of 16S rRNA gene sequence similarity was observed between strain SO07^T and '*Thiobacillus baregensis*' (90 %) than between strain SO07^T and members of the genus *Halothiobacillus* (87–89 %). High sequence similarities were obtained between strain SO07^T and partial sequences of the as-yet-uncultured bacterial clones SRang2.5 (98 %) and bacteriap48 (97 %), which were retrieved from sulfurous environments, i.e. Sulphur River in Parker Cave and a muddy hot pool in Kuirau Park, respectively (Angert *et al.*, 1998; Sunna & Bergquist, 2003). Thus, the ability of these clones to oxidize sulfur could be inferred. Characterization of strain SO07^T strongly indicated that the cluster including these environmental clones comprised chemolithoautotrophic sulfur-oxidizing bacteria. As in waste-water biofilms, the source of strain SO07^T, oxygen concentrations in these habitats would be low due to the relatively high concentrations of organic matter and sulfide.

Strain SO07^T shares the same phenotypic properties as members of the genus *Halothiobacillus*: mesophilic, neutrophilic and obligately chemolithoautotrophic, obtaining energy from reduced inorganic sulfur compounds. Both strain SO07^T and members of the genus *Halothiobacillus* contain ubiquinone Q-8 as the major isoprenoid quinone.

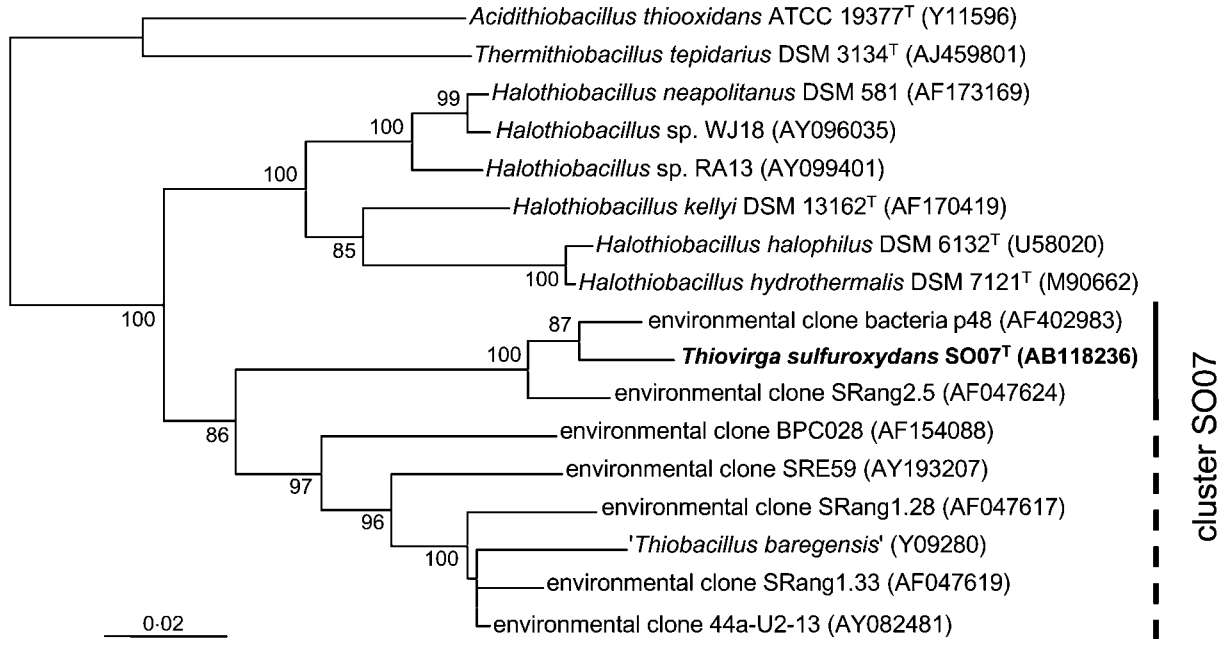

Fig. 3. Phylogenetic tree based on analysis of 16S rRNA gene sequences of strain SO07^T and representative species of some distantly related sulfur-oxidizing genera of the *γ-Proteobacteria*, constructed by using the neighbour-joining method. Nearly complete 16S rRNA gene sequences were used. Numbers at the nodes represent bootstrap values. Bar, 0·02 inferred nucleotide substitutions per nucleotide position. Further isolation and characterization of bacteria represented by environmental clones, including BPC028, SRE59 or SRang1.28, might expand the coverage of cluster SO07, as shown by the dashed line.

The presence of carboxysome-like inclusions in the cells of strain SO07^T has also been described for *H. neapolitanus* by Shively *et al.* (1973). In contrast to members of the genus *Halothiobacillus*, strain SO07^T did not require NaCl for growth and the growth of this strain was completely inhibited at NaCl concentrations greater than 180 mM (Table 1). In fact, all *Halothiobacillus* species have high NaCl tolerances (more than 860 mM NaCl and up to 4000 mM) and their optimal NaCl concentrations for growth are relatively high (more than 400 mM NaCl) (Kelly & Wood, 2000; Sievert *et al.*, 2000). These distinctive characteristics of strain SO07^T allow it to be differentiated

Table 1. Main phenotypic characteristics that differentiate strain SO07^T from mesophilic, neutrophilic and chemolithoautotrophic sulfur-oxidizing bacteria belonging to the *γ-Proteobacteria*

Species: 1, *Thiovirga sulfuroxydans* SO07^T; 2, *Halothiobacillus neapolitanus* DSM 15147^T; 3, *Halothiobacillus hydrothermalis* DSM 7121^T; 4, *Halothiobacillus halophilus* DSM 6132^T; 5, *Halothiobacillus kellyi* DSM 13162^T; 6, *Thiobacillus* sp. W5; 7, *Thiomicrospira frisia* DSM 12351^T; 8, *Thiomicrospira chilensis* DSM 12352^T. All species were obligately chemolithoautotrophic, mesophilic, neutrophilic motile rods and oxidized thiosulfate, sulfur and sulfide. Data from Kelly & Wood (2000), Sievert *et al.* (2000) and Brinkhoff *et al.* (1999a, b).

Characteristic	1	2	3	4	5	6	7	8
Conditions for optimum growth:								
Temp. (°C)	30–34	28–32	35–40	30–32	37–42	25–30	32–35	32–37
pH	7·5	6·5–6·9	7·5–8·0	7·0–7·3	6·5	7·0–7·5	6·5	7·0
NaCl (mM)	0	ND	430	800–1000	400–500	ND	470	470
Upper NaCl concn for growth (mM)	180	>860	2000	4000	2500	ND	1240	1240
Major fatty acids	$C_{12:0}$, $C_{16:0}$, $C_{18:0}$, $C_{16:1}$, $C_{18:1}$	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$, C_{17}cyc., C_{19}cyc.	ND	ND	ND	ND	ND	ND
DNA G+C content (mol%)	47·1	56·0	67·4	64·2	62·0	56·0	39·6	49·9

ND, Not determined.

from *Halothiobacillus* species. Strain SO07^T may be regarded as a freshwater species. Analysis of nearly complete 16S rRNA gene sequences revealed low levels of similarity (87–89 %) between strain SO07^T and *Halothiobacillus* species. The level of DNA–DNA hybridization between strain SO07^T and *H. neapolitanus* was less than 2 %. In addition, the DNA G+C content of strain SO07^T (47·1 mol%) is significantly lower than those of *Halothiobacillus* species, i.e. 56·0–67·4 mol% (Kelly & Wood, 2000; Sievert *et al.*, 2000). Because of the distinct differences in phylogeny, chemotaxonomy and the requirement for and tolerance of NaCl between strain SO07^T and members of the genus *Halothiobacillus*, it is proposed that strain SO07^T represents the type strain of the type species of a new genus, *Thiovirga sulfuroxydans* gen. nov., sp. nov.

Description of *Thiovirga* gen. nov.

Thiovirga (Thi.o.vir′ga. Gr. n. *thion* sulfur; L. fem. n. *virga* rod; N.L. fem n. *Thiovirga* sulfur rod).

Obligately chemolithoautotrophic, Gram-negative rod. Motile, obtaining energy from reduced inorganic sulfur compounds. Oxidase- and catalase-positive. No spore formation. No anaerobic or heterotrophic growth observed. Cells contain carboxysome inclusions. Cells store polyphosphate inclusions when grown on sulfide. Contains ubiquinone Q-8. Major fatty acids are $C_{16:0}$, $C_{18:0}$, $C_{16:1}$ and $C_{18:1}$. Member of the *γ-Proteobacteria*, which is distantly related to halotolerant sulfur-oxidizing bacteria, the members of the genus *Halothiobacillus*.

The type species is *Thiovirga sulfuroxydans*.

Description of *Thiovirga sulfuroxydans* sp. nov.

Thiovirga sulfuroxydans (sul.fur.ox′y.dans. L. n. *sulfur* sulfur; N.L. part. adj. *oxydans* oxidizing; N.L. part. adj. *sulfuroxydans* sulfur-oxidizing).

Cells are rod-shaped, 0·5–0·8 × 1·0–2·0 μm. Colonies on inorganic medium containing thiosulfate (SOB medium) are white in colour and lens-shaped (diameter of 0·5–1·0 mm). Gram-negative. Cells occur singly or in pairs and are motile by single polar flagella. Strictly aerobic. Grows chemolithoautotrophically on thiosulfate, sulfur and sulfide. Nitrate is not used as terminal electron acceptor. No heterotrophic growth occurs. Optimum growth temperature is 30–34 °C; optimum pH is 7·5; and optimum NaCl concentration is 30 mM. No growth is observed in NaCl concentrations of more than 180 mM or above 42 °C. Cells contain carboxysome inclusions. Stores polyphosphate inclusions in cells when grown on sulfide. Major isoprenoid quinone is ubiquinone Q-8. Major fatty acids are $C_{16:0}$ (19 %), $C_{18:0}$ (16 %), $C_{16:1}$ (30 %) and $C_{18:1}$ (31 %); $C_{12:0}$ (2 %) is present as a minor fatty acid.

The type strain is SO07^T (=JCM 12417^T=ATCC BAA-1033^T). The DNA G+C content of the type strain is 47·1 mol%. Isolated from a microaerobic waste-water biofilm at a waste-water treatment plant at Sapporo, Japan.

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