***Bacillus schlegelii*, a New Species of Thermophilic, Facultatively Chemolithoautotrophic Bacterium Oxidizing Molecular Hydrogen**

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A thermophilic, facultatively chemolithoautotrophic bacterium oxidizing molecular hydrogen has been isolated. It formed round, terminal endospores and had a multilayered cell wall. Although growth rates were highest under autotrophic conditions, certain organic chemicals, including short-chain fatty acids, some amino acids, 1-propanol and phenol, could serve as sole carbon and energy sources; ammonium ions, urea and L-asparagine could serve as sole nitrogen sources. The hydrogenase was membrane-bound and did not reduce NAD(P). The guanine+cytosine content of its DNA was 67 to 68 mol%. Based on its morphological and biochemical features, the organism belongs to *Bacillus*, but differs from any of the previously described species attributed to this genus. It is therefore proposed as constituting a new species, *Bacillus schlegelii*, named after the German microbiologist H. G. Schlegel. The type strain of *B. schlegelii*, strain MA48, has been deposited with the German Collection of Microorganisms, Göttingen, under the accession number DSM 2000.

INTRODUCTION

Aerobic, hydrogen-oxidizing bacteria form a physiological group comprising bacteria from various taxa (Davis *et al.*, 1969, 1970; Aragno *et al.*, 1977; Walther-Mauruschat *et al.*, 1977). Mesophilic, spore-forming, hydrogen-oxidizing bacteria, among them *Bacillus pycnoticus*, were isolated by Ruhland (1922, 1924) and Grohmann (1924). These strains were subsequently lost; besides, the descriptions by Grohmann (1924) suggest that some of them at least could have been mixed cultures. Further attempts to isolate mesophilic, spore-forming, hydrogen-oxidizing bacteria have so far failed (Wilde, 1962; Eberhardt, 1965). Thermophilic, hydrogen-oxidizing bacteria that have been described to date belong to the genera *Pseudomonas* and *Flavobacterium* (Aragno & Schlegel, 1980). However, numerous strains of a thermophilic, endospore-forming, hydrogen-oxidizing bacterium were isolated by Aragno (1978). These organisms were long, straight, peritrichously flagellated rods, with terminal round endospores swelling the sporangium, their Gram-reaction was variable and they grew optimally at 70 °C; they were considered to belong to the genus *Bacillus*. This paper is concerned with the characterization and taxonomy of two representative strains of this bacterium, MA48 and MA51.

METHODS

Organisms. The two strains of thermophilic, endospore-forming, hydrogen-oxidizing bacteria studied here, MA48 and MA51 (DSM 2000 and DSM 2001, respectively), were isolated from the surface layer of the sediment of a small eutrophic lake, Le Loclat, near St-Blaise, Neuchâtel, Switzerland. The enrichment and isolation procedures used have been described previously (Aragno, 1978).

The following strains were used for comparison: mesophilic strains of *Bacillus sphaericus*, labelled NRS1691, NRS1692, NRS1693, NRS1694, NRS1695 and NRS1696, and the type strain of *B. acidocaldarius* (ATCC 27009), received from Dr R. E. Gordon, Rutgers University, New Brunswick, U.S.A.; thermophilic strains of *B. sphaericus*, labelled S55-67, S56-67, S58-67, S60-67, S62-67, S64-67, S66-67, E1-65, H14-66 and L54-66, received from Dr F. Hollaus, Zuckerforschungs-Institut, Fuchsenbigl, Austria; *B. thermoruber* strain BT/2, received from Dr R. Craveri, Università degli Studi, Milano, Italy; *Aquaspirillum autotrophicum* type strain (DSM 732, ATCC 29984), obtained from the Culture Collection of the Institut de Botanique, Université de Neuchâtel, Switzerland.

Media and growth conditions. The basal mineral medium, after Schlegel *et al.* (1961), contained: 4.5 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 g KH_2PO_4 , 1 g NH_4Cl , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.005 g ferric ammonium citrate and 5 ml trace element solution SL6 [solution SL4 of Pfennig & Lippert (1966) minus EDTA and iron salt] in 1000 ml double-distilled water. The final pH was about 7.1.

For autotrophic growth, NaHCO_3 (0.5 g l^{-1}) was added; cultures were then incubated under 0.05 atm O_2 /0.1 atm CO_2 /0.45 atm H_2 (partial pressures measured at laboratory temperature) in desiccators, without agitation. Reduced pressure (0.4 atm at laboratory temperature) allowed incubation of the desiccators at temperatures up to 70 °C without displacing the cover. For heterotrophic growth, an appropriate organic carbon source was added and cultures were incubated under air. If required, Oxoid agar no. 1 (10 g l^{-1}) was added. Stock cultures were maintained autotrophically on mineral agar slants and were transferred to fresh medium every 3 months.

Liquid cultures were grown in 100 ml screw-capped Pyrex bottles containing 20 ml medium; they were not agitated as this inhibited growth. Growth was followed turbidimetrically at 436 nm, using 1 cm cuvettes in a Zeiss PM4 spectrophotometer.

To measure growth rates at different pH values, the pH of the medium was modified with either NaOH or HCl. The exact pH value was then measured in control media after sterilization.

Nutritional characteristics. The utilization of compounds as sole sources of carbon and energy was tested in liquid media supplemented with carbohydrates (2 g l^{-1}), phenol (0.1 g l^{-1}) or one of a number of the other carbon compounds (1 g l^{-1}). A control lacking any carbon source was also inoculated.

The utilization of compounds as sole nitrogen sources was tested in liquid media, under autotrophic conditions. The medium used was the basal mineral medium without NH_4Cl , but supplemented with either NaNO_2 (0.2 g l^{-1}) or one of the other nitrogen compounds examined (1 g l^{-1}). Control cultures contained NH_4Cl (1 g l^{-1}). Ability to fix N_2 was tested using acetylene reduction to ethylene by nitrogenase; this was done under autotrophic conditions with reduced oxygen partial pressure (p_{O_2} 0.02 atm) in nitrogen-free medium.

Biochemical characteristics. Unless stated otherwise, the following tests were performed in liquid cultures, as described above, with basal mineral medium supplemented with sodium acetate at 1 g l^{-1} (MMA). Cultures were incubated for 1 week at 65 °C under air. Uninoculated media were used as controls.

MMA supplemented with appropriate substrates was employed for the following determinations. (i) Aerobic nitrate reduction and anaerobic growth in the presence of KNO_3 (2 g l^{-1}); nitrites were detected with the Griess reagent and an inverted Durham vial was used to detect gaseous nitrogen evolution. (ii) Indole formation from Tryptone (1 g l^{-1} ; Difco), detected by production of a red colour with acidic *p*-dimethylaminobenzaldehyde. (iii) H_2S production from cysteine (1 g l^{-1}), detected by reaction with lead acetate strips suspended above the medium. (iv) Visible growth within 2 weeks in the presence of glycine (10 g l^{-1}) or NaCl (10, 30, 50 or 70 g l^{-1}); MMA without additions was used as a control. (v) Hydrolysis of soluble starch (2 g l^{-1}), tested with iodine reagent. (vi) Hydrolysis of gelatin; after 1 week incubation, MMA cultures containing gelatin (60 g l^{-1}), pH 7.2, were chilled for detection of liquefaction.

Hydrolysis of casein was tested in Petri dishes containing solidified MMA supplemented with skim milk (10 g l^{-1}). The oxidase test was performed by adding α -naphthol and *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (1%, w/v) directly to 48 h-old MMA cultures. To test for catalase, 5 drops of 3% (v/v) H_2O_2 were added to 48 h-old MMA and autotrophic agar slant cultures.

Urease activity was tested on the basal mineral medium supplemented with proline (1 g l^{-1}), urea (20 g l^{-1}) and phenol red (0.01 g l^{-1}), pH 6.8; this medium was sterilized by filtration. Urea was slowly hydrolysed at the incubation temperature, so controls lacking urea as well as uninoculated media were used.

Penicillin susceptibility was tested on MMA containing 0.015 to 15.90 units (U) penicillin G ml⁻¹, as described by Wallhäusser & Schmidt (1967). MMA without additions was used as a control. Cultures were incubated for 2 weeks.

Protein determination. The biuret method as described by Schmidt *et al.* (1963) was used, with bovine serum albumin as a standard.

Preparation of cell-free extracts. Cells were washed, suspended in 50 mM-phosphate buffer (final density about 10 mg protein ml⁻¹) and disintegrated by passage through a Sorvall RM cell fractionator at 100 MPa. Intact cells and debris were removed by centrifugation at 10000 *g* for 30 min. The supernatant cell-free extract was used for further assays.

Enzyme tests. Hydrogenase [with either methylene blue or NAD(P) as electron acceptors], malate dehydrogenase and NADH oxidase were tested at 50 °C as previously described (Aragno & Schlegel, 1978*b*).

DNA base composition. The base composition was determined from the buoyant density in CsCl according to the procedure of Schildkraut *et al.* (1962). *Micrococcus lysodeikticus* DNA (density 1.731 g ml⁻¹) was used as a standard.

Poly- β -hydroxybutyrate. Attempts to isolate and identify poly- β -hydroxybutyrate were made by the method of Law & Slepceky, as described in Herbert *et al.* (1971). Cells of *Aquaspirillum autotrophicum*, known to possess inclusions of poly- β -hydroxybutyrate (Aragno & Schlegel, 1978*a*), were used as a positive control.

Glycogen. The presence of glycogen in the cells was demonstrated by the method of Palmstierna, as described in Herbert *et al.* (1971). The precipitated glycogen was determined by the anthrone reagent.

Electron microscopy. Autotrophically grown cells were centrifuged (4000 *g*, 5 min) and washed three times in cacodylate buffer (pH 6.0, 0.2 M). Fixation was done at laboratory temperature as described by Lickfeld (1976). Cells were pre-fixed with 5% (v/v) glutaraldehyde in cacodylate buffer (pH 6.0, 0.2 M) for 2.5 h and then washed twice in RK buffer (pH 6.1) (Ryter & Kellenberger, 1958). The main fixation was with 1% (w/v) OsO₄ in RK buffer (pH 6.1) for 13 h (0.1 ml freshly prepared Difco Bacto Tryptone B123 was added). Cells were then washed twice in RK buffer and embedded in 2% (w/v) agar (Oxoid no. 1) at 47 °C before post-fixation with 4% (w/v) uranyl acetate in RK buffer for 3 h. After dehydration in a graded series of acetone/water mixtures, fixed cells were embedded in TAAB embedding resin. Ultrathin sections were cut using a Sorvall Porter Blum MT2B ultramicrotome with diamond knife. Sections were collected on copper grids and post-stained with uranyl acetate (saturated in 50% ethanol) for 10 min and lead citrate for 10 min. Electron micrographs were taken with a Philips EM201 electron microscope operating at 80 kV.

RESULTS AND DISCUSSION

Cultural characteristics

The two representative strains chosen for this study formed different types of colonies: those of MA48 were smooth, circular and convex; those of MA51 were rough, flat and spreading. Except for this feature, both strains were similar.

Dense, uniformly turbid growth occurred in liquid synthetic media, under either autotrophic or heterotrophic conditions. Shaking the cultures strongly inhibited growth.

Micromorphology

The cell wall of strain MA48 had a three-layered structure as seen in electron micrographs of thin sections (Fig. 1). The central layer was densely stained, whereas the inner layer, adjacent to the cytoplasmic membrane, and the outer layer were less dense. In many sections, the outer and inner layers appeared to be composed of regularly spaced globular units, with a centre-to-centre spacing between the units of approximately 9 nm.

A layered wall structure is not common among endospore-forming bacteria, though a similar type of wall has recently been found in three Gram-variable bacilli and in two Gram-variable clostridia (see Leduc *et al.*, 1977, for a review). The three-layered wall structure of the hydrogen-oxidizing *Bacillus* is similar to the structure found in *Bacillus* sp. C.I.P. 76-111 (Leduc *et al.*, 1977), in which a thin dense layer is sandwiched between two less dense layers.

In *Bacillus* sp. C.I.P. 76-111, as in the other organisms possessing such a multilayered wall, the thin dense layer corresponds to the peptidoglycan layer, while the less dense layers are made of protein subunits. Furthermore, all these bacteria possess a regular surface

Fig. 1. Multilayered cell wall of strain MA48 showing regularly spaced globular units: cl, central layer; ol, outer layer; il, inner layer; cm, cytoplasmic membrane. Bar marker represents $0.1 \mu\text{m}$.

pattern of subunits. We assume that the wall of strain MA48 is similar, but this needs further investigation. The wall composition and surface structure of the cell are now under study in our laboratory.

Neither mesosomes nor other intracytoplasmic membranes could be seen, except for membranes involved in septum and spore formation.

The only cytoplasmic inclusions observed were large electron-dense bodies, often in contact with cell membranes. A polysaccharide was isolated from autotrophically grown cells harvested in stationary phase; its anthrone spectrum was characteristic for glucose and so we assume that these inclusions consist of a glycogen-like glucan. In contrast to all Gram-negative, hydrogen-oxidizing bacteria investigated so far (Walther-Mauruschat *et al.*, 1977), no poly- β -hydroxybutyrate could be detected.

General properties

Compared with other hydrogen-oxidizing bacteria (Aragno & Schlegel, 1980), the organism used only a limited spectrum of organic chemicals as carbon and energy sources. Furthermore, autotrophic growth was much faster than heterotrophic growth (Aragno, 1978). Of 62 compounds tested with NH_4Cl as sole nitrogen source, the following substances were used as carbon and energy sources: acetate, butyrate, caproate, fumarate, DL- β -hydroxybutyrate, L-malate, propionate, pyruvate, succinate, valerate, L-alanine, L-cysteine, L-glutamate, L-phenylalanine, L-proline, phenol and 1-propanol; growth with phenol occurred only at concentrations up to 1 g l^{-1} . The following substances were not used: benzoate, citrate, formate, gluconate, glycollate, *p*-hydroxybenzoate, α -ketoglutarate, DL-lactate, malonate, mesaconate, D(-)-tartrate, L(+)-tartrate, *meso*-tartrate, L-arginine, L-asparagine, L-aspartate, L-citrulline, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-serine, L-tryptophan, L-valine, L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannose, L-rhamnose, saccharose, L-sorbose, trehalose, D-xylose, 1-butanol, ethanol, glycerol, mannitol and methanol.

Ammonium salts, asparagine and urea were used as sole nitrogen sources in autotrophic cultures; nitrates, nitrites and molecular nitrogen were not.

The biochemical properties of the two strains, together with their temperature and pH optima for growth, penicillin susceptibility and DNA base composition, are summarized in Table 1.

For most aerobic spore-formers, the G+C content of their DNA is between 32 and 53 mol %. Strains MA48 and MA51 have a much higher G+C content, approaching that of the recently described thermophilic bacilli, i.e. *B. acidocaldarius* (62 mol % G+C; Darland & Brock, 1971), *B. thermoruber* (67 mol % G+C; Guicciardi *et al.*, 1968), and *B. thermocatenulatus* (69 mol % G+C; Golovacheva *et al.*, 1975).

Penicillin susceptibility

While controls without penicillin showed growth after 2 d, growth did not occur until 4 d in the presence of $0.015 \text{ U penicillin G ml}^{-1}$ and 12 d in the presence of $15.90 \text{ U penicillin G ml}^{-1}$. To test whether the growth lags were due to heat destruction of the

Table 1. *Biochemical characteristics and other general properties of the hydrogen-oxidizing Bacillus*

Character	Result
Nitrate reduction to nitrite	+
N ₂ evolution from nitrate	—
Oxidase	w
Catalase	+
Urease	—
Hydrolysis of gelatin	—
Hydrolysis of casein	w
Hydrolysis of starch	—
Indole formation	—
H ₂ S from cysteine	—
Growth in the presence of 1% (w/v) glycine	—
Growth in the presence of 3% (w/v) NaCl	+
Growth in the presence of 5% (w/v) NaCl	—
Optimum temperature (autotrophic growth, pH 7.0)	70 °C
Optimum pH (growth on MMA, 65 °C)	6.0–7.0
M.i.c. of penicillin G	0.005 U ml ⁻¹
DNA base composition: strain MA48	67.7 mol % G + C
strain MA51	67.1 mol % G + C

+, Positive; w, weakly positive; —, negative.

Fig. 2. Relation between growth lag (compared with a control) and the initial concentration of penicillin G in the medium (semilogarithmic plot). Incubation temperature was 65 °C.

antibiotic, MMA containing 15.90 U penicillin G ml⁻¹ was incubated for 14 d at 65 °C before inoculation. In these media, growth was as rapid as in a control without penicillin G. There was an almost linear relationship between the logarithm of penicillin G concentration and growth lag as compared with the control (Fig. 2); this corresponds to the heat destruction curve of the antibiotic at 65 °C. From the intercept of the curve with the ordinate, the minimum inhibitory concentration (m.i.c.) of penicillin G was about 0.005 U ml⁻¹, which is very low compared with other Gram-positive bacteria.

Hydrogenase activity

Crude cell-free extracts of autotrophically grown cells contained hydrogenase activity, measured manometrically under 100% H₂ with methylene blue as electron acceptor, comparable to that of intact cells [1969 versus 2207 nmol H₂ min⁻¹ (mg protein)⁻¹]. No activity was detected using either NAD or NADP as potential electron acceptors (tested photometrically). After centrifuging the cell-free extract at 140000 *g* for 2 h, the supernatant and sediment (particulate fraction) were both tested for hydrogenase activity, and for activities of malate dehydrogenase (a soluble enzyme) and NADH oxidase (a membrane-

Table 2. *Distribution of hydrogenase, malate dehydrogenase and NADH oxidase activities between particulate and soluble fractions of cell-free extract*

The soluble and particulate fractions were obtained by centrifuging the cell-free extract for 2 h at 140000 g. Enzyme activities in these fractions are expressed as a percentage of the activities in the cell-free extract.

	Hydrogenase*	Malate dehydrogenase	NADH oxidase
Cell-free extract	100	100	100
Soluble fraction	15	100	7
Particulate fraction	72	8	78

* Tested manometrically.

bound activity). The results (Table 2) show that most of the hydrogenase activity was in the particulate fraction, i.e. a membrane-bound, non-NAD-reducing hydrogenase was present, but no cytoplasmic NAD-reducing hydrogen-dehydrogenase could be detected. This appears to be the most common pattern in hydrogen-oxidizing bacteria (Schneider & Schlegel, 1977).

Autotrophic growth of other bacilli

Other bacilli possessing characteristics in common with strains MA48 and MA51 were tested for autotrophic growth under $H_2/O_2/CO_2$ at their respective temperature and pH optima. These included thermophilic strains of *Bacillus sphaericus* (Hollaus & Klaushofer, 1970) as well as mesophilic strains of the same species previously attributed to *B. pycnoticus* (R. E. Gordon, personal communication), *B. thermoruber* (Guicciardi *et al.*, 1968; Aragozzini *et al.*, 1976) and *B. acidocaldarius* (Darland & Brock, 1971). None of these strains were able to develop autotrophically.

Proposal of a new species

The characteristics of strains MA48 and MA51 fit the description of the genus *Bacillus* as defined in the 8th edition of *Bergey's Manual* (Buchanan & Gibbons, 1974), i.e. aerobic, rod-shaped cells with peritrichous flagellation, forming one endospore per cell. In Table 3, the characteristics of strains MA48 and MA51 are compared with those of *Bacillus sphaericus* and with those of the other thermophilic bacilli with high G+C content of DNA. The much higher G+C content of strains MA48 and MA51 excludes any affinity with *B. sphaericus*; these two strains also differ considerably from other bacilli having a high G+C content, e.g. in spore shape, inability to grow on sugars, ability to develop autotrophically under $H_2/O_2/CO_2$, and so on. Strains MA48 and MA51 also differ from *B. pycnoticus* as described by Grohmann (1924); this bacterium has cylindrical spores (not formed under autotrophic conditions) and is able to use carbohydrates as carbon and energy sources. Thus, we consider that strains MA48 and MA51 represent a new species, for which we propose the name *Bacillus schlegelii*. We dedicate this new species to Professor Hans G. Schlegel, Director of the Institute of Microbiology of the University of Göttingen, Federal Republic of Germany.

Description of the species

Bacillus schlegelii sp. nov.

schle.gel'i.i. M.L. gen.n. *schlegelii* of Schlegel; named after H. G. Schlegel, a German microbiologist.

Long, straight, rod-shaped cells, $0.6\ \mu m \times 2.5$ to $5\ \mu m$, with peritrichous flagellation; spherical, terminal endospores formed, 0.8 to $1\ \mu m$ in diameter, distinctly distending the sporangium. Gram-variable.

Colonies cream-coloured, round or spreading over the agar surface. Neither a carotenoid pigment nor poly- β -hydroxybutyrate are produced.

Table 3. Characteristics useful for differentiating between strains MA48 and MA51 and other thermophilic bacilli

Character	MA48, MA51	<i>B. sphaericus</i>	<i>B. acidocaldarius</i>	<i>B. thermoruber</i>	<i>B. thermo-catenulatus</i>
G+C (mol %)	67-68	37-43	61-62	67	69
Spore shape*	S	S	O	O	O
Gram-reaction	v	v	v	v	+
Motility	+	+	.	+	+
Optimum pH	6-7	neutral	2-5/6	neutral	neutral
Optimum temperature (°C)	70	30†	65	45-50	55-70
H ₂ autotrophy	+	-	-	-	.
Pigment	-	-	-	+	-
Auxotrophy	-	+	-	+	-
Facultatively anaerobic	-	-	-	-	+
Poly-β-hydroxybutyrate	-	.	.	.	+
Carbon and energy sources:					
Acetate	+	.	-	.	.
Citrate	-	.	-	.	+
D-Gluconate	-	.	+	.	.
Succinate	+	.	-	.	.
Sugars (at least some)	-	-	+	.	+
Glycerol	-	+	+	.	+
Mannitol	-	-	.	.	+
Nitrogen sources:					
Ammonium	+	.	+	-	.
Nitrate	-	.	-	-	.
Urea	+	.	.	-	.
Nitrate reduction	NO ₂ ⁻	-	.	.	N ₂
Nitrate respiration	-	-	-	.	+
Urease	-	+	.	.	-
Hydrolysis of starch	-	-	+	w	-
Hydrolysis of gelatin	-	v	.	++	-
Hydrolysis of casein	w	v	.	++	w
Penicillin G (m.i.c., U ml ⁻¹)	0.005	.	.	8	.

+, Positive; w, weakly positive; -, negative; v, variable result for different strains.

* S, Spherical; O, oval.

† Thermophilic strains attributed to *B. sphaericus* were described by Allen (1953) and Hollaus & Klaushofer (1970).

No growth factors required.

Strictly respiratory metabolism, with oxygen as the terminal electron acceptor. Cannot grow anaerobically with nitrate. Nitrate reduced to nitrite.

Either chemolithoautotrophic, using H₂ as the electron donor and CO₂ as the carbon source, or chemoorganoheterotrophic.

Phenol, 1-propanol and a small number of amino acids and organic acids can serve as sole carbon sources, with ammonium ions as the nitrogen source. Sugars not metabolized. Ammonium ions, urea and asparagine can be utilized as sole nitrogen sources.

Hydrogenase constitutive, membrane-bound, and not NAD(P) specific; hydrogenase activity strongly thermophilic, with a temperature optimum between 70 and 75 °C. Catalase and oxidase positive. Urease not produced. Weak hydrolysis of casein. No hydrolysis of starch and gelatin. H₂S not produced from cysteine. Indole not produced. Growth in the presence of 3 % NaCl, but not 5 %. No growth in the presence of 1 % glycine. Minimum inhibitory penicillin G concentration: 0.005 U ml⁻¹.

Temperature for optimum growth about 70 °C; no growth at 37 or 80 °C. pH for optimum growth 6 to 7.

The G+C content of the DNA is 67 to 68 mol %.

Isolated from the surface layer of the sediment of a small eutrophic lake near Neuchâtel, Switzerland.

Type strain: strain MA48 is designated as the type strain for the species. It has been deposited with the Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G., under the accession number 2000. The description of the type strain is the same as that given above for the species with the following addition: the G+C content of the DNA is 67.7 mol %.

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