Clostridium hydrogeniformans sp. nov. and *Clostridium cavendishii* sp. nov., hydrogen-producing bacteria from chlorinated solvent-contaminated groundwater

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Four hydrogen-producing, aerotolerant, anaerobic bacterial strains isolated from chlorinated solvent-contaminated groundwater were characterized using a polyphasic approach. Three of the strains, designated BL-18, BL-19 and BL-20^T, were found to be identical in 16S rRNA gene sequences and in phenotypic properties. Cells of these strains are Gram-positive-staining, spore-forming, motile rods with peritrichous flagella. Growth occurred at 15–40 °C, pH 5.0–10.0 and at NaCl concentrations up to 5 % (w/v). Acid was produced in fermentation of cellobiose, fructose, galactose (weak), glucose, maltose and salicin. Products of fermentation in PYG medium were acetate, butyrate, ethanol, formate, carbon dioxide and hydrogen. Dominant cellular fatty acids when grown in PYG medium were C_{13:0} iso, C_{16:0}, C_{13:0} anteiso, C_{15:0} iso and C_{15:0} anteiso. The genomic DNA G + C content was 30.4 mol%. These isolates can be differentiated from their closest phylogenetic relative, the cluster I *Clostridium* species *Clostridium frigidicarnis* (97.2 % similar to the type strain in 16S rRNA gene sequence), on the basis of phenotypic and chemotaxonomic properties. The other strain characterized in this study, BL-28^T, was Gram-positive-staining with spore-forming, rod-shaped cells. Growth occurred at 15–46 °C, pH 6.0–8.5 and at NaCl concentrations up to 3 % (w/v). Acid was produced from cellobiose, dextran, fructose (weak), glucose, maltose, salicin and trehalose. End products of PYG fermentation included acetate, butyrate, pyruvate, carbon dioxide and hydrogen. Dominant cellular fatty acids from cells grown in PYG medium at 30 °C were C_{14:0}, C_{14:0} dimethyl aldehyde, C_{16:0} and C_{12:0}. The DNA G + C content was 28.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain BL-28^T falls within cluster I of the genus *Clostridium*, but with ≤95.2 % identity with previously described species. On the basis of results presented here, strains BL-20^T (=NRRL B-51348^T =DSM 21757^T) and BL-28^T (=NRRL B-51352^T =DSM 21758^T) are proposed as the type strains of novel species of the genus *Clostridium* with the names *Clostridium hydrogeniformans* sp. nov. and *Clostridium cavendishii* sp. nov., respectively.

During a study aimed at characterizing the microbial population in groundwater contaminated with chlorinated solvents at the PetroProcessors of Louisiana, Inc. (PPI) Superfund Site located near Baton Rouge, LA, USA, 168 bacterial strains that grouped phylogenetically in 18 operational taxonomic units (OTUs) were isolated

(Bowman *et al.*, 2006). Of these, strain BL-18 was isolated on R2A agar (Difco) supplemented with L-cysteine hydrochloride (0.5 g l⁻¹) as a reductant and resazurin (1.0 mg l⁻¹) as a redox indicator, adjusted to pH 7.0 prior to autoclaving, and incubated at 30 °C in an anaerobic chamber (Coy Laboratory Products) supplied with a gas headspace composed of 90 % N₂, 5 % CO₂ and 5 % H₂. Strains BL-19 and BL-20^T were isolated on plate count agar (PCA; Difco) amended with the same supplements and under the same incubation conditions. Strain BL-28^T was isolated on Columbia anaerobe 5 % sheep blood agar (CSBA; BD Diagnostics) under the same incubation

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BL-20^T and BL-28^T are DQ196623 and DQ196621, respectively.

The fatty acid profile of strain BL-28^T is available as supplementary material with the online version of this paper.

conditions. Purity of the strains was verified by microscopy after multiple transfers. Strains were preserved at -80°C in R2A medium (formulation of R2A agar but without solidifying agent) supplemented with 5 % (v/v) DMSO.

To characterize their potential role in supporting chlorinated solvent transformation at the site from which they were isolated, strains BL-20^T and BL-28^T were tested to determine their abilities to dechlorinate and their ability to produce hydrogen and grow in the presence of tetrachloroethene (PCE), 1,2-dichloroethane (1,2-DCA) and 1,1,2-trichloroethane (1,1,2-TCA). Neither strain dechlorinated the solvents tested, but both strains were found to produce hydrogen even when grown in the presence of near-saturation concentrations of PCE (1.1 mM) and high concentrations of 1,2-DCA (22.2 and 19.8 mM for BL-20^T and BL-28^T, respectively) and 1,1,2-TCA (4.9 and 7.4 mM) (Bowman *et al.*, 2009). Here, we report the polyphasic characterization of these isolates and establish their taxonomic status. For comparative purposes, *Clostridium frigidicarnis* DSM 12271^T, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, was included in testing.

All described procedures followed strict anaerobic protocols as described in Holdeman *et al.* (1977). Unless stated otherwise, all tests were carried out in duplicate in anaerobic PY broth with 1 % (w/v) glucose adjusted to pH 7.0 with headspace gas purged with ultra-high-purity nitrogen prior to autoclaving (Holdeman *et al.*, 1977). Colony morphology was observed after growth on anaerobic CSBA incubated for 48 h at 37°C . Cells grown in PYG broth incubated for 18 h at 30°C were Gram-stained using the method of Johnson *et al.* (1995). The Gram type was confirmed using the KOH procedure of Powers (1995). Cell morphology was determined using phase-contrast and DIC light microscopy (Nikon Microphot-FXA) and transmission electron microscopy (JEOL 100-CX TEM). Vegetative cells were imaged after 18 h of incubation and spores after 5 days of incubation at 30°C in anaerobic PYG broth. Motility was assessed by microscope observations and stab inoculation of 0.4 % (w/v) semi-solid PYG agar incubated at 37°C (Gerhardt *et al.*, 1981).

The temperature range for growth was assessed in PYG broth incubated at temperatures ranging from 4 to 50°C for 21 days. The salinity range for growth was tested in PYG broth amended with 1, 2, 3, 4, 5 and 6 % (w/v) NaCl incubated at 30°C for 14 days. The pH range for growth was assessed at 25°C in PYG medium buffered as described by Lee *et al.* (2007) with 14 days of incubation. The ability of strains to grow in PYG medium with no oxygen, with 5 % oxygen and with 21 % oxygen in the headspace was assessed following incubation at 37°C for 7 days. The ability to utilize cellobiose, microgranular cellulose (Sigma) and Avicel microcrystalline cellulose (type PH-105, lot no. 50630C; FMC BioPolymer) was evaluated in modified CM3 medium prepared as described by Ren *et al.* (2007), adjusted to pH 6.5, and incubated at 30°C for 21 days. Exponentially growing

cultures diluted to an OD₆₀₀ of 0.05 were used for inoculation (2 % v/v). Growth was measured spectrophotometrically at 600 nm (Evolution 60; Thermo Scientific). Cultures exhibiting an OD₆₀₀ ≥ 0.05 units higher than uninoculated negative controls were recorded as positive for growth.

The ability of the strains to utilize various substrates was determined in duplicate in anaerobic PY broth as described by Holdeman *et al.* (1977) with cellobiose, dextran, fructose, galactose, glucose, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose and xylose at 1 % (w/v) and adonitol, arabinose and trehalose at 0.5 % (w/v). Growth was evaluated by a decrease in pH, as described by Holdeman *et al.* (1977), after incubation at 30°C for 10 days.

Tests for gelatin, aesculin and starch hydrolysis, lecithinase and catalase activity, milk reaction and hydrogen sulfide production were performed following methods described previously (Holdeman *et al.*, 1977). Additional biochemical features were tested using the Rapid ID 32A system (bioMérieux) according to the manufacturer's recommended protocol.

Fermentation products were determined in PYG broth incubated at 30°C for 6 days. Exponentially growing cultures diluted to an OD₆₀₀ of 0.05 were used to inoculate (2 %, v/v) serum bottles containing 100 ml liquid medium and 60 ml gas headspace. Acetate, butyrate, formate, lactate, propionate and pyruvate were analysed by ion chromatography (DX500; Dionex) as described by Lin *et al.* (2007). Ethanol and butanol were determined using purge and trap gas chromatography with a flame-ionization detector as described by Yan *et al.* (2009). Gas production volume was measured as described by Owen *et al.* (1979). Hydrogen and carbon dioxide concentrations in the headspace gas were analysed by gas chromatography as described by Van Ginkel *et al.* (2001).

Hydrogen yield from glucose fermentation was assessed as described by Van Ginkel *et al.* (2001) but with the medium supplemented with yeast extract (0.5 g l^{-1}) and resazurin (1.0 mg l^{-1}), the pH adjusted to 7.0 and with the headspace gas purged with ultra-high-purity nitrogen prior to autoclaving.

For cellular fatty acid analyses, cells grown in PYG broth at 30°C were harvested in mid-exponential growth phase by centrifugation at 8000 g for 15 min. Cellular fatty acids were extracted from the cell pellet, saponified and methylated at the BCCM/LMG (Ghent, Belgium) following the Sherlock Microbial Identification System recommended protocol (MIDI) and then analysed by gas chromatography (Sasser, 1990). Compounds were identified using the MIDI MOORE library version 5.0 (for VPI broth-grown anaerobes in PYG broth). Genomic DNA G+C content was determined at the BCCM/LMG using the HPLC technique of Mesbah *et al.* (1989).

Genomic DNA extraction, PCR and sequencing of 16S rRNA genes were performed as described by Bowman *et al.*

(2006). The resulting sequences were verified manually. Pairwise nucleotide similarity values were obtained from EzTaxon analysis on nearly complete sequences for BL-20^T (1454 bp) and BL-28^T (1452 bp) (<http://www.eztaxon.org/>; Chun *et al.*, 2007). A phylogenetic tree (Fig. 1) was constructed as described by Bowman *et al.* (2009).

On anaerobic CSBA medium, strains BL-18, BL-19 and BL-20^T formed colonies that were circular with entire margins, convex, creamy opaque and 1.5–3.5 mm in diameter after 48 h at 37 °C. Under the same conditions, strain BL-28^T formed colonies that were irregular with undulate to lobate margins, convex, creamy white, semi-opaque to translucent and 3–6 mm in diameter. All four strains also grew well on anaerobic PCA.

Cells of strains BL-18, BL-19 and BL-20^T were Gram-positive-staining, motile rods, 0.7–1.1 µm in diameter and 2.0–5.5 µm long, with peritrichous flagella. Spores of BL-18, BL-19 and BL-20^T were oval and subterminal. Cells of BL-28^T were Gram-positive-staining rods, 0.4–1.0 µm in diameter and 2.2–10.0 µm long, with oval, terminal spores. Motility of cells of strain BL-28^T was not observed with light microscopy and no flagella were observed through TEM. In stab inoculation of semi-solid PYG agar, however, strain BL-28^T grew quickly throughout the medium.

The temperature range for growth of strains BL-18, BL-19 and BL-20^T was 15–40 °C, with optimum growth between 30 and 40 °C. No growth was detected at 10 or 43 °C. Strain BL-28^T grew between 15 and 46 °C, with optimum growth between 37 and 46 °C. Growth was not observed at 10 or 50 °C.

Strains BL-18, BL-19 and BL-20^T grew in NaCl concentrations ranging from 0 to 5 %; no growth was detected in the presence of 6 % NaCl. BL-28^T grew in 0–3 % NaCl, but not in 4 % NaCl. All four strains grew optimally when no additional NaCl was added to the PYG medium.

The pH range for growth of strains BL-18, BL-19 and BL-20^T was pH 5.0–10.0, with optimum growth at pH 6.9–8.9. Growth was not detected at or below pH 4.0 or at or above pH 11.0. The pH range for growth of BL-28^T was pH 6.0–8.5, with optimum growth at pH 6.9–8.0. No growth was observed at or below pH 5.0 or at or above pH 9.0.

In PYG medium, all strains grew well in the absence of oxygen and in the presence of 5 % oxygen (tested with five consecutive transfers). None of the strains grew in the presence of atmospheric oxygen levels (21 %, v/v).

Physiological properties of strains BL-18, BL-19 and BL-20^T were identical and are contained in the species description and in Table 1. Physiological properties of strain BL-28^T are contained in the species description. The ability of the strains to grow in the presence of chlorinated solvents, as reported in the species descriptions, was determined previously (Bowman *et al.*, 2009).

The end products of fermentation in PYG medium for strain BL-20^T included acetate (13.1 mM), butyrate (2.0 mM), formate (2.1 mM), ethanol (0.4 mM) and trace amounts (<0.1 mM) of butanol. For BL-28^T, the end products of fermentation in PYG were acetate (4.9 mM), butyrate (4.8 mM) and pyruvate (1.6 mM) and trace amounts (<0.1 mM) of ethanol and butanol. Hydrogen and carbon dioxide accumulated in the headspace as

Fig. 1. Neighbour-joining phylogenetic tree showing the relatedness of strains BL-20^T and BL-28^T to members of the genus *Clostridium* based on 16S rRNA gene sequences. Bar, 1 substitution per 100 nucleotide positions. Bootstrap values for nodes with significant support (>90 %) are indicated by numbers located at branching points.

Table 1. Properties that differentiate strain BL-20^T from *C. frigidicarnis* DSM 12271^T

All data were determined in this study except for the DNA G+C content of *C. frigidicarnis* DSM 12271^T, which was reported previously (Broda *et al.*, 1999).

Characteristic	BL-20 ^T	<i>C. frigidicarnis</i> DSM 12271 ^T
Cell size (µm)	0.7–1.1 × 2.0–5.5	1.2–1.6 × 4.3–8.7
DNA G+C content (mol%)	30.4	27.3
Minimum temperature for growth (°C)	15	4
Utilization of:		
Salicin	+	–
Sorbitol	–	+
Trehalose	–	+
Milk reaction	Curdled	Curdled/digested
Activity of:		
Lecithinase	–	+
α-Glucosidase	+	–
β-Glucosidase	+	–
Proline arylamidase	–	+

fermentation products for all strains tested. The yield (mol hydrogen produced per mol glucose consumed) was 1.4 for BL-20^T and 1.1 for BL-28^T.

The DNA G+C contents of strains BL-20^T and BL-28^T determined by HPLC were 30.4 and 28.5 mol%, respectively. Cellular fatty acids of strains BL-20^T and BL-28^T are reported in Table 1 and Supplementary Table S1 (available in IJSEM Online), respectively.

Nearly complete 16S rRNA gene sequences were determined for all four strains. All four strains were placed within the radiation of cluster I of the genus *Clostridium* as defined by Collins *et al.* (1994). Sequences of BL-18, BL-19 and BL-20^T were identical and were most closely related to the sequences of *Clostridium frigidicarnis* DSM 12271^T (97.2 % identity), *Clostridium septicum* ATCC 12464^T (94.7 %) and *Clostridium chauvoei* ATCC 10092^T (94.7 %) (Fig. 1). On the basis of 16S rRNA gene sequences, strain BL-28^T was found to be only distantly related to previously described *Clostridium* species. The highest sequence identity of strain BL-28^T was with *Clostridium amylolyticum* SW408^T (95.2 %), *Clostridium sartagoforme* DSM 1292^T (95.1 %), *Anaerobacter polyendosporous* (94.8 %), *Clostridium diolis* DSM 5431^T (94.7 %) and *Clostridium tertium* DSM 2485^T (94.7 %). However, strain BL-28^T branched with *Clostridium cellulovorans* DSM 3052^T on the phylogenetic tree, but shared only 94.5 % identity.

Based on their identical phenotypes and 16S rRNA gene sequences, strains BL-18, BL-19 and BL-20^T are assigned to the same species, represented by the type strain BL-20^T. The clustering of BL-20^T with *C. frigidicarnis* DSM 12271^T

is supported by a bootstrap value of 100 %. Strain BL-20^T is clearly differentiated from its closest phylogenetic relative, *C. frigidicarnis*, based on cell size and genotypic and physiological differences (Table 1). Further differences are evident in the cellular fatty acid profiles of BL-20^T and *C. frigidicarnis* DSM 12271^T when grown under identical conditions (Table 2). Of the major cellular fatty acids produced (≥10 %), only C_{16:0} (12.9 % for BL-20^T, 13.2 % for *C. frigidicarnis* DSM 12271^T) was produced by both strains. The other primary fatty acids produced by strain BL-20^T were C_{13:0} iso (16.2 %), C_{13:0} anteiso (12.1 %), C_{15:0} iso (11.0 %) and C_{15:0} anteiso (10.0 %). In contrast, *C. frigidicarnis* DSM 12271^T contained large proportions of C_{14:0} (37.2 %) and C_{16:1ω7c} (26.8 %).

16S rRNA gene sequence analysis revealed that BL-28^T branched with *C. cellulovorans* DSM 3052^T, but the pairwise sequence identity of BL-28^T with *C. cellulovorans* DSM 3052^T was lower (94.5 %) than with other *Clostridium* relatives (e.g. *C. amylolyticum* SW408^T; 95.2 %). Low bootstrap support (44 %) for the branching

Table 2. Comparison of cellular fatty acid compositions of strains BL-20^T and *C. frigidicarnis* DSM 12271^T

Values are percentages of total fatty acids and were determined in this study; fatty acids occurring at less than 1 % in both strains are not listed. –, Not detected (<1 %). DMA, Dimethyl aldehyde.

Fatty acid	BL-20 ^T	<i>C. frigidicarnis</i> DSM 12271 ^T
C _{12:0} iso	1.3	–
C _{12:0}	2.3	1.1
C _{13:0} iso	16.2	–
C _{13:0} anteiso	12.1	–
C _{14:0} iso	5.1	2.8
C _{14:0}	6.4	37.2
C _{14:0} DMA	1.6	–
C _{15:0} iso	11.0	2.0
C _{15:0} anteiso	10.0	2.3
Summed feature 4*	–	1.5
C _{16:0} aldehyde	1.0	–
C _{15:0} iso DMA	2.0	–
Summed feature 13*	1.2	–
C _{16:0} iso	1.4	–
C _{16:1ω9c}	–	1.7
C _{16:1ω7c}	–	26.8
C _{16:1ω5c}	–	3.3
C _{16:0}	12.9	13.2
C _{16:0} DMA	4.4	–
C _{16:1ω7c} DMA	–	3.4
C _{17:0} iso	2.2	–
C _{17:0} anteiso	1.5	–
C _{18:0}	1.8	–

*Summed feature 4 contains one or more of unknown ECL 14.762, C_{15:2} and C_{15:1ω8c}. Summed feature 13 contains C_{15:0} anteiso DMA and/or C_{14:0} 2-OH.

provides further evidence that strain BL-28^T is phylogenetically divergent from *C. cellulovorans* and other members of *Clostridium* cluster I. Phenotypically, BL-28^T may be differentiated from *C. cellulovorans* on the basis that the latter grows on cellulose, galactose, lactose and sucrose (Sleat *et al.*, 1984), while BL-28^T did not utilize these substrates. Additionally, BL-28^T grew on trehalose, but *C. cellulovorans* does not.

On the basis of phylogenetic, chemotaxonomic and phenotypic features obtained in this study, we propose that strains BL-20^T and BL-28^T each represent novel species within cluster I of the genus *Clostridium*. The names proposed for these novel species are *Clostridium hydrogeniformans* sp. nov. for strain BL-20^T and *Clostridium cavendishii* sp. nov. for strain BL-28^T.

Description of *Clostridium hydrogeniformans* sp. nov.

Clostridium hydrogeniformans (hy.dro.ge.ni.for'mans. N.L. n. *hydrogenum* hydrogen; L. part. adj. *formans* forming; N.L. part. adj. *hydrogeniformans* hydrogen-forming).

Cells are Gram-positive-staining, motile rods (0.7–1.1 × 2.0–5.5 µm) with peritrichous flagella. They form oval, subterminal spores. Colonies grown on anaerobic CSBA are convex, circular, creamy and opaque with entire margins. Aerotolerant anaerobes. Grows at 15–40 °C, with optimum growth at 30–40 °C. In PYG medium, grows at NaCl concentrations up to 5% (w/v). The pH range for growth at 25 °C is pH 5.0–10.0, with optimum growth at pH 6.9–8.9. Ferments cellobiose, fructose, galactose (weak), glucose, maltose and salicin, but not adonitol, arabinose, cellulose, dextran, inositol, inulin, lactose, mannitol, rhamnose, sorbitol, sorbose, sucrose, trehalose or xylose. Hydrolyses gelatin and aesculin but not starch. Produces hydrogen sulfide. Negative for lecithinase and catalase activities. Milk is curdled. In the Rapid ID 32A system, positive results are observed for D-mannose and raffinose (weak) fermentation and for α-glucosidase, β-glucosidase and pyroglutamic acid arylamidase activities, but negative results are observed for nitrate reduction, indole production, activity of alkaline phosphatase, α-arabinosidase, arginine dihydrolase, α-fucosidase, α-galactosidase, β-galactosidase, β-galactosidase 6-phosphate, β-glucuronidase, glutamic acid decarboxylase, N-acetyl-β-glucosaminidase and urease and activity of the following arylamidases: alanine, arginine, glutamyl glutamic acid, glycine, histidine, leucine, leucyl glycine, phenylalanine, proline, serine and tyrosine. Products of fermentation in PYG medium are acetate, butyrate, ethanol, formate, hydrogen and carbon dioxide. Dominant cellular fatty acids of cells grown in PYG medium at 30 °C are C_{13:0} iso, C_{16:0}, C_{13:0} anteiso, C_{15:0} iso and C_{15:0} anteiso. Grows and produces hydrogen in the presence of near-saturation concentrations of tetrachloroethene (1.1 mM) and high concentrations of 1,2-dichloroethane (22.2 mM) and 1,1,2-trichloroethane (4.9 mM).

The type strain, BL-20^T (=DSM 21757^T =NRRL B-51348^T), was isolated from groundwater. The DNA G+C content of the type strain is 30.4 mol%.

Description of *Clostridium cavendishii* sp. nov.

Clostridium cavendishii [ca.ven.di'shi.i. N.L. masc. gen. n. *cavendishii* of Cavendish, named after Henry Cavendish (1731–1810), the British chemist who has been credited with the discovery of hydrogen].

Cells are Gram-positive-staining rods (0.4–1.0 × 2.2–10.0 µm) that form terminal spores. Colonies grown on anaerobic CSBA are irregular with undulate to lobate margins, convex, creamy white and semi-opaque to translucent. Aerotolerant anaerobe. Temperature range for growth is 15–46 °C, with optimum growth at 37–46 °C. In PYG medium, grows in the presence of up to 3% (w/v) NaCl. The pH range for growth at 25 °C is pH 6.0–8.5, with optimum growth at pH 6.9–8.0. Ferments cellobiose, dextran, fructose (weak), glucose, maltose, salicin and trehalose but not adonitol, arabinose, cellulose, galactose, inositol, inulin, lactose, mannitol, raffinose, rhamnose, sorbitol or sucrose. Hydrolyses aesculin, but not gelatin or starch. Does not produce hydrogen sulfide. Positive for lecithinase but not catalase. No reaction in milk. In the Rapid ID 32A test system, positive for β-galactosidase (weak), α-glucosidase and pyroglutamic acid arylamidase activity, but negative results are observed for nitrate reduction, indole production, fermentation of mannose and raffinose, activity of alkaline phosphatase, α-arabinosidase, arginine dihydrolase, α-fucosidase, α-galactosidase, β-galactosidase 6-phosphate, β-glucosidase, β-glucuronidase, glutamic acid decarboxylase, N-acetyl-β-glucosaminidase and urease and activities of the following arylamidases: alanine, arginine, glutamyl glutamic acid, glycine, histidine, leucine, leucyl glycine, phenylalanine, proline, serine and tyrosine. End products of fermentation in PYG medium include acetate, butyrate, pyruvate, hydrogen and carbon dioxide. Dominant cellular fatty acids from cells growing exponentially in PYG medium at 30 °C are C_{14:0}, C_{14:0} dimethyl aldehyde, C_{16:0} and C_{12:0}. Produces hydrogen when grown in the presence of near-saturation concentrations of PCE (1.1 mM) and high concentrations of 1,2-dichloroethane (19.8 mM) and 1,1,2-trichloroethane (7.4 mM).

The type strain, BL-28^T (=DSM 21758^T =NRRL B-51352^T), was isolated from groundwater. The DNA G+C content for the type strain is 28.5 mol%.

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