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Fermentative degradation of 3-hydroxybenzoate in pure culture by a novel strictly anaerobic bacterium, *Sporotomaculum hydroxybenzoicum* gen. nov., sp. nov.

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A strictly anaerobic bacterium, strain BT, from termite hindgut homogenates, was isolated in pure culture and grew on 3-hydroxybenzoate as sole source of carbon and energy. No other substrate tested was degraded, sulfate, sulfite, thiosulfate, nitrate, ferric iron, oxygen or fumarate were not reduced, and no electron transfer to partner organisms was observed. 3-Hydroxybenzoate was fermented to butyrate, acetate and CO_2. Benzoate was detected in the culture supernatant as an intermediate. The isolate was a slightly motile, endospore-forming Gram-positive rod; 16S rDNA sequence analysis revealed a high similarity to members of the genus *Desulfotomaculum*. The G+C content of the DNA was 48 mol%. Strain BT differs from the members of the genus *Desulfotomaculum* significantly due to its lack of dissimilatory sulfate reduction, and is therefore described as the type strain of a new genus and species, *Sporotomaculum hydroxybenzoicum* gen. nov., sp. nov.

Keywords: *Sporotomaculum hydroxybenzoicum* gen. nov., sp. nov., 3-hydroxybenzoate, anaerobic degradation, aromatic compounds

INTRODUCTION

Anaerobic degradation of benzoate and many benzoate derivatives has been studied with various types of bacteria, and most of these compounds are degraded via the benzoyl-CoA pathway. Anaerobic degradation of hydroxybenzoates depends to a great extent on the number and position of the hydroxy substituents: either hydroxy substituents are reductively eliminated, e.g. in degradation of 4-hydroxybenzoate or gentisate (13, 14), or the substrate is decarboxylated to the corresponding hydroxybenzene as exemplified by the fermentation of gallate via the phloroglucinol pathway by *Pelobacter acidigallici* (7), and the fermentation of β- and γ-resorcylate via resorcinol to acetate, butyrate and CO_2 by the co-culture KN 245 (31).

The conversion of 3-hydroxybenzoate in anoxic environments presumably proceeds via benzoyl-CoA as well, as studies with nitrate-reducing, sulfate-reducing and fermenting bacteria in syntrophic co-cultures with a hydrogen-scavenging partner organism indicate (reviewed by 28). In the latter case, the substrate is converted to acetate, CO_2 and hydrogen, in a reaction that is endergonic under standard conditions and requires removal of e.g. hydrogen by partner organisms to become energetically feasible.

In the present study we report on the isolation and characterization of a strictly anaerobic bacterium which ferments 3-hydroxybenzoate to butyrate, acetate and CO_2 in pure culture, without cooperation with hydrogen-consuming partner organisms. Degradation of the substrate is carried out presumably via the benzoyl-CoA pathway after reductive removal of the hydroxy group. Based on 16S rRNA sequence comparison, the strain appears to be closely affiliated with the genus *Desulfotomaculum*, and is described as the type strain of a new genus and species.

METHODS

Sources of organisms. Strain BT was isolated from an enrichment culture inoculated with gut homogenates of soil-feeding termites (*Cubitermes speciosus*). The removal of the termite gut and the preparation of homogenates followed previously described techniques (8). *Methanospirillum hungatei* strain SK (DSM 3595) and *Desulfovibrio vulgaris*

The EMBL accession number for the sequence reported in this paper is Y14845.

strain Marburg (DSM 2119) were from our own culture collection.

Cultivation and isolation. All procedures for cultivation and isolation were essentially as described previously (23, 35). The mineral medium for cultivation, enrichment and isolation contained 30 mM sodium bicarbonate as buffer, 1·5 mM sodium sulfide as reducing agent, the trace element solution SL 10 (35), a selenite-tungstate solution (31) and a seven-vitamin solution (35). The medium contained 0·5 g NaCl and 0·4 g $MgCl_2.6H_2O$ per litre. The pH was adjusted to 7·6 by addition of sterile 1 M HCl or 1 M Na_2CO_3. 3-Hydroxybenzoate and other aromatic compounds tested for growth were added from filter-sterilized stock solutions which were kept under N_2 atmosphere. In addition to sulfide, some crystals of dithionite were added (final concentration about 100 μM), as a further strong reducing agent, before inoculation. Two deep agar dilution series (23) and one additional pasteurization step were applied for isolation of a pure culture. Purity was frequently checked by microscopic examination and by growth tests in complex medium (AC medium; Difco). Growth tests were carried out in 120 ml serum bottles containing 50 ml mineral salt medium under N_2/CO_2 (80/20) atmosphere, and the optical density was monitored in 1 cm light path cuvettes at 578 nm wavelength. The amount of cell matter formed in the growth test was calculated via an experimentally determined conversion factor ($OD_{578} = 0{\cdot}1 \cong 18{\cdot}2$ mg l^{-1}, which was determined by direct measurements in 1 l cultures). Substrate assimilated into cell material was calculated after the following equation: $17C_7H_5O_3^- + 54H_2O + 10H^+ \rightarrow 28\langle C_4H_7O_3\rangle + 7HCO_3^-$.

Characterization. The Gram type was determined using standard methods (1, 15). The presence of cytochromes was tested by redox difference spectroscopy: cell-free extracts were prepared by French press treatment (138 MPa), membrane and soluble fraction were separated by ultracentrifugation (120000 ***g***, 1 h), and the redox difference spectrum of each fraction (dithionite-reduced *minus* air-oxidized) was measured in a Uvikon 860 spectrophotometer (Kontron). Sulfate, thiosulfate, sulfite, nitrate, ferric iron and fumarate were tested as electron acceptors in growth medium at a final concentration of 10 mM. Sulfate as an electron acceptor was also tested in the presence of 1,4-naphthoquinone (200 μg l^{-1}) and haemin (50 μg l^{-1}), or yeast extract (5 g l^{-1}) in the growth medium amended with 5 mM 3-hydroxybenzoate or 5 mM lactate. For tests for syntrophic growth, either *Methanospirillum hungatei* or *Desulfovibrio vulgaris* was added to the culture, in the latter case with additional sodium sulfate (10 mM final concentration).

Chemical determinations. Aromatic compounds were quantified by HPLC as described previously (7). Butyrate and acetate were assayed by GC as described previously (24). Hydrogen was measured with a trace gas detector based on the HgO-to-Hg vapour-conversion technique (34). Sulfide was determined according to the method of Cline (9), and protein was determined by the Bradford method (5) with BSA as standard.

Analysis of 16S rDNA and DNA base composition. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out as described previously (25, 26). Purified PCR products were sequenced using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) as described by the manufacturer's protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A DNA Sequencer. The 16S rDNA sequence was manually aligned against representative sequences of members of the clostridia and related taxa. Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (17). The least square distance method of De Soete (10) was used in the construction of the phylogenetic dendrogram from distance matrices.

For determination of the G+C content, the DNA was isolated using the method of Murray & Thompson (22). DNA digestion and subsequent analysis by HPLC were carried out according to Mesbah *et al.* (21).

RESULTS

Enrichment and isolation

To enrich for benzoate-degrading bacteria from the guts of the soil-feeding termite *Cubitermes speciosus*, enrichment cultures containing 5 mM benzoate as sole source of carbon and energy were inoculated with gut homogenates. During the first five transfers into fresh medium, acetate and methane were detected as sole fermentation products. After several further transfers, however, acetate and methane production decreased and butyrate was detected in the culture fluid. Microscopic analysis revealed the presence of four predominant morphotypes in these cultures: a slightly curved rod, a straight rod with thin ends, a highly motile vibrio which was later isolated and charac-

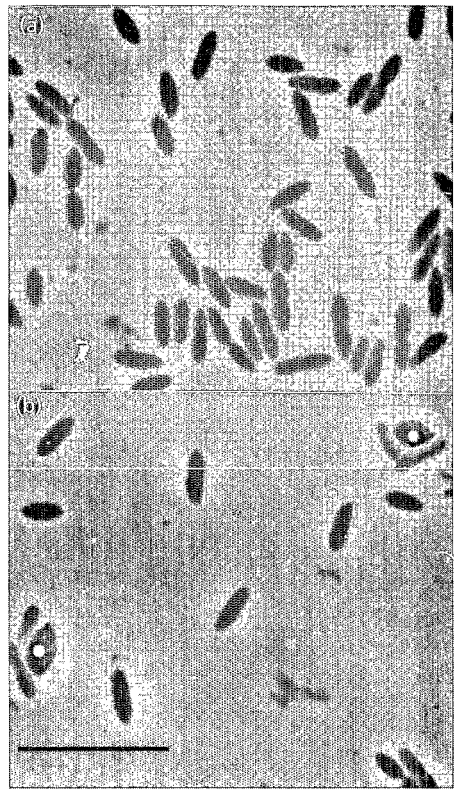

Fig. 1. Phase-contrast photomicrographs of vegetative cells of strain BT (a), and endospore formation in ageing cells (b). Bar, 10 μm.

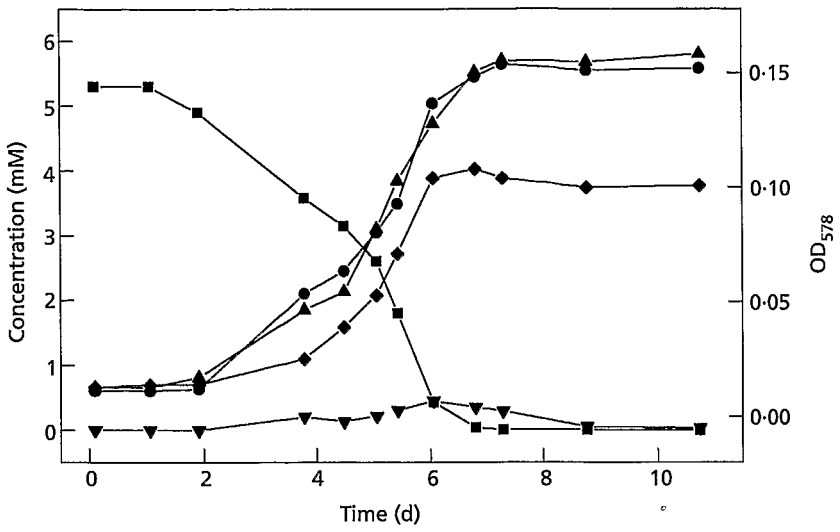

Fig. 2. Growth of strain BT with 5 mM 3-hydroxybenzoate as substrate: cell density (◆), 3-hydroxybenzoate (■), acetate (●), butyrate (▲) and benzoate (▼).

terized as a strain of *Desulfovibrio desulfuricans* (6), and a coccus resembling *Methanogenium* sp. which fluoresced in epifluorescence microscopy at 420 nm excitation wavelength. Growth of this enrichment culture was very weak, and upon transfers into fresh medium, the culture exhibited long and unpredictable lag phases. Attempts to purify this enrichment culture with benzoate in the presence of electron acceptors or partner bacteria failed.

A preliminary study of the physiological properties of this culture revealed good growth on 3-hydroxybenzoate. Microscopic examination showed predominance of a thick, rod-shaped bacterium, together with the other morphotypes, which was observed also in the enrichment culture growing on benzoate. In agar shake dilution series in the presence of 20 mM 3-hydroxybenzoate, large yellowish lens-shaped colonies were most numerous, and developed after 8–10 weeks incubation. These colonies consisted of the predominant thick rods observed in the 3-hydroxybenzoate-grown culture. A subsequent agar dilution series and an additional pasteurization step were applied to purify this strain.

Characterization and physiology

Cells of strain BT were thick rods, 2·0–3·0 × 0·6–0·8 μm in size, with pointed ends (Fig. 1a), and occurred singly or in pairs. Motility was observed only in the early exponential growth phase. Central, spherical endospores were formed (Fig. 1b) after growth in culture medium with 5 mM 3-hydroxybenzoate amended with yeast extract (5 g l^{-1}), tryptone (5 g l^{-1}), and small amounts of $MnSO_4$ (200 μM). Cells stained Gram-positive only in the early exponential growth phase. In outgrown cultures kept at 28 °C, cells lysed within 1–2 weeks. The strain was strictly anaerobic; no growth occurred after exposure of the culture to air. Addition of some crystals of dithionite (final concentration about 100 μM) was helpful for resumption of growth after transfer into fresh medium. The pH optimum was 7·3–7·6; growth was possible between pH 6·8 and 8·1. The optimal growth temperature was at 30 °C; no growth was observed below 24 °C or above 37 °C. Strain BT tolerated up to 35 mM NaCl and up to 10 mM phosphate in the growth medium.

Strain BT converted 250 μmol 3-hydroxybenzoate to 235 μmol acetate, 243 μmol butyrate, 1·4 μmol benzoate, and 0·5 μmol molecular hydrogen; 5 μmol substrate was assimilated which accounted for 0·85 mg cell dry matter formed. The electron recovery was 97% and the molar growth yield was 3·52 g dry cell material per mol 3-hydroxybenzoate. The growth rate was 0·01 h^{-1} (t_d = 70 h). A growth curve of strain BT is depicted in Fig. 2. Besides 3-hydroxybenzoate, none of the following compounds tested supported growth: pyruvate, DL-lactate, succinate, fumarate, malate, citrate, valerate, pimelate, crotonate, crotonate with H_2/CO_2-atmosphere (80:20%, v/v), caproate, adipate, hexanoate, heptanoate, cyclohexane carboxylate, ethanol, methanol, D-fructose, glucose, benzoate, 2-hydroxybenzoate, 4-hydroxybenzoate, 3-aminobenzoate, 4-aminobenzoate, 2,3- and 2,5-dihydroxybenzoate, α-, β- and γ-resorcylate, protocatechuate, gallate, syringate, ferulate, caffeate, hydroxycinnamate, terephthalate, phenylacetate, 2-, 3- and 4-chlorobenzoate, phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, 2-, 3- and 4-cresol. Strain BT did not use sulfate, thiosulfate, sulfite, nitrate, ferric iron, oxygen or fumarate as external electron acceptor. Sulfate reduction was not even observed in growth medium amended with 1,4-naphthoquinone, haemin or yeast extract. Addition of hydrogen to the headspace or consumption of hydrogen by *Methanospirillum hungatei* or *Desulfovibrio vulgaris* during growth did not change the fermentation pattern of strain BT. There was no indication of the presence of cytochromes in redox difference spectra of the soluble or the membrane fraction of cell-free extracts.

Table 1. Similarity of 16S rDNA between strain BT and related taxa

Species	Similarity (%)
Desulfotomaculum thermosapovorans	95·1
Desulfotomaculum geothermicum	91·6
Desulfotomaculum thermocisternum	87·3
Desulfotomaculum australicum	86·8
Desulfotomaculum thermobenzoicum	86·9
Desulfotomaculum nigrificans	86·0
Desulfotomaculum ruminis	85·2
Desulfotomaculum orientis	85·3
Desulfitobacterium dehalogenans	84·6
Moorella thermoacetica	86·1
Thermoanaerobacter ethanolicus	83·2
Thermoanaerobacterium thermosulfurigenes	83·3
Syntrophospora bryantii	82·3
Megasphaera elsdenii	80·8

16S rDNA sequence analysis and DNA base composition

The 16S rDNA sequence analysis of strain BT revealed a close affiliation with the genus *Desulfotomaculum*. The closest known relative of strain BT was *D. thermosapovorans* with a 16S rRNA similarity value of 95·1 % (Table 1). The level of sequence similarity with other *Desulfotomaculum* species was 91·6–85·3 %. A phylogenetic tree showing the relationship of 16S rRNA of strain BT and other related taxa is depicted in Fig. 3. The DNA G+C content of strain BT was 48 mol %.

DISCUSSION

Thus far, fermentative degradation of 3-hydroxybenzoate was reported only for a defined syntrophic co-culture (33) and for aquifer slurries (20). Strain BT, described in this paper, is the first anaerobic bacterium isolated in pure culture that ferments 3-hydroxybenzoate. Besides 3-hydroxybenzoate, strain BT does not use any other substrates, nor does it reduce any external electron acceptor. The fermentation balance agrees with the following fermentation equation (free energy change calculated after 18, 29, 30):

$$C_7H_5O_3^- + 3H_2O \rightarrow$$
$$CH_3COO^- + CH_3(CH_2)_2COO^- + CO_2 + H^+$$
$$\Delta G^{\circ\prime} = -38 \text{ kJ (mol 3-hydroxybenzoate)}^{-1}$$

Two basic features render this bacterium interesting: (i) the fermentative degradation of 3-hydroxybenzoate in pure culture, and (ii) the reductive removal of a hydroxy substituent from the aromatic ring in *meta* position to a carboxyl group, provided that 3-hydroxybenzoate is degraded by this strain via the benzoyl-CoA pathway (3, 16). The transient accumulation of benzoate during the fermentation of 3-hydroxybenzoate indicates that strain BT uses this pathway. It is likely that the pathway of benzoate degradation in fermenting bacteria follows basically the scheme described for nitrate-reducing bacteria (19), however, with modifications due to their limited energy supply (2, 27): in strain BT, the amount of energy released in the fermentation of 1 mol of 3-hydroxybenzoate to butyrate, acetate and CO_2 is sufficient to drive the synthesis of only a fraction of 1 mol of ATP; the molar growth yield of 3·5 g (mol 3-hydroxybenzoate)$^{-1}$ fits with this assumption. It is expected that strain BT gains intermediately 2 ATP-equivalents per mol substrate by substrate-level phoshorylation in the kinase reactions; additional means of energy conservation may be present, e.g. decarboxylation of glutaconyl-CoA to crotonyl-CoA to build up a sodium-ion gradient across the membrane. Nitrate-reducing bacteria consume four ATP units in activation and initial reduction of the aromatic nucleus (2). The corresponding reactions in fermenting bacteria still await elucidation; in any case, fermenting bacteria have to operate with smaller energy investments in these reactions.

It is noteworthy that strain BT forms small amounts of molecular hydrogen during growth, but co-incubation with a hydrogen-scavenging organism did not shift the fermentation balance in favour of more acetate and H_2 instead of butyrate. This is surprising because increased acetate formation could increase the total amount of ATP synthesis via acetate kinase.

Reductive elimination of the hydroxy substituent of 3-hydroxybenzoate has been postulated for various bacteria (3, 16, 33). Formation of benzoate from 3-hydroxybenzoate by strain BT gives further evidence that this compound is anaerobically degraded via benzoyl-CoA. Whereas the reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA has been demonstrated (4), removal of the hydroxy substituent of 3-hydroxybenzoate has never been shown in cell-free extracts (3, 14). It is hypothesized that such a reaction proceeds also at the CoA-ester level (16), but experimental evidence is lacking. Such an elimination reaction could even be linked to energy conservation, as was shown for the reductive removal of chloride from 3-chlorobenzoate by *Desulfomonile tiedjei* (11), and suggested for the reduction of gentisyl-CoA to benzoyl-CoA by *Syntrophus gentianae* (14).

Taxonomy

Comparison of 16S rRNA sequences of strain BT with those of clostridia and related taxa showed a close affiliation with the genus *Desulfotomaculum*, with *D. thermosapovorans* (12) as its closest relative (4·9 % sequence divergence). The physiological and phenotypical data provide evidence that strain BT is different from *D. thermosapovorans*. The most striking difference which also separates strain BT from all other members of this genus is that strain BT does not reduce sulfate, sulfite or thiosulfate, not even in the

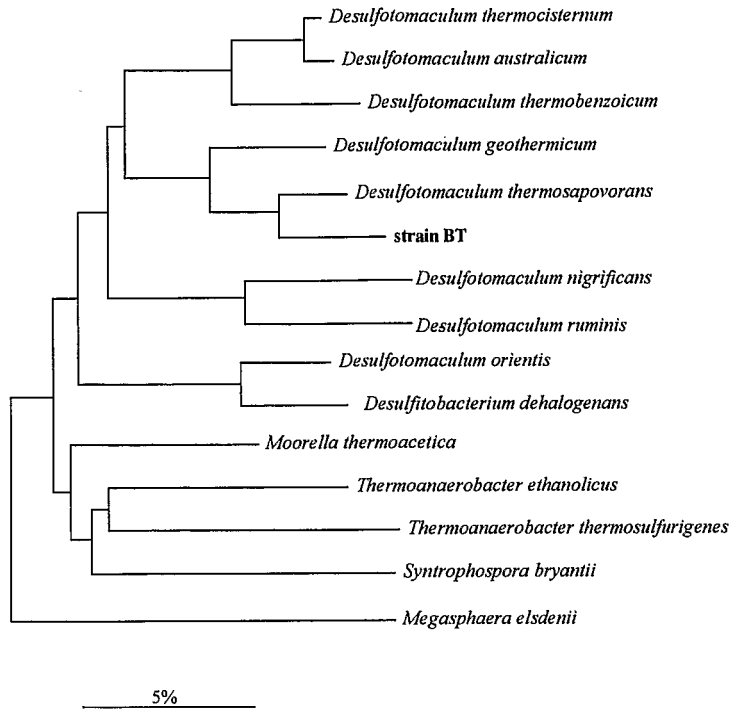

Fig. 3. Phylogenetic dendrogram indicating the position of strain BT within the radiation of the representatives of the genus *Desulfotomaculum* and related taxa. The scale bar represents 5 nucleotide substitutions per 100 nucleotides.

presence of added haemin or 1,4-naphthoquinone. Additionally, *D. thermosapovorans* utilizes a variety of organic substrates, namely long-chain fatty acids and propanediols, whereas strain BT grows only on 3-hydroxybenzoate as sole source of carbon and energy. The fact that strain BT is mesophilic but that *D. thermosapovorans* is thermophilic provides further evidence that they are two distinct species. There are also significant physiological and phylogenetical differences between strain BT and all other described members of the genus *Desulfotomaculum*. It appears therefore justified to describe strain BT as a new genus and species. A formal description follows.

Description of *Sporotomaculum* gen. nov.

Sporotomaculum (Spo.ro.to.ma'cu.lum. M.L. n. *spora* spore; L. n. *tomaculum* sausage. M.L. neut. n. *Sporotomaculum* a spore-forming sausage-shaped organism). Gram-positive, strictly anaerobic. Metabolism fermentative, inorganic electron acceptors not used. Type species is *Sporotomaculum hydroxybenzoicum*.

Description of *Sporotomaculum hydroxybenzoicum* sp. nov.

Sporotomaculum hydroxybenzoicum (hy.dro.xy.ben.-zo'i.cum. M.L. neut. adj. *hydroxybenzoicum* referring to hydroxybenzoic acid which is used as sole carbon and energy source).

Rod-shaped cells, 2·0–3·0 × 0·6–0·8 μm in size, with pointed ends, occurring singly or in pairs, Gram-positive, slightly motile in the early exponential phase. Formation of central, spherical endospores. Strictly anaerobic chemoorganoheterotroph. Growth on 3-hydroxybenzoate as sole source of carbon and energy. No growth with pyruvate, DL-lactate, succinate, fumarate, malate, citrate, valerate, pimelate, crotonate, crotonate with H_2/CO_2 atmosphere (80:20%, v/v), caproate, adipate, hexanoate, heptanoate, cyclohexane carboxylate, ethanol, methanol, D-fructose, glucose, benzoate, 2-hydroxybenzoate, 4-hydroxybenzoate, 3-aminobenzoate, 4-aminobenzoate, 2,3- and 2,5-dihydroxybenzoate, α-, β- and γ-resorcylate, protocatechuate, gallate, syringate, ferulate, caffeate, hydroxycinnamate, terephthalate, phenylacetate, 2-, 3- and 4-chlorobenzoate, phenol, catechol, resorcinol, hydroquinone, phloroglucinol, pyrogallol, 2-, 3- and 4-cresol. 3-Hydroxybenzoate is fermented to butyrate, acetate and CO_2. No reduction of nitrate, sulfate, thiosulfate, sulfite, ferric iron, oxygen or fumarate. Growth requires sulfide-reduced mineral media. Addition of small amounts of dithionite shorten the lag phase. Yeast extract and tryptone stimulate but are not required for growth. Selective enrichment in sulfide-reduced freshwater mineral medium with 3-hydroxybenzoate as substrate. Growth in the pH range of 6·8–8·1, optimum at 7·3–7·6. Temperature range: 24–37 °C, optimum around 30 °C. Cytochromes not detected. DNA G+C content: 48 mol%. Habitat: guts of *Cubitermes speciosus*. Type strain: strain BT (= DSM 5475).

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