Cellulosimicrobium variabile sp. nov., a cellulolytic bacterium from the hindgut of the termite Mastotermes darwiniensis

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A novel cellulolytic and xylanolytic bacterium, strain MX5T, was isolated from the hindgut contents of the Australian termite Mastotermes darwiniensis (Froggatt). The isolate was a facultative anaerobe and had a Gram-positive cell-wall profile. The rod-shaped bacterium formed irregular coryneform and coccoid cells during growth. Phylogenetic analysis of the 16S rDNA provided evidence that the organism was closely related to the as-yet undescribed cellulolytic strain SR272 and the non-validly described species 'Cellulomonas pachnodae' as well as Promicromonospora citrea and Promicromonospora sukumoe. Strain MX5T was assigned to the genus Cellulosimicrobium on the basis of phylogenetic and chemotaxonomic criteria. The murein of strain MX5T contained the diamino acid lysine. N-Glycolylmuramic acid, mycolic acids and hydroxy fatty acids were absent. The major neutral sugar in the cell wall was galactose and the major quinone was menaquinone MK-9(H4). The predominant fatty acids were ai-C15:0, i-C15:0, i-C16:0 and C16:0. The G+C content of the DNA was in a range 70–72 mol%. On the basis of 16S rDNA sequence similarities and chemotaxonomic features, MX5T was clearly different from Cellulosimicrobium cellulans and other validly described species within this phylogenetic group. For this reason, a novel species is described, for which the name Cellulosimicrobium variabile sp. nov. is proposed.

Keywords: Cellulosimicrobium, Cellulomonas, termites, intestinal flora, cellulose degradation

INTRODUCTION

Termites play an important role in the turnover of lignocellulolytic material. Although endogenous cellulases are active (Veivers et al., 1982; Tokuda et al., 1997; Watanabe et al., 1998), the microflora also has a significant impact on cellulose degradation (Breznak, 1982; Breznak & Brune, 1994; Varma et al., 1994). Symbiotic flagellates are major contributors to cellulose decomposition in the guts of lower termites (Cleveland, 1924; Gutierrez, 1956; Hogan et al., 1988; Hungate, 1943; Odelson & Breznak, 1985; Trager, 1934), whereas fungus-growing termites can also consume the fungal cellulases together with fungus nodules (Rouland et al., 1988).

Because many attempts to isolate cellulose degraders were unsuccessful (Cleveland, 1924; Dickman, 1931; Hungate, 1936; Schultz & Breznak, 1978), it has been concluded that bacteria are not important contributors to cellulose digestion in termites (Slaytor, 1992), and only a few cellulose-degrading bacteria have been isolated, from a few termite species (Hethener et al., 1992; Hungate, 1946; Mannesmann, 1972; Pasti & Belli, 1985; Paul et al., 1986; Saxena et al., 1993; Sebald & Prévot, 1962; Thayer, 1976).

In this study, we have identified and characterized a novel cellulolytic and xylanolytic bacterium, strain MX5T, from the Australian termite Mastotermes

Abbreviations: pNA, para-nitroanilide; pNP, para-nitrophenyl.

The EMBL accession numbers for the 16S rDNA sequences of strains MX5T, KHC4, KMaC5 and KNeC1 are respectively AJ298873, AJ315029, AJ296274 and AJ313329.
**METHODS**

**Termites.** The termites *Heterotermes indicola* (Wasman), *Mastotermes darwiniensis* (Frogatt), *Nasutitermes nigriceps* (Haldeman), *Neotermes castaneus* (Burmeister), *Schedorhinotermes intermedius* (Isoliener) and *Zootermopsis angusticollis* (Hagen) were obtained from the Bundesanstalt für Materialforschung und Materialprüfung (BAM, Berlin, Germany).

**Micro-organisms and culture conditions.** The isolation of the novel strain MX5<sup>T</sup> from gut contents of *Mastotermes darwiniensis* (Frogatt) on xylan-containing agar plates was described by Schäfer et al. (1996). *Rhodococcus rhodochrous* DSM 43241<sup>T</sup> and *Cellulosimicrobium cellulosan* DSM 43879<sup>T</sup> were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *Pseudomonas aeruginosa* strain KNB5 was isolated from the gut contents of *Nasutitermes nigriceps* (Haldeman). *Bacillus cereus* strain KD12 (Kuhnigk & König, 1997) was obtained from the gut contents of *Retiiculartermes santonensis* (Feytand). Bacteria were grown in tryptic soy broth (TSB; Difco) or on tryptic soy agar (TSA; Difco) at 28 °C. Cellulose degradation was tested on agar plates at 28 °C using the medium described by Yamin (1978) containing, in addition, 15 g agar l<sup>−1</sup>, 10 g carboxymethylcellulose l<sup>−1</sup> (instead of glucose) and 0.5 g yeast extract l<sup>−1</sup> and supplemented with vitamins and trace minerals (Balch et al., 1979). Anaerobic growth was tested in the latter medium without agar containing glucose (10 g l<sup>−1</sup>) instead of carboxymethylcellulose and supplemented with a resazurin solution (1 g l<sup>−1</sup>; 1 ml stock solution l<sup>−1</sup>) as redox indicator and Na<sub>2</sub>S (0.1 g l<sup>−1</sup>). The gas phase was N<sub>2</sub>/CO<sub>2</sub> (80:20; v/v).

**Microscopy.** Cells were observed and photographed with a phase-contrast microscope (Axioplan; Zeiss) equipped with a Plan-Neofluar objective (100×) and a camera (MC 63). Transmission electron microscopy was performed as described previously (Breunig et al., 1993).

**Physiological tests.** Physiological tests were performed as described elsewhere (Kämpfer et al., 1991). In addition, BioLog GN MicroPlates and the MicroLog computer software (BioLog identification system; Biolog Inc.) were used.

**Cell wall components.** After two cycles of washing (9 g NaCl l<sup>−1</sup>; cells collected at 4000 g, 10 min at 4 °C in an Eppendorf centrifuge ZK 401 BHG), the cells from a 500 ml culture were disrupted by two cycles through a French press (137 MPa; American Instrument Company). Residual intact cells were separated by centrifugation (4000 g; 10 min; 4 °C). Crude cell walls were harvested from the supernatant by centrifugation (45000 × g; 25 min, 4 °C, Beckman LS-60 M). The crude cell walls were suspended in 40 g SDS l<sup>−1</sup> and heated at 100 °C for 20 min. The cell walls were pelleted by centrifugation (45000 × g; 25 min, 20 °C) and washed three times with water before a final centrifugation step (45000 × g; 25 min, 20 °C) to give the cell wall preparation.

For murein analysis, the cell walls were hydrolysed with 0.3 M HCl at 100 or 120 °C for 18 h. The murein constituents were determined with an amino acid analyser (Biotronic LC 5001) programmed for murein analysis (Biotronic). For analysis of neutral sugars, the cell walls were hydrolysed with 0.5 M H<sub>2</sub>SO<sub>4</sub> at 100 °C for 2 h. The neutral sugars were separated on a Carbopac PA1 column ( Dionex) at 20 °C. NaOH (12 mM) was used as eluant at a rate of 1 ml min<sup>−1</sup>. The elution profile was recorded amperimetrically (PAD; Dionex).

In some bacteria, glycolylmuramic acid can replace acylmuramic acid. For the detection of glycolic acid, 20 mg freeze-dried cells of isolate MX5<sup>T</sup> and *Rhodococcus rhodochrous* DSM 43241<sup>T</sup> (standard) were hydrolysed (4 M HCl, 20 h, 100 °C) and further treated as described by Kutzner et al. (1986). The development of a purple colour after addition of a 2,7-naphthoindole solution was taken as indicative of the presence of glycolic acid.

**Mycolic acids.** For the detection of mycolic acids and hydroxy fatty acids, freeze-dried cells of isolate MX5<sup>T</sup> and standards *Rhodococcus rhodochrous* DSM 43241<sup>T</sup> (mycolic acids C<sub>45</sub>−C<sub>55</sub>; Rf = 0.36), *Pseudomonas aeruginosa* (2-hydroxy fatty acid methyl esters, Rf = 0.16; 3-hydroxy fatty acid methyl esters, Rf = 0.22) and *Bacillus cereus* (fatty acid methyl esters, Rf = 0.84) (Kutzner et al., 1986) were used. After transmethylation of the fatty acids, the corresponding methyl ester derivatives were extracted with n-hexane. The fatty acid methyl esters were separated on activated (20 min, 120 °C) silica G60 plates. A mixture of petrolether and diethylether (85:15, v/v) was used as eluant. Compounds were detected with molbydate reagent (Merck).

**Quinones.** Menaquinones were extracted from 100 mg freeze-dried cells of isolate MX5<sup>T</sup> or *Cellulosimicrobium cellulosan* DSM 43879<sup>T</sup> (standard) as described by Kutzner et al. (1986). Menaquinones were separated on silica GF 60 plates using a mixture of petrolether and diethylether (85:15, v/v) as eluant. The UV-positive bands were eluted with diethylether. The eluted menaquinones were further separated on a Nucleosil 100-5 C18 column (Macherey-Nagel) at 40 °C. The eluant was a mixture of acetonitrile and 2-propanol (60:35, v/v). The elution rate was 1 ml min<sup>−1</sup>. An additional separation was performed on a Nucleosil 100-10 SA column (Macherey-Nagel) at 50 °C with methanol at a flow rate of 1 ml min<sup>−1</sup>. With the latter column, menaquinones can be separated according to the position of the double bond. The elution profiles were recorded at 254 nm.

**Phospholipids.** Phospholipids were extracted from freeze-dried cells as described by Kutzner et al. (1986) and separated on silica G60 plates. Chloroform/methanol/water (65:25:4, by vol.) and chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) were used as eluants. Detection of glycolipids was carried out with anisaldehyde, ninhydrin, α-napthol and molbydate reagents (Kutzner et al., 1986).

**Fatty acids.** Fatty acid methyl esters were extracted and prepared by the standard protocol of the Microbial Identification System (MIDI; Microbial ID, Inc.). Extracts were analysed by using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector as described previously (Kämpfer & Kroppeinstedt, 1996).

**Determination of the G + C content of the DNA.** Genomic DNA was isolated as described by Johnson (1994). The DNA was digested with P1 nuclease and the nucleotides were separated on a Nucleosil NH<sub>4</sub> column (300 × 4 mm; Macherey-Nagel). Potassium phosphate buffer (25 mM),
pH 3.4, was used at an elution rate of 1.5 ml min⁻¹. DNA from calf thymus (G+C = 42 mol%) and from Escherichia coli (G+C = 50 mol%) served as standards.

**Amplification, cloning and sequencing of 16S rDNA.** Genomic DNA was isolated with InstaGene DNA purification matrix (Bio-Rad). The primers Eubak5 and Eubak3 (Berchtold et al., 1994) were used for 16S rRNA gene amplification (30 cycles: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min) resulting in nearly full-length 16S rDNA amplification products. The amplified DNA was checked by agarose gel electrophoresis and purified with the QIAquick PCR purification kit (Qiagen). The purified PCR products were ligated into pBluescript SK(+) (Stratagene) and cloned in E. coli K-12 DH5α. The plasmid was isolated with the QIAquick plasmid purification kit (Qiagen). Sequencing of the 16S rRNA gene was carried out as described by Berchtold & König (1996).

**Phylogenetic analysis.** The resulting 16S rDNA sequence was compared to sequences from the RDP (Maidak et al., 2000) and EMBL (Baker et al., 2000) databases to find the most closely related species. CLUSTAL W (Thompson et al., 1994) was used to align the MX5T sequence in the prokaryotic 16S rRNA alignment from the RDP (Maidak et al., 2000) followed by manual checking of the resulting alignment. A dataset of 26 sequences with 1458 nucleotide positions that could be aligned unambiguously was used for the construction of phylogenetic trees by distance matrix (Fitch & Margoliash, 1967; Saitou & Nei, 1987), parsimony and maximum-likelihood methods (Felsenstein, 1981) with DNADIST, NEIGHBOR, FITCH, DNAPARS and DNAML from the PHYLIP package (Felsenstein, 1993). Tree topologies were evaluated with bootstrap analyses (Felsenstein, 1985) using SEQBOOT from the PHYLIP package.

**RESULTS AND DISCUSSION**

**Morphology**

Isolate MX5T stained Gram-positive and possessed a typical Gram-positive wall structure with a thickness of about 20 nm (Fig. 1). Cultures were composed of rod-shaped cells (about 0.3 × 0.7 μm; Fig. 1) or V-shaped cells and formed cocci (about 0.25 μm in diameter) after 3 days (Fig. 2). Spores and motility were not observed. Cells grew as smooth, yellow colonies on TSA with a diameter of about 2 mm after 48 h at 28°C.

**Physiological characteristics**

The isolation and xylanase production of isolate MX5T were described by Schäfer et al. (1996). We found that strain MX5T also formed clear zones on carboxymethylcellulose-containing agar plates, indicating the production of cellulolytic activities. Strain MX5T also grew anaerobically. The isolate showed acid production from various sugars and related com-
pounds and several sugars were also utilized as sole sources of carbon (see below). Only a few organic acids, including amino acids, were utilized (for details, see species description below). Most of the tested p-nitrophenyl (pNP) derivatives were hydrolysed (see below).

G + C content of the DNA

The G + C content of the DNA of strain MX5T was within the range 70–72 mol%.

Cell wall composition

The peptidoglycan of isolate MX5T was composed of the amino acids lysine, aspartic acid, glutamic acid and alanine in a molar ratio of 1:0:0:9:2:0:1:8. When the cell walls were hydrolysed at 100 °C, partial hydrolysis products contained ε-(aminosuccinyl)-lysine (Schleifer & Kandler, 1972), indicating the presence of peptido-glycan type A4α. For complete hydrolysis, 120 °C was required. Glycic acid and mycolic acids were not detected. The main neutral sugar of the cell walls was galactose. The neutral sugars galactose, rhamnose and glucose were found in a molar ratio of 4:2:1.

Components of the cytoplasmic membrane

Hydroxy fatty acids were detected in Pseudomonas aeruginosa (used as a reference standard), but were not found in isolate MX5T. The major lipids detected were phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine. The major menaquinone of isolate MX5T co-migrated under all the chromatography systems tested with the major menaquinone of Cellulosimicrobium cellulans DSM 43879T (used for comparison), which was menaquinone MK-9(H2) (Stackebrandt & Keddie, 1986).

Strain MX5T contained the fatty acids ai15:0 (53.6 mol%), i15:0 (170 mol%), 16:0 (72 mol%), i16:0 (6.0 mol%), ai17:0 (70 mol%), 14:0 (66 mol%), i14:0 (15 mol%), i17:0 (06 mol%) and 15:0 (05 mol%). A similar fatty acid profile has been described for Cellulosimicrobium cellulans DSM 43879T (Funke et al., 1995).

Assignment of isolate MX5T to Cellulosimicrobi um

The partial 16S rDNA sequence (1472 bp) of strain MX5T was aligned with the sequences of 25 other related strains obtained from the RDP (Maidak et al., 2000). The alignment was used for phylogenetic analysis with distance-matrix, parsimony and maximum-likelihood methods. All trees obtained were nearly identical to the maximum-likelihood tree depicted in Fig. 3. The tree shows that strain MX5T clustered together with two strains from the EMBL nucleotide sequence database (strain SR272, 99% sequence similarity; ‘Cellulosimicrobium pachnodae’ strain VPCX2, 97% sequence similarity). In addition, the rRNA of both Promicromonospora citreus DSM 43110T and Cellulosimicrobium cellulans DSM 43879T showed 96% similarity to MX5T.

On the basis of the Gram-staining characteristics, cell-wall composition and G + C content, the facultative anaerobic isolate MX5T was assigned to the actinomycete branch of the Gram-positive bacteria.

N-Glycocolymuramic acid is found in coryneforms with urein type B (Aureobacterium, Micr o bacterium), in actinomycetes possessing mycolic acids (Myco bacterium, Nocardia, Rhodococcus) and among the Actinoplanaceae and Micromonosporaceae. The absence of both glycic acid and mycolic acids indicated that isolate MX5T did not belong to these genera.

The presence of cellulolytic activities and cell walls composed of the sugars galactose, rhamnose and
glucose indicated that the organism belonged to one of the families Cellulomonadaceae and Promicromonosporaceae.

Strain MX5T, together with the as-yet undescribed strain SR272, formed a separate subgroup, clustering close to the Cellulosimicrobium branch of the family Cellulomonadaceae (Rainey et al., 1995; Schumann et al., 2001). Therefore, we propose to assign isolate MX5T to a novel species of the genus Cellulosimicrobium, Cellulosimicrobium variabile sp. nov. The rationale for the assignment of the novel isolate MX5T to the genus Cellulomicrobium rather than Cellulomonas or Promicromonospora was based on distinguishing phenotypic features in addition to 16S rDNA sequence data. Like Cellulosimicrobium strains (Stackebrandt et al., 1978), strain MX5T possesses the peptidoglycan type A4z (with L-lysine as diamino acid and aspartate in the interpeptide bridge). This feature is not found in strains of authentic Cellulomonas species, which possess ornithine as diamino acid, with the exception of Cellulomonas humilata ATCC 25174T (Collins & Pascual, 2000), which contains both ornithine and lysine. Both Cellulosimicrobium cellulans DSM 43879T and strain MX5T contain galactose in their cell walls. In contrast, most authentic Cellulomonas species, with the exception of Cellulomonas biazoae, do not contain galactose.

Isolate MX5T possesses 16S rDNA sequence similarity of 95–96% to species of the related genus Promicromonospora, but can be distinguished from this genus by phenotypic features. The species of the genus Promicromonospora form persistent branching substrate and aerial mycelia which can undergo fragmentation. Promicromonospora citrea and Promicromonospora sukumoe are strict aerobes, while Promicromonospora enterophila is a facultative anaerobe. None of the three Promicromonospora species possesses an A4z-type peptidoglycan with aspartic acid in the interpeptide bridge (Kalakoutskii et al., 1989; Takakashi et al., 1987; Schumann et al., 2001).

Interestingly, the closely related non-validly described species ‘Cellulomonas pachnodae’ strain VPCX2 was isolated from the gut of the rose-chafer Pachnoda marginata, which, like termites, feeds on cellulose-rich plant material (Cazemier et al., 1999). Strain VPCX2 also possesses cellulolytic activity. Whether this indicates that this phylogenetic group is specially adapted to the gut systems of plant-feeding insects remains to be examined.

In conclusion, according to the available 16S rDNA sequence data, MX5T does not possess a sequence similarity of more than 96% to any validly described species within the radiation of the Cellulosimicrobium and Cellulomonas lineages. Organisms with less than 97% 16S rDNA sequence similarity possess a DNA–DNA reassociation of less than 60% (Stackebrandt & Goebel, 1994; Fröhlich et al., 1999). In addition, there are several phenotypic features that allow the clear differentiation of MX5T from Cellulosimicrobium cellulas (Table 1). On the basis of these results, a novel species is described, for which the name Cellulosimicrobium variabile sp. nov. is proposed.

**Distribution of cellomonads in the termite gut**

Strains related to Cellulomonas cartae (reclassified as Cellulosimicrobium cartae; Schumann et al., 2001) (100% 16S rDNA sequence similarity; 400–450 nucleotides) were isolated from Mastotermes darwiniensis (strain KMaC5), Neotermes castaneus (strain KNeC1) and Heterotermes indicola (strain KHC4). Other strains showing the same 16S rDNA restriction fragment length pattern with restriction enzymes BsuRI and HpaII (BsuRI: 290–220–150; HpaII: 600–180) as the above-mentioned Cellulosimicrobium cellulans-related strains were obtained from Mastotermes darwiniensis (strain X6), Schedorhinotermes intermedius (strains KScC2, KScC6), Nasutitermes nigriceps (strain NA2) and Zootermopsis angusticollis (strain ZC1). Strain KScC3 from Schedorhinotermes intermedius had the same 16S rDNA restriction fragment length pattern (BsuRI: 310–240–150; HpaII: 480–200) as Cellulosimicrobium variabile strain MX5T. This indicates that Cellulosimicrobium-related strains are widespread in termites.

**Description of Cellulosimicrobium variabile sp. nov.**

Cellulosimicrobium variabile (va.ri.a’bi.le. L. neut. adj. variabile variable, because the cells form rods or cocci).

Cells are rod-shaped (0.3 × 0.7 μm), V-shaped or coccoid (diameter 0.25 μm) and non-motile. Cells stain Gram-positive. Yellow, smooth colonies on TSA (Difco) about 2 mm in diameter after 48 h at 28 °C. Facultatively anaerobic, cellulolytic and xylanolytic activities are produced. The murein contains the amino acids lysine, aspartic acid, glutamic acid and alanine in a molar ratio of 1:0:9:2:0:1:8 and belongs to the peptidoglycan type A4z. N-Glycolylmuramic acid, mycolic acids and hydroxy fatty acids are absent. The neutral sugars galactose, rhamnose and glucose are found in a molar ratio of 4:2:1. The main quinone is menaquinone MK-9(H2). Major fatty acids are ai15:0 (53-6 mol %), i15:0 (17-0 mol %), 16:0 (7-2 mol %), i16:0 (6-0 mol %), ai17:0 (7-0 mol %), 14:0 (6-6 mol %), i14:0 (1-5 mol %), i17:0 (0-6 mol %) and 15:0 (0-5 mol %). Acid is produced from d-glucose, l-arabinose, raffinose, l-rhamnose, d-xyllose, d-cellbiose, d-melibiose, d-arabitol and d-mannose. No acid production is observed from sucrose, d-mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, maltose, methyl β-D-glucoside or erythritol. The following substrates are used as sole sources of carbon: N-acetyl D-glucosamine, l-arabinose, p-arbutin, d-cellbiose, D-fructose, D-galactose, gluconate, D-glucose, D-maltose, D-mannose, d-melibiose, l-rhamnose, D-ribose, sucrose, salicin, D-trehalose, maltitol, mannitol, sorbitol, acetate, fumarate (weak), D-L-hydroxybutyrate, pyruvate, l-histidine and L-proline. N-Acetyl D-
The 16S rDNA sequence of the DNA is 70–72 mol%. The 16S rDNA sequence of strain $\text{MX}^T$ (accession number AJ298873) has high similarity to those of two as-yet undescribed isolates, strain SR 272 (99%; accession number X87317) and strain VPCX2 (‘Cellulomonas pachnodae’; host, larvae of the rose-chafer $\text{Pachymeria marginata}$) (97%; accession number AF105422) from the EMBL nucleotide sequence database. Strains can be distinguished from Cellulosimicrobium cellulans and from Cellulomonas species by the phenotypic features compiled in Table 1.

Habitat: hindgut contents of the Australian termite Mastotermes darwiniensis (Froggatt). The type strain is $\text{MX}^T$ (= DSM 10177$^T$ = ATCC BAA-303$^T$).

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Table 1. Phenotypic characteristics that differentiate strains of the genera Cellulosimicrobium and Cellulomonas

Strains: 1, $\text{MX}^T$; 2, Cellulosimicrobium cellulans DSM 43879$^T$; 3, Cellulomonas fermentans DSM 3133$^T$; 4, Cellulomonas humilata ATCC 25174$^T$; 5, Cellulomonas hominis DSM 9581$^T$; 6, Cellulomonas persica ATCC 700642$^T$; 7, Cellulomonas iranensis ATCC 700643$^T$; 8, Cellulomonas biazotea DSM 20112$^T$; 9, Cellulomonas cellasea DSM 20118$^T$; 10, Cellulomonas flavigena DSM 20109$^T$; 11, Cellulomonas fimi DSM 20113$^T$; 12, Cellulomonas gelida DSM 20111$^T$; 13, Cellulomonas uwa DSM 20107$^T$. Phenotypic characteristics of described species are from Elberson et al. (2000), Funke et al. (1995), Kämpfer et al. (1993), Schaal (1986) and Stackebrandt & Keddie (1986). Abbreviations: ND, not determined; +, positive; +, strongly positive; –, negative; Lys, L-lysine; Orn, L-ornithine; Y, yellow; Y-W, yellow-white; w, white.
(BAM, Berlin) for the termite cultures. We thank Peter Schumann and Erko Stackebrandt for information on the proposal of reclassification of Cellulomonas cellulans in the new genus Cellulosimicrobium prior to publication.

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