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Ereboglobus luteus gen. nov. sp. nov. from cockroach guts, and new insights into the oxygen relationship of the genera *Opitutus* and *Didymococcus* (*Verrucomicrobia*: *Opitutaceae*)

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ABSTRACT

We isolated a novel member of the phylum *Verrucomicrobia* from the hindgut of the cockroach *Shelfordella lateralis*. Strain Ho45 is a yellow-pigmented, motile coccus that represents a new genus-level lineage with less than 93% sequence similarity to the 16S rRNA genes of other species in the family *Opitutaceae*. Ultrastructural analysis revealed a Gram-negative cell envelope with an outer membrane and a periplasmic space. In its ability to ferment sugars to propionate and acetate as major products, strain Ho45 resembles its closest relative, *Opitutus terrae*. However, the strains differed in their relationship to oxygen. Although strain Ho45 grew and consumed oxygen at sub-atmospheric concentrations (1–4%), both growth rate and cell yield decreased strongly with increasing oxygen concentration in the headspace. By contrast, *O. terrae*, previously described as an obligate anaerobe, proved to be facultatively aerobic, with highest growth rates and cell yields at 2% and 16% oxygen, respectively. Also the closely related *Didymococcus* (*Diplosphaera*) *colitermitum*, previously described as an obligately aerobic microaerophile, showed a fermentative metabolism under anoxic conditions, forming the same products from glucose as strain Ho45 and *O. terrae*. Based on phenotypic and phylogenetic evidence, we propose strain Ho45 as the type strain of a novel genus, *Ereboglobus luteus* gen. nov. sp. nov., and provide an emended description of the family *Opitutaceae* and the genera *Opitutus* and *Didymococcus*.

Introduction

The phylum *Verrucomicrobia* is presently composed of seven class-level groups, some of them still without or with only few cultured representatives [21]. The family *Opitutaceae* was proposed by Choo et al. [12] based on a comparative analysis of the 16S rRNA gene of few isolated microorganisms and a large number of sequences of the former verrucomicrobial subdivision 4 obtained from various environments, including terrestrial and aquatic habitats [21]. Furthermore, 16S rRNA gene sequences of *Opitutaceae* have been detected among the gut microbiota of invertebrates, such as termites and ants (e.g., Refs. [24,29,64,31]).

In comparison to other *Verrucomicrobia* families, the number of sequences classified as *Opitutaceae* is rather small [14]. The few isolates of *Opitutaceae* described so far are Gram-negative, non-spore-forming cocci with a relatively high G+C content of 60.5–67.0 mol% [49,33,44] and appear to differ considerably in their physiology, particularly their energy metabolism and response to oxygen.

Alterococcus agarolyticus, a moderate thermophile isolated from a hot spring [54], and *Opitutus terrae*, a mesophile isolated from rice field soil [10], have been described as facultatively or obligately anaerobic bacteria that form propionate as a major fermentation product. By contrast, *Didymococcus* (formerly *Diplosphaera*) *colitermitum*, a microaerophilic isolate from termite guts [64,65] that shows optimal growth rates between 2–8% oxygen, has been described as an obligate aerobe that possesses a cytochrome cbb3 oxidase [64,26]. The same is true for strain TAV1, a so far undescribed relative of *D. colitermitum* isolated from the same termite

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species [58,64]. Also members of the recently described genera *Cephaloticoccus* (isolated from ant guts) and *Lacunisphaera* (isolated from a freshwater lake) are considered to be aerobic [33,44].

Here, we report the isolation and detailed ultrastructural characterization of a novel member of *Opitutaceae* from the hindgut of the cockroach *Shelfordella lateralis*. A comparative analysis of its energy metabolism to that of *O. terrae* and *D. colitermitum* provides new insights into the oxygen relationship of members of the *Opitutaceae*. Based on phenotypic and phylogenetic evidence, we propose that strain Ho45 be classified as a species of a novel genus and provide emended descriptions of the family *Opitutaceae* and the genera *Opitutus* and *Didymococcus*.

Materials and methods

Microbiological media

All microorganisms used in this study were cultivated in medium AM-5, an anoxic, bicarbonate-buffered mineral medium supplemented with vitamins and other growth factors [60], unless indicated otherwise. The medium was routinely amended with yeast extract and Casamino acids (0.1% each), cysteine and DTT (1 mM each) as reducing agents, and resazurin (0.8 mg/L) as redox indicator. This "basal medium" was dispensed (4.7 ml) into 16-ml rubber-stoppered culture tubes and gassed with a headspace of N_2/CO_2 (80:20, v/v). Glucose (5 mM) was routinely added as substrate, the medium was inoculated with a fresh preculture (0.3 ml), and tubes were incubated at 30 °C.

The pH range of growth was analyzed with cultures grown in MM-5 medium [60], which received the same amendments as AM-5 "basal medium" and was incubated under a N_2 headspace (100%). The pH of the medium was adjusted by adding alternative buffer systems: sodium phosphate buffer, pH 2.0, 6.0, 7.0, and 12.0; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0; sodium acetate buffer, pH 4.0; and sodium carbonate buffer, pH 9.0 and 10.0; each at a final concentration of 20 mM.

Growth on Fastidious Anaerobe Agar [2] was tested by transferring the strains to the agar plates (under air) and incubating them under air or in an anoxic jar under N_2/CO_2 (80:20, v/v) headspace. Additional growth tests were done in liquid R2 B medium, which was identical to R2A [45] but lacked agar and was incubated under N_2 headspace.

Isolation and cultivation

S. lateralis was obtained from a commercial breeder and maintained on a diet of chicken feed (Gold Plus, Versele-Laga, Deinze, Belgium) as previously described [51]. An adult female cockroach was dissected and the hindgut was placed in a 16-ml culture tube with 1 ml basal medium and 1 g of 2-mm glass beads. The tube was closed with a rubber stopper, gassed with N_2/CO_2, and vortexed for 10 min. The resulting gut homogenate was serially diluted in AM-5 basal medium, and pure cultures were subsequently isolated by two consecutive agar dilution series [42], which contained AM-5 basal medium amended with 1% washed agar.

O. terrae (DSM 11246) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *D. colitermitum* (ATCC BAA-2264) and strain TAV1 were kindly provided by J.L.M. Rodrigues of the University of Texas, Arlington (Texas, USA). Purity and identity of all cultures were confirmed by microscopy and 16S rRNA gene sequencing with *Bacteria*-specific primers [59].

It is noteworthy that the culture of strain TAV1 included in this study is not the same as the original isolate (AY587231; [58,64]). Its 16S rRNA gene sequence is identical to those in the genomes of strain TAV1 (AHKS00000000; [25]) and strain TAV5 (CP007053; [30]). Based on tetranucleotide usage patterns and average nucleotide identity, Rodriguez and Isanapong [49] concluded that the genomes reported for the isolates TAV1 and TAV5 belong to the same species.

Growth and physiology

Growth on various sugars, sugar alcohols, sugar acids, carboxylic acids, amino acids (each 10 mM; carboxylates supplied as sodium salts), cellulose (0.2%; Sigmacell, type 20, Sigma–Aldrich, Hamburg, Germany), starch (0.2%; potato, soluble GR for analysis, Merck, Darmstadt, Germany), and pectin (0.5%; Pectin C, Roth, Karlsruhe, Germany) was tested in basal medium under anoxic conditions.

The temperature range for growth was determined between 10 and 45 °C at 5-degree intervals, and at 37 °C. Oxygen tolerance was tested in tubes with non-reduced medium (without DTT and cysteine), which received different amounts of oxygen in the headspace (added through a sterile filter; 0.2 μm pore size) and were incubated on a roller mixer (60 rpm). Salt tolerance was tested under anoxic conditions by adding different amounts of NaCl (0–4%, at intervals of 0.5%) to the medium.

Growth was measured photometrically by following the increase in optical density at 578 nm (OD_{578}) using a culture tube photometer (Spectronic 20$^+$, Milton Roy; path length 1.3 cm). For dry weight determination, cultures were grown in 500 ml basal medium with glucose (10 mM). The cells were harvested by centrifugation (14,000 × *g*; 10 min), washed with ammonium acetate solution (20 mM), and dried overnight at 80 °C.

Gram reaction was tested by Gram staining according to Murray et al. [38] and by the KOH method [20]. *Bacillus subtilis* JH642 and *Escherichia coli* DH5α were used as controls. Motility was tested in stab culture tubes containing AM-5 basal medium amended with glucose (5 mM) and washed agar (1%) under a headspace of N_2/CO_2 (80:20, v/v). Oxidase activity was tested with liquid cultures using oxidase test strips (Bactident, Merck, Darmstadt, Germany), using *B. subtilis* and *E. coli* as controls. The presence of catalase was assayed by observing gas formation upon adding a drop of H_2O_2 (3%) to bacterial cell pellets that were harvested by centrifugation; *E. coli* and *Clostridium mangenotii* (DSM 1289) were used as controls.

Utilization of alternative electron acceptors was tested in basal medium amended with 5 mM glucose and 5 mM sulfate or nitrate. Nitrite formation was analyzed using test strips (Quantofix, Macherey-Nagel, Düren, Germany). Sulfide was analyzed colorimetrically after precipitation with zinc acetate (1 mM), using the methylene blue method of Cline [13].

Metabolic products

The cell-free supernatants of fully grown cultures (centrifuged at 14,000 × *g* for 10 min) were acidified with H_2SO_4 (50 mM final concentration) and analyzed using an HPLC system equipped with an ion-exclusion column and a refractive index detector [51]. Hydrogen concentrations in the culture headspace were analyzed by gas chromatography using a molecular sieve column and a thermal conductivity detector [52]. Products formed in controls without glucose were subtracted.

Since the bicarbonate buffer did not allow the analysis of CO_2 formation, carbon recovery was calculated with the assumption that acetate production was accompanied by the formation (and malate or succinate production by the consumption) of one CO_2. For the calculation of electron recovery, all metabolites were formally oxidized to CO_2, and the number of valence electrons theoretically released from the respective amounts of products was compared with that of the dissimilated substrate [61].

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