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'Candidatus Troglogloea absoloni' - novel lineage of *Nitrospirae*, forming sprout-like bacterial aggregates in Dinaric Karst subterranean stream

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Subterranean habitats, including karst caves, are unique environments characterized by constant climatic parameters, absence of light and a limited availability of nutrients. Although cave microorganisms were often neglected, several microorganisms capable of formation of macroscopic formations were described and characterized in cave environments in past few decades [1].

One of the earliest mentions of macroscopic microbial structures in cave environments dates almost a century ago, when gross morphology of whitish, sprout-like aggregates attached to rocky streambed of subterranean stream of cave Vjetrenica in Herzegovina were described [2]. A single sprout-like aggregate measured 1–2 cm in length, several millimeters in diameter at the base and narrows progressively towards the tip. The aggregates occurred in groups, ranging from few to thousands, covering considerable parts of the stream bed in meadow-like formations.

Since these aggregates were not further characterized after initial description, we applied the combination of transmission electron microscopy (TEM), field emission scanning electron microscopy (FE-SEM), cloning, sequencing and fluorescent *in situ* hybridization (FISH) to describe the architecture, ultrastructure and microbial composition of these formations.

For scanning electron microscopy the samples were fixed in 1% glutaraldehyde and 0,4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7,2) at the sampling spot. Samples were postfixed OsO_4, dehydrated in a graded series of ethanol, critical point dried mounted on aluminum holders, sputter coated with platinum and examined by Jeol JSM-7500F FESEM. The samples for transmission electron microscopy were fixed in 3,5% glutaraldehyde in 0,1 M sodium cacodylate buffer (pH 7,2). Prior to embedding in Agar 1000 medium the samples were washed, postfixed and dehydrated as described above. After staining with uranyl acetate and lead citrate, the ultrathin sections were examined with Philips CM100 microscope. For fluorescence *in situ* hybridization (FISH) the samples were fixed in 4% paraformaldehyde in 0,1 M sodium phosphate buffer (pH 7,2), dehydrated and embedded in paraffin. Histological sections were rehydrated in ethanol series and hybridized to oligonucleotide probes that target the largest phylogenetic groups detected by sequence analysis [3] and observed on AxioImager Z.1 microscope (Zeiss) upgraded by Apotome system, for optical sectioning by structured illumination [3].

Ultrastructural analysis revealed complex structure of sprout-like aggregates. Their core consists of rod-shaped bacteria embedded in a thick extracellular matrix (Figure 1A). A closer examination of these bacteria revealed small protuberances on the cell surface, expansions of periplasmic space, as well as electron-dense spherical inclusions and translucent vesicles in the cytoplasm (Figure 1B). The core of the aggregates is covered by an electron denser crust, consisting of mineral inclusions and complex community of predominantly of filamentous bacteria (Figure 2A). The crust is thicker at the base and gets gradually thinner towards the tip of the aggregate, where it is completely missing, exposing the rod-shaped bacteria in the core (Figure 2B). FISH experiments and phylogenetic analysis based on 16S rRNA affiliated the rod-like bacteria in the core to novel lineage of the bacterial phylum *Nitrospirae* provisionally named "*Candidatus* Troglogloea absoloni", while most of bacteria in the crust was successfully hybridized by probe specific to *Betaproteobactera*. (Figure 3)

Although a possible ecological role of *Ca. Troglogloea absoloni* remains unknown, the surprising discovery of this novel *Nitrospirae* lineage in the sprout-like formations demonstrates our limited knowledge of the microbial biodiversity in subterranean habitats and contributes to biodiversity of subterranean ecosystems in Dinaric Karst, which is already recognized as one of the reaches in the world.

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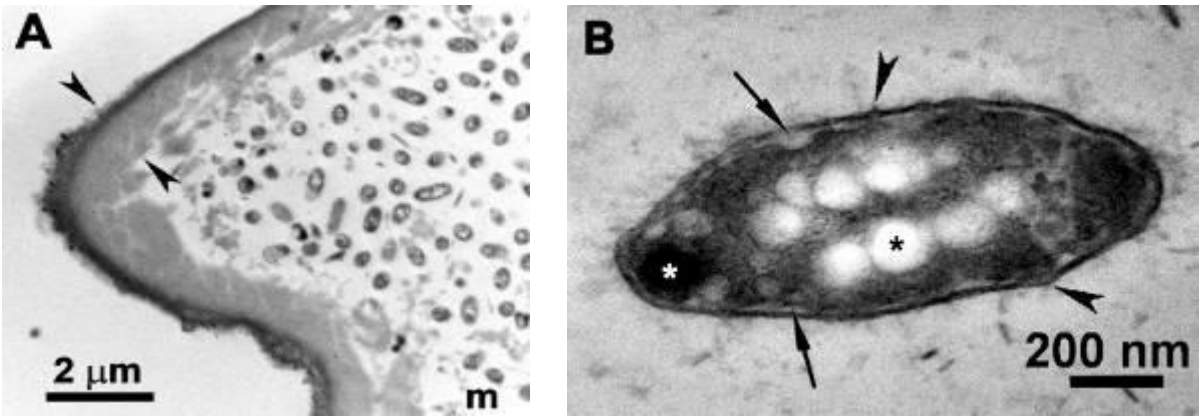

Figure 1. A. Cross-section of single sprout-like aggregate, showing rod -like bacteria embedded in extracellular matrix (m) and covered by crust (between arrowheads). B. Ultrastructure of single rod-like bacterium, showing protuberances on its surface (black arrowheads), expansions of periplasmic space (black arrows), cytoplasmic vacuoles (black asterisk) and dense inclusions (white asterisk).

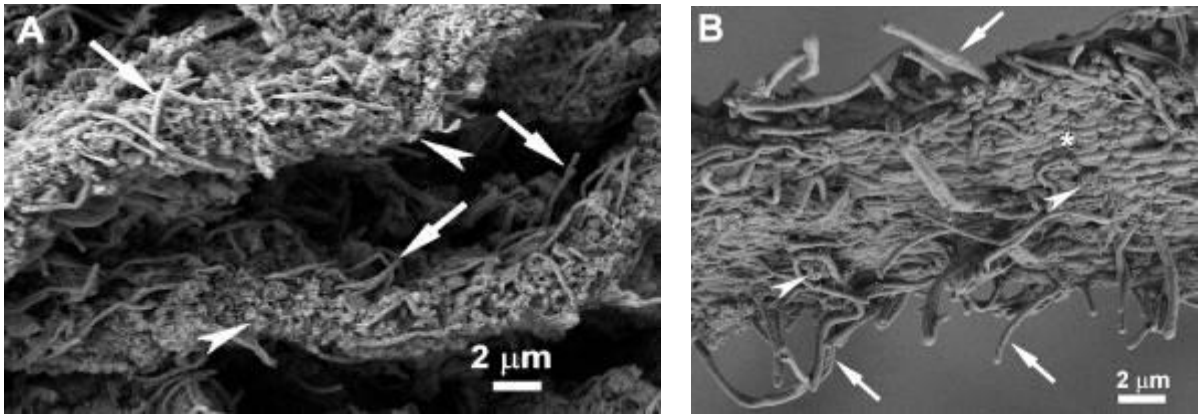

Figure 2. A. Basal part of sprout-like aggregate covered by crust consisting filamentous bacteria (arrows) and mineral inclusions (arrowheads). B. Apical part of the aggregate with exposed rod-like bacteria in the core (asterisk), filamentous bacteria (arrows) and mineral inclusions (arrowheads) on the surface.

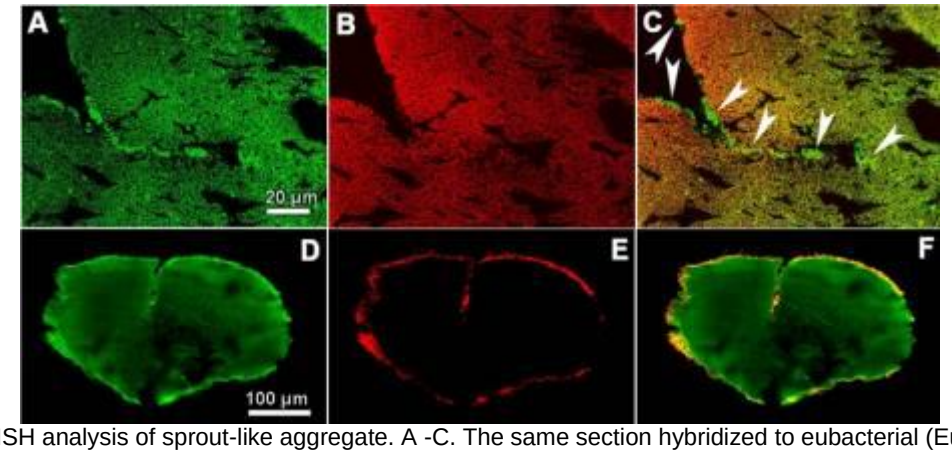

Figure 3. FISH analysis of sprout-like aggregate. A -C. The same section hybridized to eubacterial (Eub338) probe (A) or to probe specific to Ca. *T. absoloni* (B). A combination of both images (C) shows the bacteria not hybridized by probe specific to Ca. *T. absoloni* on the surface of aggregate (arrows). Panels D to F show the same section hybridized to eubacterial probe (D) or to probe specific for *Betaproteobacteria* (E). A combination of both images (F) shows the absence of *Betaproteobacteria* in the core and their location on the surface of the sprout-like structure.