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Microbial assemblages within crustacean calcium bodies

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Different crustacean groups have evolved various forms of transient calcium deposits, enabling them to minimize calcium loss during molt [1]. Trichoniscids, a group of terrestrial isopod crustaceans, store calcium in special organs, the calcium bodies (Figure 1). These organs are epithelial sacs containing mineral concretions and are located in the hemolymph space alongside the digestive system [2,3,4]. In our recent work on the subterranean trichoniscid *Titanethes albus,* we established that calcium bodies contain high densities of bacteria [4]. Unlike most animal tissues, which provide a constant environment for symbiotic microorganisms, periodical deposition and resorption of minerals in calcium bodies subjects the microorganisms within them to high solvent concentrations and strong fluctuations of their chemical environment.

Since the observation of bacteria within calcium bodies evoked further questions, we aimed to establish which microorganisms inhabit the calcium bodies, whether they are present in different crustacean species and whether or not calcium bodies are colonized by a single microbial species or by a complex microbial community.

To confirm the presence of microorganisms within calcium bodies and classify them to the level of domain, we used fluorescence *in situ* hybridization on paraffin sections of fresh *T. albus* specimens fixed overnight in formaldehyde. We used Cy3-labeled fluorescent probes complementary to archeal or eubacterial 16S rRNA sequences.

The morphological characteristics of microorganisms within *T. albus* calcium bodies were studied with scanning electron microscopy (SEM). For this, calcium bodies were isolated from narcotized specimens of *T. albus* and immediately fixed in a mixture of glutaraldehyde and formaldehyde in cacodylate buffer. The organs were then postfixed with OsO_4, dried in HMDS, sputter coated with platinum and observed with a JSM-7500F field emission scanning electron microscope (JEOL). For ultrastructural observations of the bacteria with transmission electron microscopy (TEM), *T. albus* specimens were dissected, fixed as described above, embedded in Spurr's resin and sectioned. Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed with a CM 100 transmission electron microscope (FEI).

To investigate the possible presence of bacteria in calcium bodies of trichoniscids other than *T. albus*, specimens of *Hyloniscus riparius*, *Haplophthalmus mengii*, *Trichoniscus noricus* and *Androniscus roseus* were prepared as described above, sectioned and observed with TEM. For SEM observation of small species in which manipulation of calcium bodies is difficult, whole animals were fixed, embedded in paraffin and sectioned. Sections and cut paraffin blocks were then deparaffinized and prepared for SEM observation as described for *T. albus*.

As demonstrated with *in situ* hybridization, calcium bodies of *T. albus* are inhabited by eubacteria. TEM and SEM analyses showed that the calcium bodies of this species contain predominantly rod-shaped Gram-negative bacteria. The morphological examination demonstrated the presence of several bacterial morphotypes within an individual calcium body (Figure 2), indicating the presence of a diverse bacterial community rather than a single species. This observation was further supported by preliminary analyses of 16S rDNA clone libraries constructed from DNA isolated from *T. albus* calcium bodies. Further analyses of bacterial communities within calcium bodies and additional work on how bacteria colonize these organs during ontogenetic development are needed; however, the consistent presence of large bacterial populations in calcium bodies of all examined trichoniscids strongly indicates that bacteria are involved in calcification processes in this group of crustaceans.

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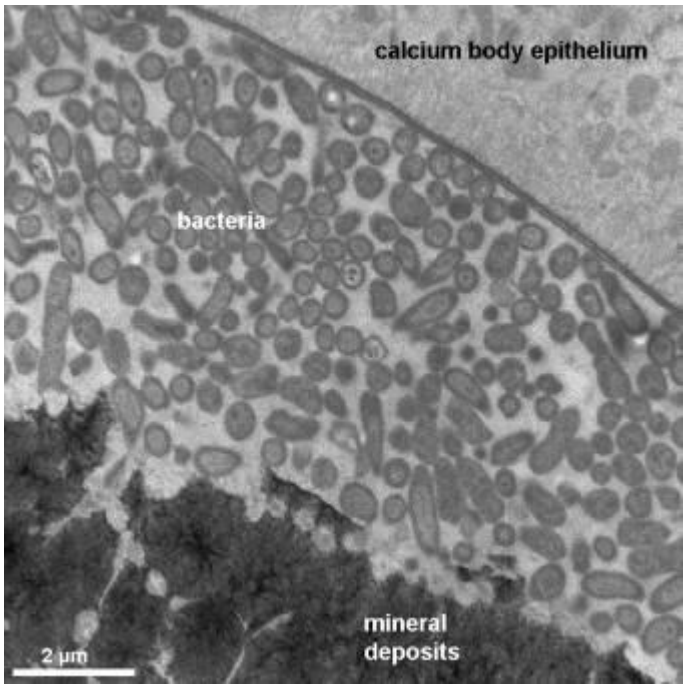

Figure 1. Section through a posterior calcium body of *Hyloniscus riparius*. Bacteria fill the lumen of the organ and surround the mineral deposits at its center.

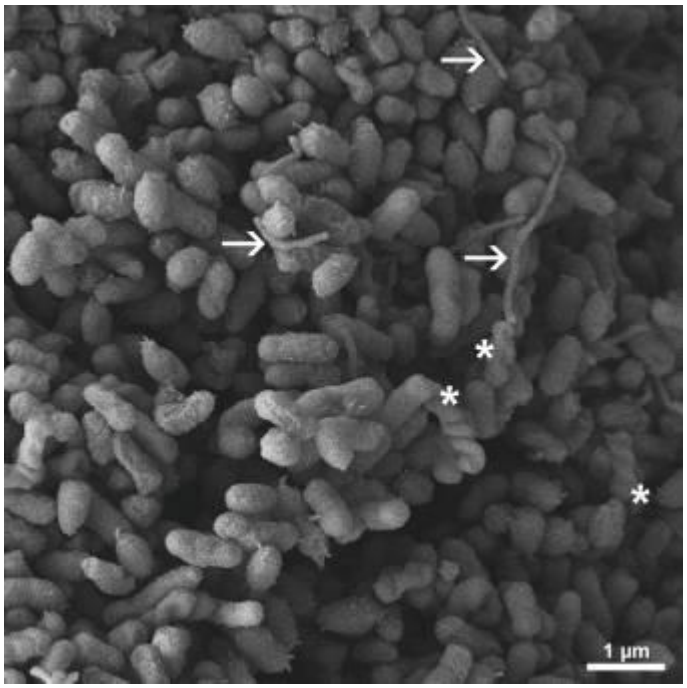

Figure 2. SEM image of the bacterial community within a calcium body of *Titatnethes albus*. In addition to numerous rod-shaped bacteria, filamentous (arrows) and helical (asterisks) forms are visible.