



Cutting out the middle clam: lucinid endosymbiotic bacteria are also associated with seagrass roots worldwide

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Abstract

Seagrasses and lucinid bivalves inhabit highly reduced sediments with elevated sulphide concentrations. Lucinids house symbiotic bacteria (*Ca. Thiodiazotropha*) capable of oxidising sediment sulphide, and their presence in sediments has been proposed to promote seagrass growth by decreasing otherwise phytotoxic sulphide levels. However, vast and productive seagrass meadows are present in ecosystems where lucinids do not occur. Hence, we hypothesised that seagrasses themselves host these sulphur-oxidising *Ca. Thiodiazotropha* that could aid their survival when lucinids are absent. We analysed newly generated and publicly available 16S rRNA gene sequences from seagrass roots and sediments across 14 seagrass species and 10 countries and found that persistent and colonising seagrasses across the world harbour sulphur-oxidising *Ca. Thiodiazotropha*, regardless of the presence of lucinids. We used fluorescence in situ hybridisation to visually confirm the presence of *Ca. Thiodiazotropha* on roots of *Halophila ovalis*, a colonising seagrass species with wide geographical, water depth range, and sedimentary sulphide concentrations. We provide the first evidence that *Ca. Thiodiazotropha* are commonly present on seagrass roots, providing another mechanism for seagrasses to alleviate sulphide stress globally.

Introduction

Seagrasses are marine flowering plants that cover an estimated global area of 300,000–600,000 km² and are crucial

to the health of shallow coastal ecosystems worldwide [1]. Seagrasses typically thrive in highly reduced sediments where sulphide concentrations can accumulate to phytotoxic levels (>10 μmol L⁻¹ [2]), which presents an ongoing enigma as to how they survive. Several hypotheses have been presented, including oxidation of sulphides internally in the aerenchyma and externally in the rhizosphere by radial oxygen loss and by root associated sulphide oxidising bacteria [3–5]. Bivalves belonging to the family Lucinidae have also been proposed as a mechanism for sulphide detoxification in seagrass beds globally [6]. Lucinids, unlike most animals, thrive in sulphide-rich sediments as they house symbiotic chemoautotrophic bacteria (*Ca. Thiodiazotropha*) inside their gills which oxidise sulphides to provide energy for CO₂ fixation. In this tripartite relationship, seagrasses benefit from minimised sulphide intrusion, whilst lucinids and their symbionts profit from enhanced sulphide production arising from seagrass organic matter accumulation, which fuels microbial sulphate reduction [3]. This relationship has been recently tested in the field, where increased densities of the lucinid *Loripes orbiculatus* correlated with reduced sulphide intrusion in the seagrass *Zostera noltei* [7]. Whilst this study provides compelling

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evidence for sulphide detoxification for seagrass systems with high densities of lucinid bivalves, it is not adequate for explaining sulphide detoxification for systems lacking lucinids.

Lucinids acquire their endosymbionts in free-living stage from the environment, believed to be a result of chance encounters with potential symbionts during the juvenile phase [8, 9]. It follows that seagrass roots, which provide a chemical environment that bears a striking resemblance to lucinid gills (i.e., gradients of oxygen, organic matter, sulphide and carbon dioxide availability) [10, 11], would also provide a suitable niche to attract and possibly attain these bacteria. As part of recolonisation of the marine environment, seagrasses require adaptations to cope with high sulphide environments, one of which could be supported via their root microbiota. Thus, we hypothesized that seagrass roots harbour sulphur-oxidising *Ca. Thiodiazotropha*, which could explain their successful colonisation even in sulphide-rich sediments where lucinids are absent.

Methods

To explore the possibility that seagrass roots serve as a suitable habitat for *Ca. Thiodiazotropha*, we analysed 16S rRNA gene sequences (Illumina MiSeq platform) recovered from publicly available and newly generated data on seagrass roots and sediments across 14 seagrass species and 10 countries (Fig. S1; Supplementary methods, Table S1 and Table S2; Supplementary datasets). All raw sequences were processed through the DADA2 pipeline [12] using SILVA 132 database [13] to assign taxonomy (R markdown file in Supplementary). Amplicon Sequence Variants (ASVs) classified as *Ca. Thiodiazotropha* were compared to SILVA 138 using blastn (v 2.2.29+) [13] and a phylogenetic tree was constructed using PhyML [14] with 1000 bootstraps. We also applied fluorescence in situ hybridisation (FISH) to visualise *Ca. Thiodiazotropha* on roots of the seagrass *Halophila ovalis* following the protocol outlined in [11] (detailed methods in Supplementary, Table S3 and Table S4, and Fig. S2 and Fig. S3).

Results and discussion

We recovered *Ca. Thiodiazotropha* sequences from roots of 12 of the 14 seagrass species analysed, comprising four seagrass families and three independent lineages of seagrass evolution [15]. When present, *Ca. Thiodiazotropha* sequences were consistently more abundant in seagrass

root samples compared to the surrounding sediment, where often no sequences were recovered at all (Fig. 1b). *Ca. Thiodiazotropha* sequences were also found in seagrasses of all functional types; from large, slow growing seagrass species that develop persistent meadows such as *Posidonia* spp. to smaller fast growing species that form transient meadows such as *Halophila* spp. (Fig. 1). Data are skewed towards temperate systems, however, *Ca. Thiodiazotropha* sequences were also obtained from tropical seagrass roots in Florida, as well as the tropical Paracel Islands in the South China Sea (Fig. S1 and interactive map; Supplementary material). Notably, comparable levels of relative abundance of *Ca. Thiodiazotropha* from seagrass roots appeared independent of the presence or absence of lucinids (Fig. 1c). Together, this suggests that *Ca. Thiodiazotropha* colonise seagrasses irrespective of their geography, lifestyle, evolutionary history and proximity to lucinids likely because the root environment and its mosaic of chemical gradients (e.g. oxygen, pH, metals and nutrients) [10, 11] provides a suitable niche for these chemoautotrophs.

Most seagrass species exhibited a relatively diverse set of ASVs aligning to known endosymbionts of lucinid species (Fig. S4; Supplementary). Some lucinid species also exhibit diversity in their endosymbionts, possibly as a reflection of the diversity of free-living symbionts present in the environment [8, 9]. To date, functional analysis of three gill endosymbionts (including *Ca. Thiodiazotropha* endolucinida indicated in Fig. S2), revealed not only genes relating to chemoautotrophy, but also diazotrophy, heterotrophy and oxidation of C1 compounds [8, 16–18]. Hence these bacteria may not only provide a means for lowering sediment sulphide levels, but may also provide an additional source of NH_4^+ via nitrogen fixation; the preferred source of N for seagrass uptake [19].

FISH with a *Ca. Thiodiazotropha*-targeted oligonucleotide probe combined with class- and domain-level probes showed that *Ca. Thiodiazotropha* forms dense colonies on the surface of *Halophila ovalis* roots, particularly in axial grooves between epidermal cells and on the base of root hairs (Fig. 2, Fig. S5; Supplementary). Strikingly, a morphological diversity of *Ca. Thiodiazotropha* was apparent in FISH images—with smaller single-cell rods (~1 μm) present on the root surface (e.g. Fig. S5a; Supplementary), as well as larger coccoid shaped (~2 μm) cells (e.g. Fig. S5d; Supplementary) that form dense colonies in axial grooves and root hairs. Such differences in morphology have been previously observed between extracellular (those found in the sediment) and intracellular lifestyles (those located inside lucinid gills) for the symbionts of the lucinid *Codakia orbicularis* [20]. We cannot say if our observations of

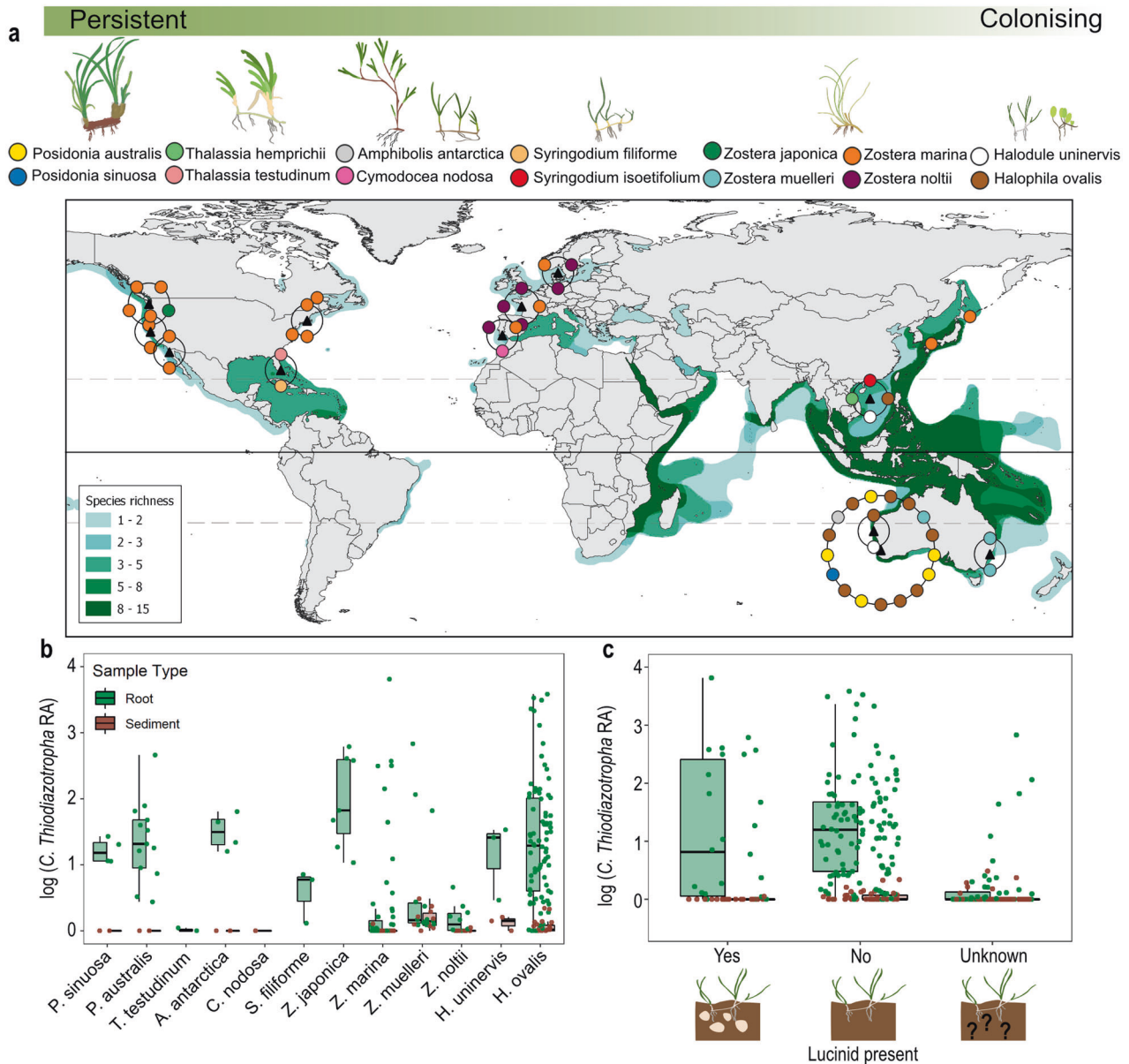


Fig. 1 Global distribution of seagrass samples and the relative abundance of *Ca. Thiodiazotropha*. **a** Seagrass species and sample locations analysed in this study and the global distribution of seagrass species. Seagrasses are classified by functional type; with seagrasses on the left classified as persistent (large, slow growing persistent meadows), and those on the right as colonising (small, fast growing transient meadows). Global seagrass distribution data was acquired from Green EP and Short FT, World atlas of seagrasses, 2003. Black triangles indicate a cluster of sites and/or seagrass species that were

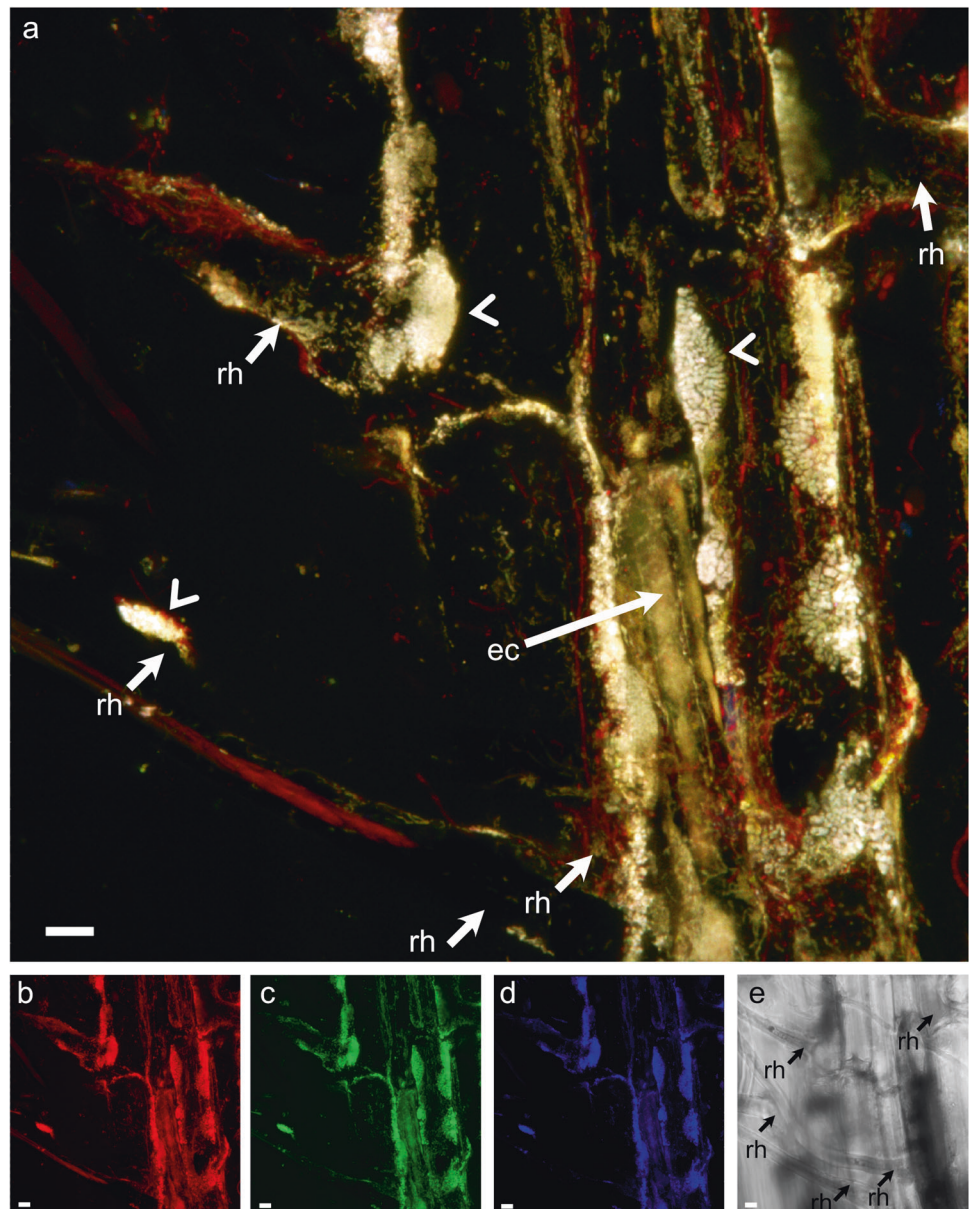
collected in close proximity. Location meta-data can be found in Table S1 and Table S2, as well as collection details and primer information. **b** Relative abundance of *Ca. Thiodiazotropha* sequences (natural log scale +1) recovered from all seagrass roots and sediments. **c** Relative abundance of *Ca. Thiodiazotropha* sequences (natural log scale +1) recovered from seagrass roots and sediments from environments with and without lucinids present (lucinid presense/absence references available in Table S2).

Ca. Thiodiazotropha is a reflection of morphological plasticity or closely related strains with differing morphologies. Regardless, we provide the first in situ evidence that *Ca. Thiodiazotropha* is colonising seagrass roots in high abundances. Together with amplicon sequencing based evidence that *Ca. Thiodiazotropha* are

associated with seagrasses of varying life-strategies and evolutionary histories worldwide, our data suggests that this relationship may be both general and another essential mechanism for seagrasses to thrive in sulphide-rich sediments, thus extending the proposed lucinid-seagrass relationship.

Fig. 2 Projected images of *Halophila ovalis* roots (collected from the Swan River, Western Australia, Australia) with associated populations of *Ca. Thiodiazotropha*. **a** overlay of all probes. Large clusters of *Ca. Thiodiazotropha* can be seen in yellow/white (white arrow heads) and populations of other bacteria (particularly filamentous bacteria) can be seen in red. Several root hairs are present (rh; white arrow) and the epidermal cells of the seagrass are also visible (ec; white arrow). N.B. *H. ovalis* possesses no lateral roots, so all 'branching' are root hairs (i.e., not lateral roots). **b** Bacteria (probe: EUBI-III-ATTO 647), **c** Gammaproteobacteria (probe: GAM42a-ATTO 565), **d** *Ca. Thiodiazotropha* (probe: THIO-847) **e** transmission image with the bases of root hairs (rh) indicated with black arrows.

Scale bars: 10 μ m.



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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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