Nitrogen fixation in a chemoautotrophic lucinid symbiosis

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The shallow water bivalve *Codakia orbicularis* lives in symbiotic association with a sulfur-oxidizing bacterium in its gills. The endosymbiont fixes CO₂ and thus generates organic carbon compounds, which support the host's growth. To investigate the uncultured symbiont's metabolism and symbiont–host interactions in detail we conducted a proteogenomic analysis of purified bacteria. Unexpectedly, our results reveal a hitherto completely unrecognized feature of the *C. orbicularis* symbiont's physiology: the symbiont's genome encodes all proteins necessary for biological nitrogen fixation (diazotrophy). Expression of the respective genes under standard ambient conditions was confirmed by proteomics. Nitrogenase activity in the symbiont was also verified by enzyme activity assays. Phylogenetic analysis of the bacterial nitrogenase reductase NifH revealed the symbiont's close relationship to free-living nitrogen-fixing Proteobacteria from the seagrass sediment. The *C. orbicularis* symbiont, here tentatively named '*Candidatus* Thiodiazotropha endolucinida', may thus not only sustain the bivalve's carbon demands. *C. orbicularis* may also benefit from a steady supply of fixed nitrogen from its symbiont—a scenario that is unprecedented in comparable chemoautotrophic symbioses.

Mutualistic associations between marine invertebrates and sulfur-oxidizing (thiotrophic) bacteria are a well-documented and widespread phenomenon in a variety of sulfidic habitats ranging from hydrothermal vents to shallow-water coastal ecosystems^{1–3}. Thioautotrophic symbionts generate energy through sulfide oxidation and provide their hosts with organic carbon. In the Lucinidae, a diverse family of marine bivalves, all members are obligatorily dependent on their bacterial gill endosymbionts after larval development and metamorphosis⁴. The shallow-water lucinid *Codakia orbicularis*, which lives in the sediment beneath the tropical seagrass *Thalassia testudinum* along the Caribbean and Western Atlantic coast⁵, harbours a single species of endosymbionts in its gills⁶. The symbiont has been shown to be newly acquired by each clam generation^{7,8} from a pool of free-living symbiosis-competent bacteria in the environment⁹, rather than being inherited from clam parents. *C. orbicularis* appears not to release its endosymbionts, even under adverse conditions, but can digest them as a source of nutrition^{10–12}. Moreover, bacterial cell division seems to be inhibited inside the host tissue. The majority of the symbiont population was shown to be polyploid (that is, containing multiple genome copies), while dividing symbiont cell stages are very rarely observed in host bacteriocytes¹³. The host undoubtedly benefits from the symbiont both by way of detoxification of its sulfidic environment and by supply of organic compounds through the bacterial Calvin–Benson cycle. It remains questionable, however, whether the symbiont gains any advantage from this association in evolutionary terms¹¹.

Biological nitrogen fixation (diazotrophy) is the conversion of molecular nitrogen (N₂) into ammonia¹⁴. It provides the basis for the biosynthesis of organic nitrogen compounds and thus of

indispensable cellular components such as proteins and nucleic acids. Diazotrophy occurs exclusively in the prokaryotic kingdom¹⁵; nitrogen fixation in eukaryotes is therefore restricted to symbioses with bacteria¹⁶. Prokaryotic nitrogen fixers are associated with a diversity of eukaryotic hosts in a wide range of habitats, including the well-studied *Rhizobium* and *Bradyrhizobium* genera that form root-nodule symbioses with legumes¹⁷ and the actinomycetal *Frankia* species associated with woody plants¹⁸. In marine environments, symbiotic nitrogen-fixers are known to be associated with a variety of invertebrates, such as wood-boring bivalves (shipworms)¹⁹, corals, sponges and sea urchins²⁰.

Marine ecosystems are frequently nitrogen-limited and therefore considered to be favourable niches for diazotrophic organisms²¹. Coastal seagrass beds, such as those inhabited by *Codakia* species, are areas of particularly high microbial nitrogen fixation activity¹⁴, as the plants' rhizosphere is densely populated by a complex diazotrophic bacterial community^{22,23}. The majority of these nitrogen-fixers are anaerobic, heterotrophic sulfate reducers, which decompose decaying seagrass leaves, producing large amounts of toxic sulfide²⁴. Thiotrophic members of the rhizosphere community oxidize and thus detoxify the sulfide²⁵. Unlike most other animals, for which sulfide is toxic, lucinids thrive in this habitat. Moreover, through the sulfide-oxidation activity of their symbionts, the bivalves contribute to sulfide detoxification of the seagrass bed ecosystem and have therefore been suggested to be part of a beneficial association with the seagrass ('tripartite symbiosis')²⁶. However, nitrogen fixation in their sulfur-oxidizing symbiont has to our knowledge never been reported for *C. orbicularis*, nor yet for any other thiotrophic marine invertebrate symbiosis, despite the broad scientific attention these systems have attracted^{1–3}.

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Figure 1 | Nitrogen fixation gene clusters in the *Codakia orbicularis* symbiont and related diazotrophic organisms. The '*Ca. T. endolucinida*' COS *nif* and *rf* gene clusters (middle) were compared to the genomes of *Allochromatium vinosum* DSM 180 (top) and *Sedimenticola thioaurini* SIP-G1 (bottom) using BL2seq (BLASTn, E-value $1e^{-5}$). Gene functions: red, nitrogenase and nitrogenase reductase; blue, electron transfer, cofactor biosynthesis and other nitrogen fixation-specific functions; yellow, transcriptional and post-translational regulation of nitrogen fixation; grey, function not necessarily related to nitrogen fixation; green, *rf* genes. White arrows with solid outlines indicate unknown function, and white arrows with broken outlines indicate other functions (these genes of *A. vinosum* and *S. thioaurini* do not have a homologue in the *C. orbicularis* symbiont's *nif* cluster). Sequence similarities are symbolized by red hues for direct comparisons and blue hues for reversed comparisons. Darker colours correspond to higher identities. For protein functions see Supplementary Table 3. *Identified as a protein in this study. Sequence data and the BLAST comparison files were drawn with the R package genoPlotR (ref. 57) and edited in Inkscape.

Figure 2 | Nitrogenase activity assay. The production of ethylene as a consequence of acetylene reduction by the nitrogenase enzyme complex was measured in gill tissue samples from freshly collected *C. orbicularis* specimens, in purified gill symbionts, in symbiont-free foot tissue, in the water column and in *T. testudinum* sediment. Gill tissue and isolated symbionts were assayed under aerobic (+O₂) conditions and under microaerobic (-O₂) conditions ('+O₂' and '-O₂' refer to initial oxygen concentrations at the start of the assay). All other sample types were incubated under aerobic conditions. Purified symbiont fractions contained approximately 5×10^8 to 8×10^8 bacteria per assay and gill tissue samples contained approximately 3×10^8 bacteria per assay (see Methods for details). The ethylene production rate is given in nmol h⁻¹ mg⁻¹ as average values ($n = 5$), and error bars show standard deviation. No ethylene production was detected in gill tissue samples under microaerobic conditions, in symbiont-free foot tissue and in the water column.

Here, we show, in a combined proteogenomic and experimental approach, that the thioautotrophic gill endosymbiont of *C. orbicularis*, '*Candidatus Thiodiazotropha endolucinida*', expresses nitrogen

fixation proteins and exhibits nitrogenase activity under its natural ambient conditions in the host. This finding adds a novel feature to the picture of the *Codakia* symbiosis and enhances our understanding of its role in the seagrass ecosystem.

Results and discussion

Detection of nitrogen fixation in the *C. orbicularis* symbiont. To reconstruct the *C. orbicularis* symbiont's metabolism, we sequenced the genome of purified bacteria to provide a basis for subsequent global proteomic analyses. As expected, the symbiont's genome encoded all the enzymes necessary for sulfide oxidation via the Dsr-APS-Sat pathway and for carbon fixation through the Calvin-Benson cycle. The respective enzymes were expressed in high concentrations, as detected at the protein level (these results will be the subject of a separate publication). Interestingly, we also detected a complete gene cluster for the fixation of dinitrogen in the symbiont's genome (CODIS_20190–20650), including the highly conserved nitrogenase enzyme complex.

The *C. orbicularis* symbiont's nitrogen fixation (*nif*) cluster comprises 47 genes (Fig. 1), 26 of which were found to be expressed as proteins in this study (Supplementary Tables 3 and 4). The actual nitrogenase enzyme complex consists of two components: a dinitrogenase (component I), which is a molybdenum-iron protein and heterotetramer of two NifD and two NifK subunits, and a dinitrogenase reductase (component II), a homodimer of two NifH subunits¹⁵. All three proteins were found to be expressed in *C. orbicularis* symbiont samples isolated from freshly collected clams (see Supplementary Table 4 for protein concentrations). Nitrogen fixation-related electron transfer proteins, regulatory proteins, as well as proteins needed for nitrogenase cofactor biosynthesis are also encoded among the *nif* genes and many of them were

Figure 3 | Immunodetection of nitrogenase expression. **a**, Western blot analysis of NifH expression in gill extracts (50 mg of protein extract per lane) of the asymbiotic tellinid *Arcopagia crassa* (left, negative control) and of *C. orbicularis* (right). The NifH band migrates at around 34 kDa. The full western blot is displayed in Supplementary Fig. 6. **b**, Goldner staining of gill filaments from a freshly collected *C. orbicularis* bivalve. Each gill filament possesses a ciliated zone (CZ) devoid of bacteria. The lateral zone contains mostly bacteriocytes (BC) harbouring the bacterial symbionts and granule cells (GC, stained in orange), which are free of bacteria. Dark brown dots are *C. orbicularis* cell nuclei. Scale bar, 30 μm . **c**, DAPI staining of the lateral gill filament zone. Large bright blue dots indicate the location of nuclei in bacteriocytes and granule cells. Symbiont DNA is visible as small light blue dots all over the bacteriocyte area but not in the granule cell area. Sp: space between the gill filaments. Scale bar, 20 μm . **d**, Immunolocalization of the *C. orbicularis* symbiont's nitrogenase protein in the same gill section as in **c**, hybridized with the Alexa Fluor 546-labelled NifH antibody (yellow fluorescence). NifH fluorescence is visible in bacteriocytes but not in granule cells. Sp: space between gill filaments. Scale bar, 20 μm . **e**, Magnification of a detail from **d** (shown by a rectangle), highlighting the localization of NifH labelling in the bacteria. Scale bar, 2 μm .

detected as proteins under *in situ* conditions. In addition to these *nif*-encoded proteins, we also identified a number of genes and proteins that are apparently indirectly related to nitrogen fixation in the *C. orbicularis* symbiont (see Supplementary Results and Discussion for details). First, immediately adjacent to the *nif* genes, the *C. orbicularis* symbiont's genome encodes a set of Rnf electron transfer proteins (CODIS_20660–20720), which are putatively involved in electron transfer towards the nitrogenase complex. Second, the high abundance of glutamine synthetase (CODIS_03590) and the P-II signal transduction protein GlnK (CODIS_03150) suggests that nitrogen fixation-derived ammonium is assimilated through the GS/GOGAT pathway and that the symbionts analysed in this study may have experienced nitrogen limitation. The two-component nitrogen regulation (Ntr) system, which controls ammonium assimilation in Proteobacteria²⁷, appears also to be present and active in the *C. orbicularis* symbiont. Third, we also detected a rubrerythrin (Rbr, CODIS_04230) as one of the most abundant proteins in the '*Ca. T. endolucinida*' proteome.

Rubrerythrins provide protection against hydrogen peroxide-mediated oxidative stress²⁸ and have been suggested to play a role in shielding the highly oxygen-sensitive nitrogenase protein from oxidative damage²⁹. We observed that both the nitrogenase proteins and Rbr were detected with significantly lower protein concentrations during diazotrophy-inhibiting conditions (that is, energy limitation caused by sulfide starvation; Supplementary Fig. 5 and Supplementary Results and Discussion). These results may suggest a co-regulation of Rbr and nitrogenase expression and support the idea that Rbr might be involved in protecting the nitrogenase from oxidative stress.

The functionality of the nitrogenase enzyme complex was confirmed by an acetylene reduction assay³⁰. We detected the nitrogenase-dependent conversion of acetylene to ethylene in *C. orbicularis* gill tissue samples and in purified symbionts obtained from freshly collected individuals (Fig. 2). Ethylene accumulation rates in gill samples ranged from 8.65 to 15.41 $\text{nmol h}^{-1} \text{mg}^{-1}$ of tissue under aerobic conditions, while no ethylene production was detected in

Figure 4 | In situ habitat concentrations of NH₄⁺, NO₃⁻ and NO₂⁻. Gas concentrations (in μM) in pore water samples from *T. testudinum* sediments are given as averages of six individual sediment cores ($n = 6$). Standard deviations are indicated by error bars. The light grey background indicates the sediment depth in which *C. orbicularis* burrows. Sediment samples were taken within a radius of 20 cm around *C. orbicularis* specimens.

gills incubated under low oxygen conditions. The latter might be due to the fact that host bacteriocytes probably die quickly in the absence of oxygen, which may also be detrimental for their intracellular symbionts. On the other hand, symbionts that were isolated from their bacteriocytes showed distinctly higher nitrogenase activities (Fig. 2) with an average ethylene production rate of 43 nmol h⁻¹ mg⁻¹ under aerobic conditions. Under microaerobic conditions, the nitrogenase activity of purified symbionts was even higher (57.5 nmol C₂H₄ h⁻¹ mg⁻¹) than in the aerobic approach, suggesting that the thioautotrophic symbiont's nitrogenase enzyme complex may—as most other nitrogenases—be oxygen-sensitive and work more efficiently under low-oxygen conditions¹⁶. Low ethylene production (~0.24 nmol C₂H₄ h⁻¹ mg⁻¹) was observed in crude sediment collected from *T. testudinum* seagrass beds, presumably indicating the presence of free-living nitrogen-fixing bacteria in the sediment. No ethylene production was detected after incubation of sea water with acetylene and after incubation of symbiont-free foot tissue with acetylene, respectively. This clearly indicates that nitrogenase activity is exclusively present in symbiont-containing tissue in *C. orbicularis*.

To support this observation, we performed a western blot analysis based on an antibody raised against NifH (~34 kDa). As displayed in Fig. 3a, hybridization of the NifH antibody was observed in extracts from freshly collected *C. orbicularis* gills, while no signal could be detected in the negative control, that is, gill tissue from the asymbiotic marine bivalve *Arcopagia crassa* (Tellinidae). As described by Frenkiel and Mouëza³¹, gills of freshly collected individuals of *C. orbicularis* consist of an arrangement of filaments organized in three zones, the lateral of which contains the bacteriocytes (BCs), filled with intracellular bacterial symbionts, and granule-containing cells (GC) without endosymbionts (Fig. 3b). To allocate the expression of the nitrogenase protein NifH to the symbionts in the bacteriocytes, we conducted immunofluorescence microscopy on *C. orbicularis* gill histological sections using an anti-NifH antibody and 4',6-diamidino-2-phenylindole (DAPI) counterstaining. The DAPI staining (Fig. 3c) allowed for the localization of host nuclei (large light blue dots) in bacteriocytes and granule cells all along the lateral gill filament zone but also of bacterial DNA (small light blue dots), only within the bacteriocytes. The NifH signals (yellow fluorescence, Fig. 3d) cover the bacteriocyte area but are absent from the granule cells and thus evidently emanate from the bacterial endosymbionts located in the cytoplasm of the bacteriocytes (Fig. 3e), even if not all the bacteria from a single

bacteriocyte seem to be positive. In the asymbiotic tellinid *A. crassa*, which was used as a negative control, no NifH immunofluorescence was observed (Supplementary Fig. 2). Moreover, trophosome tissue sections of the deep-sea tube worm *Riftia pachyptila* (Vestimentifera, negative control 2), did not hybridize with the NifH antibody either. *R. pachyptila* harbours a sulfur-oxidizing symbiont in its trophosome, which is closely related to the *C. orbicularis* symbiont, but which is not capable of diazotrophy^{32,33}. These results clearly reveal that *nifH* expression in *C. orbicularis* can be specifically attributed to the symbionts inside the host bacteriocytes.

Availability and use of other nitrogen sources in the seagrass sediment. Our sediment pore water analyses show that ambient inorganic nitrogen concentrations are comparatively low in the direct vicinity of *C. orbicularis* (that is, within a radius of 20 cm from the animals), suggesting that diazotrophy is of particular advantage for the bacterial symbiont in this habitat. In marine systems, the main sources of nitrogen available for bacterial growth (besides N₂) are NH₄⁺ and free amino acids³⁴, as well as—to a lesser extent—NO₃⁻, NO₂⁻ and urea³⁵. In seagrass bed sediments in particular, NH₄⁺ is considered to be the dominating nitrogen source, with concentrations of up to 175 μM in some areas³⁶. We detected NH₄⁺ levels from 1 to 10 μM in the *T. testudinum* sediment directly surrounding *C. orbicularis*, with the highest concentrations measured in the deeper layers of the sediment (Fig. 4). These values are low compared to previously reported NH₄⁺ concentrations of up to 120 μM in *T. testudinum* sediments in subtropical Florida³⁷. However, sediment pore water ammonium concentrations have been shown to vary substantially between sampling sites and times^{36,38}. In tropical *T. testudinum* seagrass beds of the Caribbean, NH₄⁺ levels were observed to be particularly low, that is, below 25 μM in the upper 10 cm of sediment³⁹. In this presumably ammonium-limited environment, nitrogen fixation is probably the preferable strategy for bacterial nitrogen acquisition.

Interestingly, a number of genes required for uptake and assimilation of nitrate and/or nitrite were found to be expressed in the *C. orbicularis* symbiont (including a nitrate transporter and assimilatory nitrate and nitrite reductase subunits, Supplementary Table 4). Unlike ammonium, nitrate and nitrite are usually considered not to play a substantial role as nitrogen sources in seagrass sediments, with NO₃⁻ + NO₂⁻ concentrations ranging from 0 to 10 μM (ref. 36). In accord with previous studies, we detected NO₃⁻ and NO₂⁻ concentrations of up to 9 μM and below 3 μM, respectively, in our pore water analysis in the immediate surroundings, that is, within a radius of 20 cm around *C. orbicularis* specimens (Fig. 4). The detection of assimilatory nitrate/nitrite reductase subunits indicates that, despite the relatively low ambient concentration, nitrate (and nitrite) may be relevant as nitrogen sources for the *C. orbicularis* symbiont after all, in addition to N₂ fixation. This seems to be surprising at first, as nitrogen fixation is often repressed in the presence of alternative nitrogen sources in free-living diazotrophs⁴⁰. The simultaneous expression of both *nif* genes and nitrate assimilation genes might be due to less stringent regulation in *C. orbicularis* symbionts, as also suggested for other symbiotic nitrogen fixers, which provide for their hosts' nitrogen supply⁴¹. However, the most likely explanation would be that the environmental nitrate and nitrite levels are too low to trigger repression of the N₂ fixation genes.

Overall, in view of the specific nitrogen-limited ambient conditions in the seagrass bed habitat, nitrogen fixation in the *C. orbicularis* symbiont emerges as a most advantageous feature. Moreover, employing bacterial endosymbionts that can fix N₂ in this environment might also be highly profitable for *C. orbicularis*. Nitrogen incorporated by the symbiont through N₂ fixation most probably supplements the bivalve's nitrogen diet after symbiont

Figure 5 | NifH-based phylogenetic tree of the *Codakia orbicularis* symbiont and its closest relatives. The tree was inferred based on maximum likelihood. Numbers given on the branches are bootstrap proportions as a percentage of 1,000 replicates for values $\geq 50\%$. Taxonomic classes of the phylum Proteobacteria are given on the right. Although the original inference used full-length NifH amino acid sequences, partial NifH sequences from uncultured marine isolates were added by maximum parsimony (dotted branches). Note that some of the full-length sequences had up to 80 partial sequences affiliated to them. For clarity's sake, only one partial sequence per full-length sequence is displayed (that is, the one with highest sequence similarity). For a comprehensive list of all partial sequences that were affiliated to the *C. orbicularis* symbiont '*Candidatus Thiodiazotropha endolucinida*' COS see Supplementary Table 5. '*Ca. T. endolucinida*' COS (this study) is shown in bold. Orange dots denote known diazotrophic sulfur oxidizers. (Note that no information regarding potential thiotrophy is available for the uncultured seagrass bed isolates. Some of these clones might be sulfur oxidizers, although they are not marked with orange dots.) Accession numbers or locus tags in parentheses refer to the International Nucleotide Sequence Database Collaboration (INSDC). The sequence marked with an asterisk was only available as a nucleotide sequence and was translated for this analysis (frame 3). The NifH sequences of *Anabaena variabilis* ATCC 29413 (AAA93020) and *Frankia alni* Ar13 (AAA96262) were used as outgroup (shown in Supplementary Figure 4d).

digestion. Thus supplied, the host would circumvent competition with seagrass root tissues for other, limited nitrogen sources. (See Supplementary Fig. 3 for a model of interactions between bivalves, symbionts, seagrass and the free-living microbial community.)

Phylogeny of nitrogen fixation in the *C. orbicularis* symbiont. Our phylogenetic analyses of the nitrogenase reductase NifH and of the nitrogenase subunits NifD and NifK place the *C. orbicularis* symbiont in a group with free-living diazotrophic Gammaproteobacteria that are known to be sulfur oxidizers, such as *Sedimenticola thiotaurini*, *Thiorhodococcus drewsii* and *Allochrochromatium vinosum* (Fig. 5 and Supplementary Fig. 4b–d). Moreover, particularly high similarities were observed between the *C. orbicularis* symbiont's NifH and NifH sequences of a variety of uncultured diazotrophic isolates from seagrass beds and mangrove sediments, some of them classified as presumptive Beta- or Gammaproteobacteria⁴² (see Supplementary Results and Discussion and Supplementary Table 5

for details). Although these uncultured isolates are not characterized with regard to their 16S rRNA genes (nor their genetic potential for thiotrophy), this pronounced NifH-based identity implies a high degree of physiological similarity to '*Ca. T. endolucinida*'. At the level of 16S rRNA sequence phylogeny, the *C. orbicularis* symbiont clusters most closely with other yet uncultured thiotrophic lucinid symbionts from seagrass bed habitats, many of which are known Gammaproteobacteria (Supplementary Fig. 4a). None of these related lucinid symbionts has been characterized with regard to potential diazotrophy, but considering their close phylogenetic relatedness and their very similar ecosystems it seems quite likely that these symbionts, too, are capable of nitrogen fixation.

These observations suggest the presence of a hitherto unrecognized group of sulfur-oxidizing diazotrophic Gammaproteobacteria in seagrass sediment ecosystems, whose members can be free-living, like the uncultured clones, or live in symbiosis with *C. orbicularis* and (possibly) other lucinids. The phylogenetic relatedness of

thiotrophic lucinid symbionts and seagrass sediment bacteria has been suggested previously^{23,43}. The *C. orbicularis* symbiont exists as a free-living bacterium in the environment before colonization of its bivalve host⁹ and therefore presumably belongs to this microbial seagrass community. Moreover, free-living sulfide oxidizers are known to be part of the complex bacterial population in the seagrass rhizosphere (Supplementary Fig. 3)²⁵, although their potential involvement in plant-associated nitrogen fixation has not been addressed so far. It will be highly interesting to investigate this hypothetical gammaproteobacterial community of thiotrophic diazotrophs in seagrass beds in further studies, to verify its existence, abundance and composition.

Conclusion

The results presented in this study provide evidence that the thiotrophic *C. orbicularis* gill symbiont '*Ca. T. endolucinida*' is a diazotroph. The bacterium is thus perfectly adapted to the prevalent low NH_4^+ and NO_3^- concentrations in its seagrass habitat. As *C. orbicularis* digests parts of its symbiont population for nutrition, it seems very likely that diazotrophy-derived nitrogen compounds are transferred from the symbiont to the host, supporting the bivalve's nitrogen diet. Future studies will be needed to confirm this proposition. Harboring a 'multi-talented' symbiont, which not only detoxifies sulfide and sustains its host's carbon needs, but which presumably also provides nitrogen compounds, seems to be most advantageous for *C. orbicularis* and may enable the bivalve to prevail in its highly sulfidic and nitrogen-limited environment. The interesting question of how the symbiont might benefit from this association in evolutionary terms—considering the highly restrictive regime of its host—remains to be elucidated in future studies (Supplementary Discussion). As the specific ambient conditions in sulfidic seagrass beds are a common feature shared by many lucinid symbioses, it seems quite likely that future studies will reveal diazotrophy in other thiotrophic lucinid symbionts as well. Follow-up studies are required to elucidate the composition of a putative population of sulfur-oxidizing diazotrophic Gammaproteobacteria in the seagrass bed sediment and their specific role in the rhizosphere ecosystem.

Marine chemoautotrophic symbioses have been studied intensively over the past decades, as has nitrogen fixation in marine and terrestrial habitats. However, the combination of both chemoautotrophy and nitrogen fixation in a microbial population or even in a single organism has rarely been considered. The recent discovery of diazotrophic *Thiothrix* species living as amphipod ectosymbionts in a sulfidic cave⁴⁴ and the coexistence of chemoautotrophy and diazotrophy in the microbial community of cold water corals⁴⁵, along with the results of this study, suggest that chemoautotrophic diazotrophs may be more widespread than previously anticipated. Other invertebrate models colonizing sulfidic environments may rely on chemoautotrophic symbionts that are also capable of diazotrophy.

Methods

Sampling. Adult individuals of *C. orbicularis* (Linné, 1758) were collected by hand at a depth of 5–10 cm in the sediment of *T. testudinum* seagrass beds in Guadeloupe (French West-Indies, Caribbean). For details of sampling sites, times and replicate numbers see Supplementary Table 1. Specimens designated for immediate dissection (for proteomics, genome sequencing, acetylene reduction assays, western blot and immunohistochemistry) were transported to the laboratory in seawater at ambient temperature within 1 h after collection. *C. orbicularis* individuals designated for incubation (starvation experiment, see below) were transported to the laboratory within 5–6 h. Sediment samples were collected off 'Îlet cochon', directly adjacent to *C. orbicularis* individuals (that is, not more than 20 cm away from the animals) using translucent polypropylene sediment corers (80 mm diameter). The cores (up to 16 cm in depth) were sliced at 4 cm intervals in the field, stored at 4 °C in an ice chest for transport to the laboratory and were subjected to pore water extraction within 1 h (see section 'Chemical analysis of pore water samples').

Incubation experiments with *C. orbicularis*. For incubation experiments, freshly collected *C. orbicularis* specimens were randomly separated into two batches (a control batch and a starvation batch). Specimens in the control batch were dissected immediately, while those in the starvation batch were kept in sterile (0.22 µm-filtered) sea water in 50 l plastic tanks at 26 °C for seven days. The water was oxygenated using an aquarium air pump. To simulate starvation conditions, no organic particles and no reduced sulfur compounds were added throughout the experiment. Bivalves were killed after one week of incubation, and symbionts were purified by density gradient centrifugation (see section 'Symbiont enrichment') and stored at –20 °C.

Chemical analysis of pore water samples. Nitrate, nitrite and ammonium concentrations in pore water from sediment cores were analysed as follows. Pore water was extracted from the sliced cores by filtration under vacuum using a 5 µm filter. Collected pore water samples were then passed through 0.45 µm membrane syringe filters, frozen immediately and stored frozen before colorimetric tests. Nitrate and nitrite concentrations were analysed according to the standard colorimetric Griess method⁴⁶ with the use of vanadium as a reduction agent⁴⁷, and ammonium was measured by the indophenol blue method⁴⁸.

Symbiont enrichment. Bacterial symbionts were purified from healthy *C. orbicularis* gills with a light beige colour using Percoll density gradient centrifugation as described previously^{13,49}. Briefly, after dissection of the animals, gill tissue was homogenized in a Dounce homogenizer in cold sterile seawater (salinity 35 g l⁻¹) and the resulting homogenate was centrifuged at 30g for 1 min (4 °C) to remove crude tissue debris. The supernatant was centrifuged again (400g, 2 min, 4 °C) to pellet the bacterial cells, which were subsequently resuspended in sterile sea water and layered on top of a Percoll cushion (60% Percoll in imidazole-buffered saline, containing 490 mM NaCl, 30 mM MgSO₄, 11 mM CaCl₂, 3 mM KCl and 50 mM imidazole, pH 7.5). During centrifugation (4,000g, 10 min, 4 °C), the bacteria—because of their elemental sulfur inclusions—accumulate below the cushion, while host tissue fragments stay on top. The bacteria were collected, washed twice in sterile sea water, and stored at –20 °C.

Genome sequencing and genomic analysis. Symbionts for genome sequencing were isolated from one single *C. orbicularis* host individual immediately after collection of the animal (as described in section 'Sampling') in March 2012. Genomic DNA was isolated from the purified bacteria using the MasterPure DNA Purification Kit (Epicentre). The genome was sequenced using Illumina sequencing technology. A Nextera shot-gun library was generated for a 112 bp paired end (PE) sequencing run on a Genome Analyzer IIx. For an overview of all subsequent steps involved in assembly, binning and annotation, see Supplementary Fig. 1. Sequencing resulted in 2 × 6,108,111 raw reads, which were checked with FastQC version 0.11.3 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and subsequently trimmed with Trimmomatic version 0.35 (ref. 50) (settings: remove Nextera adapters and leading/trailing low quality bases, trim using MAXINFO:40:0.8 and keep sequences ≥36 nt). As preliminary assemblies had shown some eukaryotic contaminations in the sequenced data, trimmed reads (2 × 5,729,978 reads + 355,335 single reads) were assembled in a two-step process of binning and mapping to obtain a clean symbiont draft genome. First, Ray version 2.3.1 (ref. 51; k = 37) was used and the resulting 1,018 contigs were visually binned with VizBin (ref. 52; Bin 'COS': 83 contigs; Bin 'Other': 935 contigs). The trimmed reads used for assembly were then mapped against Bin COS with the help of BBSplit version 35.85. In a second step, SPAdes version 3.7.0 (ref. 53) used those mapped reads (2 × 5,131,806 reads + 336,019 single reads) to perform the final assembly (k = 21, 33, 55, 77, 'careful' option and minimum coverage of 30). This time, contigs were binned with MetaWatt version 3.5.2 (ref. 54), basically splitting contigs into bins with known and unknown taxon assignments. The final draft genome comprises 55 contigs (total length = 4,489,993 bp; largest contig = 297,909 bp; N50 = 164,003 bp), as evaluated by QUAST version 3.2 (ref. 55). The rapid annotation tool Prokka version 1.11 (ref. 56) was used for automatic open reading frame (ORF) calling and annotation.

Gene cluster comparison. All genes in the *nif* gene cluster of '*Ca. T. endolucinida*' COS (CODIS_20190–20650, Fig. 1) were compared to the genomes of *Allochrochium vinosum* DSM 180 (NC_013851) and *Sedimenticola thiotaurini* SIP-G1 (NZ_CP011412) using BL2seq (BLASTn, E-value 1e-5). Sequence data and the BLAST comparison files were drawn with the R package genoPlotR version 0.8.4 (ref. 57) and edited in Inkscape version 0.91. Blast results were automatically edited, so that short hits contained in longer hits were removed. They were further curated manually, so that each of the '*Ca. T. endolucinida*' genes matched only one gene in its relatives.

Proteomic analyses. Mass spectrometry (MS) analyses of *C. orbicularis* symbiont proteins were performed in two parallel approaches: (1) in an identification-centred (that is, qualitative), gel-based approach and (2) in a quantitative gel-free approach (see section 'Gel-free protein quantification'). Measurements for the identification-centred approach were performed in three biological replicates (that is, for symbionts of three individual *C. orbicularis* hosts) for (1) soluble symbiont proteins from freshly collected *C. orbicularis*, (2) symbiont membrane proteins from freshly

collected hosts and (3) symbiont membrane proteins from *C. orbicularis* specimens collected after one week of starvation. For the quantitative approach, three measurements (three technical replicates) were acquired for each of the three biological replicates of (1) symbiont proteins from freshly sampled *C. orbicularis* specimens and (2) symbiont proteins from starved *C. orbicularis* after one week of starvation. For a detailed overview of replicate numbers in all MS measurements performed in this study, see Supplementary Table 2.

Protein extraction. Symbiont cell pellets for gel-based MS analysis (approach (1)) of soluble proteins and membrane proteins were resuspended in lysis buffer (10 mM Tris, 1 mM EDTA, Roche complete protease inhibitor cocktail) before cell disruption by sonication (3 × 20 s) under permanent cooling. Cell debris was removed by centrifugation (10 min, 4 °C, 12,000g for soluble protein extraction/8,000g for extraction of membrane proteins), leaving the protein raw extract in the supernatant. Raw extracts designated for the analysis of soluble proteins were subjected to acetone precipitation overnight (−20 °C), the resulting protein pellets were washed with ethanol, and soluble proteins were resuspended in 8 M urea/2 M thiourea solution. For membrane protein analysis, the protein raw extract was subjected to repeated ultracentrifugation and solubilization steps as described by Eymann *et al.*⁵⁸, before solubilization of the enriched membrane protein fraction in 50 mM TEAB (triethylammonium bicarbonate) buffer. For quantitative gel-free proteome analysis (approach (2)), symbiont cell pellets were resuspended in 50 mM TEAB buffer and cells were disrupted by sonication (3 × 20 s). In either case, protein concentrations were determined using a Bradford assay⁵⁹.

Gel-based protein identification. Aliquots of 20 µg soluble protein and 20 µg membrane protein fraction, respectively, were loaded onto 12% polyacrylamide mini gels and subjected to gel electrophoresis at 150 V for 1 h. After Coomassie staining, individual gel lanes were excised and divided into ten equally sized slices (subsamples) each. Gel pieces were destained (200 mM NH₄HCO₃, 30% acetonitrile) and dried before overnight digestion with trypsin solution (1 µg ml^{−1}, sequencing grade, Promega) at 37 °C. Peptides were eluted from the gel pieces in a sonication bath and purified using ZipTips (Millipore) according to the manufacturer's recommendations. As described previously⁶⁰, peptide mixes were subjected to separation using reversed-phase C18 column chromatography on a nanoACQUITY-UPLC system (Waters). Mass spectrometry (MS)- and tandem mass spectrometry (MS/MS) data were acquired using an online-coupled LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). MS spectra were searched against a target-decoy protein sequence database including all sequences from the *C. orbicularis* symbiont database (see 'Genome sequencing and genomic analysis' section) and common laboratory contaminants using the SEQUEST Sorcerer platform (Sage-N). Scaffold (version 4.2, Proteome Software) was used to validate MS/MS-based peptide and protein identifications using the XCorr-based filter settings described previously⁶¹. Proteins were considered identified if they passed the filter criteria in at least one of the three biological replicates. Peptide false discovery rates (FDRs) were <2% and protein FDRs were 0.0% throughout all samples.

Gel-free protein quantification. As described by Muntel *et al.*⁶², 250 µg protein (in TEAB buffer) was treated with RapiGest SF Surfactant solution (Waters), before reduction with TCEP (tris(2-carboxyethyl)phosphine, final concentration 5 mM), alkylation with iodoacetamide (final concentration 10 mM) and digestion with activated trypsin (5 h, 37 °C). After removal of RapiGest by acidification and repeated centrifugation, peptides were purified using StageTips (Proxeon) as suggested by the manufacturer. Trypsically digested bovine haemoglobin (Waters, MassPREP Bovine Hemoglobin Standard) was added to the protein samples as an internal peptide standard at a final concentration of 50 fmol µl^{−1}. Samples were stored at −80 °C until MS analysis. Peptide mixtures were analysed using the nanoACQUITY-UPLC system (Waters), coupled to a Synapt G2 mass spectrometer (Waters), equipped with a NanoLockSpray source in positive mode. The analyser was set to resolution mode and operated with ion mobility separation as described by Huja and co-authors⁶³. The ProteinLynx Global Server (PLGS) 2.5.3 was used for processing raw data and carrying out the database searches against the randomized *C. orbicularis* symbiont database with added common laboratory contaminants and the bovine haemoglobin sequence⁶³. Proteins were considered identified if they were detected in one of the respective technical or biological replicates with a minimum of two peptides at a maximum FDR of 5% on the protein level. For quantification, only those proteins were included for which a minimum of two peptides were detected and which were identified in a minimum of two technical replicates in at least two biological replicates. For the proteins that met those criteria, average values of protein concentrations (in fmol ng^{−1}) were calculated in two subsequent steps, that is, first across all available technical replicates for each biological replicate and subsequently across all biological replicates using the average values from step 1 (ref. 64). Protein FDRs were below 1%. To identify significant differences in protein expression between fresh control samples and samples obtained after starvation, mean protein abundances calculated across all three technical replicates of each biological replicate were used for statistical testing in TM4 MeV (TM4 Software Suite's Multi-Experiment Viewer; <http://www.tm4.org/mev.html>). After *z*-score transformation, an unpaired permutational *t*-test with Welch approximation (using all permutations, *P* value of 0.01, adjusted Bonferroni correction) was applied.

Microscopy and immunohistochemistry. For an overview and histological information about the *C. orbicularis* gill ultrastructure, a gill (dissected from one *C. orbicularis* individual) was fixed in Bouin's fluid for 24 h at room temperature and then embedded in Paraplast. Tissue sections (7 µm thick) were stained by Goldner's trichrome⁶⁵ to allow for clear differentiation between granule cells and bacteriocytes.

For immunolocalization, gills of a freshly collected *C. orbicularis* specimen were dissected and fixed in 2% paraformaldehyde (in 0.22 µm-filtered seawater) and dehydrated in ethanol before being embedded in paraffin. Gill tissue of the asymbiotic bivalve *Arcopagia crassa* (family Tellinidae) was used as a negative control and treated accordingly. As an additional negative control, pieces of trophosome tissue of the vestimentiferan tube worm *Riftia pachyptila* were fixed in 4% paraformaldehyde immediately after collection (in November 2014, Supplementary Table 1) and stored in 70% ethanol before embedding in paraffin. Histological sections (7 µm thick) were deparaffinized before rehydration. After non-specific binding site saturation, sections were incubated with anti-NifH (nitrogenase iron protein) antibody from Agrisera, at a dilution of 1:500 in blocking solution containing 3% bovine serum albumin (BSA) in 1 × PBS (phosphate-buffered saline) for 2 h at room temperature. Subsequently, sections were incubated in the secondary antibody, horseradish peroxidase-conjugated anti-hen IgY (Santa Cruz Biotechnology) at a 1:200 dilution in blocking solution for 1 h at room temperature. Slides were incubated in amplification buffer with Alexa Fluor 546, mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI, VectorLabs) and observed under an epifluorescence microscope (Nikon Eclipse 80i).

Western blot analysis. Gill tissues from fresh *C. orbicularis* and *A. crassa* specimens (negative control) were crushed on ice before protein extraction in lysis buffer as described in the 'Proteomic analyses' section. The total protein raw extract was homogenized in Laemmli buffer (Sigma-Aldrich), and denatured at 95 °C for 10 min before storage at −20 °C. Proteins were separated on precast 4–12% NuPage polyacrylamide mini gels (Invitrogen) and blotted to PVDF membranes (Immobilon) for 1.5 h. The proteins were probed with a hen anti-NifH antibody (Agrisera) at a dilution of 1:10,000. Blots were incubated in the secondary antibody (horse radish peroxidase-conjugated anti-hen IgY) diluted to 1:50,000. HRP activity was detected using the ECL kit (GE Healthcare) according to the manufacturer's instructions and documented on Kodak X-OMAT autoradiography film (Fisher-Scientific). The specificity of the antibody raised against NifH was verified using *C. orbicularis* whole gill protein extracts.

Nitrogenase activity assay. Nitrogenase activity was tested in both dissected gills and in purified gill endosymbiont fractions obtained by the Percoll cushion method (see 'Symbiont enrichment' section). The isolated symbiont fractions contained ~5 × 10⁸ bacteria (per assay), while the number of bacterial cells in the gill samples was approximately 3 × 10⁸ per assay (assuming a bacterial density of 10⁶ bacteria per mg of gill tissue; 242–352 mg of gill tissue was used for each assay). Tissue and cell samples, respectively, were incubated in 22 ml glass tubes containing 2 ml of 0.22 µm-filtered sea water in contact with a headspace (20 ml) of air supplemented with 20 µmoles of acetylene (aerobic conditions at the beginning of the incubation). A parallel incubation of gill tissue and purified symbionts was started under low-oxygen conditions, for which air in the head space was replaced by O₂-free argon and supplemented with the same quantity of acetylene. The samples were incubated at 24 °C and shaken continuously (60 r.p.m.) using a shaking water bath (SWB25 Thermo Scientific) throughout the experiment to equilibrate the gas and bacteria/tissue samples. After 24 h of incubation, ethylene production was measured as follows. A 500 µl gas sample was withdrawn from the headspace using a gas-tight hypodermic glass syringe (Interchim) and analysed using a CP-3800 gas chromatograph (Varian). The apparatus was equipped with a flame ionization detector (FID) for acetylene and ethylene detection, and thermal conductivity detectors (TCDs) for air nitrogen detection. Gas analysis was performed on a Rt-QS-Bond capillary PLOT fused-silica column (15 cm × 0.53 mm × 20 µm; Restek) under the following conditions: column input flow rate 9.1 ml min^{−1}, oven temperature 40 °C, FID temperature 150 °C, helium as gas carrier (30 ml min^{−1} flow) and make-up (20 ml min^{−1} flow), hydrogen at 4.5 bar and air at 300 ml min^{−1} flow. The column retention time for acetylene and ethylene was monitored with standards throughout the experiments. Sea water and symbiont-free *C. orbicularis* foot tissue were assayed as negative controls, as was a sediment sample from the *T. testudinum* bed (larger seagrass roots were removed before analysis). In addition, gill and foot tissue samples, purified symbionts, sea water and sediment samples were incubated without acetylene and assayed for ethylene production to exclude false positive results. No ethylene production was detected in any of these samples incubated without acetylene.

Phylogenetic analysis

NifH phylogeny. The NifH tree was created in two consecutive steps. First, full-length NifH amino acid sequences of the *C. orbicularis* symbiont and of related cultured diazotrophic organisms were aligned and the phylogeny was inferred based on maximum likelihood. Second, partial NifH sequences of environmental clones from various marine habitats were added using ARB's interactive parsimony (see later in this paragraph). The NifH protein of '*Ca. T. endolucinida*' was identified by

searching the Pfam⁶⁶ model Fer4_NifH (PF00142, available from <http://pfam.xfam.org/family/PF00142/hmm>) against all annotated proteins of the *C. orbicularis* symbiont's draft genome with hmmssearch (Expect value 1e-10) from HMMER version 3.1b2 (ref. 67). Its sequence was subsequently searched with HMMER's phmmer (Expect value 1e-10) against a local version of NCBI's nr database (version 2015-11-17), and the top 100 hits, that is, the 100 most closely related NifH sequences from other organisms, were retrieved. Additional (partial) NifH sequences of bacterial isolates from tropical seagrass beds⁴², oligotrophic open ocean⁶⁸, salt marsh ecosystems^{69,70}, mangrove root sediments^{71,72} and sulfidic cave water⁴⁴ (1,007 sequences in total) were included. Some of these NifH sequences⁷¹ were only available as nucleotide sequences and were therefore translated using EMBOSS Transeq⁷³ and the correct reading frame was selected manually. Four additional NifH protein sequences from known thiotrophs and two sequences from the outgroup taxa *Anabaena variabilis* ATCC 29413 (NifH: AAA93020) and *Frankia alni* Ar13 (NifH: AAA96262) complemented the data set, giving a total of 1,114 sequences for the NifH-based phylogenetic analysis. Sequence entries from the PDB and PRF databases as well as sequences containing stop codons or ambiguous amino acids (X) were removed, leaving 1,073 sequences that were clustered at 100% identity with CD-HIT (refs 74,75) to remove redundancies. All 834 representative non-redundant sequences (93 full length, 741 partial length) were automatically aligned with MAFFT version 7.221 (2014/04/16)⁷⁶ using the L-INS-i algorithm and the 'leave gappy region' option. The alignment was imported into ARB version 5.5 (ref. 77) using a custom filter, and a protein database was created. A total of 55 full-length amino-acid sequences were selected and exported. ProtTest version 3 (refs 78,79) was employed to select the best-fit model of amino-acid replacement from 120 models (15 matrices: +G, +I or +G+I; +F) with a starting topology based on maximum likelihood. As the Bayesian information criterion (BIC; also known as the Schwarz criterion, or SC)⁸⁰, the corrected Akaike information criterion (AICc)^{81,82} and the decision theory framework (DT)⁸³ favoured LG+I+G, the phylogeny was then inferred using RAXML version 8.2.4 (ref. 84) with the LG amino acid matrix⁸⁵, a gamma model of rate heterogeneity and an estimate of proportion of invariable sites. The best tree was chosen from 1,000 independent inferences that were executed on the original alignment (169 alignment patterns) using 1,000 distinct randomized maximum parsimony (MP) trees and was imported into ARB version 6.0.3. All 741 partial length NifH sequences were added by ARB's interactive parsimony. Sequences that could not be unambiguously inserted at a specific position in the tree were removed. To identify the partial length NifH sequences with the highest identity to the full-length sequences, a BLAST+ (ref. 86) protein database of all partial sequences was created and the full-length sequences that had partial sequences assigned by parsimony were searched against this database using BlastP version 2.2.31+. For each full-length sequence, only the best matching partial sequence (that is, the one with highest identity) was kept in the tree, and only if sequence identity between both was 95% or higher. The final tree (Fig. 5) thus comprises the *C. orbicularis* symbiont's NifH, 54 full-length NifH sequences of cultured organisms and 21 partial NifH sequences mostly from uncultured clones.

NifDK phylogeny. The NifD and NifK trees were constructed based on maximum likelihood as described above and include the respective amino acid sequences of the *C. orbicularis* symbiont and of the same cultured organisms that are displayed in the NifH tree, but with two exceptions: *Methylogaea oryzae* JCM 16910 is not part of the NifK tree and *Agarivorans gilvus* WH0801 could not be included in the NifD tree because the respective genes (locus tags JCM16910_RS02630 and AR383_RS01715) are disrupted. *Anabaena variabilis* ATCC 29413 (NifD: AAA93021; NifK: AAA93022) and *Frankia alni* Ar13 (NifD: AAA96263; NifK: AAA96264) were used as outgroup taxa. Note that the uncultured clones from seagrass and mangrove sediments (dotted branches in the NifH tree) are not included in the NifD and NifK trees, because no NifD or NifK sequence information was available for these isolates.

16S rRNA phylogeny. The 16S rRNA-coding region of the '*Ca. T. endolucinida*' genome was identified using barnmap version 0.6 (available from <https://github.com/tseemann/barnmap>) and automatically aligned against the SILVA SSU Ref NR 99 Release 123 database (available from <http://www.arb-silva.de>)⁸⁷ using the SILVA Incremental Aligner (SINA) version 1.2.11 (ref. 88). The SINA-aligned sequence was imported into the ARB software package version 5.5 (ref. 77). Besides the *C. orbicularis* symbiont's 16S ribosomal RNA nucleotide sequence and its closest homologues (by NCBI blastn), the data set contained additional 16S rRNA sequences of bivalve and gastropod symbionts, free-living sulfur oxidizers and some diazotrophic bacteria, whose NifH sequences were also included in the NifH tree. The alignment of these 58 sequences was manually refined taking into account the secondary structure information of the rRNA. Phylogenetic reconstruction was performed using a maximum likelihood method. The final tree was calculated with RAXML version 8.2.4 (GTRGAMMA model)⁸⁴ and was based on an alignment with 750 distinct alignment patterns. The best tree from 1,000 independent inferences using 1,000 distinct randomized maximum parsimony trees is presented. *Frankia alni* ACN14a (CT573213) was used as outgroup.

Accession codes and data availability. Sequence data that support the findings of this study, that is, the '*Ca. T. endolucinida*' Whole Genome Shotgun project, have been deposited at DDBJ/ENA/GenBank under project accession

no. [MARB00000000](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA284177). The version described in this paper is version MARB01000000 (NCBI BioSample [SAMN03435122](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA284177), BioProject [PRJNA284177](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA284177)). All nitrogen metabolism-related protein identifications obtained in this study are available in Supplementary Table 4. Additional data (for example, MS raw data) are available on request from the corresponding author (S.Ma.).

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Author contributions

S.K. and O.G. planned and performed the experiments with *C. orbicularis*. Sediment chemistry analyses were conducted by J.T., western blots and immunohistochemistry by M.V., and acetylene reduction measurements by O.G. and D.M.-M. Proteome analyses were planned by S.K., T.S., S.Ma. and D.B., and conducted by S.K., S.Me. and D.B. A.T., A.P. and R.D. performed the genome sequencing and submitted the sequences to NCBI. S.K. and S.E.H. assembled and annotated the genome. S.E.H. performed the phylogenetic analyses and gene cluster comparisons. O.G., T.S. and S.Ma. coordinated and supervised the laboratory work. S.K. and S.Ma. analysed the proteome data. S.Ma. wrote the

manuscript. T.H. was involved in manuscript writing, figure generation and, together with R.P., in MS data evaluation.

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Competing interests

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