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SHORT COMMUNICATION

Imaging the uptake of nitrogen-fixing bacteria into larvae of the coral *Acropora millepora*

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Diazotrophic bacteria are instrumental in generating biologically usable forms of nitrogen by converting abundant dinitrogen gas (N_2) into available forms, such as ammonium. Although nitrogen is crucial for coral growth, direct observation of associations between diazotrophs and corals has previously been elusive. We applied fluorescence *in situ* hybridization (FISH) and nanoscale secondary ion mass spectrometry to observe the uptake of ^{15}N-enriched diazotrophic *Vibrio* sp. isolated from *Acropora millepora* into conspecific coral larvae. Incorporation of *Vibrio* sp. cells was observed in coral larvae after 4-h incubation with enriched bacteria. Uptake was restricted to the aboral epidermis of larvae, where *Vibrio* cells clustered in elongated aggregations. Other bacterial associates were also observed in epidermal areas in FISH analyses. Although the fate and role of these bacteria requires additional investigation, this study describes a powerful approach to further explore cell associations and nutritional pathways in the early life stages of the coral holobiont.
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Introduction

Nitrogen is essential for growth, development and maintenance of all living cells, yet it is typically limited in nutrient-poor systems such as waters surrounding coral reefs. Consequently, symbioses with nitrogen-fixing organisms (diazotrophs) that convert the abundant gas dinitrogen (N_2) into more usable forms like ammonium (NH_4^+) provide important supplemental sources of nitrogen. Although evidence is mounting that nitrogen-fixing bacteria are associated with corals (Shashar *et al.,* 1994; Lesser *et al.,* 2004, 2007; Kvennefors and Roff 2009; Olson *et al.,* 2009; Kimes *et al.,* 2010; Lema *et al.,* 2012; Santos *et al.,* 2014; Lema *et al.,* 2014a,b), to date the location of nitrogen-fixing bacteria within coral tissues and evidence that fixed nitrogen is available to corals or their endosymbiotic algae, *Symbiodinium,* remains elusive. Techniques such as nanoscale secondary ion mass spectrometry (NanoSIMS), which is able to map enriched stable isotope (for example, ^{13}C, ^{15}N and so on) tracers at the cellular scale, provides a powerful tool for co-locating diazotrophs within coral tissues. When combined with fluorescence *in situ* hybridization (FISH), the presence and functional role(s) of bacteria within the coral host can be identified.

Rapid uptake of ammonium by the coral animal and transfer to symbiotic dinoflagellates have been demonstrated through molecular studies (Yellowlees *et al.,* 2008; Stambler, 2011) and hypothesized to be key strategies in the nutritional economy of cnidarians that have evolved in nutrient-poor waters (Pernice *et al.,* 2012; Kopp *et al.,* 2013). Support for this is provided by recent work that used NanoSIMS to demonstrate ammonium assimilation from ^{15}N-labelled ammonium ($^{15}NH_4Cl$) in cells of both the coral host (that is, *Acropora aspera* and *Isopora palifera*) and *Symbiodinium* symbionts after just 1-h incubation (Pernice *et al.,* 2012; Pernice *et al.*, 2014). NanoSIMS technology has also been

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used to show the incorporation and translocation of labelled ammonium ($^{15}NH_4$) originating from nitrogen-enriched cultured bacteria (*Vibrio* sp. and *Alteromonas* sp.) into coral larval tissues and associated *Symbiodinium* cells within 8 h of coral-bacterial incubations (Ceh *et al.,* 2013). Although the latter demonstrated incorporation of labelled nitrogen from bacteria into coral and *Symbiodinium* cells, the presence of these bacteria in coral host tissues could not be confirmed and the capacity of these bacteria to fix dinitrogen gas was not investigated.

Materials and methods

In the present study, our goal was to observe the uptake of nitrogen-fixing bacteria (labelled with ^{15}N) into aposymbiotic (that is, *Symbiodinium*-free) larvae of the broadcast spawning coral *Acropora millepora*. The uptake of diazotrophs was initially identified through FISH, and the distribution of the ^{15}N label was observed using NanoSIMS (see Supplementary Methods for details of techniques). Briefly, a nitrogen-fixing *Vibrio* sp. (GenBank accession number KF691569) was isolated from *A. millepora* juveniles and grown with 99% $^{15}N_2$ gas for isotopic labelling (Cambridge Isotope Laboratories Inc., Cambridge, MA, USA; gas cylinder) (Supplementary Figure S1; Supplementary Table S2). Nitrogen-enriched bacteria (1×10^6 bacteria ml^{-1}) were incubated with aposymbiotic larvae of *A. millepora* that were all at a similar developmental stage (that is, 4–5 days after coral spawning; larvae elongated with a distinct oral pore that was not fully developed to avoid predation capacity) in six-well plates filled with 0.2 μm-filtered seawater ($n = 10$ larvae per well). The control treatments were: larvae incubated with unlabelled (that is, natural abundance $^{15}N/^{14}N$) *Vibrio* sp. cells (C1); and larvae incubated with the supernatant of sonicated ^{15}N labelled *Vibrio* sp. cells (C2) (see Supplementary Materials). Swimming larvae were sampled after 4 h of incubation to avoid dilution of the ^{15}N signal and fixed in 4% paraformaldehyde in filtered seawater.

Results

Bacteria within coral larvae were located using FISH probing performed on whole larvae samples, involving either an equimolar mix of the universal eubacterial EUB338 and specific *Vibrio*-GV probes, or a negative control non-EUB338 probe at a final concentration of 5 ng μl^{-1} (see Supplementary Materials and Supplementary Table S1 for details on probe sequence and fluorophore emission spectra). Imaging performed on whole *A. millepora* larvae ($n = 16$) using confocal laser scanning

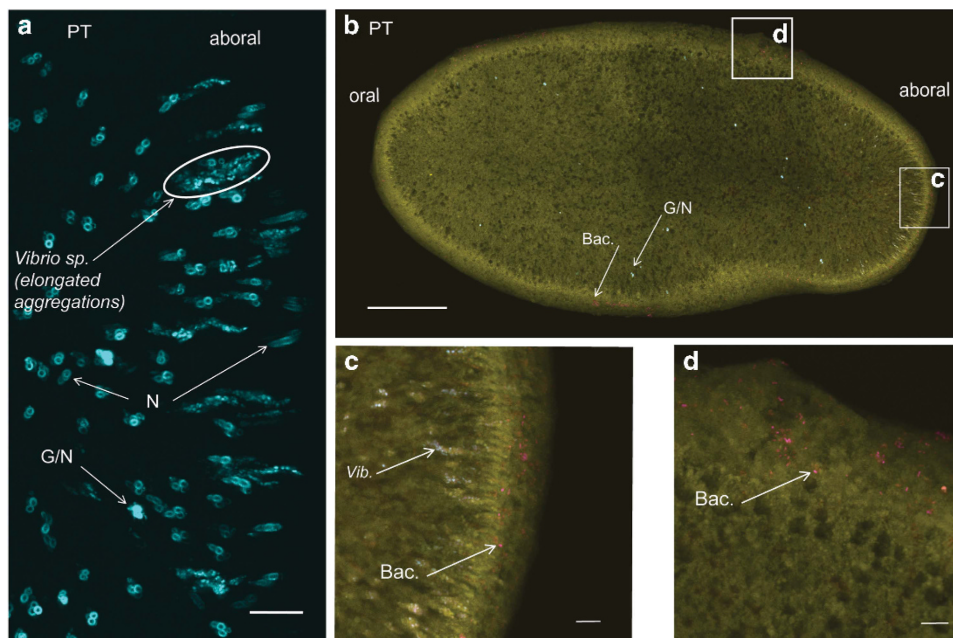

Figure 1 Maximum intensity projection confocal images of two representative 4-day old *Acropora millepora* larvae incubated with ^{15}N-enriched nitrogen-fixing *Vibrio* sp. Larval samples were hybridized with probes *Vibrio*-GV (ATTO647) (emits in far red laser emission channel; coloured as cyan), and EUB338 (AlexaFluor 546) (emits in orange emission channel; shown as magenta) (see Supplementary Table S2 for details on emission wavelengths). Images show: (**a**) aboral end of larva, identifying *Vibrio* sp. bacterial agglomerates (circled), unspecific binding of nematocysts (N) and possible gland cells (G); (**b**) larva with location of insets, where **c** shows *Vibrio* sp. (*Vib.*) aggregates in the aboral epidermis (cyan structures); and **d** shows bacterial aggregations (Bac.) in epidermis (magenta structures). PT: positive treatment (that is, larvae incubated with ^{15}N-enriched *Vibrio* sp. cells). Scale bars, (**a**) 20 μm, (**b**) 100 μm and (**c** and **d**) 5 μm.

microscopy (Nikon A1Si, Tokyo, Japan) and three-dimensional imaging revealed elongated aggregations of bacterial cells only in the epidermal cell layer at the aboral end of the larvae (Figures 1a and c; Supplementary Figures S2). These were identified as *Vibrio* sp. through specific binding of the *Vibrio*-specific probe and the general eubacterial probe (EUB338) (Supplementary Figures S2e and f). Additional bacteria (revealed by the general eubacterial probe EUB338) were also observed within coral cells. In our study, bacteria (positively labelled by probe EUB338) were located in the epidermal layer, but were found throughout the whole larvae (that is, both ends of the larvae aboral and oral, as well as middle section) (Figures 1b–d).

One experimental larval sample was selected for ^{15}N NanoSIMS analysis (Cameca NanoSIMS 50 ion microprobe, Cameca, Courbevoie, France) together with two control samples, C1 and C2. The selected samples contained *Vibrio* sp. aggregations, as assessed by FISH probing. NanoSIMS analyses, undertaken in the aboral epidermal regions where

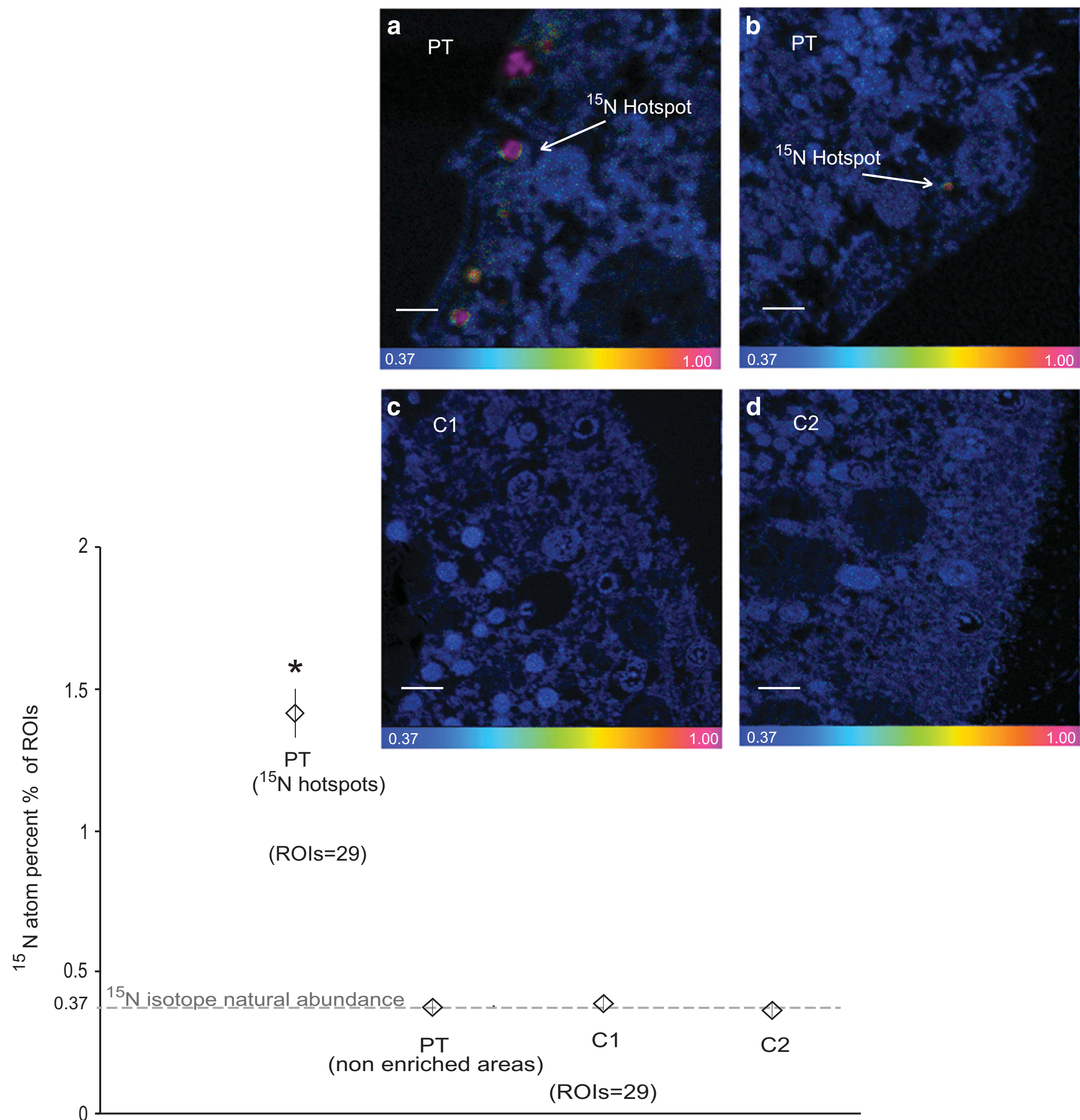

Figure 2 NanoSIMS $^{15}N/^{14}N$ ratio images of *Acropora millepora* larvae (aboral epidermis) after 4 h incubation with ^{15}N-enriched *Vibrio* sp., and a plot showing the average ($n = 29$) atom % and associated standard error from regions of interest in positive and control treatment images. The ratio is expressed as a hue saturation intensity image, where blue represents the natural isotopic abundance of nitrogen (^{15}N atom % = 0.37) and enrichment is shown as a shift towards magenta (colour scale label in atom %). Dashed line indicates the natural isotopic abundance of ^{15}N at 0.37 atom %; asterisks (*) denote significant differences between positive samples and controls, as assessed by one-way permutational multivariate analysis of variance ($P < 0.001$). Scale bars, (**a**) 2 μm, (**b**), (**c**) and (**d**) 5 μm. PT: positive treatment (that is, larvae incubated with ^{15}N-enriched *Vibrio* sp. cells); C1 (control 1): larvae incubated with unlabelled *Vibrio* sp. cells; and C2 (control 2): larvae incubated with the surnatant of sonicated ^{15}N-enriched *Vibrio* sp. cells.

Vibrio sp. aggregations were observed by FISH analysis, revealed the presence of ^{15}N-enriched hotspots (1.41 ± 0.08 ^{15}N atom % levels recorded; cf. 0.37 ^{15}N atom % for natural isotopic abundance), with sizes matching those of bacteria in FISH images (Figure 2; Supplementary Figures S1 and S6; Supplementary Table S2). However, no translocation of ^{15}N could be observed into surrounding areas of larval tissue, where N levels (0.377 ± 0.001 atom%) were close to natural abundance (Figure 2; Supplementary Table S2; Supplementary Figure S6). Similarly, in controls (C1 and C2), no ^{15}N-enriched regions were detected, and all regions of interest (that is, aboral epidermal areas) displayed N values close to natural abundance (C1: 0.377 ± 0.001 ^{15}N atom %; C2: 0.379 ± 0.001 ^{15}N atom %; Figure 2; Supplementary Table S2; Supplementary Figure S6). Differences in ^{15}N abundance between enriched hotspots and non-enriched tissue areas, and between enriched hotspots versus controls, were statistically significant (Figure 2; Supplementary Table S2; permutational multivariate analysis of variance; $P < 0.001$).

Discussion

This study demonstrates that aposymbiotic (that is, *Symbiodinium*-free) larvae of the coral *A. millepora* are able to uptake nitrogen-fixing bacteria (*Vibrio* sp.) originally isolated from conspecific coral juveniles. Both FISH and NanoSIMS analyses confirmed the presence of *Vibrio* sp. cells and enriched ^{15}N hotspots, respectively, in the aboral epidermis of larvae after 4 h of co-incubation. Further experiments are now required to ascertain if nitrogen fixed by these bacteria is subsequently available to the coral host, as well as the subsequent fate of and extent to which this *Vibrio* sp. is beneficial to the coral host through nutritional supplementation. Our study provides a reliable approach to detect and observe rapid incorporation of specific bacteria into the coral holobiont and further explore nutritional pathways in the early life stages of these complex symbiotic relationships. This knowledge will advance understanding of the role bacteria have in coral larval survival, a critical stage in the resilience and success of coral reefs that face increasing anthropogenic stress.

Conflict of Interest

The authors declare no conflict of interest.

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