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Biogeosciences Discussions

Interactive comment on "16S rRNA gene metabarcoding reveals a potential metabolic role for intracellular bacteria in a major marine planktonic calcifier (Foraminifera)" *by* Clare Bird et al.

Anonymous Referee #2

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This MS is a challenging study to unveil bacterial endosymbiosis in planktonic foraminifera. The topic is interesting for a wide range of people, if the results of this study have certainly demonstrated the presence of endosymbionts and their metabolisms. Here, I would like to mention four major questions about the methods and results of this study.

(1) Amplicon sequencing does not exactly show the quantity of DNA in the cell. The authors used amplicons, which were amplified by PCR, for 16S rDNA sequencing. In this process, the primers never randomly attach to DNA molecules. Even though

16S amplicon shows high abundance of DNA sequences of the cyanobacteria (Result 3.3), this does not mean high abundance of cyanobacteria in the foraminiferal cell. A large variation (37–87%) of the abundances of the cyanobacterial DNA sequences possibly shows a bias of PCR amplification. The authors removed some contaminated sequences from the samples based on the three negative controls (Method 2.6.1). However, we still find the sequences of the class Alphaproteobacteria in Fig. 3. What are they? Are they contaminates, preys, or symbionts?

(2) The data of 16S rDNA sequences does not show "living" bacteria in the foraminiferal cell. The DNA fragments are highly remained in the cell, if the cell (foraminifer) takes bacteria through endocytosis. As mentioned in the method section 2.2, the samples were immediately put in the buffer after collection. In this case, foods were not digested in a planktonic foraminifer. If G. bulloides is a bacteria-feeder, the DNA fragments of foods could be detected in 16S rDNA sequencing. This hypothesis (bacteria-feeder) is also reasonable to explain why the components of 16S rDNA sequences without cyanobacteria were different among the specimens (Fig. 3).

(3) The images of DAPI and TEM are not enough to support the presences of "living" bacteria in the foraminiferal cell. As the image of DAPI (Fig. 2) was unclear, it's difficult for me to follow the description in the result section 3.2. Although the TEM image was clear, there is no explanation about cytoplasm. Which are vacuoles? Are there phagosomes? The TEM images indicated the cell structures, which have carboxysomes and thylakoid membranes, as a character of the cyanobacteria. However, these images were not enough to certify that Synechococcus are living in the foraminiferal cell. As the body size of bacteria is very small rather than foraminifera, they can be remained in the foraminiferal cell. At least, the authors will need to count the number of bacteria-like cells in a planktonic foraminifer and show how these bacterial cells are universal. Moreover, I recommend the authors to use the FISH (Fluorescence in situ hybridization) method for detecting the "living" bacterial cells.

(4) General characters of Synechococcus did not prove their metabolic functions in the

foraminiferal cell. Synechococcus generally take or use nitrogen, carbon, and phosphorus. Their uptakes are different depending on species or clades. The current data and discussion (4.2) have no evidence which Synechococcus clade (I to IV) take these elements. Moreover, the authors should investigate intracellular distributions and assimilations of these elements. Please see one good example: Nomaki et al. (2016) published in Frontiers in Microbiology.

Because of these reasons, the title of this MS is not supported by any results. Although this study may show the presence of bacteria in a planktonic foraminifer, it is open to question whether those bacteria are endosymbionts or not. If the authors won't show any additional information to answer the questions as mentioned above, the title, abstract, and main text should be modified to report finding bacterial cells in a planktonic foraminifer. In particular, please delete the descriptions concerning the carbon isotope of the foraminiferal shells, because no data is suggesting this topic.

Minor comments:

a) Abstract (lines 3-4): Not "unusual". G. bulloides is one of spinose species without algal symbionts.

b) Abstract (line 8): There is no direct and own data about the bacterial populations in the water column.

c) Abstract (line 16): This study does not show the presence of bacterial enobiont.

d) Abstract (lines 17-19): Please delete.

e) Keywords: "symbiosis", "endobiont", and "carbon isotope" are deleted.

f) Introduction: The first two paragraphs should be omitted. Instead of them, the authors need to describe how the other studies demonstrated the presence of symbionts in Foraminifera and/or other organisms.

g) 2.2 Sample collection: Samples were collected from the vertical net-towing or sur-

face water. In this case, it is very difficult to compare the hosts with bacterial components in different depths.

h) 2.3 Decalcification: Have the authors decalcify and wash the cell for all specimens?

i) 2.6. DNA extraction: Why did the authors use only one comparison (N. dutretrei)?

j) Discussion 4.1, 4.1.4: Based on amplicon sequencing, it is difficult to discuss the "abundance" of bacteria. Please see my comment (1).

k) Discussion 4.1.3: The authors used "selective uptake" in line 28. It's wrong, because there is no evidence.

l) Discussion 4.1.4: A visual coloration of planktonic foraminiferal cytoplasm does not demonstrate the characters of Synechococcus clades.

m) Figure 2: Unfortunately, Fig. 2 was somehow incomplete. Especially, I cannot find black arrows in Fig. 2a.

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