Biogeosciences Discuss., 10, C247–C252, 2013
www.biogeosciences-discuss.net/10/C247/2013/

Biogeosciences
Discussions

Interactive comment on "Differential gene expression in the mussel *Bathymodiolus azoricus* from the Menez Gwen and Lucky Strike deep-sea hydrothermal vent sites" *by* R. Bettencourt et al.

Anonymous Referee #2

Received and published: 8 March 2013

The manuscript by Bettencourt et al provides data regarding the symbiont population and expression of genes associated with immune response in mussels sampled at two vent sites differing in depth, Menez Gwen and Lucky Strike. Overall the writing is very confusing, questions are not clear, and several parts suffer from significant problems. In the abstract, the question is not clear and the procedure is not well explained, so one hardly understands what the manuscript is about. This needs to be carefully formulated. The question as it stands is "In the present study, the specific gene expression levels for both bacterial genes and host-immune related genes, were compared between animals from the shallower Menez Gwen and the deeper Lucky Strike vent sites, to address the hypothesis that distinct hydrothermal vent sites can be experi-

mentally differentiated on the basis of gene expression patterns and in the vent most emblematic species, the. . .". I do not understand the rationale for experimentally differenciating on the basis of gene expression patterns (with immune response genes). I would rather think that authors want to test whether living in distinct sites results in distinct gene expressions because of physic-chemical and/or symbiont densities difference. The whole point indeed remains obscure: do authors want to point to site-related differences arguing that host species and symbionts are similar on both sites? And the link between the immune system and symbionts is not clear to me. For sure the immune system has something to do with the presence of bacteria but this is not really presented, nor discussed, and there lacks a clear question to which the design of the study answers.

Samples: A major problem is that we have no idea of the physiological state of the animals at the time of sampling. We have no information regarding their habitat, and its heterogeneity. Linking differences to the distinct depths or sites is a bit short, it could simply be linked to different levels of exposure to fluids, parasites, symbionts, or even stress (including during recovery). This is hard to test for of course, but anyway the design of the study as is would not allow it. In many instances (gill microbiome structure, qPCR), it is not clear how many specimens were investigated, how were replicate specimens treated, and which type of statistical support is reached.

FISH: The FISH part suffers several significant problems. Probes used for methanos and thios are not properly referenced. I had to Google them to find out that these were from a 2006 Environ Microbiol paper but this is not cited in this part, and the names under which probes were initially published (BMARm and BMARt) is not even mentioned. Also, universal primer sequences 8F and 1492R have never been properly tested as probes, and should not be used without proper testing. Universal probes exist for bacteria, such as Eub338 for example in Amann et al. papers, 1990. Methanotrophs were not detected in LS animals but then it is said that they assumed a more random distribution and lesser signal intensities. Then universal primers (as probes) seem to

target methanotrophs, not thios. I do not understand. Another big issue it that apparently, authors assume the published probes to target functional genes, but they target 16S rRNA. Caption of Fig. 2 and discussion indicate that “Methane monooxygenase-, Sulfur oxidation Sox B and 16S rRNA-based fluorescent probes were used”. Only 16S rRNA-based probes are mentioned in the material and methods. Probes employed to target functional genes (and thus mRNA) are usually polynucleotidic, as described in Pernthaler et al 2004, and this is a different approach. There is no such thing as a methane monooxygenase gene-specific probe in this study. The whole paragraph and the discussion are thus very confusing. Misunderstandings also extend to the microbiology. For example, MMO (methane mono-oxygenase) is absent from methylotrophs (which use C1 compounds but not methane), and present only in methanotrophs. It is the first enzyme by which methane is oxidized to methanol. This ability separates methanotrophs from methylotrophs, which can process methanol but not methane. Both methylos and methanos have the subsequent enzymes, including methanol dehydrogenase. And RubisCO is more often associated with thiotrophs (it catalyses CO2 fixation from the CBB cycle), meanwhile only one study (Elsaied 2006) suggests RubisCO could be present in the methanotroph. The FISH results somehow illustrate some points, but signals are not self-evident, in particular for LS methanos and thios. In my opinion, FISH has not worked properly. Authors should re-check the protocols used for FISH and try again. If material for histology was indeed stored in 10% formalin as indicated in the material and methods, this is not unexpected, as this mixture does not work well for FISH. Other, more reliable fixation protocols exist (using 4% formaldehyde for a few hours, then rinse and dehydrate).

Bacterial community

Regarding proportions of sequences in the 454 analysis, they very unlikely correlate with those of bacteria in gill tissue. Indeed, a RT-PCR was done which will favour the more active bacteria (more 16S rRNA because more ribosomes are present). The result will be a mixture between bacterial densities and activities, to which we must

add RT-PCR bias. I however agree that if the 454-estimated structure is similar, it can be assumed that the communities must not be completely different. But any further interpretation should be very cautious.

qPCR Authors use 28S rRNA as an internal standard for gene expression studies, which is fine for animal genes. A big concern is that they use this gene also for the bacterial part. A single copy-or-so bacterial gene (16S, 23S) should be employed. Indeed, expression needs to be measured against the number of cells, which can be very different from one mussel to the next, and one site to the next. In this regard it’s intriguing that 12 out of 14 bacterial genes are “overexpressed” in LS mussels. A simple reason could be that these contain more bacteria per host cell unit (as estimated by 28S, and as acknowledged in the discussion). This does not mean that each bacterium actually expresses more this or that gene. Some studies have employed symbiont probes coupled to universal primers to estimate bacterial densities by qPCR (works by Tanguy, Boutet. . .). This approach may be criticized but at least provides a first clue. This could also allow comparing host immune response against distinct bacterial loads. As a consequence I disagree with the conclusion that the similar percentage of thios vs methanos at LS and MG support that the change is due to higher expression. Because the sequencing only tells (at best) something about relative abundances (see above). In Fig. 3, one hardly sees any trend in the results: in some cases LS>MG, and in other cases LS<MG. We don’t know if differences are any significant. It is usually assumed that there should be at least a factor 2 between the values, unless proper statistics based on replicates can be obtained. In that respect error bars seem to represent +-5% of the value rather than standard deviation from the 6 replicates, please check. If we stick to that empirical rule, not many of the differences are “significant” (whatever that means without statistics), but maybe sticking to this would make the story clearer.

Overall, the whole writing of the results makes them very hard to follow. Some points are unclear: what does it mean that “PGRP was highly induced in MG animals”, compared to what? Unstressed animals, LS specimens? It could well be that it is repressed

in LS. There are many long sentences that would best be cut into two sentences.

Discussion The whole discussion did not answer my own question: what is the hypothesis behind testing immune response genes? Differences are acknowledged to be difficult to explain in the context of this study (p.2024). Again I do not understand the link between this and symbionts. A wild guess would be that symbiont presence, and/or density might influence marker genes of the immune response, and thus if bacteria are more abundant, the system is up-regulated. Such a hypothesis might have been tested in some insect symbioses, and probably also in the bobtail squid (Ruby and McFall Ngai groups) and it would be worth checking, as this might shed light on what's going on in mussels. Here the hypothesis is not clear, and the generally uncomfortable feeling is that we're missing a piece of the puzzle. The discussion regarding immunity feels very disconnected from the mussels and their symbionts, and reads like a list of gene abilities that remain to be investigated in mussels. For example the concluding sentence says one thing and its opposite "It also points to the possibility of using specific immune or stress-related genes to characterize transcriptional statuses in deep sea hydrothermal vent animals particularly when aiming at Bathymodiolus mussel's acclimatization studies in land based aquaria systems. However, no clear immune gene expression signature was able to be depicted from our study given the variability of expression observed within and between the different functional immune gene categories for both MG and LS samples Âż.

To conclude, this manuscript provides a set of rather independent experiments, with little connection between them. Results are not integrated into a proper discussion, mostly because we lack a clear question, or at least a starting point. The link between immune response and symbiosis should be investigated first from the literature, which provides some clues that might be used as hypotheses here. But in my opinion the design of the study does not allow to explore site- or symbiont-related difference in gene expression.

Minor points: -In the abstract please define MG and LS (I guess it stands for Menez

Gwen and Lucky Strike) -Geographically distinct mussels can't be distinguished based on 16S rRNA amplicon sequencing, at least from the point of view of bacteria. -2.1: authors should mention depths for MG and LS, as this is an important point they try to make. -2.2: authors should clarify whether RNA was extracted onboard, or later on back to the lab. -P2025 l5: could indicated, please correct -Fig. 1: where the data comes from (pure fluid, fluid/seawater mixing zone, or around animals) should be indicated. There are many more references available than indicated here, and it would be nice to specify ranges of values rather than single values which we have no idea which of the papers they come from.

Interactive comment on Biogeosciences Discuss., 10, 2013, 2013.