

Rebuttal Anonymous Referee #1

We thank Referee #1 for taking the time to review our discussion paper.

We understand the paper in its present form, reveals some inconsistencies and confusing interpretations partially due to an apparent lack of “story-line” and incoherent flow between different sections and experiments reported. This was reconsidered and carefully amended in a revised version of the manuscript but readers and public audience should be aware that the work presented is of descriptive nature and not aiming at the characterization of physiological reactions and molecular mechanisms in light of differences found in gene expression between Menez Gwen and Lucky Strike *B. azoricus* mussels. In the contrary, our manuscript was aiming at the finding of “signatures” or “markers” of descriptive nature, supported by differences found at gene expression levels, in-situ hybridization results and 16S amplicon sequencing results. All three have the potential to show differences of such markers or signatures pointing at the geographical origin of both Menez Gwen and Lucky Strike populations and interpret our results in light of our long-standing knowledge of immune genes in *B. azoricus* and more recently our microbial community studies from gill tissues.

We believe the paper was harshly misevaluated due to an error on our part in writing the probe sequences used in in-situ hybridization experiments. We have in most of the cases and several years, used oligonucleotide probes aimed at nuclear genes instead of ribosomal genes. While the so called “correct general “standard FISH probes have been extensively popularized, and Duperron’s probes are well known for targeting 16S rRNA, nothing really impedes someone of using other probes of different sequences and test them in FISH experiments as they might work just as well. Not only we wanted to use probes targeting nuclear genes (this somewhat novel but it has now become more and more in use, especially in Nicole Dubilier’s lab) we wanted also to base our probes on bacterial sequences that we have revealed from our own transcriptome studies in *B. azoricus* not someone else’s sequences. Ribo probes work as well as cDNA probes as long as they follow the rules of base complementarity, RNA integrity (whether or not the target RNA is intact) and are targeting coding sequences since this is what we wanted to target, after all, expressed mRNA. In the present study we wanted to target the MMO and sulfur oxidation genes and used probes that were designed to target the respective nuclear genes:

MMO- CACTAACTATGCTAACCGCGATGTCA

SOX- CGACTAGGAGCACATCTATTAGGTTT

The sequences for our MMO probe design came from our sequence >mussel_c5320 length: 933 **methane monooxygenase protein A** [Methylococcaceae bacterium SF-BR] ensued from our transcriptomics studies already published and referred in the discussion paper as Bettencourt et al. 2010 BMC Genomics BMC Genomics, 11, 559, doi:10.1186/1471-2164-11-559, 2010 and Egas et al. 2012 Mar. Drugs, 10, 1765–1783, 2012.

The sequence for our SOX probe design came from our sequence [>mussel_c3834](#)
[length: 922 sulfur oxidation protein SoxY \[Sulfurovum sp. NBC37-1\]](#)

Ensued form the same published studies as above

A BLAST search in NCBI confirmed that our complementary sequence resulted in hits
within Bathymodiolus MMO gene

Bathymodiolus brooksi gill symbiont clone GoM_Chap_pmoA_2.1
particulate methane monooxygenase A (pmoA) gene, partial cds
Sequence ID: [gb|JN021262.1](#)|Length: 467Number of Matches: 1
Related Information
Range 1: 58 to 83[GenBankGraphics](#)Next MatchPrevious Match[First Match](#)
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
52.0 bits (26)	7e-05 ()	26/26 (100%)	0/26 (0%)	Plus/Minus	

Features:
Query 1 GTGATTGATACGATTGGCGCTACAGT 26
|||||
Sbjct 83 GTGATTGATACGATTGGCGCTACAGT 58

[GenBankGraphics](#)NextPrevious[Descriptions](#)
Endosymbiont of Bathymodiolus puteoserpentis partial pmoA gene for
particulate methane monooxygenase subunit A, sequence ID #7986
Sequence ID: [emb|FR865039.1](#)|Length: 471Number of Matches: 1
Related Information
Range 1: 31 to 56[GenBankGraphics](#)Next MatchPrevious Match[First Match](#)
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
52.0 bits (26)	7e-05 ()	26/26 (100%)	0/26 (0%)	Plus/Minus	

Features:
Query 1 GTGATTGATACGATTGGCGCTACAGT 26
|||||
Sbjct 56 GTGATTGATACGATTGGCGCTACAGT 31

A BLAST search in NCBI confirmed that our sequence [>mussel_c3834](#) length: 922
[sulfur oxidation protein SoxY \[Sulfurovum sp. NBC37-1\]](#) from which our SOX in-
situ probe was designed is indeed matching proteins hits containing the SOX Y domain
from SOX Y superfamily

[GenPeptGraphics](#)NextPrevious[Descriptions](#)
sulfur oxidation protein SoxY [Sulfurovum sp. AR]
Sequence ID: [ref|ZP_10062574.1](#)|Length: 155Number of Matches: 1
Related Information
Range 1: 6 to 155[GenPeptGraphics](#)Next MatchPrevious Match[First Match](#)
Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps	Frame
205	2e-	Compositional	119/150 (79%)	135/150 (90%)	0/150 (0%)	+1
bits(522)	62()	matrix adjust.				
Features:						
Query	301					
FLKSICAasavvatv	vsp	llva	KDAPKGGNALS	YDAA	vvtitggkvvtg	SDKIKLTVPEI 480
	F+KSICAASAV	ATV+PS	L AK+APKGGN	LSYDAAV	ITGGK V	SDK+
LTVPEI						
Sbjct	6					
FIKSICAASAVAATV	T	PSALFAKEAPKGGNVLS	YDAAVAAITGGKAVAD	SDKVNLT	TVPEI	65
Query	481					
AENGAVVPVKVNVES	PMTDADY	VKAIHVL	TTKNSNARCADV	MLTPLNGKGY	FATR	VKLGG 660
	AENGAVVPVKV+V+	PM +	+YVKAIHVL+TKN			
NARCADVMLTPLNGKGY	FATR+KLGG					
Sbjct	66					
AENGAVVPKVDVDHP	MEENNYVKAIHVL	STKNGNARCADV	MLTPLNGKGY	FATRIK	KLGG	125
Query	661	TQDVVALVEMSDGS	FLRAAKPVKVTIGGCG	750		
	TQDVVALVE+S+G+F+++AK	VKVTIGGCG				
Sbjct	126	TQDVVALVELSNGT	FIKSAKSVKVTIGGCG	155		

[GenPeptGraphics](#)[Next](#)[Previous](#)[Descriptions](#)

sulfur oxidation protein SoxY [Sulfurovum sp. NBC37-1]

Sequence ID: [ref|YP_001357815.1|](#) Length: 159 Number of Matches: 1

Related Information

[Gene](#)-associated gene details

Range 1: 32 to 159 [GenPeptGraphics](#)[Next Match](#)[Previous Match](#)[First Match](#)

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps	Frame
178	7e-	Compositional	90/128 (70%)	105/128 (82%)	0/128 (0%)	+1
bits(452)	52()	matrix adjust.				
Features:						
Query	367					
KDAPKGGNALS	YDAA	vvtitggkvvtg	SDKIKLTVPEIA	AENGAVVPVKVNVES	PMTDADY	546
	K PKG NALS +AA+	ITGGK	SDK+KLT	VPEIAENGAVVPVKVNV+	PM +	
+Y						
Sbjct	32					
KAVPKGNALS	VEAAIDA	ITGGKGAKESDKV	KLT	VPEIAENGAVVPVKVNV	DHPMEEGNY	91
Query	547					
VKAIHVL	TTKNSNARCADV	MLTPLNGKGY	FATR	VKLGGTQDVVALVEMSDGS	FLRAAKPV	726
	VKAIHVL KN N+RC	DVMLT	NGK YFATR+KLG	TQ+V+ +	E+SDG+F+++AAK	
V						
Sbjct	92					
VKAIHVLA	AKNGNSRCVDV	MLTPANGKAY	FATRIK	LGSTQE	VIGVAELSDGTFIKA	AKSV 151
Query	727	KVTIGGCG	750			
	KVTIGGCG					
Sbjct	152	KVTIGGCG	159			

So in conclusion, our probes are theoretically good for FISH experiments and results obtained do show signal specificity.

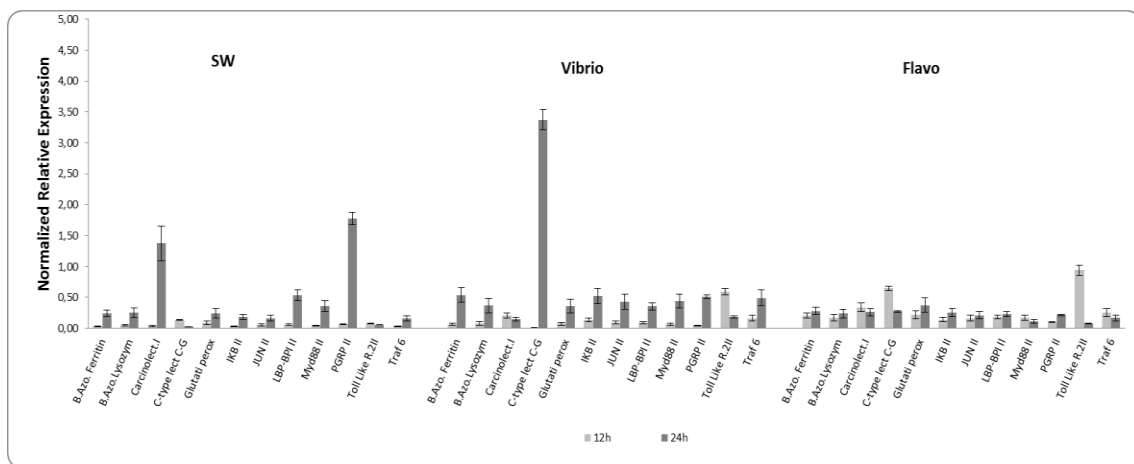
The following comment by the anonymous referee #1 raises some concerns

The cDNA library used for qPCR of bacterial genes was inappropriate as this was subjected to poly-A selection, a treatment designed to remove ribosomal RNA, but which also removes bacterial mRNA. For this reason, the V6 sequencing experiment also has little value, as the PCR products for sequencing were amplified from the same cDNA library

We have devoted 1 full-length article to this question apart from our work published in 2010 Bettencourt et al. BMC Genomics in which we described in detail how the cDNA library was built and how we ended up with 3000 bacterial genes sequences which made through the poly-A selection during the course of library preparation and prior to 454 sequencing. Bacterial mRNA do not have poly-A tails, right, but it is not true that bacterial mRNA will be removed after poly-A selection, actually some bacterial RNAs are poly-adenylated too. The point here is not to debate whether or not our initial cDNA was “contaminated” with bacterial mRNA but to deal with the fact that in our initial work on the transcriptome sequencing of *B. azoricus* gill tissues (vide Bettencourt et al. 2010 BMC Genomics) some 3000 cDNA sequences were revealed pointing at functional bacterial genes that were subjected to the MG RAST, the Metagenomics RAST server, an automated analysis platform for metagenomes providing quantitative insights into microbial populations based on sequence data. This is a fact that cannot go unnoticed and that prompted us to dedicate another work published in Marine Drugs by Egas et al. 2012 with the title “The Transcriptome of *Bathymodiolus azoricus* Gill Reveals Expression of Genes from Endosymbionts and Free-Living Deep-Sea Bacteria”. I do agree with the anonymous reviewer in that the V6 sequencing experiment also has little value, given the possibility that cDNA libraries were poorly represented by bacterial mRNA, however it was not clearly written or stated in our paper that both LS and MG cDNA libraries were obtained by using random primers and not oligo-dT during the process of reverse-transcription and that would significantly change the outcome of our analyses. The same total RNA was used but reverse-transcribed differently, using random primers instead of oligo-dT. Moreover, the 16S amplicon sequencing was strictly meant for gill’s microbiome structure analyses, not for quantifying bacterial gene expression.

Furthermore, the cDNA library used for qPCR of bacterial genes might have been inappropriate to the reviewer’s view, as this was subjected to poly-A selection but it was primarily generated for host gene expression studies, nonetheless given our previous results on the transcriptome sequencing of *B. azoricus* gill tissues and the bacterial genes that were revealed then, we felt compelled to pursue these qPCR experiments even with the same cDNA libraries. It is not uncommon that mispriming events do occur with RNA species and often cases, even ribosomal RNA will be misprimed with oligo-dT and reverse-transcribed into cDNA. rRNA is expressed at a very high level, so even a little leaky priming by nonspecific priming would work rather well, but not as well as the random priming. Such abundant RNA species (rRNA) almost invite mispriming merely by being highly present and thus a small amount will always be reverse transcribed into cDNA, including bacterial mRNA, because of normal, expected low-level RT mis-priming events... One has to assume in the present case that bacterial mRNA was highly present in our gill total RNA extractions!

Another point of criticism referred by the anonymous reviewer was related to the immune gene as not being validated or shown to respond to infection or microbial stimulation. The reason why we did not present evidence of gene expression upon immune stimulation is because we have submitted another paper to the same BG special issue that is regarded as a companion paper and dealing precisely with this subject. It has been submitted by Martins et al. from my group with the following title “Finding immune gene expression differences induced by marine bacterial pathogens in the Deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*”. However, I will be glad to include in the present paper, results from an infection experiment using *Vibrio parahaemolyticus* and *Flavobacterium* that was conducted in parallel, for validation purposes during the course of these studies (see below)



In conclusion the manuscript was substantially re-written in view of the criticism conveyed by anonymous referee #1. The title was change to make our point clearer that there are site-related differences in gene expression and bacterial densities in the mussel *Bathymodiolus azoricus* from the Menez Gwen and Lucky Strike deep-sea hydrothermal vent sites and these differences may serve as “markers” to differentiate *B. azoricus* populations from either vent site. The confusion between methanotrophs and methylotrophs is no longer present in this reviewed manuscript and the expression of methanol dehydrogenase gene is no longer claimed as an evidence for these bacteria not being methanotrophs because of the fair argument made by the referee that the expression of the methanol dehydrogenase gene simply cannot rule out the presence of methanotrophs. In view of this our manuscript has been amended.