Dear Editor,

Here I hope to address the remaining comments of the reviewers.

1 Round 3: D. Baurain

- COMMENT: About rooting, I can accept the author's logic for the sake of the argument, but please be more cautious in the phrasing: "this [reflects =¿ MIGHT REFLECT] functional differences rather than evolutionary history".
- REPLY: Still, I think that we can only know a gain of function by mapping the functions onto the history, and not the other way around. However, in the interest of compromise, this text has been changed to "which may be the result of functional divisions rather than evolutionary history.".
- COMMENT: Related to this point, I would like to get some argument in the text to better rule out ancestral paralogy in LUCA for SHC/OSC (e.g., not found at all in Archaea). This should come around these sentences: "Even if unintended by the authors, this implies a parallel origin of the two enzymes relative to the unknown outgroup, which would be outside of both bacteria and eukaryotes. It is unlikely that the tree should be rooted at this position, or at least should not be drawn this way."
- REPLY: A good point. This has been clarified to mention a scenario where most Archaea possessed OSC and Bacteria possessed SHC, though is unsupported by the absence of OSC or SHC in all Archaea. I now state: "Even if unintended by the authors, this implies a parallel origin of the two enzymes relative to the unknown outgroup, which would be outside of both bacteria and eukaryotes - potentially in the last universal common acenstor (LUCA). Such scenario would be plausible if SHC were present in early Bacteria and OSC were present in early Archaea. However, given the complete absence of either enzyme in known Archaea, this is unlikely. Thus, the tree should not be rooted at this position, or at least should not be drawn this way. "
- COMMENT: Two other comments in my original review have not been addressed yet. They pertain to Santana-Molina et al. 2020: 1) "SHC was the original enzyme, distributed across many bacterial lineages" (SM2020 says: "maybe not ancestral to all lineages");
- REPLY: My wording does not imply all lineages or the ancestral state, but this has been clarified to say "SHC was the original enzyme, distributed across many bacterial lineages (though not necessarily all)."
- COMMENT: 2) about a mitochondrial origin of OSC, SM2020 says that alpha-proteobacteria mostly lack OSC and I am not convinced by the author's answer "a single, ancient loss can remove the gene from nearly all alpha proteobacteria", except if the alpha-proteobacterial endosymbiont is assumed to be very deeply branched within the phylum.
- REPLY: This indeed appears to be the case, but the root is disputed between either very-deep or sister-to-the-rest. See both Martijn 2018 Nature and Fan 2020 Nature Eco Evo. Furthermore, similar circumstances of losses appear to be reported elsewhere, see Hugoson 2022 MBE regarding ancestral gene content of Legionellales.
- COMMENT: Regarding endoymbiosis and inheritance, this might sound like nitpicking but, to be me, a gene is acquired from an endosymbiont, not inherited, the latter being vertical in nature. Thus, I would amend the following two sentences for clarity: 1) "and then being [inherited =¿ ACQUIRED] by a pre-eukaryotic host from a bacterial endosymbiont"; 2) "This is explained by [vertical inheritance =¿ HORIZONTAL ACQUISITION] from bacteria at the origin of eukarotes (probably endosymbiosis)."

- REPLY: This is a tough question, and I see your point. To me it makes the most sense to say "endosymbiotically acquired", since it is not HGT as normally imagined, nor is it really vertical. This has been changed in the text.
- COMMENT: I am not sure about the meaning of Figure 1D: does the blue triangle correspond to additional eukaryotes? Or is it meant to depict the source of HGT to eukaryotes (with no arrow to highlight the events)?
- REPLY: I changed the caption to say "secondarily gained in select groups from a later horizontal gene transfer".
- COMMENT: Please clarify the extent of the synonymy between SHC and STC at first occurrence in the text and/or add "stc" gene to Figure 1A, as done for the synonyms of OSC. Indeed, in the headings and figures, both STC and SHC are used interchangeably and are sometimes mentioned together as synonyms. This is somewhat confusing.
- REPLY: My apologies, again these were originally named by biochemical activity, so the names are confusing. For clarity, I added the SHC in parentheses, changing the caption "Squalene-tetrahymanol cyclase (STC) does not strictly make hopene, but is included for clarity and would grop with SHC rather than OSC.".

I have also corrected the typos indicated by the reviewer.

2 Round 3: S Abalde

- COMMENT: However, it is worth mentioning that the major issue remains: the strength of the hypothesis depends on how reliable the rooting of the tree is.
- REPLY: This is ultimately a good consideration, but even as the methods that you had cited were at their infancy, and according to their own authors do not answer the question reliably, I think this would be beyond the scope of this paper.

3 Round 3: JR. Pardos-Blas

- COMMENT: I consider that the author has contributed to improve the manuscript with the comments. However, I would like to emphasize in one of them. I mentioned that the methods about the trimming process for the matrix should be incorporated into the manuscript so I recommend to the author to add his reply to the method section.
- REPLY: The trimming step has been added to the methods.