nature portfolio

Peer Review File

Evidence of an acetone carboxylation pathway in photoheterotrophic bacteria from the Arctic Ocean

Corresponding Author: Dr David Walsh

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

A version of this paper was originally rejected for publication by Nature Communications, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript suggests the presence of abundant Porticoccus arcticus bacteria that assimilate CO2 via acetone carboxylation and also have phototrophic capability. The existence of such activities would be important to understanding the ecology of organisms specializing in uptake and metabolism of low concentration volatile compounds, such as acetone. The P. arcticus genome was assembled by metagenomics and is closely related to P. hydrocarbonoclasticus, which also encodes the genes for acetone carboxylase and metabolism to acetyl-CoA for entry into central carbon metabolism, but does not encode genes enabling phototrophy.

P. hydrocarbonoclasticus was tested for its ability to grow using acetone and CO2 as sole carbon sources. Figure 1 shows robust growth (reaching OD600 of 0.65), but either the units for acetone concentration are incorrect (reported throughout as 67.5 nM acetone) or there are other unknown carbon sources. A quick calculation shows that 70 nM acetone cannot support an OD600 over 0.1.

For acetone at 70 nM = 210 nM C…210 nM C x 12 g/mol ~ 2.5 micrograms C/L…Estimating on the high side, 50% yield, 0.5 x 2.5 ug ~1.25 ug/L. OD600 = 1 ~ 0.3 g/L estimate.
OD600 ~ (1.25 x 10^-6 g/L)/(0.3 g/L per OD) ~ 4 x 10^-6 which is below the limit of detection by spectrophotometry.

Another possibility is that I do not understand the Figure 1B legend, “Growth curves for P. hydrocarbonoclasticus strain MCTG13d in relation to initial acetone concentration.” Materials and Methods states that cells grown on pyruvate were pelleted, washed, and resuspended in ONR7 liquid medium containing acetone at concentrations ranging from 67.5 to 270 nM acetone. The legend suggests these amounts are in addition to other sources of acetone.

For this reason, I cannot support publication of this work until the problem is resolved and acetone uptake confirmed via other biochemical approaches.

This work would also benefit from demonstrating CO2 incorporation using isotopically labeled bicarbonate. The calculation given above is for 3C acetone, but the addition of a 4th C from CO2 still cannot overcome the problem, but an experiment quantifying CO2 incorporation relative to acetone use would be helpful to demonstrating stoichiometric use of substrates.

P. arcticus also encodes proteorhodopsin, suggesting phototrophic growth via a light-driven proton pump. The title and text imply light-driven acetone carboxylation (Lines 167-169), but making this linkage requires experimental demonstration, such as through culture or field-based light/dark incubations.

A minor point is the use of ‘mixotrophy’ in the place of photoheterotrophy. Plankton ecologists usually reserve mixotrophy for protistan use of both photosynthesis and preformed organic matter, especially via phagocytosis.

There is no information given for acetone concentrations in the Arctic that are thought to be supporting Porticoccus sp. and presumably contribute to the net negative air-sea flux in the Arctic.

Reviewer #2

(Remarks to the Author)
OVERVIEW: In this paper, the authors combine isolate growth assays and metagenomics/metatranscriptomics to explore an unusual metabolic strategy in a marine bacterium from the Arctic ocean. Primarily focusing on a single MAG, the study explores a possible bacterial physiology that relies heavily on ATP derived from proteorhodopsin and carbon derived from acetone as well as CO2 from anaplerotic reactions. I commend the authors on an intriguing study and for their generally robust methodology; however, I have identified several technical and conceptual issues that should be addressed before publication.

The main issue I perceive, as detailed below in the MAJOR COMMENTS, is the way the authors have framed the potential metabolic strategy of P. arcticus as a mixotrophic one involving carbon fixation via acetone degradation. The common understanding of mixotrophy in the microbial ecology literature is a carbon assimilation strategy that involves a mix of inorganic carbon fixation through autotrophy and organic carbon assimilation via heterotrophy. While I do not contest that CO2 assimilation via anaplerotic reactions could be considered a form of fixation, it is clear in this case – as the authors note – that this fixation is not autotrophic in nature, as it is inherently accompanied by the uptake of acetone. Therefore, I do not find it appropriate to call this a novel mixotrophy, and would suggest instead revising the framing to instead focus on the seemingly novel combination of photoheterotrophy and acetone degradation. While potentially geochemically relevant, CO2 fixation is a byproduct here, and therefore also does not feel like an appropriate primary framing for the manuscript.

This framing not only suffers from conceptual pitfalls but also technical ones - the entire inference of metabolic features hinges on one incomplete MAG, which, even if largely complete, does not permit a definitive accounting of which genes/capacities are absent. More specifically, the existing evidence does not support the claim that P. arcticus relies solely on acetone/CO2 to meet its carbon demands and light to meet its energy demands. While I concede that the growth assays and high levels of transcription from bulk metatranscriptomes are suggestive, I do not think these pieces of evidence are adequate to definitively establish that energy/electron flux is linked between these pathways in a novel, uncultivated environmental species.

Additionally, there are several places in the manuscript detailed below where authors make inadequately supported claims about gene function, ecological relevance, and correlations with environmental geochemistry. In these cases, the manuscript (and central argument) would benefit greatly from major amendments to the analyses presented.

MAJOR COMMENTS:

l 87-89: I appreciate that authors acknowledge the difficulty of establishing function of a metagenomically-derived gene cluster from a broader superfamily and the steps they took to account for uncertainty. However, it is currently very difficult to assess the phylogenetic claims made by the authors for two reasons: 1) gene naming scheme is not consistent between the tree and text (e.g. does Port-CB9S-26_00193 == Port-CB9S-26?) and 2) genes that have been biochemically characterized are not indicated on the tree. Thus, it is impossible to assess whether the authors' claim that two homologs "were positioned within a well-supported clade that contained experimentally validated acetone carboxylase enzymes and the enzyme from P. hydrocarbonoclasticus." Please address this in the next version of the manuscript.

l 147-148 - As noted above, the inference of metabolic absence from a single MAG is highly risky. Are there additional, related MAGs of P. arcticus that could be analyzed in tandem to strengthen these inferences? If not, I do not think it appropriate to infer that the MAG is unable to use sugars as growth substrates, nor that its glycolytic pathway is necessarily incomplete (even if so, aren't workarounds possible?). Given this, I would revise the language here to account for the possibility that the organism can derive organic carbon from sources beyond acetone. However, I think it would still be reasonable to infer that acetone is the/one of the primary substrates.

l 153-154 - Again, it is difficult to independently evaluate the claim that these rhodopsins are proton pumps without any indication of function on the phylogenetic tree. Please add rhodopsin type/substrate onto this tree where known, as well as whether these pumps are likely to pump protons inwards or outwards.

l 192-194 - as noted above, without evidence of canonical autotrophic pathways, I do not think it appropriate to refer to this as mixotrophy. Both proteorhodopsin-mediated ATP generation and acetone degradation, even with anaplerotic CO2 assimilation, are both associated with heterotrophic processes. Is it possible that photo(hetero)trophy and photoautotrophy are being conflated here? However, I agree with the characterization of anaplerotic CO2 assimilation as supplementary, and think this should be better reflected in the paper title rather than centering CO2 fixation as a primary metabolism, as this implies autotrophy.

line 205-206 - claim about acetone distribution either needs a citation, or, more ideally, an actual geochemical profile collected contemporaneously with the molecular data.

l 222 - this paper would benefit from analyzing the co-occurrence of the other acetone degradation with the carboxylase-based one described here. Doing so would help establish the ecological relevance of the latter pathway, contributing to overall study impact.

MINOR COMMENTS:

l 95-96: What is this MAG collection, and how was it generated? Beyond a brief reference in the data availability statement, I see no description of this in either the main text or methods.

l 99-100: “Nearly identical’ is non quantitative and cannot be inferred from the tree alone. Please provide numerical support (e.g., % identity) to support this claim.

l 105-111: It is quite difficult to assess what these TAD80 numbers mean intuitively, and thus claims about ecological dominance or rarity. The authors should consider reporting abundance as a % of coverage of all AcxB-encoding MAGs or perhaps making some sort of comparison to TAD80 of other functional groups present in the MAG catalogue.

figure 2 - remove the generic Lorem Ipsum text inserted by illustrator.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have mostly refined the manuscript in line with reviewers’ concerns.
Thank you for correcting the unit problems for growth of P. hydrocarbonoclasticus on acetone. Presumably, growth on acetone should also show increasing growth rate and maximum cell density at lower concentrations. Since mM concentrations are unrealistic in marine systems, it would be useful to observe growth at lower concentrations (i.e., showing Monod-type growth behaviors) in culture.

The authors responded to my earlier review with useful data from Wohl et al. on acetone concentrations in the Arctic. “They report a mean seawater acetone concentration of 9 nM, with elevated concentrations (>40 nM) at the surface, which would be sufficient to support growth of P. arcticus.’.. Through a combination of depth profile distributions and underway measurements, this work demonstrated mean seawater acetone concentrations of 9 nM, with elevated concentrations at the surface. We have now included these details and the reference in the revised manuscript.”

However, the new version only refers to the Arctic as having a “relatively high” concentration of acetone, which is not sufficiently informative to the broader community.

L216-218 are unclear: “Hence, the association with acetone-enriched Arctic surface waters13, and a general rarity at the SCM, fits with acetone playing a central role in P. arcticus metabolism.” Please clarify the association is with P. arcticus abundances. Here it would be useful to emphasize that acetone concentrations in the nM range require rapid turnover of this acetone pool if its use by this metabolism is occurring.

L61 – citric cycle should be citric acid cycle
L87 – Please add some quantitative information about the range of known acetone concentrations in the Arctic.
L119 – From the previous sentences, the acxABC, scoAB and atoD genes are all necessary for growth on acetone.

Reviewer #2

(Remarks to the Author)

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The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.
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Reply: We are grateful to both reviewers for their critical, but constructive, comments on our study. We have carefully considered all comments and incorporated many of the suggested changes and additions to the revised manuscript.

A general concern raised by both reviewers was our use of the term "mixotrophy" to refer to the novel metabolism that we described in *P. arcticus*. In addition, both reviewers were concerned by our claim of "light-driven acetone carboxylation" and voiced specific concerns that we were inaccurately implying that *P. arcticus* is capable of autotrophic carbon fixation. To address these general concerns, we have made significant modifications to the abstract, introduction, and the interpretation of our findings, which are best captured by our revised title: *"Evidence of an acetone carboxylation pathway in photoheterotrophic bacteria from the Arctic Ocean"*.

The opening paragraph in the introduction now presents the diversity of carboxylation reactions that exist in nature. Specifically, we define and differentiate between autotrophic, anaplerotic, and assimilatory carboxylases using the functional classifications defined in: *Erb (2011) Carboxylases in natural and synthetic microbial pathways. Applied and Environmental Microbiology*. We then present acetone carboxylase as an assimilatory carboxylase involved in assimilation of organic compounds. We have modified any text that may have originally implied autotrophic growth. We are grateful to both reviewers for these critical comments and anticipate they will be more supportive of our description of what is still an unusual and novel carbon and energy metabolism in marine bacteria.

As pointed out by the editor, an important concern raised by Reviewer #1 was the "missing" carbon required to explain the growth curves of *P. hydrocarbonoclasticus* presented in Figure 1b. The acetone concentrations presented in the figure legend and the methods section were incorrect. Acetone concentrations were in fact orders of magnitude higher that reported in the manuscript (mM versus nM) and can explain the observed cell densities. This error was corrected in the revised manuscript. We have also modified the text in the methods to make it clear that acetone was the sole source of organic carbon in a synthetic seawater medium.

In addition, the editor highlighted a concern raised by Reviewer #2 regarding our findings of metabolic specialization in *P. arcticus*. Rightly so, one must be cautious in reporting on gene absence from an incomplete genome or, in this case, a metagenome-assembled genome (MAG). The reviewer proposed that we include additional *P. arcticus* genome in our analysis, and (rather serendipitously) there is a closely related (>99% 16S rRNA identity) publicly available genome from a mixed enrichment culture (*Porticoccus* sp. Uisw_501_02) recently reported in: *Sadler and Morris (2025) Genomic diversity and adaptation in Arctic marine bacteria. mBio.* Most importantly, the Uisw_501_02 genome is closed and complete. We have revised our comparative genomics/metabolic reconstruction analysis to include Uisw_501_02 (Figure 3a and 3b), which confirm an absence of genes required for sugar metabolism as previously concluded. The "missing" pyruvate dehydrogenase gene was present in Uisw_501_02, so we have cautiously removed this result from the revised manuscript.

The editor pointed out that the reviewers share concern about the wider environment relevance of this metabolism. Our understanding is this concern is related to a lack of information on the concentration and distribution of acetone in the Arctic Ocean, precluding any conclusions that acetone carboxylation could be a relevant metabolic strategy. This was an oversight on our part, and we have now included more detail (and references) in the introduction and discussion on acetone concentrations and distributions in the ocean and the Arctic specifically. Measuring acetone is difficult, requiring Proton Transfer Reaction Mass Spectrometry (PTR-MS) instrumentation. Nevertheless, there is one highly informative study directly relevant to our work:

Wohl et al. (2022) Sea ice concentration impacts dissolved organic gases in the Canadian Arctic. Biogeosciences. **They report a mean seawater acetone concentration of 9 nM, with elevated concentrations (>40 nM) at the surface, which would be sufficient to support growth of *P. arcticus*. Based on these biogeochemical observations, we have also expanded the discussion section slightly to point out that photochemical degradation of dissolved organic matter in surface water is a likely source of acetone for *P. arcticus*.**

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript suggests the presence of abundant Porticoccus arcticus bacteria that assimilate CO2 via acetone carboxylation and also have phototrophic capability. The existence of such activities would be important to understanding the ecology of organisms specializing in uptake and metabolism of low concentration volatile compounds, such as acetone. The P. arcticus genome was assembled by metagenomics and is closely related to P. hydrocarbonoclasticus, which also encodes the genes for acetone carboxylase and metabolism to acetyl-CoA for entry into central carbon metabolism, but does not encode genes enabling phototrophy.

P. hydrocarbonoclasticus was tested for its ability to grow using acetone and CO2 as sole carbon sources. Figure 1 shows robust growth (reaching OD600 of 0.65), but either the units for acetone concentration are incorrect (reported throughout as 67.5 nM acetone) or there are other unknown carbon sources. A quick calculation shows that 70 nM acetone cannot support an OD600 over 0.1.

For acetone at 70 nM = 210 nM C…210 nM C x 12 g/mol ~ 2.5 micrograms C/L…Estimating on the high side, 50% yield, 0.5 x 2.5 ug ~1.25 ug/L. OD600 = 1 ~ 0.3 g/L estimate.
OD600 ~ (1.25 x 10^-6 g/L)/(0.3 g/L per OD) ~ 4 x 10^-6 which is below the limit of detection by spectrophotometry.

Reply: As described in our general reply to editor/reviewer comments above, there was a mistake in the acetone concentrations reported in the original manuscript. The true concentrations ranged between 13.4 - 53.7 mM and this mistake was corrected in the revised Figure 1b and the methods section.

Another possibility is that I do not understand the Figure 1B legend, “Growth curves for P. hydrocarbonoclasticus strain MCTG13d in relation to initial acetone concentration.” Materials and Methods states that cells grown on pyruvate were pelleted, washed, and resuspended in ONR7 liquid medium containing acetone at concentrations ranging from 67.5 to 270 nM acetone. The legend suggests these amounts are in addition to other sources of acetone.

For this reason, I cannot support publication of this work until the problem is resolved and acetone uptake confirmed via other biochemical approaches.

Reply: ONR7a is a defined synthetic seawater medium with no carbon source. In our experiments (Figure 1b), the only added carbon source was the acetone, at a purity of >99.9%. The growth observed was directly due to the acetone as the sole organic carbon substrate. The legend was corrected to the accurate acetone concentrations used in the experiment. Additional details were added to the figure title and the methods to clarify that acetone was the sole source of organic carbon in the medium.

This work would also benefit from demonstrating CO2 incorporation using isotopically labeled bicarbonate. The calculation given above is for 3C acetone, but the addition of a 4th C from CO2 still cannot overcome the problem, but an experiment quantifying CO2 incorporation relative to acetone use would be helpful to demonstrating stoichiometric use of substrates.

Reply: We agree that demonstrating CO_2 incorporation using carbon isotopes would be a valuable addition to the study but feel it is currently beyond the scope of the manuscript. Based on the findings reported in this manuscript, we’re now working to establish enrichment/pure cultures of *P. arcticus* from Arctic seawater using acetone and light and (if successful) to then use carbon isotopes to demonstrate carbon assimilation. In theory, this experiment could be performed using *P. hydrocarbonoclasticus*. However, *P. hydrocarbonoclasticus* is an obligate heterotroph (no evidence of phototrophy in the genome), so CO_2 that is assimilated during acetone carboxylation may subsequently be respired back to CO_2 through the citric acid

cycle. Nevertheless, we thank the reviewer for this suggestion, and we hope to report on experimental characterization of the acetone carboxylation metabolism in future work.

P. arcticus also encodes proteorhodopsin, suggesting phototrophic growth via a light-driven proton pump. The title and text imply light-driven acetone carboxylation (Lines 167-169), but making this linkage requires experimental demonstration, such as through culture or field-based light/dark incubations.

Reply: As detailed in the reply to the comment above, our next aim is to experimentally demonstrate the predicted photoheterotrophic metabolism. In the meanwhile, we have revised the title to be more conservative and not imply light-mediated carbon fixation. We think the reviewer supports our interpretation that *P. arcticus* is a photoheterotroph and therefore a larger fraction of acetyl-CoA (end product of the acetone carboxylation pathway) can be funneled to biosynthesis if light is used for ATP synthesis, compared to strictly heterotrophic growth. We have modified the text accordingly. Specifically, we removed part of the opening sentence where we implied that light may drive the production of ATP required for acetone carboxylation). We also reworded the final sentence where we originally stated that light produced the ATP to fuel carbon assimilation

A minor point is the use of 'mixotrophy' in the place of photoheterotrophy. Plankton ecologists usually reserve mixotrophy for protistan use of both photosynthesis and preformed organic matter, especially via phagocytosis.

Reply: In light of the reviewer's comment, we have replaced "mixotrophy" with "photoheterotrophy" in the title and throughout the text.

There is no information given for acetone concentrations in the Arctic that are thought to be supporting Porticoccus sp. and presumably contribute to the net negative air-sea flux in the Arctic.

Reply: We thank the reviewer for noting this omission. To the best of our knowledge there is one study that measured acetone concentrations in the Arctic Ocean: Wohl et al. (2022) Sea ice concentration impacts dissolved organic gases in the Canadian Arctic. Biogeosciences. Through a combination of depth profile distributions and underway measurements, this work demonstrated mean seawater acetone concentrations of 9 nM, with elevated concentrations at the surface. We have now included these details and the reference in the revised manuscript.

Reviewer #2 (Remarks to the Author):

OVERVIEW: In this paper, the authors combine isolate growth assays and metagenomics/metatranscriptomics to explore an unusual metabolic strategy in a marine bacterium from the Arctic ocean. Primarily focusing on a single MAG, the study explores a possible bacterial physiology that relies heavily on ATP derived from proteorhodopsin and carbon derived from acetone as well as CO2 from anaplerotic reactions. I commend the authors on an intriguing study and for their generally robust methodology; however, I have identified several technical and conceptual issues that should be addressed before publication.

Reply: We thank the reviewer for their commendation and are pleased that they see the "intriguing" results we present here using a "robust methodology". We have carefully considered the reviewers thoughtful comments and have addressed them below.

The main issue I perceive, as detailed below in the MAJOR COMMENTS, is the way the authors have framed the potential metabolic strategy of P. arcticus as a mixotrophic one involving carbon fixation via acetone degradation. The common understanding of mixotrophy in the microbial ecology literature is a carbon assimilation strategy that involves a mix of inorganic carbon fixation through autotrophy and organic carbon assimilation via heterotrophy. While I do not contest that CO2 assimilation via anaplerotic reactions could be considered a form of fixation, it is clear in this case – as the authors note – that this fixation is not autotrophic in nature, as it is inherently accompanied by the uptake of acetone. Therefore, I do not find it appropriate to call this a novel mixotrophy, and would suggest instead revising the framing to instead focus on the seemingly novel combination of photoheterotrophy and acetone degradation. While potentially geochemically relevant, CO2 fixation is a byproduct here, and therefore also does not feel like an appropriate primary framing for the manuscript.

Reply: As detailed in our general reply to the editor's comments, we have revised the manuscript to remove all mention of mixotrophy and to clarify the differences between autotrophic, anaplerotic, biosynthetic and assimilatory carboxylation reactions. We agree that the amount of CO_2 assimilation via acetone carboxylation is likely negligible compared to autotrophic CO_2 fixation pathways. Nevertheless, we frame assimilatory carboxylation as a previously undescribed evolutionary adaptation to oligotrophic (low organic carbon) conditions. We anticipate that the changes to the title, abstract, and interpretation of results is in line with the reviewer's comment here.

This framing not only suffers from conceptual pitfalls but also technical ones - the entire inference of metabolic features hinges on one incomplete MAG, which, even if largely complete, does not permit a definitive accounting of which genes/capacities are absent. More specifically, the existing evidence does not support the claim that P. arcticus relies solely on acetone/CO2 to meet its carbon demands and light to meet its energy demands. While I concede that the growth assays and high levels of transcription from bulk metatranscriptomes are suggestive, I do not think these pieces of evidence are adequate to definitively establish that energy/electron flux is linked between these pathways in a novel, uncultivated environmental species.

Reply: We completely agree that the existing evidence does not support the claim that *P. arcticus* relies solely on acetone/CO_2 and light. We never made this claim, nor do we believe it to be true. However, based on this comment, it's clear we need to reword some of our results. We experimentally show that *P. hydrocarbonoclasticus* is able to grow on acetone as a sole organic carbon source (not that it relies on acetone). The MAG and gene expression analysis suggests an importance of acetone and light in supporting growth, but not that *P. arcticus* growth relies on acetone/CO2 and light. In fact, we report the presence of other organic carbon compound transporters in the *P. arcticus* genome. We also refer to P. arcticus as a specialist, which may be misleading. We did not intend to mean a specialist with an obligate metabolism based on acetone/CO_2 and light. We have modified the title, abstract, and main manuscript to address this comment.

Additionally, there are several places in the manuscript detailed below where authors make inadequately supported claims about gene function, ecological relevance, and correlations with environmental geochemistry. In these cases, the manuscript (and central argument) would benefit greatly from major amendments to the analyses presented.

MAJOR COMMENTS:

l 87-89: I appreciate that authors acknowledge the difficulty of establishing function of a metagenomically-derived gene cluster from a broader superfamily and the steps they took to account for uncertainty. However, it is currently very difficult to assess the phylogenetic claims made by the authors for two reasons: 1) gene naming scheme is not consistent between the tree and text (e.g. does Port-CB9S-26_00193 == Port-CB9S-26?) and 2) genes that have been biochemically characterized are not indicated on the tree. Thus, it is impossible to assess whether the authors' claim that two homologs "were positioned within a well-supported clade that contained experimentally validated acetone carboxylase enzymes and the enzyme from P. hydrocarbonoclasticus." Please address this in the next version of the manuscript.

Reply: Thank you for this helpful comment. First, PortCB9S-26 is the MAG identifier, whereas Port-CB9S-26_00193 is the locus tag of the *acxB* gene in the PortCB9S-26 MAG. We introduce confusion by using the locus tag ID in the text, but the MAG identifier in the figure. We have changed the figure to be consistent with the text. Second, we inadvertently omitted an important detail in the Figure title. Proteins in bold are those that are biochemically characterized as acetone carboxylases. We have added this information to the figure title.

l 147-148 - As noted above, the inference of metabolic absence from a single MAG is highly risky. Are there additional, related MAGs of P. arcticus that could be analyzed in tandem to strengthen these inferences? If not, I do not think it appropriate to infer that the MAG is unable to use sugars as growth substrates, nor that its glycolytic pathway is necessarily incomplete (even if so, aren't workarounds possible?). Given this, I would revise the language here to account for the possibility that the organism can derive organic carbon from sources beyond acetone. However, I think it would still be reasonable to infer that acetone is the/one of the primary substrates.

Reply: Rightly so, one must be cautious in reporting on gene absence from an incomplete genome or, in this case, a metagenome-assembled genome (MAG). The reviewer proposed that we include additional *P. arcticus* genome in our analysis, and (rather serendipitously) there is a closely related (>99% 16S rRNA

identity) publicly available genome from an enrichment culture (*Porticoccus* sp. Uisw_501_02) recently reported in: *Sadler and Morris (2025) Genomic diversity and adaptation in Arctic marine bacteria. mBio.* Most importantly, the Uisw_501_02 genome is closed and complete. We have revised our comparative genomics/metabolic reconstruction analysis to include Uisw_501_02 (Figure 3a and 3b), which confirm an absence of genes required for sugar metabolism as previously concluded. The "missing" pyruvate dehydrogenase gene was present in Uisw_501_02, so we have cautiously removed this result from the revised manuscript.

l 153-154 - Again, it is difficult to independently evaluate the claim that these rhodopsins are proton pumps without any indication of function on the phylogenetic tree. Please add rhodopsin type/substrate onto this tree where known, as well as whether these pumps are likely to pump protons inwards or outwards.

Reply: We have added the term "proton translocating" to Figure 3e to clarify which clade is involved in pumping proteins across the cellular membrane. Also, we previously provided further structural evidence that the *P. arcticus* proteins are proton pumps: "Both rhodopsins were predicted proton pumps based on phylogenetic position within the proteorhodopsin clade and amino acid identities at positions 97 (aspartate), 101 (threonine), and 108 (glutamate)". To the best of our knowledge all proteorhodopsins pump protons outwards, whereas the inward pumping rhodopsins are distantly related to the proteorhodopsin clade.

l 192-194 - as noted above, without evidence of canonical autotrophic pathways, I do not think it appropriate to refer to this as mixotrophy. Both proteorhodopsin-mediated ATP generation and acetone degradation, even with anaplerotic CO2 assimilation, are both associated with heterotrophic processes. Is it possible that photo(hetero)trophy and photoautotrophy are being conflated here? However, I agree with the characterization of anaplerotic CO2 assimilation as supplementary, and think this should be better reflected in the paper title rather than centering CO2 fixation as a primary metabolism, as this implies autotrophy.

Reply: As described above, we have removed all reference of mixotrophy and modified the paper title.

line 205-206 - claim about acetone distribution either needs a citation, or, more ideally, an actual geochemical profile collected contemporaneously with the molecular data.

Reply: We have added a citation (Wohl et al. (2022) Sea ice concentration impacts dissolved organic gases in the Canadian Arctic. Biogeosciences) that as far as we know is the sole study to measure acetone in the Arctic Ocean. Through a combination of depth profile distributions and underway measurements, this work demonstrated mean seawater acetone concentrations of 9 nM, with elevated concentrations (>40 nM) at the surface. We have now included these details and the reference in the revised manuscript.

l 222 - this paper would benefit from analyzing the co-occurrence of the other acetone degradation with the carboxylase-based one described here. Doing so would help establish the ecological relevance of the latter pathway, contributing to overall study impact.

Reply: The discovery of the acetone carboxylase was associated with a larger survey of metabolic pathways for volatile organic carbon degradation, including acetone, methanol, and acetaldehyde. The acetone monooxygenase was rare in the MAG collection and restricted to deeper waters. We've thought about how we can incorporate this observation into the current manuscript and have opted to keep it out. We are performing another study of global diversity of acetone metabolism in the oceans where we will compare diversity, distribution, and ecological importance of the two acetone degradation pathways.

MINOR COMMENTS:

l 95-96: What is this MAG collection, and how was it generated? Beyond a brief reference in the data availability statement, I see no description of this in either the main text or methods.

Reply: The methodology for generation of the MAG collection was provided in the Supplementary Material.

l 99-100: "Nearly identical' is non quantitative and cannot be inferred from the tree alone. Please provide numerical support (e.g., % identity) to support this claim.

Reply: The additional AcxB homologs recovered from the metagenome assemblies were 98% identical in amin acid sequences to the *P. arcticus* AcxB homolog. This detail has been added to the revised manuscript.

l 105-111: It is quite difficult to assess what these TAD80 numbers mean intuitively, and thus claims about ecological dominance or rarity. The authors should consider reporting abundance as a % of coverage of all AcxB-encoding MAGs or perhaps making some sort of comparison to TAD80 of other functional groups present in the MAG catalogue.

Reply: We agree that absolute TAD80 values are not intuitive and that relative abundances are more interpretable. In this case, the dominance of *P. arcticus* is pretty clear, as it is an order of magnitude or more abundant that other acetone-carboxylating populations. So, we'd prefer to report the TAD80 values in absolute terms. With respect to the relative abundance of *P. arcticus* in the bacterioplankton community, we refer the reviewer to the 16S rRNA data presented in Figure 4, where we show that *P. arcticus* can represent nearly 10% or more of the 16S rRNA sequences in the surface water communities.

figure 2 - remove the generic Lorem Ipsum text inserted by illustrator.

Reply: Thank you. This text has been removed.

Reviewer #1 (Remarks to the Author):

The authors have mostly refined the manuscript in line with reviewers' concerns.
Thank you for correcting the unit problems for growth of P. hydrocarbonoclasticus on acetone. Presumably, growth on acetone should also show increasing growth rate and maximum cell density at lower concentrations. Since mM concentrations are unrealistic in marine systems, it would be useful to observe growth at lower concentrations (i.e., showing Monod-type growth behaviors) in culture.

Reply: We thank you reviewer for the suggestion to perform growth experiments at lower acetone concentrations. The objective of the growth experiments in the manuscript was to demonstrate that AcxABC-harbouring marine bacteria do indeed have the capacity to use acetone as a carbon and energy source, which then led us to confidently use these genes as markers for acetone use and putative carboxylation in the metagenomic survey. Indeed, in future work, we plan to further characterize growth kinetics in response to acetone concentration, as well as light availability, using *P. arcticus* enrichment cultures from the Arctic Ocean.

The authors responded to my earlier review with useful data from Wohl et al. on acetone concentrations in the Arctic. "They report a mean seawater acetone concentration of 9 nM, with elevated concentrations (>40 nM) at the surface, which would be sufficient to support growth of P. arcticus.'.. Through a combination of depth profile distributions and underway measurements, this work demonstrated mean seawater acetone concentrations of 9 nM, with elevated concentrations at the surface. We have now included these details and the reference in the revised manuscript."

However, the new version only refers to the Arctic as having a "relatively high" concentration of acetone, which is not sufficiently informative to the broader community.

Reply: Thank you for this comment and the reviewer is correct that we still omit quantitative information on acetone concentration in Arctic seawater, even though we added these details to our reply letter. In the introduction, we have now added details on the acetone concentration (> 40nM) in Arctic surface waters (line 64).

L216-218 are unclear: "Hence, the association with acetone-enriched Arctic surface waters13, and a general rarity at the SCM, fits with acetone playing a central role in P. arcticus metabolism." Please clarify the association is with P. arcticus abundances. Here it would be useful to emphasize that acetone concentrations in the nM range require rapid turnover of this acetone pool if its use by this metabolism is occurring.

Reply: We have added specific details on the concentration and depth distribution of acetone that supports are claim that *P. arcticus* should be more abundant in Arctic Ocean surface water where acetone concentrations are elevated and acetone can be produced through UV degradation of dissolved organic matter (liens 227-229).

L61 – citric cycle should be citric acid cycle

Reply: Thank you for catching this error. The correction is included in the final revision.

L87 – Please add some quantitative information about the range of known acetone concentrations in the Arctic.

Reply: Quantitative information on acetone concentrations was added to the introduction (line 64).

L119 – From the previous sentences, the acxABC, scoAB and atoD genes are all necessary for growth on acetone.

Reply: Yes, correct. Likely the full pathway is required, and we show its presence in the *P. arcticus* genome. The point we are trying to make on L119 is that AcxABC are likely accurate markers for the capacity to use acetone, so we can use them as markers in the metagenome survey.