

Reversing Methanogenesis to Produce Ethanol

Corresponding Author: Professor Thomas Wood

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Decision Letter:

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Dear Professor Wood,

Your manuscript titled "Reversing Methanogenesis to Produce Ethanol" has now been seen by 3 reviewers, and we include their comments at the end of this message. They find your work of interest, but some important points are raised. We are interested in the possibility of publishing your study in Communications Earth & Environment, but would like to consider your responses to these concerns and assess a revised manuscript before we make a final decision on publication.

We therefore invite you to revise and resubmit your manuscript, along with a point-by-point response that takes into account the points raised. Please highlight all changes in the manuscript text file.

Please submit your point-by-point responses as a separate file, distinct from your cover letter where you can add responses to the Editors' comments that you do not want to be made available to the reviewers. Word files are preferred. We recommend that any figures, tables or graphs that are included in the response to reviewers are also included in the main article or Supplementary Information.

We are committed to providing a fair and constructive peer-review process. Please don't hesitate to contact us if you wish to discuss the revision in more detail.

Please use the following link to submit your revised manuscript, point-by-point response to the referees' comments (which should be in a separate document to any cover letter), a tracked-changes version of the manuscript (as a PDF file) and the completed checklist:

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We hope to receive your revised paper within six weeks; please let us know if you aren't able to submit it within this time so that we can discuss how best to proceed. If we don't hear from you, and the revision process takes significantly longer, we may close your file. In this event, we will still be happy to reconsider your paper at a later date, as long as nothing similar has been accepted for publication at Communications Earth & Environment or published elsewhere in the meantime.

Please do not hesitate to contact us if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Best regards,

Marisa McDonald, PhD

Associate Editor, Communications Earth & Environment
Consulting Editor, Communications Sustainability

On Bluesky:

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REVIEWER COMMENTS:

Reviewer #1 (Remarks to the Author):

Comments for Authors:

This manuscript presents an interesting study on engineering *Methanosarcina acetivorans* to reverse methanogenesis for ethanol production. The demonstration of converting methane to ethanol using ANME-1 Mcr, and the finding that the host's native pathway is more efficient than a heterologous one, is a significant contribution to C1 biocatalysis. However, several sections require clarification. The authors need to address the following specific comments to improve the overall quality of the manuscript:

1. Abstract: The abstract suggests that the native host converts acetate "more efficiently" than the strain with heterologous

Car and Adh, stating this is "most likely" via the native AOR/Adh pathway. Please clarify if this conclusion of "efficiency" is supported by comparative enzyme activity assays, or if it is inferred solely from the lower final ethanol yields in the engineered strain. If it is the latter, the authors might consider clarifying that the "detrimental" effect could also be due to metabolic burden (introduction of new plasmid) or lack of heterologous protein expression.

2. Introduction: Authors are recommended to strengthen the introduction, by including (i) quantitative data on the annual global mean emission of CO₂ from anthropogenic sources, and (ii) the global warming potential (GWP) of methane relative to CO₂ in the first paragraph to better contextualize the urgency of the study.

3. Materials and methods: Under the section "culture conditions", authors are recommended to provide details on the strain ID of *M. acetivorans* and the repository/culture collection centre from where it was acquired, or the source from where it was isolated?

4. Materials and methods: Regarding supplementation of 0.5% methanol and 0.025% Na₂S·9H₂O, please specify are these in %v/v or %w/v. Moreover, what was the pH of medium used for routine cultivation?

5. Materials and methods: For the ferrozine assay, humic acids are known to be dark-colored and potentially interfering substances. Did the authors perform any blank corrections or controls to ensure the humic acids did not interfere with the colorimetric absorbance reading at 562 nm?

6. Materials and methods: For GC analysis, please clarify if sample injections were performed manually or via an autosampler. If manual, did the authors use an internal standard (e.g., isobutanol) to correct for injection variability?

7. Materials and methods: The liquid phase was sparged with methane for 5 minutes. Please specify the final headspace pressure of the vials after sealing. Was the headspace maintained at atmospheric pressure, or was it pressurized to enhance methane solubility?

8. Materials and methods: Please specify how statistical analysis was performed and the software used?

9. Results: The authors observe that 100 mM Fe(III) resulted in a final pH of < 2, leading to low yields. Since *Methanosarcina* is neutrophilic, this acidification is a confusing variable that prevents any conclusion about the specific effects of high iron concentration on the metabolic pathway. The claim that "excessive iron(III) concentrations disrupt this balance" is unsupported because the effect cannot be distinguished from simple acid toxicity. Did the authors attempt this condition with a stronger buffer (e.g., higher concentration PIPES or MES) to decouple the iron concentration from the pH drop?

10. Results: The authors assert that "70% of the total ethanol... is contributed by methane" and "14.5% is contributed by yeast extract". These precise percentages appear to be derived from simple linear subtraction of yields from different control conditions. This calculation assumes that metabolic fluxes from different carbon sources (methane vs. yeast extract) are additive and non-interacting, which is rarely the case in complex regulatory networks. The authors should acknowledge that these values are estimates based on an unproven assumption of additivity, or provide isotopic data to support the specific carbon routing.

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Reviewer #2 (Remarks to the Author):

The abstract should be rewritten to provide some data about the results obtained. For example, how much ethanol did you produce?

In the introduction the authors start describing the methodology and justification of their previous work and show the stoichiometry in the introduction, which makes no sense. Overall, the introduction is badly written. The authors are not making an introduction to the topic, rather summarizing their previous research which is not appropriate for the introduction. In the end of introduction instead of stating the problem to be studied the authors presented the results of their study. The introduction should be totally rewritten.

Line 61 you stated you are following the procedure described by Soo et al with modifications. Which modifications?

Did you perform your experiments in triplicate? Clarify this information in the materials and methods section.

What is the industrial relevance of this research?

Compare the productivity with other methane-to-ethanol approaches

Reviewer #3 (Remarks to the Author):

In this manuscript, Mitra et al build on prior reverse methanogenesis work by using *Methanosarcina acetivorans* expressing ANME-1 Mcr to further convert methane and CO₂ into ethanol. They propose that a native host AOR/Adh-mediated pathway converts acetate to acetaldehyde and then to ethanol and show that ethanol production is enhanced by adding Fe(III), yeast extract, and humic acids. This study is mechanistically interesting and extends the use of *M. acetivorans* as a model for reversible methanogenesis and is potentially relevant for biological methane valorization. However, several scientific issues need to be addressed, particularly regarding quantitative analysis (including time courses, carbon/electron balances, and ethanol source attribution), before the work can be considered for publication.

- Fig 1 and Fig S2, it is recommended to include at least one set of time course data (e.g. 0, 2, 4, 6, 8 days) for methane consumption, acetate and ethanol production for key strains under the optimal ethanol production conditions. This would substantially strengthen the mechanistic interpretation.
- Please provide a summary table in main text or supplementary information, for each major condition, reports methane consumed, CO₂ produced/consumed, Fe(III) reduced, acetate and ethanol formed, and the resulting carbon and electron recoveries (in %). This will greatly enhance the credibility of thermodynamic and stoichiometric analysis.
- Line 206-210, please strengthen quantitative analysis for the ethanol source attribution. It would be helpful to provide a clear quantitative derivation, for example in Supplementary methods, detailing how the percentages were calculated and how uncertainties were propagated. In the main text, use more cautious wording such as “we estimate that approximately X% of ethanol carbon derives from methane” and explicitly reference the corresponding figure or table.
- Fig 1 and Fig 2, since C2A/pES1(Pmat) also produces ethanol, could you clarify the proposed pathway by which ethanol is formed in this strain? It would also be helpful to include methane consumption, acetate and ethanol production data for the wild type C2A strain.
- Fig 2 and Table 2, In the pathway schematic, explicitly annotating MA_2962, MA_0403, and the key ΔG° values would help readers integrate the transcriptomic and thermodynamic information.
- The finding that the strain carrying heterologous car/adh produces less ethanol and consumes less methane than the ANME-1 Mcr only strain is an important negative result. However, without direct supporting evidence such as expression levels, enzyme activities, intracellular redox measurements, or growth defects, attributing this phenotype primarily to the thermodynamic limitations of the NAD(P)H-dependent Car and to metabolic burden from the foreign Adh remains speculative. I suggest softening these mechanistic statements or providing additional supporting evidence.
- Abstract: Please be cautious with the novelty claim of “the first demonstration of methane derived biofuel production by *M. acetivorans*”, since Soo, et al. 2016 has already shown that *M. acetivorans* expressing ANME-1 Mcr can convert methane and CO₂ into acetate.
- Line 70-74, For the plasmid pES1-EtOH, how did you verify recombinant expression of the car and adh genes? For example, did you perform a Western blot or introduce some tag to confirm protein expression?
- Line 82-86, Did you plate the transformed *M. acetivorans* and isolate a single colony before proceeding?

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Decision Letter:

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Dear Professor Wood,

Your manuscript titled "Reversing Methanogenesis to Produce Ethanol" has now been seen by our reviewers, whose comments appear below. In light of their advice we are delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Earth & Environment.

We therefore invite you to revise your paper one last time to address the remaining concerns of our reviewers. At the same time we ask that you edit your manuscript to comply with our format requirements and to maximise the accessibility and therefore the impact of your work.

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Best regards,

Marisa McDonald, PhD

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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

My comments from the previous review report have been well addressed.

Reviewer #2 (Remarks to the Author):

The authors failed to address all the comments proposed by the reviewer.

Reviewer #3 (Remarks to the Author):

The revisions have significantly improved the manuscript, and almost all my previous comments have been addressed. However, I still have several concerns that should be addressed before publication.

- Page 5, Line 101-106, I remain concerned that PCR verification alone only confirms the presence of the plasmid in the host *M. acetivorans*, but does not demonstrate that ANME Mcr, Car, and Adh are actually expressed or functionally active in the host. I recommend either providing additional evidence at the protein or activity level (e.g., expression or functional biochemical assays), or explicitly stating in the text that the current data confirm plasmid presence but not gene expression or function, and softening any conclusions that rely on assuming expression.
- The transformed *M. acetivorans* is not from a single colony, which could further complicate interpretation of the phenotype. While Western blotting can confirm that a FLAG-tagged subunit is expressed, it does not directly address whether this subunit is correctly assembled into an active, functional complex, nor whether the recombinant proteins are stable or rapidly degraded. Some discussion of these limitations, or additional functional data, would strengthen the conclusions regarding the activity of ANME Mcr, Car, and Adh in the host.
- ANME-1 communities in Black Sea sediments typically experience in situ temperatures of approximately 8–9 °C, whereas the experiments here are conducted at 39 °C. It would be helpful for the authors to briefly comment on the temperature dependence of ANME-1 Mcr activity and the physiological relevance of expressing this enzyme under mesophilic conditions in *M. acetivorans*.
- Page 12, for the time-course experiment under the “best conditions” for ethanol production from methane, the description should explicitly state that this condition is “in the presence of glass wool.” I suggest adding this phrase both to the legend of Fig. 2 and to the corresponding description in the main text (lines 265–267).

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Comments for Authors

This manuscript presents an interesting study on engineering *Methanosarcina acetivorans* to reverse methanogenesis for ethanol production. The demonstration of converting methane to ethanol using ANME-1 Mcr, and the finding that the host's native pathway is more efficient than a heterologous one, is a significant contribution to C1 biocatalysis. However, several sections require clarification. The authors need to address the following specific comments to improve the overall quality of the manuscript:

1. Abstract: The abstract suggests that the native host converts acetate "more efficiently" than the strain with heterologous Car and Adh, stating this is "most likely" via the native AOR/Adh pathway. Please clarify if this conclusion of "efficiency" is supported by comparative enzyme activity assays, or if it is inferred solely from the lower final ethanol yields in the engineered strain. If it is the latter, the authors might consider clarifying that the "detrimental" effect could also be due to metabolic burden (introduction of new plasmid) or lack of heterologous protein expression.
2. Introduction: Authors are recommended to strengthen the introduction, by including (i) quantitative data on the annual global mean emission of CO₂ from anthropogenic sources, and (ii) the global warming potential (GWP) of methane relative to CO₂ in the first paragraph to better contextualize the urgency of the study.
3. Materials and methods: Under the section "culture conditions", authors are recommended to provide details on the strain ID of *M. acetivorans* and the repository/culture collection centre from where it was acquired, or the source from where it was isolated?
4. Materials and methods: Regarding supplementation of 0.5% methanol and 0.025% Na₂S·9H₂O, please specify are these in %v/v or %w/v. Moreover, what was the pH of medium used for routine cultivation?
5. Materials and methods: For the ferrozine assay, humic acids are known to be dark-colored and potentially interfering substances. Did the authors perform any blank corrections or controls to ensure the humic acids did not interfere with the colorimetric absorbance reading at 562 nm?
6. Materials and methods: For GC analysis, please clarify if sample injections were performed manually or via an autosampler. If manual, did the authors use an internal standard (e.g., isobutanol) to correct for injection variability?
7. Materials and methods: The liquid phase was sparged with methane for 5 minutes. Please specify the final headspace pressure of the vials after sealing. Was the headspace maintained at atmospheric pressure, or was it pressurized to enhance methane solubility?
8. Materials and methods: Please specify how statistical analysis was performed and the software used?
9. Results: The authors observe that 100 mM Fe(III) resulted in a final pH of < 2, leading to low yields. Since *Methanosarcina* is neutrophilic, this acidification is a confusing variable that prevents any conclusion about the specific effects of high iron concentration on the metabolic pathway. The claim that "excessive iron(III) concentrations disrupt this balance" is unsupported because the effect cannot be distinguished from simple

acid toxicity. Did the authors attempt this condition with a stronger buffer (*e.g.*, higher concentration PIPES or MES) to decouple the iron concentration from the pH drop?

10. Results: The authors assert that "70% of the total ethanol... is contributed by methane" and "14.5% is contributed by yeast extract". These precise percentages appear to be derived from simple linear subtraction of yields from different control conditions. This calculation assumes that metabolic fluxes from different carbon sources (methane vs. yeast extract) are additive and non-interacting, which is rarely the case in complex regulatory networks. The authors should acknowledge that these values are estimates based on an unproven assumption of additivity, or provide isotopic data to support the specific carbon routing.

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Response for manuscript COMMSENV-26-0374-T, “Reversing Methanogenesis to Produce Ethanol”

We wish to thank the editor and the three reviewers for their help with this manuscript and feel the manuscript is much-improved due to their careful review.

We have addressed all the issues as indicated below and made all the suggested changes. In summary, we performed 8 additional sets of experiments. Specifically, we (i) revised the pathway schematic (**now Fig. 3**) to show the pathway for how ethanol is made without ANME-1 Mcr; i.e., to show how native (chromosomal) Mcr primarily makes methanol but is reversible and can capture methane, which is a major discovery of this paper, (ii) added 5 sets of results to ensure there are 3 independent cultures for each condition (experiments conducted for conditions of only methane, methane + iron, no methane + YE + iron, and + methane + YE + HA + 10 mM iron, measuring total protein, methane consumed, and acetate and ethanol produced for each condition), (iii) tested *M. acetivorans* C2A without a plasmid (methane & methane + iron) for R3 (measuring total protein, methane consumption, acetate and ethanol production for 3 independent cultures), which confirms the host C2A converts consistently methane to ethanol without Mcr (**Fig. S2**), and (iv) conducted a time course experiment (over 8 days) with *M. acetivorans* C2A/pES1-MATmcr3 & *M. acetivorans* C2A/pES1(Pmat) (measuring total protein, methane consumption, acetate and ethanol production for 3 independent cultures) (**Fig. 2**). We also have indicated the standard deviations for all quantities in the Results. We also tried to grow the host *M. acetivorans* as colonies on solid agar but failed at both Penn State and the U of AZ.

Our responses to the comments are underlined below, and the changed text is shown as tracked changes in the revised manuscript. Note the line numbers below refer to the tracked changes version.

Reviewer #1

This manuscript presents an interesting study on engineering *Methanosarcina acetivorans* to reverse methanogenesis for ethanol production. The demonstration of converting methane to ethanol using ANME-1 Mcr, and the finding that the host's native pathway is more efficient than a heterologous one, is a significant contribution to C1 biocatalysis. However, several sections require clarification. The authors need to address the following specific comments to improve the overall quality of the manuscript:

1. Abstract: The abstract suggests that the native host converts acetate "more efficiently" than the strain with heterologous Car and Adh, stating this is "most likely" via the native AOR/Adh pathway. Please clarify if this conclusion of "efficiency" is supported by comparative enzyme activity assays, or if it is inferred solely from the lower final ethanol yields in the engineered strain. If it is the latter, the authors might consider clarifying that the "detrimental" effect could also be due to metabolic burden (introduction of new plasmid) or lack of heterologous protein expression.

As suggested, we now indicate that “efficiency” is based on final ethanol yields (line 12) and that the lower ethanol level may be due to metabolic burden, lack of heterologous protein production, or activity (line 346).

2. Introduction: Authors are recommended to strengthen the introduction, by including (i) quantitative data on the annual global mean emission of CO₂ from anthropogenic sources, and (ii) the global warming potential (GWP) of methane relative to CO₂ in the first paragraph to better contextualize the urgency of the study.

As suggested, the annual global mean emission of CO₂ from anthropogenic sources was added (line 19) along with the global warming potential of methane relative to CO₂ (line 20).

3. Materials and methods: Under the section “culture conditions”, authors are recommended to provided details on the strain ID of *M. acetivorans* and the repository/culture collection centre from where it was acquired, or the source from where it was isolated?

As suggested, we have added twice that the *M. acetivorans* C2A culture is from the DSMZ Collection (#2834) (line 54 and Table S1).

4. Materials and methods: Regarding supplementation of 0.5% methanol and 0.025% Na₂S·9H₂O, please specify are these in %v/v or %w/v. Moreover, what was the pH of medium used for routine cultivation?

As suggested, we have specified that the supplementation of methanol is 0.5% v/v and Na₂S·9H₂O is 0.02% w/v (line 57, 67, 68, 82, 87, 88, 90, and 91). We have also added that the pH of medium was 6.5 during routine cultivation (line 58).

5. Materials and methods: For the ferrozine assay, humic acids are known to be dark-colored and potentially interfering substances. Did the authors perform any blank corrections or controls to ensure the humic acids did not interfere with the colorimetric absorbance reading at 562 nm?

Since the manuscript does not contain any data from the ferrozine assay, this assay has been removed from the Materials and Methods section. We apologize for this oversight.

6. Materials and methods: For GC analysis, please clarify if sample injections were performed manually or via an autosampler. If manual, did the authors use an internal standard (e.g., isobutanol) to correct for injection variability?

As suggested, we have now added that all GC sample injections were made manually (line 122). While an internal standard was not used, to ensure precise and reproducible readings, all injections were performed in triplicates using a high-precision syringe (Hamilton Co., Reno, NV, USA). For methane measurements, exactly 30 µL of headspace methane was injected three times for each sample, to obtain a consistent and reproducible peak area. For ethanol and acetate measurements, exactly 2 µL of sample was injected for three times.

7. Materials and methods: The liquid phase was sparged with methane for 5 minutes. Please specify the final headspace pressure of the vials after sealing. Was the headspace maintained at atmospheric pressure, or was it pressurized to enhance methane solubility?

As suggested, we have now specified that, the sealed vials were sparged with methane using both inlet and outlet needles to allow continuous gas exchange and prevent pressure buildup (line 72). After 5 minutes of sparging at 70 mL/min, the headspace (28 mL) was fully replaced with methane and the pressure was maintained at atmospheric pressure (~1 atm). No additional pressurization was applied to enhance methane solubility.

8. Materials and methods: Please specify how statistical analysis was performed and the software used?

As suggested, we have specified that the statistical analysis was done using Graphpad Prism 10, and all data are now presented as the mean ± standard deviation of at least three independent cultures throughout the Results, as indicated in the tracked changes. Student's T-test was used to evaluate the difference between data sets (probability values (p) < 0.05 were considered significant) (line 126).

9. Results: The authors observe that 100 mM Fe(III) resulted in a final pH of < 2, leading to low yields. Since *Methanosarcina* is neutrophilic, this acidification is a confusing variable that prevents any conclusion about the specific effects of high iron concentration on the metabolic pathway. The claim that "excessive iron(III) concentrations disrupt this balance" is unsupported because the effect cannot be distinguished from simple acid toxicity. Did the authors attempt this condition with a stronger buffer (e.g., higher concentration PIPES or MES) to decouple the iron concentration from the pH drop?

To provide an appropriate growth condition for *M. acetivorans*, all experiments were conducted in 50 mM PIPES buffered medium with pH maintained at 6.5, which is suitable for the growth of the strains (now indicated in line 55). However, when a high concentration of iron was added (100 mM Fe(III)), the medium pH dropped to below 2 despite the buffering capacity, which ultimately may have had a detrimental effect on the cells and its ethanol production. We have not attempted additional experiments using stronger buffers to maintain pH at 6.5 under such high iron concentrations as this might alter the optimal growth conditions of the strain and would interfere with comparisons of all other conditions.

10. Results: The authors assert that "70% of the total ethanol... is contributed by methane" and "14.5% is contributed by yeast extract". These precise percentages appear to be derived from simple linear subtraction of yields from different control conditions. This calculation assumes that metabolic fluxes from different carbon sources (methane vs. yeast extract) are additive and non-interacting, which is rarely the case in complex regulatory networks. The authors should acknowledge that these values are estimates based on an unproven assumption of additivity, or provide isotopic data to support the specific carbon routing.

As suggested, we have now indicated that the contribution of ethanol from different sources such as methane, yeast extract, and humic acids is estimated based on the assumption that the metabolic flux from different sources is additive in the line 220.

Krishna Kalyani Sahoo, Ph.D.
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Cornell University

Reviewer #2

The abstract should be rewritten to provide some data about the results obtained. For example, how much ethanol did you produce?

As suggested, we have added some data to the abstract, such as amount of methane captured (line 7) and ethanol produced (line 8) in the abstract.

In the introduction the authors start describing the methodology and justification of their previous work and show the stoichiometry in the introduction, which makes no sense. Overall, the introduction is badly written. The authors are not making an introduction to the topic, rather summarizing their previous research which is not appropriate for the introduction. In the end of introduction instead of stating the problem to be studied the authors presented the results of their study. The introduction should be totally rewritten.

We disagree with the reviewer and note the corresponding author has 11 years experience as an editor along with decades of experience as an editorial board member, so he is familiar with how an Introduction should be structured. In the Introduction, we indicate clearly the environmental motivation for removing methane and carbon dioxide as greenhouse gases in the first paragraph and what could be more logical than that? This first paragraph is not based on our research, but inspires our work so it is relevant. Then we indicate that you cannot culture anaerobic methane oxidizers as pure cultures so it is difficult to capture methane with them in biotechnological applications. Then we explain how methanogenesis was reversed to allow conversion of methane in a pure culture and to make the synthesis of other compounds possible from methane. However, to make it clear anaerobic processes are more efficient than aerobic ones, we have added this to the Introduction (line 26) and added information about CO₂ emissions vs. methane emissions (lines 19-21).

Line 61 you stated you are following the procedure described by Soo et al with modifications. Which modifications?

The modification to the procedure described by Soo et al. was the addition of 50 mM PIPES as a buffer to the high salt media. As suggested, we have now specified this in line 62 and removed 'with modifications'. Thank you for catching this previously vague text.

Did you perform your experiments in triplicate? Clarify this information in the materials and methods section.

As suggested, so that all the results are based on at least 3 independent cultures (of course each independent culture has replicates for all relevant measurements, too), we have performed 5 additional sets of experiments with *M. acetivorans* C2A/pES1-MATmcr3 and *M. acetivorans* C2A/pES1(Pmat) with the following conditions: (i) only methane, (ii) methane + 10 mM iron(III), (iii) no methane + YE + 10 mM iron(III), and (iv) methane + YE + HA + 10 mM iron(III) and (v) 1 additional set with *M. acetivorans* C2A/pES1-EtOH with the condition methane + YE + HA + 10 mM iron(III) + glass wool. We measured total protein, methane consumed, acetate and ethanol produced for each set, to ensure all experiments in the manuscript have at least 3 independent cultures. We have also now clarified that all data presented in the manuscript are mean $\pm$ standard deviation of at least three independent cultures (line 126).

What is the industrial relevance of this research?

As suggested, we now indicate the industrial relevance of this research is that it creates the first pure culture that converts methane to ethanol, since aerobic methane oxidation produces only methanol and since archaeal microorganisms cannot be

grown in pure culture and produce only acetate, not ethanol (line 357).

Compare the productivity with other methane-to-ethanol approaches.

There are no other *anaerobic* biological methane to ethanol approaches to which to compare; that is the point of this novel research. So this manuscript is the first report of biologically converting methane into ethanol under anaerobic conditions. No other group has been able to reverse methanogenesis, so there are no reports of significant methane uptake by methanogens to make ethanol, and since no one can culture ANME-1, which would make acetate, not ethanol, there are no other anaerobic examples to compare.

Furthermore, there are no *aerobic* biological methane to ethanol approaches to which to compare; that is, methanotrophs are less efficient than anaerobic ones (now indicated line 26). Moreover, there are no known processes for converting methane to ethanol with methanotrophs since methanol is the product, not ethanol; so once again, there is nothing to compare since our work is novel. To make this clear, we have added this to the Discussion (line 357).

Reviewer #3

In this manuscript, Mitra et al build on prior reverse methanogenesis work by using *Methanosarcina acetivorans* expressing ANME 1 Mcr to further convert methane and CO₂ into ethanol. They propose that a native host AOR/Adh mediated pathway converts acetate to acetaldehyde and then to ethanol and show that ethanol production is enhanced by adding Fe(III), yeast extract, and humic acids. This study is mechanistically interesting and extends the use of *M. acetivorans* as a model for reversible methanogenesis and is potentially relevant for biological methane valorization. However, several scientific issues need to be addressed, particularly regarding quantitative analysis (including time courses, carbon/electron balances, and ethanol source attribution), before the work can be considered for publication.

- Fig 1 and Fig S2, it is recommended to include at least one set of time course data (e.g. 0, 2, 4, 6, 8 days) for methane consumption, acetate and ethanol production for key strains under the optimal ethanol production conditions. This would substantially strengthen the mechanistic interpretation.

As suggested, we have measured time course data for methane consumption, acetate production, and ethanol production for *M. acetivorans* C2A/pES1-MATmcr3 & *M. acetivorans* C2A/pES1(Pmat) with the best condition for producing ethanol (CH₄, YE, HA, 10 mM Fe(III)). The time course data has been included in line 255 and as new figure (Fig. 2).

- Please provide a summary table in main text or supplementary information, for each major condition, reports methane consumed, CO₂ produced/consumed, Fe(III) reduced, acetate and ethanol formed, and the resulting carbon and electron recoveries (in %). This will greatly enhance the credibility of thermodynamic and stoichiometric analysis.

As suggested, we have now included a summary table in the supplementary information (Table S3) for the methane only condition with and without 10 mM iron(III) and have summarized the methane consumed, ethanol produced, and the resulting carbon and electron recoveries for C2A/pES1-MATmcr3 and C2A/pES1(Pmat). We have also added the recovery percentages in the main text in line 311 and 320.

- Line 206-210, please strengthen quantitative analysis for the ethanol source attribution. It would be helpful to provide a clear quantitative derivation, for example in Supplementary methods, detailing how the percentages were calculated and how uncertainties were propagated. In the main text, use more cautious wording such as “we estimate that approximately X% of ethanol carbon derives from methane” and explicitly reference the corresponding figure or table.

As suggested, we have added standard deviations to all values in the main text. In addition, we have now show the quantitative analysis for the ethanol source attribution in the supplementary information as a new table (Table S2) detailing how the percentages were calculated and uncertainties were propagated. Also, we have revised the main text to use more cautious wording (line 220 and 224).

- Fig 1 and Fig 2, since C2A/pES1(Pmat) also produces ethanol, could you clarify the proposed pathway by which ethanol is formed in this strain? It would also be helpful to include methane consumption, acetate and ethanol production data for the wild type C2A strain.

As suggested, we revised the methane to ethanol pathway figure (now **Fig. 3**) to indicate how ethanol is produced is C2A/pES1(Pmat) and by C2A by adding an arrow showing some methane uptake by the chromosomal Mcr that primarily produces methane for the methanogenic C2A host. We also indicate more clearly the host Mcr can be reversed to a degree in the text (line 338). Basically, the chromosomal copy of *mcr* makes an enzyme that *primarily* produces methane but is capable of working in the reverse and when methane is the only carbon and energy source, as shown in **Fig. 1** and **Fig. S2**, so Mcr from the chromosome captures methane and produces ethanol using the pathway now shown in **Fig. 3**. Note, as shown, this is less than the methane captured when the cloned ANME-1 Mcr is used. This discovery that the *M. acetivorans* native Mcr is reversible is a major contribution of our paper.

As suggested, we conducted an additional set of experiments with C2A by testing two conditions for *M. acetivorans* C2A without a plasmid (methane & methane + iron) to confirm the host Mcr can be reversed to capture some methane and produce ethanol, like that previously shown for C2A/pES1(Pmat), the empty plasmid case. As shown in **Figure R1** below in the response, we measured total protein, methane consumption, acetate and ethanol production for three independent cultures of wild type C2A for each of these two conditions and compared with that of C2A/pES1(Pmat). The day 6 total protein for C2A and C2A/pES1(Pmat) was similar confirming that experiment was performed at similar turbidity of 40 at OD₆₀₀. Methane consumed and ethanol produced by C2A and C2A/pES1(Pmat) was almost identical in both conditions. Intermediate acetate was higher for C2A/pES1(Pmat) than C2A in only methane, however, in methane + iron condition, the intermediate acetate for both C2A and C2A/pES1(Pmat) was within experimental error. Since, addition of iron is more reasonable as growth of *M. acetivorans* on methane proceeds via iron reduction and iron acts as an electron acceptor for the electrons released during methane oxidation, we have only added the “methane + iron” result to the paper. The new results are added in the main text in line 164 and as new figure in the supplementary information (**Fig. S2**).

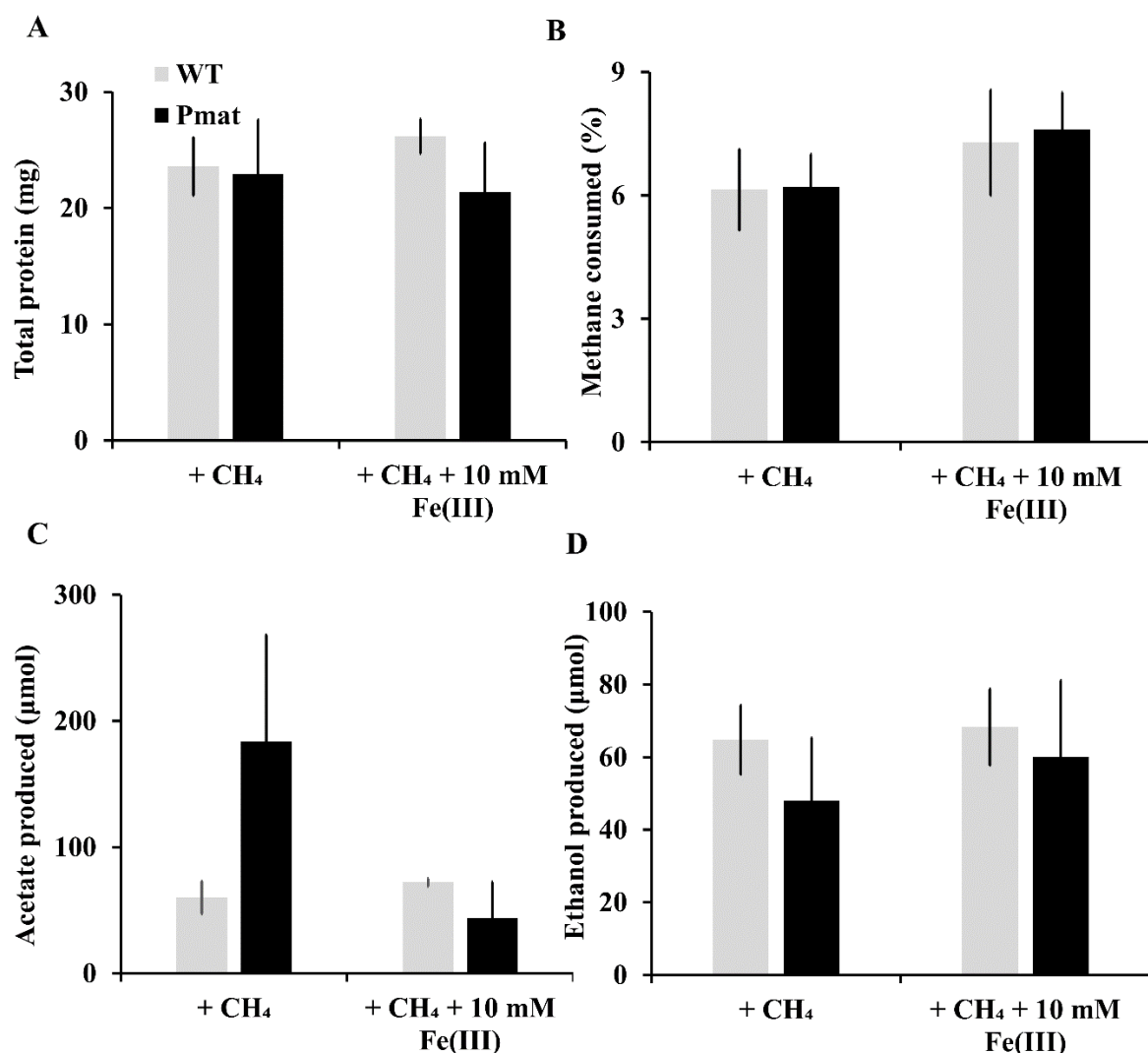


Figure R1. Ethanol production using methane with or without 10 mM iron(III) by C2A at a turbidity of 40 after six days of incubation and comparison with that of C2A/pES1(Pmat). **A.** Total protein of *M. acetivorans* at day 6. **B.** Methane consumed by *M. acetivorans* at day 6. **C.** Acetate produced by *M. acetivorans* at day 6. **D.** Ethanol produced by *M. acetivorans* at day 6. C2A represents wild type C2A without plasmid pES1(Pmat). Pmat represents C2A/pES1(Pmat). Bars and error bars are the mean and standard deviation of three independent cultures.

• Fig 2 and Table 2, In the pathway schematic, explicitly annotating MA_2962, MA_0403, and the key ΔG° values would help readers integrate the transcriptomic and thermodynamic information.

As suggested, we have added ΔG° values to the pathway schematic (now Fig. 3) to link the figure to the ΔG° values of Table 2. Thank you for this suggestion!

• The finding that the strain carrying heterologous car/adh produces less ethanol and consumes less methane than the ANME 1 Mcr only strain is an important negative result. However, without direct supporting evidence such as expression levels, enzyme activities, intracellular redox measurements, or growth defects, attributing this phenotype primarily to the thermodynamic limitations of the NAD(P)H dependent Car and to metabolic burden from the foreign Adh remains speculative. I suggest softening these mechanistic statements or providing additional supporting evidence.

As suggested, we have clarified the text by indicating the lower ethanol level may be due to metabolic burden of the additional cloned genes, the lack of heterologous protein production, or their activity (line 346).

• Abstract: Please be cautious with the novelty claim of “the first demonstration of methane derived biofuel production by *M. acetivorans*”, since Soo, et al. 2016 has already shown that *M. acetivorans* expressing ANME 1 Mcr can convert methane and CO₂ into acetate.

As suggested we have softened the text by deleting ‘for the first time’ and changed ‘biofuels’ to ‘ethanol’ (line 14).

• Line 70-74, For the plasmid pES1-EtOH, how did you verify recombinant expression of the car and adh genes? For example, did you perform a Western blot or introduce some tag to confirm protein expression?

Although we verified the construct was correct by sequencing the whole plasmid, we did not have resources to perform a Western blot for Car and Adh. However, we previously demonstrated that the same promoter, P_{mcr}, was active and showed production of McrA via a FLAG tag and Western using pES1-MATmcr3 (Soo, et al. 2016).

• Line 82-86, Did you plate the transformed *M. acetivorans* and isolate a single colony before proceeding?

Unfortunately, neither our lab nor anyone else’s, to our knowledge, can make *M. acetivorans* C2A grow as colonies on solid medium (we tried both at Penn State and at the U of AZ and both groups failed); as suggested, we now indicate this on line 90. Moreover, transformation of C2A is rather difficult with both lipid transformation and electroporation difficult. That is why we switched from lipid-based transformation in this work.

Response for manuscript COMMSENV-26-0374A, “Reversing Methanogenesis to Produce Ethanol”

We wish to thank the editor and the Reviewer 3 for their help with this manuscript; we have addressed the issues raised by Reviewer #3 and made the suggested changes.

Our responses to the comments are underlined below, and the changed text is shown as tracked changes in the revised manuscript. Note the line numbers below refer to the tracked changes version.

Reviewer #3

The revisions have significantly improved the manuscript, and almost all my previous comments have been addressed. However, I still have several concerns that should be addressed before publication.

- Page 5, Line 101-106, I remain concerned that PCR verification alone only confirms the presence of the plasmid in the host *M. acetivorans*, but does not demonstrate that ANME Mcr, Car, and Adh are actually expressed or functionally active in the host. I recommend either providing additional evidence at the protein or activity level (e.g., expression or functional biochemical assays), or explicitly stating in the text that the current data confirm plasmid presence but not gene expression or function, and softening any conclusions that rely on assuming expression.

We previously demonstrated via FLAG tag and Western that Mcr was actually produced by C2A/pES1-MATmcr3 (Soo, et al. 2016); however, future analysis will be important to confirm if Car and Adh is functionally active in the host. As suggested, we have softened the text by indicating that our PCR verification only confirms the presence of the plasmid in the host *M. acetivorans* and does not demonstrate gene expression or function of Car and Adh (l 358).

- The transformed *M. acetivorans* is not from a single colony, which could further complicate interpretation of the phenotype. While Western blotting can confirm that a FLAG-tagged subunit is expressed, it does not directly address whether this subunit is correctly assembled into an active, functional complex, nor whether the recombinant proteins are stable or rapidly degraded. Some discussion of these limitations, or additional functional data, would strengthen the conclusions regarding the activity of ANME Mcr, Car, and Adh in the host.

As suggested, we have discussed the limitation that since the transformed *M. acetivorans* is not from a single colony, it could further complicate phenotype interpretation in l 367. We have also discussed that future functional and biochemical analysis is required to directly address whether the recombinant proteins are stable or whether their subunits are correctly assembled into an active, and functional complex in l 369.

- ANME-1 communities in Black Sea sediments typically experience in situ temperatures of approximately 8–9 °C, whereas the experiments here are conducted at 39 °C. It would be helpful for the authors to briefly comment on the temperature dependence of ANME-1 Mcr activity and the physiological relevance of expressing this enzyme under mesophilic conditions in *M. acetivorans*.

We recognize that the Black Sea sediments typically experience in situ temperatures of approximately 8–9 °C and thus, 39°C, which is the optimum temperature for cultivating *M. acetivorans* is not an optimal temperature for the ANME-1 Mcr. However, enzyme activity generally increases with temperature and our results that the methane capture is higher with ANME-1 Mcr compared to C2A/pES1(Pmat) or wild type C2A clearly indicate that ANME-1 Mcr is functional in *M. acetivorans*. We have briefly addressed the temperature dependence of ANME-1 activity in the discussion section (l 345).

- Page 12, for the time-course experiment under the “best conditions” for ethanol production from methane, the description should explicitly state that this condition is “in the presence of glass wool.” I suggest adding this phrase both to the legend of Fig. 2 and to the corresponding description in the main text (lines 265–267).

The time-course experiment was conducted in the absence of glass wool as it was not necessary for ethanol production (indicated in l 194 and shown in Fig. 1); hence, we conducted the requested time-course experiment under “optimal” conditions for ethanol production and now, to avoid confusion make that clear on line 260, and 262 and in legend of Fig. 2, as suggested.