We thank the reviewers for their constructive and encouraging feedback on our manuscript "Bioengineering the Ice Worm Complex V ATP6 subunit to Increase Energy Production". We are pleased that the reviewers recognized the strengths of this work, particularly the clear demonstration of elevated ATP synthesis rates driven by the 13 aa carboxy-terminal extension and the structural insights gained from our systematic mutagenesis approach.

Below, we provide a point-by-point response to the major and minor comments, detailing the revisions and clarifications integrated into the updated manuscript.

Reviewer 1

Mutants 3 and 6 are shown to have the highest normalized ATP synthesis rates. However, these are the two mutants that showed very faint Western blot bands that were almost faded to the background (supplementary figure A1). Therefore, it is concerning whether the ATP synthesis rates for these mutants were accurate upon normalization. If the ATP synthesis activities of Mutants 3 and 6 are not accurate, the conclusions made by the authors in lines 308 and 328 (regarding flexibility being more critical than the number of histidine residues) must be re-evaluated.

We understand that faint immunoblots could introduce mathematical artifacts or inflate normalized values if the signal approaches the background detection floor. All Western blots were imaged using a Bio-Rad ChemiDoc MP system, which utilizes a cooled CCD camera with dynamic range greater than 4 orders of magnitude. Quantification was conducted via ImageJ software (NIH, Bethesda, MD, USA) using an automated rolling-ball background subtraction algorithm. Importantly, AtpB bands for Mutant-3 and Mutant-6 fall well within the dynamic range of our camera, thus represent genuine biological profiles rather than technical artifacts, loading discrepancies, or transfer limitation.

Line 491 - The authors suggest that Mutants 3 and 6 had lower expression of ATP synthase because "fewer ATP synthase complexes were necessary to meet cellular energy demands". It is also possible that expression levels were low because the mutant protein was unstable, not because the cell did not need as many ATP synthase complexes.

We acknowledge that low steady-state protein levels in heterologous expression systems can be caused by structural instability or proteotoxic stress. Our experimental obersvations, however, strongly point toward metabolic adaptation rather than cellular stress or an instability issue. Specifically--as stated in Section 2.4--all engineered genomic knock-in strains (including Mutant-3 and Mutant-6) robustly and consistently grew to an advanced stationary phase optical density of A_{600}= 1.5 at 37°C in standard media without displaying phenotypic delays, extended lag phases or premature lysis. Structural instability or stress-inducing mutations would inherently impair cell fitness, making it unlikely for the cultures to routinely achieve such high biomass densities. Moreover, because these constructs are stable chromosomal knock-ins operating under native operon control rather than overexpressed plasmids, the cellular metabolic network remains tightly regulated. As established by Rabbers and Bruggeman (2022), *E. coli* dynamically adjusts its ATP synthase abundance to maximize

growth rates and optimize resource allocation. Therefore, the lower steady-state expression of these highly efficient variants likely represent a controlled homeostatic downregulation to prevent hyperpolarization of the membrane, rather than a protein stability failure.

Rabbers, I. and Bruggeman, F.J., 2022. *Escherichia coli* robustly expresses ATP synthase at growth rate-maximizing concentrations. *The FEBS Journal*, *289*(16), pp.4925-4934.

To truly substantiate the mechanism proposed in the final paragraph, a proton flux assay (such as an ACMA quenching assay) would directly quantify the relationship between the increased ATP synthesis rates and the proton transfer rates through the FO This would significantly add to the mechanistic understanding of the unique characteristics of this ATP synthase species and the function of the FO motor in general. However, it is understandable if this is outside of the scope of this study.

We would like to clarify a specific technical limitation regarding the application of the ACMA fluorescence quenching assay to this particular engineered system. ACMA quenching typically measures the proton gradient generated when the F_1F_0 complex operates in reverse, driving ATP hydrolysis. In our previous characterization of the parent 18 aa extension variant (Dunkley et al., 2025), we demonstrated that the C-terminal histidine-rich domain displayed a quenching profile identical to wild-type. The functional advantage of the extension domain is strictly operative in the forward ATP synthesis direction, using the proton motive force to accelerate proton delivery into mtiochondrial lumen. Because ACMA assays are inherently linked to the hydrolysis pathway, they would not capture the synthesis-specific mechanism proposed here.

Dunkley, T., Shain, D.H. and Klein, E.A., 2025. A histidine-rich extension of the mitochondrial F 0 subunit ATP6 from the ice worm Mesenchytraeus solifugus increases ATP synthase activity in bacteria. *Febs Letters*, *599*(8), pp.1113-1121.

Line 286 – The authors suggest that the 13 aa variant became dominant over the 18 aa in certain regions through natural selection for smaller mitochondrial genome size. However, it is not clear if the 15 bp difference in mitochondrial genome size could impose a significant selective pressure. The authors should acknowledge genetic drift as an equally plausible mechanism for fixation in this section.

We agree that a 15 bp deletion may not exert a selection coefficient large enough to independently drive fixation through natural selection alone. Given the isolated nature of geographic ice worm populations on distinct glaciers, random genetic drift within small founder populations represents a plausible mechanism for the fixation of the shorter 13 aa variant in specific lineages. We will revise the Discussion section (Lines 288–294) accordingly.

Fig 1 - The neon green color for 13exAtpB is difficult to see. It is suggested to use a darker green, similar to Figure 3.

We have adjusted the neon green color to a darker, high-contrast forest green in Figure 1 to ensure legibility and visual consistency across all Figures.

Line 227 – The phrase "all constructs were 'knocked-in' the bacterial genome" is grammatically awkward. Consider rephrasing to "all constructs were introduced into the bacterial genome via knock-in."

The sentence has been rephrased to: *"all constructs were introduced into the bacterial genome via knock-in"* to improve grammatical flow.

Line 275 sentence starting at "We can also…" – The structure of this sentence is a bit confusing. The process of how the 13 and 18 aa variants arose could be fully described with more clarity, i.e. stating clearly that the 18 aa variant likely derived from the original LGT event and the 13 aa variant arising via a subsequent deletion, versus the less likely alternative scenario in which 13 aa variant occurred first, followed by a 5 aa insertion into the same gene that gave rise to the 18 aa variant.

Reworded as suggested.

Line 313 – The following sentence is not very clear: "Instead, the presence of distal H residues was the common denominator in constructs that functioned significantly higher than WT and the 13/18 variants." Do the authors mean that any additional histidine increases activity relative to WT? The inclusion of "and the 13/18 variants" implies a shared feature among the high-performing mutants that distinguishes them from the 13/18 variants, which is not readily apparent from the text. Please clarify.

We do not mean that simply adding any extra histidine residue increases activity relative to wild-type. Instead, the exceptional performance of Mutant-3 and Mutant-6 relative to both the WT and the natural 13/18 aa variants is driven by alterations in the length and compositional flexibility of their tethers. These specific modifications optimize the spatial orientation and geometric accessibility of the distal histidine residues relative to the F_0 exit pore, thereby accelerating the proton-transfer frequency. We will rewrite this sentence in the revised manuscript to explicitly differentiate how the engineered tethers mechanically outperform the natural variants.

Reviewer #2

We thank the reviewer for her/his assessment about reaching an innovation threshold suitable for publication.

Reviewer #3

Preparations of inverted membrane vesicles from bacteria often contain background levels of ATP and ATP synthesis activity independent of F1Fo ATP synthase, e.g. adenylate kinase,

which must be subtracted to accurately determine the ATP synthesis activity of the engineered enzymes. The authors should measure ATP production in each of their vesicle preparations in the presence of a protonophore, like carbonyl cyanide m-chlorophenylhydrazone (CCCP), to determine the gradient-independent background. Subtraction of this background would be essential prior to normalization of activity to expression.

We propose that the gradient-independent background suggested does not significantly alter our core findings (moreover, if applicable they would increase our reported values of C-terminal extensions over wild-type). Specifically, our assays were driven by NADH to selectively trigger the respiration-dependent proton motive force. Since the background vesicle matrix remained identical across all samples, any baseline enzymatic activity was effectively cancelled out by our cross-normalization protocol, which equalized all mutant values against side-by-side wild-type *E. coli* controls. Note also that inverted vesicles were washed multiple times during the preparation process (i.e., with MOPS buffer following high speed centrifugation) and thus cytosolic proteins (e.g., AK) would be mostly removed.

The Western blots suggest that atpB mutants are expressing much lower amounts of subunit a. The authors postulate that mutants, being hyperactive, can sustain cells with lower expression, but the data do not necessarily support this conclusion. The mutant subunit a may be unstable or less able to assemble the Fo complex. To support their conclusion, the authors should determine whether their mutant E. coli strains can grow aerobically in non-fermentable minimal medium at the same level as wildtype.

We highlight an inherent screening step in our methodology that addresses this concern. As described in Section 2.3 (Homologous Recombination), all of our engineered *E. coli* knock-in strains were selected and recovered by plating them on M9 minimal media supplemented with 0.2% acetate as the sole carbon source. Because acetate is a non-fermentable substrate, successful colony formation strictly requires a functionally assembled and operational F_1F_o ATP synthase complex to support aerobic respiration. Strains with unstable or poorly assembled subunits would fail to grow under these selective conditions. Therefore, because these mutants robustly grew on M9-acetate supports our conclusion that the lower protein expression observed via Western blot represents a cellular down-regulation in response to hyperactive ATP production, rather than a structural assembly defect.

The normalization of ATP synthesis activity to subunit a expression level is understandable (and key to the authors' conclusion). The authors need to ensure that their analysis is completely transparent:

a. *The Western blots (Fig. A1) show significant variability across biological replicates. Please clarify in the text how band intensities are used for normalization. Are they compared to the intensities of LamB? In section 2.6, line 164-166, it suggests that the intensities of WT bands are somehow used for normalization.*

   Dunkley (2025) established a linear relationship between an internal LamB antiserum band and BCA (bicinchoninic acid) assay-determined protein concentration (i.e., equal amounts of protein were loaded in each lane, corresponding linearly to LamB band density), which was implemented in Dunkley et al. (2025). In the current experiments, equal amounts of total protein were loaded on each lane across all samples; thus, AtpB band densities were compared to wild-type bands to reflect the relative number of F_1F_O complexes,

which were then coupled with Michaelis-Mentoen kinetic curves to generate normalized ATP synthetic rates. All Western blots were imaged using a Bio-Rad ChemiDoc MP system equipped with a cooled CCD camera with a dynamic range of greater than four orders of magnitude. Quantification was conducted via ImageJ software (NIH, Bethesda, MD, USA) utilizing an automated rolling-ball background subtraction algorithm.

Dunkley, T., 2025. *Engineering ATP Synthase for Increased ATP Production* (Doctoral dissertation, Rutgers, The State University of New Jersey-Camden).

Dunkley, T., Shain, D.H. and Klein, E.A., 2025. A histidine-rich extension of the mitochondrial F 0 subunit ATP6 from the ice worm Mesenchytraeus solifugus increases ATP synthase activity in bacteria. *Febs Letters*, *599*(8), pp.1113-1121.

b. *When expression level is very low (Mutants 3 and 6), amplification of error upon normalization would be significant. Additionally, background ATP synthesis activity (see Comment 1) would be significantly amplified. It may be useful to include the unnormalized activity values and error in a supplementary table.*

   We acknowledge that low expression levels can amplify mathematical error or baseline noise; however, the reproducible Michaelis-Menten kinetic curves and consistent K_m values across biological replicates rule out random artifacts. Likewise, equal amounts of total protein were loaded across all samples (described above) and band intensities were well within the dynamic range of our CCD camera.

c. *If the ATP synthesis activities are normalized to subunit a level using relative density on a Western blot, then this value is no longer an absolute specific activity, making the y-axis label (nmol ATP/s/mg mem prot) in Figures 1 and 3 misleading. It would be more accurately reported as specific activity relative to whatever intensity (WT? LamB?) was used for normalization.*

   We agree that this process yields calibrated relative activities rather than absolute values; therefore, we have updated the y-axis labels in Figures 1 and 3, as well as the column headers in Tables A2 and A3, to read "Normalized ATP synthesis rate (relative units/mg membrane protein)" to reflect this metric more precisely.