nature portfolio

Peer Review File

ATHENA: a dynamic metabolic hub balancing carbon economy and energy supply in microbial synthesis

Corresponding Author: Professor Guang Zhao

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have read the paper with interest. This paper introduces an interesting system that dynamically regulate carbon conservation and energy synthesis in an adaptive manner. The authors named the system as ATHENA. The intent is to develop strategies to overcome the challenge of balancing carbon efficiency and energy requirements in microbial chemical production. The paper has focused on E. coli. The presented system separates the carbon-conserving and energy-generating modules and employs a dynamic genetic circuit that sense energy levels (NADH/NAD+) in vivo and regulate the two modules according to the level of NADH. To this end, the study first engineered and studied two carbon conservation modules (CCMs) to convert the glycolytic intermediate PEP into acetyl-CoA alongside the fixation of one CO2 molecule. The two modules were studied in depth. The experimental results presented showed that while CCM2 demonstrated in vivo functionality, it yielded lower cell growth, CO2 fixation, and acetyl-CoA production compared to CCM1. As a result, the authors focused only on CCM1 for subsequent part of their study. The study then went on to develop NADH-responsive positive and negative genetic circuits using the BsRex sensor (based on Rex protein from B. subtilis) and antisense RNA based on transcription attenuator pT1812. Extensive number of hybrid promoters and antisense RNA designs were constructed and tested. Interesting functional designs were identified. Upon successfully engineered the NADH responsive circuit, the authors created the NADH-responsive asRNA-mediated bifunctional regulatory system. The system was applied to activate and repress downstream genes so as to dynamically adjust metabolic flow based on the energy states. The authors demonstrated that the system improved the yields of five distinct acetyl-CoA based chemicals through dynamic regulation between the carbon-conserving and energy-generating modules. Experimental results showed the dynamic behaviour of their systems in regulating the modules. This is an extensive study. A substantial number of mutant strains and gene circuits were constructed and evaluated in this work. I have the following comments and suggestions for improvement:

Comments:
• Abstract: It is not clear in the abstract which organism the study is engineering. Key quantitative results should also be explicitly mentioned here.
• Line 213: “saturation mutagenesis was applied to residues 257 and 258 of P. putida NadB, yielding the L257C/I258C mutant with significantly enhanced activity”. Please specify quantitatively by how much the activity was enhanced.
• Line 218: “In addition, overexpression of AceA together with NadB promoted the glutamate biosynthesis and enhanced the cell density”. Similarly, although details are reported in the supplementary, it will be informative to state in the main manuscript by how much cell density was enhanced and by how much glutamate biosynthesis has been promoted.
• Figure 1d: The small inset photograph is difficult to see and should be enlarged or clarified. Line 304, among the mutant strains, Q6578 showed optimal growth with a final cell concentration comparable to that of the wild-type strain. However, it has a long lag phase. Is there plausible reason for the long lag phase? Similarly, Q6580 also reached the similar final cell concentration comparable to that of the wild-type strain but has a longer lag phase than Q6578. Please discuss.
• Figure 4a: five secondary structures of each sense target RNA were shown. What do the arrows indicate? Furthermore, regarding the secondary structures for ReT181Sc-f, it appears that the different stem structures refer to different segments. Please clarify this. It would be helpful to label the segments as was done for ReT181Sb.
• Line 320: What is the rationale of choosing natural transcription attenuator pT1812 as the mode to regulate gene expression for metabolic flux control?
• Line 384: There is an internal promoter in a reverse direction within the segments. What is the function of the internal promoter? How would repressive efficiency be enhanced by this promoter as mentioned?
• Line 413: It was mentioned that the asRNA-activated systems that only operate solely at the transcriptional level were

constructed. What was removed as compared to the dual activation one or the differences between the two?
• Line 452: What is the rationale of introducing the imperfect palindrome (IP-Rex, TGTGAA-(4 nt)-TGAGCA) with lower affinity? How did the authors determine the sequence of the imperfect palindrome to use? It is interesting to see that eventually the mix of the operator sites in the hybrid promoter xvi gave the best performance.
• Line 475: “a strong positive correlation was observed between the intracellular NADH/NAD+ ratios and GFP fluorescence (Fig. 5d).” Please report this correlation quantitatively (e.g., provide the R^2 value).
• Line 1063: “The ratio of NADH/NAD+ was determined using a fluorimetric detection assay kit (Yeasen Biotechnology).” Please provide the name of the assay kit used and the protocol used.
• Line 737: Discussion: “Compared to previously reported carbon conservation strategies, such as NOG pathway and SCTPK”. References should be cited.
• Discussion: Interesting that the synthesis of acetate and MVA using glucose as sole carbon source has yields exceeding their natural theoretical yields. What would be the new theoretical yield using the ATHENA system?

Minor comments
• Line 51, change “are produces” to “are produced”

Reviewer #2

(Remarks to the Author)
Chemical biosynthesis requires sufficient precursor acetyl-CoA and reducing powers, while it is challenging to balance carbon atom economy and energy supply. This study developed a dynamic regulation system to balance the cell growth and product biosynthesis by using a NADH responsive biosensor and antisense RNA. This system significantly improved yields and titers of five products with differential acetyl-CoA and energy demands.

1. I really like this dynamic regulation principle but the authors claim the orthogonal modularization of acetyl-CoA and NADH synthesis pathways for carbon conservation and energy generation, which is not really correct, since this system is interacted with central metabolism and is not orthogonal. Please remove this claim in all the text.
2. Figure 2a, e and f, please add stoichiometric information, which will help audience to understand the mass and energy balance.
3. In figure 6, the acetate production is well balanced with the dynamic regulation system and the NADH/NAD level and is well correlated this dynamic regulation. This is very well designed. While in Figure 7, 3-HP, MVA and PHB biosynthesis are consuming NADPH other than NADH and there is no data to reflect the NADPH/NADP ratio. How the cellular metabolism transforms NADH to NADPH? I suggest the authors quantifing the NADPH/NADP profile at the whole cultivation phase as they did in Fig. 6g, j.
4. Line28-30 in the Abstract, the authors claim that: In the 5-L bioreactor, acetate yield markedly exceeded its native theoretical yield, while that of mevalonate nearly approached this theoretical upper limit. Did the mevalonate production perform in 5-L bioreactor or shake flask with fed-batch fermentation? From figure 7c, the mevalonate titer is much lower than previous studies such as: 10.1016/j.ymben.2021.09.011; https://journals.asm.org/doi/10.1128/aem.02178-16. There is make no sense to achieve high yield with low titer. Is it possible to increase the titer with keeping high yield by using this dynamic regulation system?
5. The authors define this dynamic regulation system as autonomous tunable hybrid central network apparatus (ATHENA), I appreciate the new definition, which however can’t really reflect the study key finding. I suggest just using ‘an autonomous central metabolic balancing’ or some other direct words to show the autonomous balancing between cell growth and product biosynthesis.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my comments.

Reviewer #2

(Remarks to the Author)
The authors almost addressed my comments; however, stoichiometry of the metabolic pathways is not correct in figure 2e, figure 6b, figure 6d
1. Figure 2e, 1 molecule of glucose can’t generate 6 molecules (2+4) of Ac-Coa. Furthermore, in the left part of the figure, one pyruvate can’t produce 2 Ac-CoA. In the right part of CCM, please add the number of CO_2 fixation to balance the carbon.
2. Figure 6b, please add the number of fixed or released CO_2 to balance the carbon
3. Figure 6d, please add number ‘2’ to the Ac-CoA molecule

Ref: NCOMMS-26-039198-T

ATHENA: a dynamic metabolic hub for balancing carbon economy and energy supply in microbial synthesis

Response to the reviewers' comments:

We thanks the reviewers for their constructive comments and appreciation for the importance of our study: "*This paper introduces an interesting system that dynamically regulate carbon conservation and energy synthesis in an adaptive manner*." (Reviewer 1); "*This study developed a dynamic regulation system to balance the cell growth and product biosynthesis by using a NADH responsive biosensor and antisense RNA. This system significantly improved yields and titers of five products with differential acetyl-CoA and energy demands*." (Reviewer 2).

We responded to the reviewer's comment in a point-by-point form.

We appreciate the opportunity to resubmit our study to Nature Communications and are happy to make any additional modifications suggested by the Editors and Reviewers.

Reviewer #1

1. Abstract: It is not clear in the abstract which organism the study is engineering. Key quantitative results should also be explicitly mentioned here.

Reply: This study was carried out in E. coli, and this information has been supplemented in Abstract section in the revised manuscript (page 2, line 23).

2. Line 213: "saturation mutagenesis was applied to residues 257 and 258 of P. putida NadB, yielding the L257C/I258C mutant with significantly enhanced activity". Please specify quantitatively by how much the activity was enhanced.

Reply: In this assay, the activity of NadB protein was not detected directly, and a genetic selection system was developed based on the glutamate auxotrophy, in which the citrate synthase gene *gltA* and *prpC* were knocked out, and citrate biosynthesis was complemented via NadB and isocitrate lyase AceA. So, the

activity of NadB plays an essential role in the production of glutamate, further affecting the survival and growth rate of host strains in minimal medium. A positive correlation exists between bacterial growth and NadB activity: better grows corresponds to higher NadB catalytic activity. As shown in Extended Data Fig. 2d, the L257C/I258C mutant presented 1.36-time higher cell density than its original protein. This information has been supplemented in the revised manuscript (page 8, line 167).

3. Line 218: "In addition, overexpression of AceA together with NadB promoted the glutamate biosynthesis and enhanced the cell density". Similarly, although details are reported in the supplementary, it will be informative to state in the main manuscript by how much cell density was enhanced and by how much glutamate biosynthesis has been promoted.

Reply: Thanks for your comment. Overexpression of AceA alongside PpNadB mutant increased the final cell density by 1.46-fold relative to expression of PpNadB mutant individually. This information has been supplemented in the revised manuscript (page 9, line 172).

4. Figure 1d: The small inset photograph is difficult to see and should be enlarged or clarified.

Reply: We believe the reviewer is referring to Fig. 2d in this context. This photo in Fig 2d has been enlarged.

5. Line 304, among the mutant strains, Q6578 showed optimal growth with a final cell concentration comparable to that of the wild-type strain. However, it has a long lag phase. Is there plausible reason for the long lag phase? Similarly, Q6580 also reached the similar final cell concentration comparable to that of the wild-type strain but has a longer lag phase than Q6578. Please discuss.

Reply: In this experiment, Q6578 and Q6580 achieved final cell densities comparable to that of the wild-type strain. Their only drawback was a longer lag phase, which we hypothesized arose from the metabolic burden caused by

overexpression of multiple heterologous genes. This speculation is supported by strain Q6579, which overexpressed all target genes from a high copy number plasmid and exhibited the longest lag phase. This has been supplemented in the revised manuscript (page 12, line 241).

6. Figure 4a: five secondary structures of each sense target RNA were shown. What do the arrows indicate? Furthermore, regarding the secondary structures for ReT181Sc-f, it appears that the different stem structures refer to different segments. Please clarify this. It would be helpful to label the segments as was done for ReT181Sb.

Reply: The arrows indicate the nucleotides modified in each step. This has been added in figure legend. The Segments I-IV were labeled for ReT181Sc-f in the revised Fig. 4a and its legend (page 58, line 1254).

7. Line 320: What is the rationale of choosing natural transcription attenuator pT181 as the mode to regulate gene expression for metabolic flux control?

Reply: We selected the natural attenuator pT181 based on the following reasons: First, pT181 represents an asRNA-mediated expression regulatory strategy. asRNA-based circuits feature high design flexibility and can be seamlessly integrated into complex biological frameworks, offering promising avenues for constructing synthetic biosystems. Unlike CRISPR tools that require heterologous dCas protein expression, asRNA functions without translation and imposes minimal cellular resource burden. This advantage is critical for engineering compact genetic programs confined within single cells. Additionally, RNA signaling networks operate with faster kinetic responses than protein-based regulatory cascades.

Second, the transcriptional attenuation mechanism of pT181 has been thoroughly characterized. This well-elucidated foundation facilitates our construction of dual-function regulatory modules capable of both activating and repressing gene expression, which we applied to precisely control metabolic pathways.

Lastly, synthetic biosystems demand regulatory tools with high performance,

robust orthogonality, and abundant available components. Investigating the native transcription attenuator pT181 enables us to establish a universal design framework, which can be extended to engineer other transcription attenuators and validate their orthogonality. This work lays a solid foundation for future tunable metabolic pathway regulation.

8. Line 384: There is an internal promoter in a reverse direction within the segments. What is the function of the internal promoter? How would repressive efficiency be enhanced by this promoter as mentioned?

Reply: In the natural staphylococcal plasmid pT181, antisense RNA regulates the expression of the plasmid initiator protein (RepC) through transcriptional attenuation, thereby modulating plasmid replication (Cell, 1989, 59: 395; Mol Microbiol, 2000, 35: 1469). The *repC* mRNA can potentially adopt two distinct conformations in which an anti-terminator or a terminator of transcription can be alternatively form. The internal reverse promoter transcribes antisense RNA (RNAI). In the absence of RNAI, the nascent *repC* transcript forms preferentially into a conformation that permits transcription throughout the *repC* mRNA. In the presence of RNAI, it binds to a hairpin structure of *repC* mRNA. The formed complex modifies the folding process of the mRNA during transcription so that a Rho-independent terminator structure is stabilized, resulting in a premature termination of transcription. Therefore, the intrinsic reverse promoter acts as a negative control factor by transcribing asRNA, interfering with the expression of RepC, and thereby regulating plasmid replication. In our study, in order to achieve controlled expression, we expressed the pT181 regulatory region and the asRNA on two separate plasmids, respectively, and mutated the reverse promoter within pT181 to eliminate its interference. The resultant ReT181Sb presented 1.98-fold repression and maximum GFP fluorescence 1.55-time higher than that of ReT181Sa. The function of promoter P1 was added in the revised manuscript (page 13, line 272).

9. Line 413: It was mentioned that the asRNA-activated systems that only operate solely at the transcriptional level were constructed. What was removed as compared to the

dual activation one or the differences between the two?

Reply: In the transcription activation system, segments I and II of the transcription attenuator were removed, while segments III and IV, which form a terminator, were retained. In addition, GFP is translated from an independent downstream RBS. The asRNA is complementary to segment III and upstream region. In the absence of the asRNA, segments III and IV form a terminator, inhibiting transcriptional elongation. In the presence of the asRNA, it complementarily pairs with the 5' half of the terminator stem, preventing terminator formation and allowing transcription to proceed.

In the dual activation system, we introduced translation elements into segments III and IV. Specifically, we introduced an RBS sequence and a translation enhancer into the stable upper stem-loop of the terminator hairpin structure, and a start codon into the double-stranded stem. Additionally, we remove the original independent RBS and start codon sequences of GFP, ensuring that GFP initiation depends solely on the translation element embedded within the terminator hairpin—unlike the independent translation element in the transcriptional activation system. This architecture ensures that, in the absence of asRNA, a terminator hairpin forms and sequesters the translation element; whereas, in the presence of asRNA, the terminator structure is disrupted via a strand-displacement reaction, exposing the translation element and enabling downstream gene expression. Fig. 4d has been revised to clearly display the difference between transcriptional activation system and dual activation system.

10. Line 452: What is the rationale of introducing the imperfect palindrome (IP-Rex, TGTGAA-(4 nt)-TGAGCA) with lower affinity? How did the authors determine the sequence of the imperfect palindrome to use? It is interesting to see that eventually the mix of the operator sites in the hybrid promoter xvi gave the best performance.

Reply: Hybrid promoters can be constructed by fusing the core promoter sequence with one or more operators to obtain new properties. To construct an NADH biosensor in *E. coli*, we first selected the natural *cydA* promoter from *Bacillus subtilis*, which is regulated by Rex and shares similar characteristics with the *E. coli* promoter. This promoter contains an IP-Rex sequence (*cydA*

promoter: TT**TTGACA**CATTTGTGAAATATTGAGC**AATATT**TTTTTCGTC, -35 and -10 regions in bold, IP-Rex underlined). This information has been supplemented in the revised manuscript (page 17, line 363).

We attempted to recreate the sequence of this feature and then develop a Rex-regulated hybrid promoter in *E. coli*. In the system we initially built, the dynamic range remained limited, but we found that inserting the IP-Rex sequence downstream of the -10 region could enhance its expression strength (as shown in vi).

Subsequently, we consider adding PP-Rex to the promoter to increase its dynamic range. We did find that when the PP-Rex is in the same position as the IP-Rex, the former had a broader range than the latter, especially when PP-Rex is present between the -35 and -10 regions. We think this is related to the occupancy hypothesis, which posits that the sequence of an operator simply determines its binding affinity for its target regulatory proteins (Cell Rep, 2012, 2: 150; Science, 2009, 324: 407). It serves as a docking point for regulatory proteins, recruiting them to the promoter region and determining the occupancy of the operator. The stronger the operator's affinity, the potentially higher the occupancy and the larger the dynamic range is expected to be. As shown in xviii and xix, the dynamic range has been increased by approximately 6 times.

Additionally, we also note that: (1) Studies have shown that operator sequences can alter gene expression independent of protein occupancy (Cell Rep, 2012, 2: 150; Nat Commun, 2017, 8: 52); (2) Operator sequences with low affinity may generate high reporter signals, as the vi we obtained; (3) There are reports of using different binding boxes inserted into promoters to improve performance (ACS Synth Biol, 2021, 10: 2076; ACS Synth Biol, 2021, 10: 132). Therefore, building upon the promoter containing PP-Rex, we further incorporated IP-Rex to balance the dynamic range and reporter signal, resulting in the xvi with optimized performance.

11 Line 475: "a strong positive correlation was observed between the intracellular NADH/NAD+ ratios and GFP fluorescence (Fig. 5d)." Please report this correlation quantitatively (e.g., provide the R^2 value).

Reply: Thanks for your suggestion. We have calculated the coefficient of determination (R^2), which reached 0.9422, demonstrating a strong positive correlation between intracellular NADH/NAD+ ratios and GFP fluorescence. This has been supplemented in revised Fig. 5d.

12 Line 1063: "The ratio of NADH/NAD+ was determined using a fluorimetric detection assay kit (Yeasen Biotechnology)." Please provide the name of the assay kit used and the protocol used.

Reply: The ratio of NADH/NAD^+ was determined using the NAD/NADH Ratio Fluorimetric Detection Assay Kit (50130ES, Yeasen Biotechnology) according to the manufacturer's instruction. Briefly, cell pellets were collected by centrifugation at 10,000 × g for 15 min at 4°C. Lysis buffer was then added, and incubated at room temperature for 15 min. Afterward, the samples were centrifuged at 2,500 × g at room temperature for 5-10 min. The supernatant was collected for analysis. For NAD^+, the NAD extraction solution was added and incubated at room temperature for 10-15 min, followed by the addition of the NADH extraction solution. For NAD^+ and NADH, the NAD/NADH control solution was added and incubated at room temperature for 10-15 min, followed by the addition of further NAD/NADH control solution. To prepare the NAD/NADH reaction mixture, the NADH sensor buffer was added to the NAD/NADH recycling enzyme mix and mixed thoroughly. To each reaction system, the NAD/NADH reaction mixture was added and incubated at room temperature away from light for 15 min-2 h. The fluorescence (excitation, 540 nm; emission, 590 nm) was measured using 96-well black plates (Corning 3603) and a microplate reader (Spark, Tecan). The NAD^+ and NAD^+/NADH levels were determined based on the NADH standard curve at different concentrations. The amount of NADH was obtained by subtracting the level of NAD^+ from the total NAD^+/NADH. The ratio of NADPH/$NADP^+$ was determined using the NADP/NADPH Ratio Fluorimetric Detection Assay Kit (50140ES, Yeasen Biotechnology) according to the manufacturer's instruction.

The procedure is similar to that mentioned above. Specifically, the NADPH sensor buffer was added to the NADP/NADPH recycling enzyme mix to prepare the NADP/NADPH reaction solution. This has been supplemented in the revised manuscript (page 43, line 936).

13 Line 737: Discussion: "Compared to previously reported carbon conservation strategies, such as NOG pathway and SCTPK". References should be cited.

Reply: The references for NOG pathway and SCTPK have been added in the revised manuscript (page 28, line 597).

14 Discussion: Interesting that the synthesis of acetate and MVA using glucose as sole carbon source has yields exceeding their natural theoretical yields. What would be the new theoretical yield using the ATHENA system?

Reply: When CCM1 is applied, the theoretical yields of acetate and MVA are 1.33 g/g glucose and 0.75 g/g glucose, and their experimental yields accounted for 78% and 75% of the theoretical maximum yields, respectively. This has been supplemented in the revised manuscript (page 31, line 673).

Reviewer #2

1. I really like this dynamic regulation principle but the authors claim the orthogonal modularization of acetyl-CoA and NADH synthesis pathways for carbon conservation and energy generation, which is not really correct, since this system is interacted with central metabolism and is not orthogonal. Please remove this claim in all the text.

Reply: We sincerely appreciate this critical and valuable comment. We fully agree with the reviewer′s opinion. The statement claiming the orthogonality of acetyl-CoA and NADH synthetic pathways has been completely deleted throughout the entire revised manuscript, as the regulatory circuit indeed interacts extensively with endogenous central carbon metabolism and cannot be defined as orthogonal. All related descriptions have been revised accordingly to avoid inappropriate wording.

2. Figure 2a, e and f, please add stoichiometric information, which will help audience to understand the mass and energy balance.

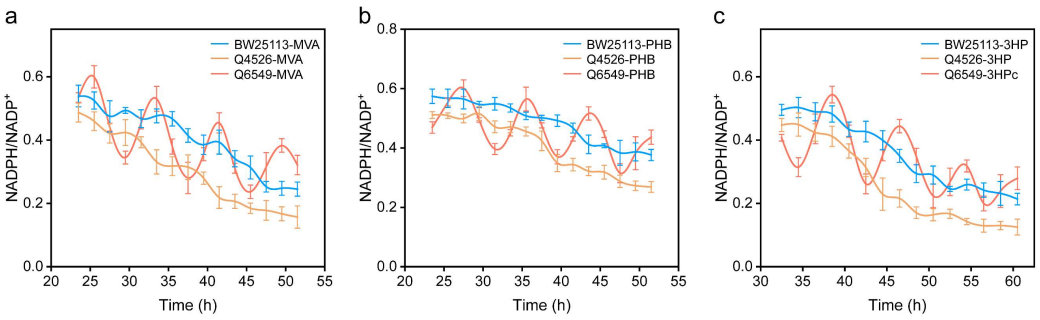
Reply: Thanks for your suggestion, and the corresponding figures have been revised.

3. In figure 6, the acetate production is well balanced with the dynamic regulation system and the NADH/NAD level and is well correlated this dynamic regulation. This is very well designed.

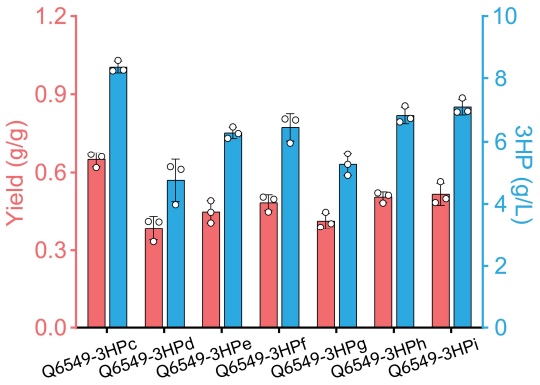
While in Figure 7, 3-HP, MVA and PHB biosynthesis are consuming NADPH other than NADH and there is no data to reflect the NADPH/NADP ratio. How the cellular metabolism transforms NADH to NADPH? I suggest the authors quantifing the NADPH/NADP profile at the whole cultivation phase as they did in Fig. 6g, j.

Reply: Thank you for your constructive suggestion. The biosynthesis of 3HP, MVA, and PHB requires NADPH as a cofactor. The intracellular transhydrogenase in *E. coli* (encoded by *pntAB*) is responsible for the conversion of NADH to NADPH.

First, we investigated the changes of NADPH/NADP$^+$ during the cultivation of these three products. We found that the NADPH/NADP$^+$ in the strain carrying ATHENA fluctuated, whereas in the other two strains, it showed a gradual decline. Furthermore, chemicals with higher energy requirements (such as 3HP and MVA) exhibited greater fluctuations and shorter oscillation periods. Also, we observed that the amplitude of the NADPH/NADP$^+$ decreased during the later cultivation of 3HP synthesis. These results were added in the revised manuscript (page 26, line 550 and Fig. 7o-q).

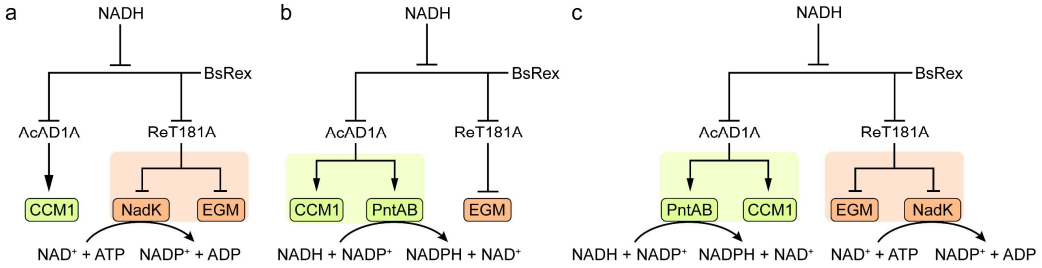


Since 3HP has the highest energy requirements among the three compounds, we then performed constitutive expression of *nadK* ($NAD^+ + ATP \rightarrow NADP^+ + ADP$), *pntAB* ($NADP^+ + NADH \rightarrow NAD^+ + NADPH$), *nadK* and *pntAB*, respectively, based on the Q6549-3HPc strain, generating strains Q6549-3HPd, Q6549-3HPe, and Q6549-3HPf. We attempted to further increase the concentration of the kinase or/and transhydrogenase to investigate whether this could further promote 3HP synthesis. However, the results showed that the highest production was only 77% of that of Q6549-3HPc (when *nadK* and *pntAB* were co-overexpressed). This is similar to a previous study, in which increasing the $NADPH/NADP^+$ did not promote the production of MVA (Biotechnol Bioeng, 2020, 117: 2153).



Subsequently, we explored another model involving dynamic regulation of *pntAB*, *nadK*, *pntAB* and *nadK*. Specifically, we combined *pntAB* with CCM1, both of which are positively regulated by the NADH biosensor-mediated RNA regulators. When intracellular NADH is high, CCM1 is activated and PntAB promotes the conversion of NADH to NADPH. Consumption of NADH represses CCM1 and *pntAB*, while upregulating EGM and *nadK*. EGM enhances NADH production, while NadK promotes the transformation of NAD^+ into $NADP^+$. We dynamically regulated *nadK* in coordination with EGM, *pntAB* in coordination with CCM1, or both simultaneously, generating the strains Q6549-3HPg, Q6549-3HPh, and Q6549-3HPi, respectively. However, the production was not restored. The yield and titer of Q6549-3HPi were 79.5% and 84.6% of those of Q6549-3HPc, respectively. Additionally, the production of the dynamically regulated strains was approximately 1.1 times that of the constitutive overexpression strains. This result was added in the revised

manuscript as Supplementary Fig. 12.



Collectively, because NADPH/NADP$^+$ exhibited fluctuating patterns, and either constitutive expression or dynamic regulation of the enzymes involved in NADH conversion affected the original performance. Possible reasons include the fact that gene overexpression imposes a greater metabolic burden; and since CCM1 requires NADH, excessive NADH transformation impairs CCM1 performance. Therefore, it is essential to maintain the transhydrogenase at an appropriate level. We believe that under these conditions, intracellular transhydrogenases play a crucial role in this process. To further improve the production, more finely tailored regulation could be achieved by coupling other sensors, such as FapR for malonyl-CoA. Alternatively, establishing an NADPH sensor using SoxR or Yap1p may further improve the production performance.

4. Line28-30 in the Abstract, the authors claim that: In the 5-L bioreactor, acetate yield markedly exceeded its native theoretical yield, while that of mevalonate nearly approached this theoretical upper limit. Did the mevalonate production perform in 5-L bioreactor or shake flask with fed-batch fermentation? From figure 7c, the mevalonate titer is much lower than previous studies such as: 10.1016/j.ymben.2021.09.011; https://journals.asm.org/doi/10.1128/aem.02178-16. There is make no sense to achieve high yield with low titer. Is it possible to increase the titer with keeping high yield by using this dynamic regulation system?

Reply: The production of mevalonate was carried out in 5-L bioreactor, as described in the Abstract and Results (page 2, line 30 and page 24, line 516).

In this study, bioproductions of diverse chemicals, including acetate, ethanol, 3HP, MVA, and PHB, were adapted as proxy to elucidate the function of ATHENA, to demonstrate that this is a universal strategy for constructing high

carbon-yield microbial cell factories. As expected, ATHENA enhanced production and yield of all tested chemicals.

Regarding MVA, its biosynthetic pathway used here consists of HMG-CoA synthase (MvaS) and HMG-CoA reductase (MvaE) from *Enterococcus faecalis*. This configuration is different from the pathway described in the mentioned reports, which employs *E. coli* AtoB, and *Lactobacillus casei* MvaS and MvaE. Notably, prior work has demonstrated that *L. casei*-derived MvaS and MvaE drive more robust MVA biosynthesis than orthologs from other microbial species (PNAS, 2014, 111: 8357), which may explain their higher MVA titers relative to our system.

To further increase chemical production, optimization can be performed in the following ways, like enhancing the uptake and utilization of substrates, improving the catalytic capacity of pathway enzymes. Dynamic regulatory systems provide a model for precise temporal control in the construction of cell factories. If combined with improvements in the catalytic capacity of underlying elements, this may further increase titer of target chemical. Overall, we consider that by dynamically regulation and coordinating with other approaches, it is possible to increase titer while maintaining production yield in the future.

5. The authors define this dynamic regulation system as autonomous tunable hybrid central network apparatus (ATHENA), I appreciate the new definition, which however can't really reflect the study key finding. I suggest just using 'an autonomous central metabolic balancing' or some other direct words to show the autonomous balancing between cell growth and product biosynthesis.

Reply: Thanks for your valuable suggestion. We have renamed our system the **autonomous hybrid energy-carbon balancing apparatus** (ATHENA) to clarify its core function for readers. Corresponding revisions have been made throughout the entire manuscript (page 2, line 21; page 5, line 90; page 20, line 423; and so on).

Ref: NCOMMS-26-039198-A

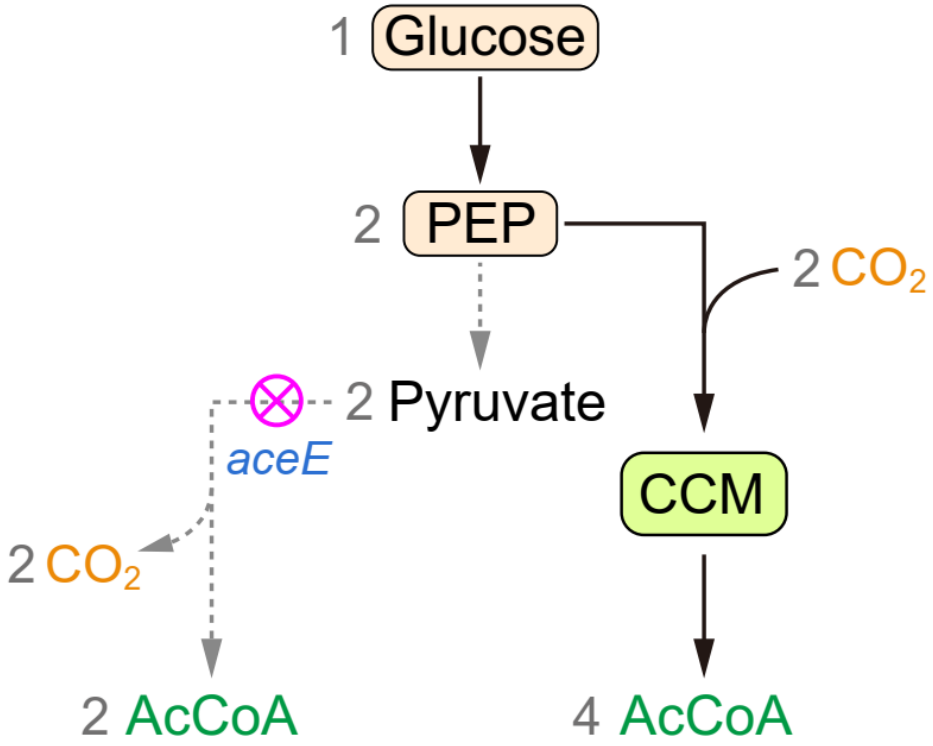
ATHENA: a dynamic metabolic hub balancing carbon economy and energy supply in microbial synthesis

Response to the reviewers' comments:

Reviewer #2

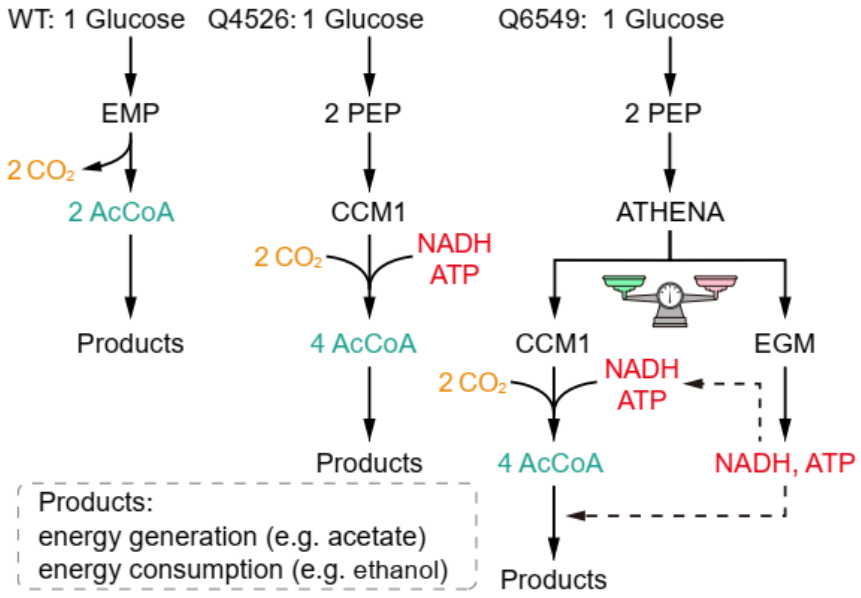
1. Figure 2e, 1 molecule of glucose can't generate 6 molecules (2+4) of Ac-Coa. Furthermore, in the left part of the figure, one pyruvate can't produce 2 Ac-CoA. In the right part of CCM, please add the number of CO2 fixation to balance the carbon.

Reply: Thanks for the comment. Fig. 2e has been revised accordingly. The molecule numbers of PEP, pyruvate, CO_2, and AcCoA were added. To clearly show the pathway in left panel was deleted, dashed lines were used. The revised Fig. 2e is shown below:



2. Figure 6b, please add the number of fixed or released CO2 to balance the carbon

Reply: As suggested, the numbers of fixed or released CO_2 were added.



3. Figure 6d, please add number '2' to the Ac-CoA molecule

Reply: The number of Ac-CoA molecule was added in Fig. 6d.