nature portfolio

Peer Review File

A nonclassical biosynthetic route enables tocopherol synthesis in bacteria

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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript reports the heterologous biosynthesis of tocopherols, including alpha-tocopherol, in photosynthetic bacteria Rhodobacter. UbiE was identified as a methyltransferase with bifunctional γ/β-tocopherol methyltransferase activity. Through random chromosomal integration of the tocopherol synthetic module combined with enhanced precursor availability from MEV pathway and shikimate pathway, the authors achieved a tocopherol titer of 295 mg/L. Further process optimization via supplementation of SAM and its precursor, and antioxidants increased the total tocopherol titer to 620 mg/L. Fed-batch fermentation yielded 3.51 g/L of tocopherols. This represents the first report of de novo tocopherol biosynthesis in heterologous bacteria, and the titer achieved is impressive. However, for a manuscript intended for Nature Metabolism, the mechanistic understanding of the underlying metabolism remains insufficient.
Major concerns:
1. As the central finding of this study, the functional properties of UbiE warrant deeper investigation. The authors should provide a rigorous comparative mechanistic analysis between UbiE and other characterized methyltransferases (MTs) in the tocopherol biosynthetic pathway. Specifically, what distinguishes UbiE from MTs that catalyze only one methylation step? Current evidence of bifunctional γ/β-tocopherol methyltransferase activity requires more detailed biochemical characterization.
2. Reliance on computational simulations and predicted kinetic parameters is insufficient to support the manuscript's mechanistic claims. Based on our experience, such in silico predictions frequently lack accuracy. We recommend that the authors conduct the following: (1) protein crystallography or cryo-electron microscopy to elucidate the three-dimensional structure of UbiE; (2) determination of kinetic parameters using purified recombinant enzyme; and (3) substrate specificity assays to validate the proposed bifunctional activity.
3. To eliminate the possibility that other methyltransferases in the host chromosome contribute to α-tocopherol synthesis, targeted knockout of UbiE should be performed. This genetic confirmation is essential to establish UbiE as the sole enzyme responsible for the observed tocopherol production phenotype.
4. There is debate on the major source of PDP in photosynthetic organisms, whether primarily derived from chlorophyll degradation or from GGPP reduction. This distinction is critical, as heterologous overexpression of geranylgeranyl reductase (GR) in non-photosynthetic hosts has consistently failed in previous studies. Therefore, the authors must address whether GR overexpression also enhances tocopherol production—independent of and in parallel with GGPP supply enhancement. This experiment would clarify the contribution of the GR-mediated pathway.
5. The substantially improved tocopherol titer resulting from random chromosomal integration of the synthetic biosynthetic module suggests significant position-dependent effects. The authors should investigate the genomic context of integration sites and identify the underlying factors—such as proximity to transcriptional hotspots, effects on local chromatin structure, or influence from nearby genomic elements—that enhance production.
6. The in vivo evaluation using Caenorhabditis elegans is informative; however, the current analysis is incomplete. While the authors emphasize the importance of feeding ratios between tocopherol-producing and wild-type bacteria, they overlook a critical variable: the impact of tocopherol composition. Since α-tocopherol is widely recognized as the most biologically active form, experiments specifically comparing batches with higher α-tocopherol content to those with lower α-tocopherol proportions would be expected to demonstrate enhanced bioactivity. This compositional effect warrants explicit investigation.
Minor issue:
1. The claim that "a new methyltransferase … is identified" is not strictly accurate. UbiE itself is not a novel methyltransferase; rather, a previously uncharacterized bifunctional activity of this enzyme has been newly demonstrated. The authors should revise this statement to reflect that the novel finding concerns the γ/β-tocopherol methyltransferase activity of UbiE, not the identification of an entirely new enzyme.

Reviewer #2

(Remarks to the Author)
The manuscript by Zhang et al. describes the characterization and engineering of Rhodobacter sphaeroides as a production platform for tocopherols. The authors demonstrate the ability of native ubiquinone/menaquinone methyltransferase UbiE to convert heterologous δ-tocopherol into α-tocopherol via γ/β-tocopherols, thereby simplifying tocopherol biosynthesis in R. sphaeroides by bypassing the 2,3-dimethyl-5-phytyl-l-4-benzoquinone step of the classical pathway. Based on molecular docking and -dynamics simulations, they propose catalytically competent binding conformations which rationalize the identified enzyme activities.
In addition to reconstructing tocopherol biosynthesis in R. sphaeroides, the authors employ established engineering strategies to improve the supply of the central precursors, geranylgeranyl diphosphate and homogentisic acid. Further media optimization efforts showcase the benefit of supplementing methyl donors and antioxidants in the fermentation process, ultimately enabling gram-scale tocopherol production in a fed-batch bioreactor setup. Finally, the authors explore the dietary effects of the tocopherol-producing bacteria on the fitness of C. elegans. While the results largely suggested the equivalence of engineered R. sphaeroides to both the wild type and commonly-fed E. coli, a slight benefit to reproductive fitness was observed at moderate tocopherol levels.
The experiments were well-executed, and the data were well-presented and convincing. The study not only establishes R. sphaeroides as a platform for microbial tocopherol production, but also makes UbiE available as a potential tool for synthetic biology. In this regard, an immediate question arising from this work is whether the geranylgeranyl diphosphate reductase from R. sphaeroides could facilitate the heterologous production of phytyl diphosphate in more common hosts. As the authors state in lines 103-105, insufficient PDP amounts are thought to be a major bottleneck in other systems — and the main reason the search for suitable hosts was expanded here. It would be very valuable if the authors could look into the possibility of identifying GGR homologs in the transcriptome at hand. At least though, I suggest that the authors revisit this GGR topic in the discussion.
Another concern is that, especially throughout the conclusion and the abstract, some of the claims made should better reflect the experimental outcomes. For example, the phrasing of “novel biosynthetic pathway” and “new bifunctional methyltransferase” is overstated considering the final product of α-tophecorol exists, the enzymes are known, but the primary discovery is UbiE’s novel activity. Similarly, comparison of feedstocks between the wildtype and modified R. sphaeroides for C. elegans suggests little difference to the benefit of the growth of the organism outside of the lengthening of the reproductive periods (though with less viable eggs) and may detract from the outcome. The observation that the feedstock mixture conferred an increase to the reproductive fitness of C. elegans is interesting but requires more testing to show the significance of this preliminary experiment. If you were to add α-tophecorol directly to the wildtype feedstock would it have a similar effect on the C. elegans as the biosynthesized α-tophecorol?

Minor Issues:
Phrasing around anti-cardiac benefits (line 39) suggests negative effects towards the cardiovascular system.
Inconsistent writing style for casing and italicization of gene names (for example line 166 and line 270) can be remedied.
The sentence in line 163 “... indicating that the nonclassical biosynthetic route might be widespread in other bacteria” seems confusing given that these bacteria do not produce tocopherols. What was found to be widespread is the capability of ubiE to convert tocopherol substrates.
Correcting the vocabulary around line 306, “To access the the impact of SCPs…” can better inform the reader on the aim of the experiment.

Reviewer #3

(Remarks to the Author)
Zhang et al. report a nonclassical biosynthetic route for tocopherol production in Rhodobacter species and describe the identification of a putative bifunctional γ/β-tocopherol methyltransferase that may enable an alternative α-tocopherol synthesis route bypassing the classical DMPBQ intermediate. This topic is interesting and potentially relevant for microbial production of high-value tocopherols. The authors further combine enzyme characterization, metabolic engineering, and fermentation optimization to improve product titers. Overall, the study presents a potentially useful direction for tocopherol biosynthesis research. However, some key experimental data and supporting validations are currently insufficient, and additional evidence is required to further strengthen the main conclusions. The following concerns should be addressed before publication.
Major Comment:
1. The authors state that vitamin E is classified into eight compounds, namely α, β, γ, δ-tocopherols and tocotrienols. According to established biosynthetic pathways, homogentisic acid (HGA) can condense not only with phytyl diphosphate (PDP) but also with geranylgeranyl diphosphate (GGPP) via HPT to form intermediates leading to tocotrienols. However, throughout the manuscript, only tocopherols are discussed, and no data are provided regarding the possible formation or exclusion of tocotrienols.It is therefore important to clarify whether tocotrienols are produced in the engineered strains. The authors should provide corresponding analytical evidence and explicitly distinguish tocopherols from tocotrienols. In particular, please clarify whether the chromatographic and mass spectrometric profiles of tocopherols and tocotrienols were resolved and how compound identities were assigned. In addition, chromatograms and mass spectra of authentic standards for the reported products should be provided and directly compared with the fermentation samples to support compound identification.
2. More detailed descriptions are also needed regarding data processing of the MS spectra shown in Figure S1 and Figure S4, including whether background subtraction or signal correction was performed. In addition, beyond LC-MS analysis of the fermentation products, further structural confirmation is recommended. The authors are requested to provide NMR characterization data (e.g., ^1H and/or ^13C NMR) of the key products to support compound identification.

3. Regarding the identification of the putative γ-TMT and MPBQMT homologues in R. sphaeroides, more comprehensive bioinformatic evidence is required. The authors should provide detailed sequence analyses in the Supplementary Information, including BLAST outputs, sequence alignments, coverage and identity metrics, conserved motif comparison, and phylogenetic analysis with characterized γ-TMT, MPBQMT, and related methyltransferases. The criteria for selecting the seed sequences used in the homology search should also be clearly described and justified.
4. A key claim of this work is the identification of a putative bifunctional γ/β-tocopherol methyltransferase (UbiE). However, its functional characterization could be better. The enzyme(s) from the relevant sources could be expressed and purified, and experimental kinetic parameters (Km, kcat, kcat/Km) measured. Predicted values alone by Deepmolecules (Table S2) are not sufficient to support the claimed bifunctional activity.
5. The proposed molecular mechanism is based only on docking and MD simulations, without experimental structural validation. Experimental evidence (e.g., structural determination or site-directed mutagenesis with activity assays) is needed to support the mechanistic conclusions.
6. Since the metabolic engineering was mainly performed via Tn5-based integration, the authors could provide experimental evidence for the copy number of the integrated gene(s) in the final selected strains (e.g., qPCR/ddPCR-based copy-number quantification or an equivalent genomic validation).
7. In Figure 4A, the meaning and design of “MEV1–13” are not defined, and the corresponding construction rationale and dataset are not clearly presented. Please provide a clear description and the underlying data supporting these engineered strains. In Figure 4B, the purpose of distinguishing Library A and Library B is unclear. If hppd-hpt-vet-aroG*-tyrA* were not independently regulated, the specific role and necessity of Library A in the experimental design should be clarified.
8. In Figure 4C, the origin and genotype of strains VE22–25 are not clearly indicated, and the linkage between strain construction and the presented data lacks continuity. Please clarify where these strains are defined and how they were derived. It is recommended to provide a flowchart of strain engineering and key modification steps in the Supplementary Information, as well as a complete genotype table for all constructed strains, to improve clarity and reproducibility (see, for example, Zhu et al., Nat Catal 3, 64–74 (2020), Supplementary Fig. 14 and Supplementary Table 3).
9. VE01 (plasmid-based expression) reportedly produces only α-tocopherol, whereas Tn5-integrated strains generate multiple tocopherol species; this difference should be explained and supported with data (e.g., expression level and flux effects). Please also provide in vitro enzyme data showing how product distribution varies with substrate (and SAM) concentrations, and discuss whether balancing HGA, PDP, and SAM supply in vivo could drive more complete conversion to α-tocopherol.
10. In Fig. 6B and 6D, the tocopherol composition clearly shifts after SAM regulation, and δ-tocopherol is no longer detected. This is intriguing and suggests that sufficient SAM supply can effectively promote further substrate conversion. However, it is unclear why the authors did not further strengthen endogenous UbiE expression to take advantage of this effect. In addition, for strain VE30, please provide the 5-L fed-batch results under MET supplementation alone (if tested), and include a more complete fermentation characterization, such as key by-product profiles, methionine and related metabolite changes , and yield calculations.
11. The authors should also provide complete plasmid maps for all constructs and clearly indicate their correspondence with the plasmid names and descriptions in the main text and Supplementary Information.
Minor comments
1. In the main text (Line 8) and Supplementary Information (Line 11), please remove the extra space after “2”.
2. In Fig. 1, it would be helpful to label proteins together with their corresponding gene names to improve clarity and readability.
3. In Supplementary Table S1 and the related discussion, please include and discuss the most recent relevant literature (e.g., https://doi.org/10.1016/j.bej.2026.110079), which appears to be directly pertinent to the topic.
4. Line 96: revise to “cyclization and/or methylation”.
5. The manuscript structure could be improved by presenting the complete synthetic biology and pathway engineering section first, and then the application results, to enhance clarity and narrative flow.
6. In Fig. S7A, please remove the extra “v” at “mvk”.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All my concerns have been properly addressed.

Reviewer #2

(Remarks to the Author)
No further remarks.

Reviewer #3

(Remarks to the Author)
I do not have further concerns and recommend acceptance of the paper in its present state.

Response to the Editor's comments:

1. We agree with the reviewers that it's crucial to provide compelling mechanistic insight and functional validation of the bifunctional γ/β-tocopherol methyltransferase activity of UbiE. In alignment with this, we suggest that you investigate how chromosomal integration of UbiE affects tocopherol production and exclude the contribution from other characterized methyltransferases to the production enhancement. Please follow the comments of reviewers #1 and #3 in this regard.

Response: Based on reviewers' comments about UbiE, we carried out the following experiments:

(1) We compared the reactions between the well-studied γ-tocopherol methyltransferase from *A. thaliana* and UbiE from *Rhodobacter* by expressing in the heterologous *E. coli*. As shown in Fig. 2D, we found that only *Rhodobacter* UbiE expression could result in α-tocopherol production by δ-tocopherol feeding, whereas γ-TMT from *A. thaliana* could only convert δ-tocopherol to β-tocopherol. Further molecular docking revealed that γ-TMT from *A. thaliana* could not give favorable docking of β-tocopherol (as shown in the detailed response below), which explains γ-TMT from *A. thaliana* only catalyze one methylation step.

(2) UbiE is involved in the biosynthesis of coenzyme Q, which is essential for cell survival. Our attempts to knockout the *ubiE* gene were unsuccessful. Therefore, we have employed CRISPR interference (CRISPRi) to repress the expression of UbiE for the functional validation.

"Since UbiE is involved in coenzyme Q synthesis which is an essential step for cell survival, attempt to knockout *ubiE* was difficult. We next constructed engineered *R. sphaeroides* with UbiE repression for biocatalysis analysis. The cell lysate of wild-type *R. sphaeroides* could convert δ/β/γ-tocopherols to α-tocopherol, whereas its repression reduces α-tocopherol formation (Figure 2C). Therefore, we confirmed that UbiE from *R. sphaeroides* has the uncharacterized promiscuous γ/β-TMT activities involving in α-tocopherol biosynthesis, albeit the possibility of other MTs besides UbiE could not be completely ruled out as UbiE repression still produced a small amount of β/γ-tocopherols." (Line 169-177)

(3) We employed the site-directed mutagenesis of the conserved residues in *Rhodobacter* UbiE to further elucidate the catalytic mechanism.

"Furthermore, multiple sequence alignment of RspUbiE with other homologs (Supplementary Fig. S6) revealed that RspUbiE shares the conserved catalytic amino acid residues (Y36, R143, N144). The molecular docking results revealed suitable conformational proximity of the β-tocopherol substrate and SAM to the conserved active sites (Fig. 3A). Based on the common methyltransferase mechanism in UbiE homologs [34], we proposed using β-tocopherol as an example: R143 or Y36 functions as a general base to abstract a proton from a water molecule, forming a negatively charged oxygen atom; this oxygen atom subsequently interacts with the hydrogen atom at the C22 position of β-tocopherol, inducing the deprotonation of C22 before methyl transfer; α-tocopherol is then produced following the methyl transfer reaction, while N144 could stabilize the reaction intermediate (Fig. 3B). Further mutation experiments (Y36A, N144A and R143A in RspUbiE) revealed that R143A and N144A completely abolished the methyltransferase activity on β-tocopherol substrate, whereas Y36A retained partial activity (Fig. 3C). These findings indicate that R143 serves as the essential general base for substrate activation, N144 plays an indispensable role in stabilizing the key reaction intermediate, and Y36, while not absolutely required, contributes a supporting role in substrate orientation or transition state stabilization. Therefore, biochemical verification results confirmed that UbiE from *R. sphaeroides* represents the first-time reported β-TMT as depicted in Fig. 1." (Line 191-209)

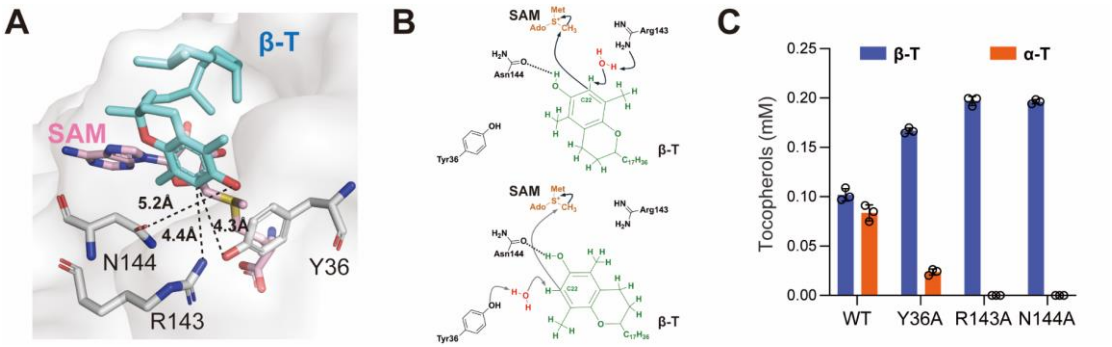

Fig. R1 Mechanism analysis of UbiE through molecular docking, mutation study and molecular dynamics (MD) simulations. A, Predicted binding pose of β-tocopherol (β-T) in the RspUbiE active site. Key residues Y36, R143 and N144 are shown as sticks, with distances (in Å)

to the substrate indicated by dashed lines. **B,** Hypothesized methyltransferase mechanism using β-tocopherol as a model based on the conserved UbiE-family mechanism and the docking pose shown in panel **A**. **C,** Mutation analysis to confirm the key amino acid residues involved in the β-TMT activity. UbiE with Y36A, N144A and R143A was tested for converting β-tocopherol to α-tocopherol at 24 h. WT, wild-type UbiE. All experiments were carried out in triplicates and data represent the average ± SD from three biological repeats.

(4) As the β-TMT activity represents the first-time reported during tocopherol biosynthesis, we next attempted to carry out biochemical verification by monitoring β -tocopherol substrate depletion rate. Further time course analysis using β-tocopherol feeding revealed that UbiE from *R. sphaeroides* gradually converted β-tocopherol to the desired α-tocopherol (Figure 2E), and approximately 65% β-tocopherol was converted to α-tocopherol in 48 h.

2. As highlighted by reviewers #2 and #3, please investigate the contribution of geranylgeranyl reductase to heterologous tocopherol production and explore its generality in other hosts.

Response: Since tocotrienol biosynthesis was not detected in the engineered strain, we hypothesized that geranylgeranyl pyrophosphate reductase (GR) not only converts GGPP to PDP but also catalyzes the conversion of tocotrienols to tocopherols. Accordingly, we have examined the effect of GR encoded by *bchP* on tocotrienol conversion in *R. sphaeroides* and further verified its catalytic activity in *E. coli*. The main results are as follows:

"Based on the LC-MS results, we found that all forms of δ/β/γ/α-tocotrienols were not detected, indicating that tocotrienols might be converted to tocopherols by endogenous *R. sphaeroides* metabolism. As shown in Fig. 4A, we found that the wild-type *R. sphaeroides* transformed δ-tocotrienol to different forms of tocopherols, suggesting that its endogenous metabolism is capable of tocotrienol reduction. Since GR was reported to not only catalyze GGPP to PDP, but also reduce GGPP-derived later intermediates [27,35], we suspected that BchP (the GR from *R. sphaeroides* involved in bacteriochlorophyll synthesis) [27] might participate in converting tocotrienols to tocopherols (Fig. 4B)." (Line 229-237)

"To provide biochemical evidence of BchP's engagement in tocotrienol reduction, we further attempted to heterologously validate the BchP from *R. sphaeroide* in *E. coli*. It was found that the whole cell lysate of *E. coli* expressing *bchP* alone could not produce δ-tocopherol from δ-tocotrienol feeding (Fig. 4C). Additional expression of its coenzymes such as FdxA (ferredoxin II) and Fdr (NADPH-ferredoxin reductase) substantially enhanced the BchP-mediated reduction of δ-tocotrienol to δ-tocopherol (Fig. 4C). These findings suggested the failure of previous efforts towards functional expression of GR homologs might be attributed to the lack of compatible coenzymes in the heterologous hosts, whereas additional supplementation of their native FdxA and Fdr is expected to address this issue [35]. Our results on successful validation of BchP-mediated tocotrienol reduction shed light on future biomanufacturing of tocopherols in other microorganisms such as *E. coli* and yeast." (Line 238-249)

3. As requested by reviewer #3, please clearly distinguish between the production of tocotrienols and tocopherols in the engineered strains.

Response: The LC-MS method described in the original manuscript allows for the separation and identification of tocopherols versus tocotrienols. Moreover, upon extracting the molecular ion peaks of target compounds from the fermentation samples, no characteristic ions corresponding to δ/β/γ/α-tocotrienols were detected in the mass spectra.

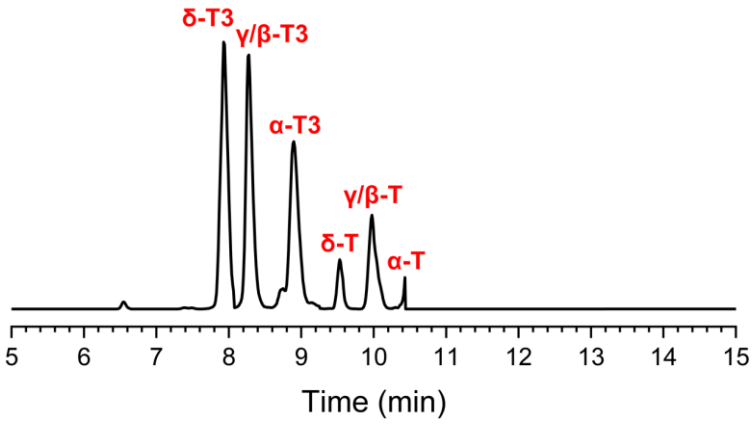

Fig. R2 Representative LC-MS chromatogram of different forms of tocopherols and tocotrienols. δ/β/γ/α-tocotrienols (δ/β/γ/α-T3); δ/β/γ/α-tocopherols (δ/β/γ/α-T).

4. As noted by reviewers #1 and #2, please investigate the tocopherol composition in

the feedstock mixture and provide further evidence to support the beneficial effect of tocopherol-producing bacteria on reproductive fitness of C. elegans.

Response: In *C. elegans*, α-tocopherol is the most extensively studied, with roles primarily in antioxidation, stress protection, and lifespan regulation, and exhibits a dose-dependent effect (Mech Ageing Dev 2007, 128:222-226; Indian J Biochem Biophys 2011, 48:191-196; Int J Food Sci Nutr 2019, 70:172-181)[2-4]. γ-Tocopherol shows stronger potential for lifespan extension and antioxidation than α-tocopherol, with no obvious toxicity at high doses (Mech Ageing Dev 2007, 128:222-226)[2]. Studies focusing individually on β-tocopherol or δ-tocopherol are scarce, and they are usually investigated together with mixed vitamin E preparations (J Gerontol A Biol Sci Med Sci 2000, 55:B280-B285; PLoS One 2013, 8:e71180)[5,6]. We agree with that the tocopherol compositional effect is worth studying for therapeutic applications. However, the *in vivo* evaluation using *Caenorhabditis elegans* is for the purpose of safety evaluation of *Rhodobacter*-based single cell proteins for animal feeds, rather than to investigate the effects of tocopherols on therapeutic applications in *C. elegans*. Considering the space limit after adding additional experiments on characterization of UbiE and BchP, we decided to remove this section to avoid misleading information to the readers.

5. As suggested by reviewer 2, please tone down the conclusion throughout the text to align more precisely with the experimental evidence.

Response: We have revised the statement for more accurate expression.

"In summary, we report a nonclassical α-tocopherol biosynthetic pathway and establish a promising approach to produce natural tocopherols by microbial fermentation. In particular, we identified UbiE as a bifunctional methyltransferase involved in transforming individual δ, γ, and β-tocopherols to α-tocopherol, which is expected to simplify α-tocopherol synthesis via a more concise route (Fig. 1). UbiE could not catalyze the MPBQ substrate, and the cyclization-mediated by TC to give δ-tocopherol is a prerequisite for the subsequent methylations to occur in *Rhodobacter* species. Functional reconstitution of BchP-mediated tocotrienol reduction in the heterologous host of *E. coli* paves the way for future biomanufacturing of tocopherols in other

microorganisms. *De novo* biosynthesis of tocopherols in *R. sphaeroides* is substantially enhanced through systematic metabolic engineering, and one of the engineered strains produced 3.51 g/L tocopherols in a fed-batch bioreactor, which represents the highest titer reported to date. Taken together, our work lays a foundation for the future commercialization of microbial synthesis of tocopherols."

6. Finally, we strongly encourage you to pay special attention to our formatting guidelines, which will be required should the manuscript be accepted for publication at a later stage. As a minimum, please prepare your figures in editable formats, present data as scatter plots, state the number of samples in the figure legends, provide source data for each figure, and provide uncropped scans of all blots and gels. Additionally, please take full advantage of our Extended figures (max. of 10) before making use of Supplementary figures (unlimited). The difference between them is that Extended figures are more easily available to readers, as they appear in the PDF and the HTML versions of the paper, whereas Supplementary figures need to be downloaded separately.

Response:

Response: We have prepared all required materials for this manuscript.

7. When preparing your revised manuscript, please be aware of our guidance on Sex and Gender reporting). Please note that:

1). If the research findings apply to only one sex or gender, that must be indicated in the title and/or abstract.

2a). For studies involving vertebrates animal and cell lines- The Reporting Summary should include whether sex was were considered in the study design.

2b). For studies involving human research participants- The Reporting Summary should include whether sex and/or gender was considered in the study design and whether sex and/or gender of participants was determined based on self-report or assigned (and methodology used).

3). Data should be reported disaggregated for sex and gender where this information has been collected and consent has been obtained for reporting and sharing individual-level data; disaggregated numbers for individual experiments must be provided in the source data as appropriate whereas overall numbers may be provided in the Nature

Portfolio Reporting Summary.

Response: We have removed the contents about the animal experiments using the hermaphrodite *Caenorhabditis elegans*, thus this study does not involve animal/gender. And we have revised this information in the Nature Portfolio Reporting Summary.

Response to Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript reports the heterologous biosynthesis of tocopherols, including alpha-tocopherol, in photosynthetic bacteria Rhodobacter. UbiE was identified as a methyltransferase with bifunctional γ/β-tocopherol methyltransferase activity. Through random chromosomal integration of the tocopherol synthetic module combined with enhanced precursor availability from MEV pathway and shikimate pathway, the authors achieved a tocopherol titer of 295 mg/L. Further process optimization via supplementation of SAM and its precursor, and antioxidants increased the total tocopherol titer to 620 mg/L. Fed-batch fermentation yielded 3.51 g/L of tocopherols. This represents the first report of de novo tocopherol biosynthesis in heterologous bacteria, and the titer achieved is impressive. However, for a manuscript intended for Nature Metabolism, the mechanistic understanding of the underlying metabolism remains insufficient.

Major concerns:

1. As the central finding of this study, the functional properties of UbiE warrant deeper investigation. The authors should provide a rigorous comparative mechanistic analysis between UbiE and other characterized methyltransferases (MTs) in the tocopherol biosynthetic pathway. Specifically, what distinguishes UbiE from MTs that catalyze only one methylation step? Current evidence of bifunctional γ/β-tocopherol methyltransferase activity requires more detailed biochemical characterization.

Response: We sincerely thank you for this valuable suggestion.

(1) We compared the reactions between the well-studied γ-tocopherol methyltransferase from *A. thaliana* and UbiE from *Rhodobacter* by expressing in the heterologous *E. coli*. As shown in Fig. 2D, we found that only *Rhodobacter* UbiE expression could result in α-tocopherol production by δ-tocopherol feeding, whereas γ-TMT from *A. thaliana* could only convert δ-tocopherol to β-tocopherol.

Further molecular docking revealed that γ-TMT from *A. thaliana* could not give favorable docking of β-tocopherol, which explains γ-TMT from *A. thaliana* only

catalyze one methylation step:

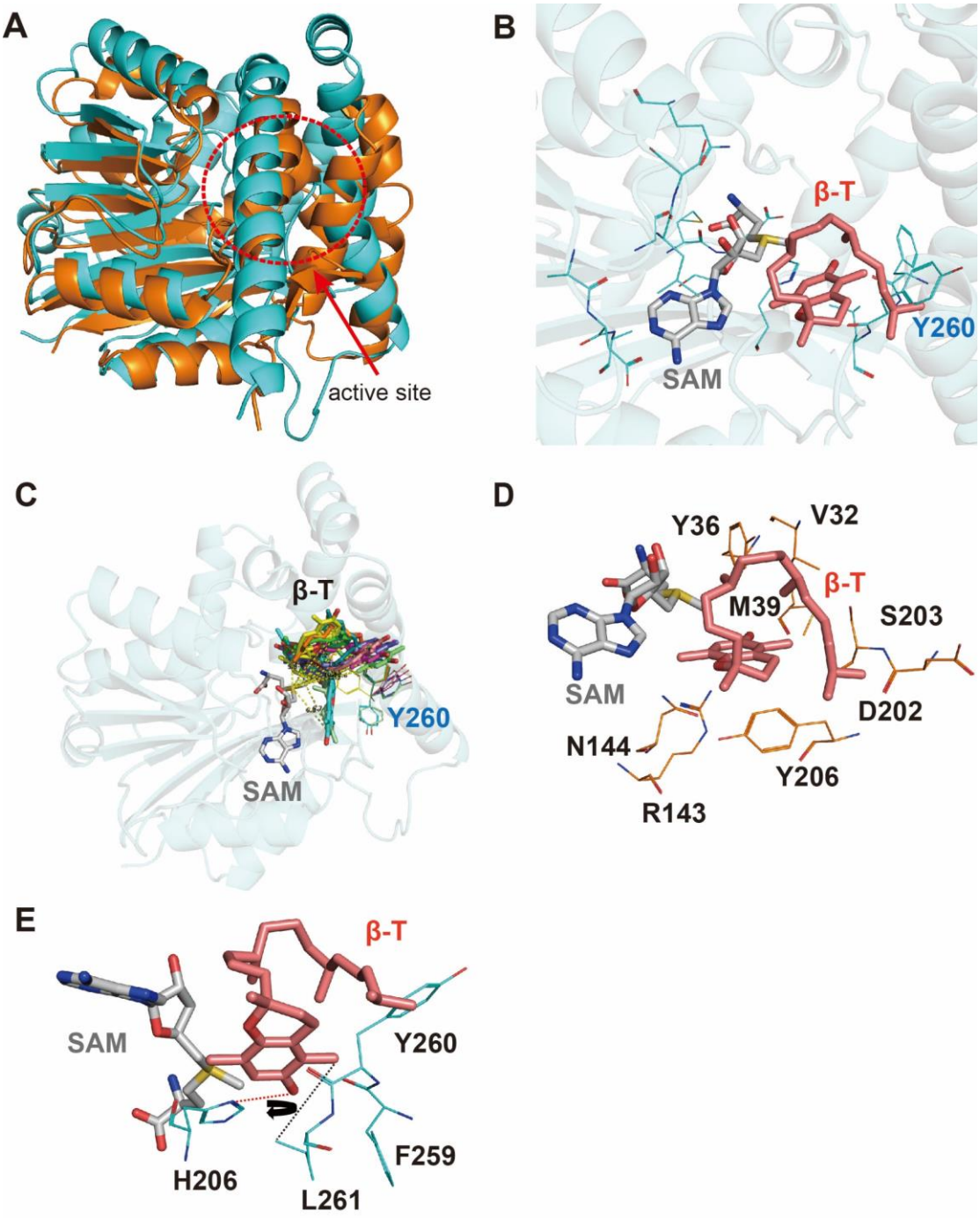

Fig. R3 Mechanistic insight into the inability of *Arabidopsis* γ-TMT to catalyze β-tocopherol. (A) Structural superposition of RspUbiE (teal) and *Arabidopsis* γ-TMT (orange). The two structures exhibit significant divergence with a backbone RMSD > 3 Å, particularly in the active site region (highlighted by a red dashed circle). (B) The docked pose of SAM and β-tocopherol from RspUbiE, when superposed onto the *Arabidopsis* γ-TMT structure, reveals a steric clash between the active site residue Y260 and β-tocopherol, indicating incompatibility of the RspUbiE binding mode in γ-

TMT. (C) Flexible docking of SAM and β-tocopherol into the Arabidopsis γ-TMT active site with residue Y260 set as flexible. Analysis of the top 10 lowest-energy conformations from 1000 docking runs shows a catalytic distance consistently greater than 5 Å, which is insufficient for methyl transfer. (D-E) Local microenvironment of the β-tocopherol binding site in (D) RspUbiE and (E) *Arabidopsis* γ-TMT. Putative hydrogen bonds and hydrophobic interactions are indicated by red and black dashed lines, respectively. The microenvironment differs significantly: the RspUbiE site is predominantly lined with polar residues, conferring binding flexibility conducive to catalysis. In contrast, in *Arabidopsis* γ-TMT, specific interactions (a hydrogen bond with H206 and hydrophobic contact with L261) rigidly fix the substrate, resulting in an unfavorable catalytic distance.

Structure superposition: The productive docking pose of SAM and β-tocopherol in RspUbiE was superposed onto the structure of mono-functional γ-TMT. This revealed severe steric clashes in the γ-TMT active sites, preventing productive binding of the intermediate (β-tocopherol) for a second methylation (Fig. R3A and B).

Flexible docking: When key γ-TMT active-site residues were allowed flexibility, the resulting optimal poses for SAM and β-tocopherol maintained a catalytic distance (>5 Å) too long for efficient methyl transfer (Fig. R3C), explaining the lack of a second catalytic step.

Active site microenvironment: Comparative analysis of the substrate-binding pockets revealed a fundamental architectural difference. The RspUbiE pocket is lined with polar residues, creating a flexible microenvironment that permits substrate repositioning between catalytic steps (Fig. R3D). In contrast, the γ-TMT pocket utilizes a rigid combination of specific hydrogen-bonding and hydrophobic interactions that "lock" the substrate (Fig. R3E), making it incompatible with the conformational changes required for the second methylation.

(2) We employed the site-directed mutagenesis of the conserved residues in *Rhodobacter* UbiE to further elucidate the catalytic mechanism. The results have added in the main text as follows:

"Furthermore, multiple sequence alignment of RspUbiE with other homologs (Supplementary Fig. S6) revealed that RspUbiE shares the conserved catalytic amino acid residues (Y36, R143, N144). The molecular docking results revealed suitable

conformational proximity of the β-tocopherol substrate and SAM to the conserved active sites (Fig. 3A). Based on the common methyltransferase mechanism in UbiE homologs [34], we proposed using β-tocopherol as an example: R143 or Y36 functions as a general base to abstract a proton from a water molecule, forming a negatively charged oxygen atom; this oxygen atom subsequently interacts with the hydrogen atom at the C22 position of β-tocopherol, inducing the deprotonation of C22 before methyl transfer; α-tocopherol is then produced following the methyl transfer reaction, while N144 could stabilize the reaction intermediate (Fig. 3B). Further mutation experiments (Y36A, N144A and R143A in RspUbiE) revealed that R143A and N144A completely abolished the methyltransferase activity on β-tocopherol substrate, whereas Y36A retained partial activity (Fig. 3C). These findings indicate that R143 serves as the essential general base for substrate activation, N144 plays an indispensable role in stabilizing the key reaction intermediate, and Y36, while not absolutely required, contributes a supporting role in substrate orientation or transition state stabilization. Therefore, biochemical verification results confirmed that UbiE from *R. sphaeroides* represents the first-time reported β-TMT as depicted in Fig. 1." (Line 191-209)

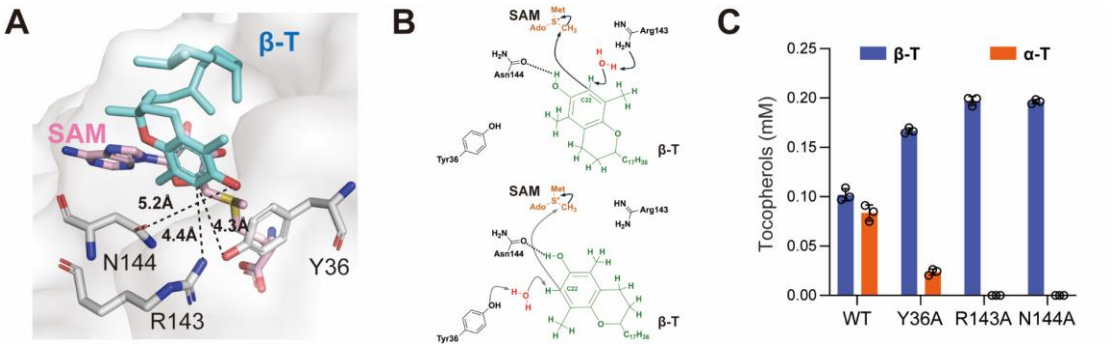

Fig. R4 Mechanism analysis of UbiE through molecular docking, mutation study and molecular dynamics (MD) simulations. A, Predicted binding pose of β-tocopherol (β-T) in the RspUbiE active site. Key residues Y36, R143 and N144 are shown as sticks, with distances (Å) to the substrate indicated by dashed lines. **B,** Hypothesized methyltransferase mechanism using β-tocopherol as a model based on the conserved UbiE-family mechanism and the docking pose shown in panel **A**. **C,** Mutation analysis to confirm the key amino acid residues involved in the β-TMT activity. UbiE with Y36A, N144A and R143A was tested for converting β-tocopherol to α-tocopherol at 24 h. WT, wild-type UbiE. All experiments were carried out in triplicates and data

represent the average ± SD from three biological repeats.

(3) We sought to purify *Rhodobacter* UbiE from *E. coli* for kinetics characterization. However, repeated attempts to use his-tag for purifying UbiE could not give high-purity UbiE (Figure below). The poor purity of UbiE prevented us kinetics characterization. As the β-TMT activity represents the first-time reported during tocopherol biosynthesis, we next attempted to carry out biochemical verification by monitoring β-tocopherol substrate depletion rate. Further time course analysis using β-tocopherol feeding revealed that UbiE from *R. sphaeroides* gradually converted β-tocopherol to the desired α-tocopherol (Figure 2E), and approximately 65% β-tocopherol was converted to α-tocopherol in 48 h. At this moment, we are confident that the novel β-TMT activity of UbiE is confirmed based on CRISPRi experiments and heterologous expression in *E. coli* via time course experiments. Meanwhile, we are still attempting to use different tagging systems and cell-free expression system to have good quality of purified UbiE, and the kinetics data will be reported in due course.

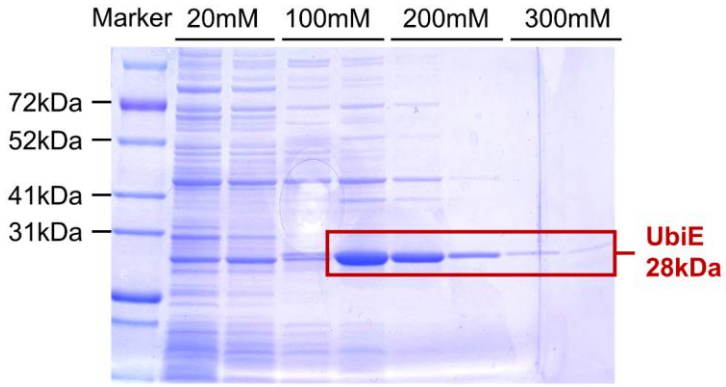

Fig. R5 SDS-PAGE gel of purified UbiE protein

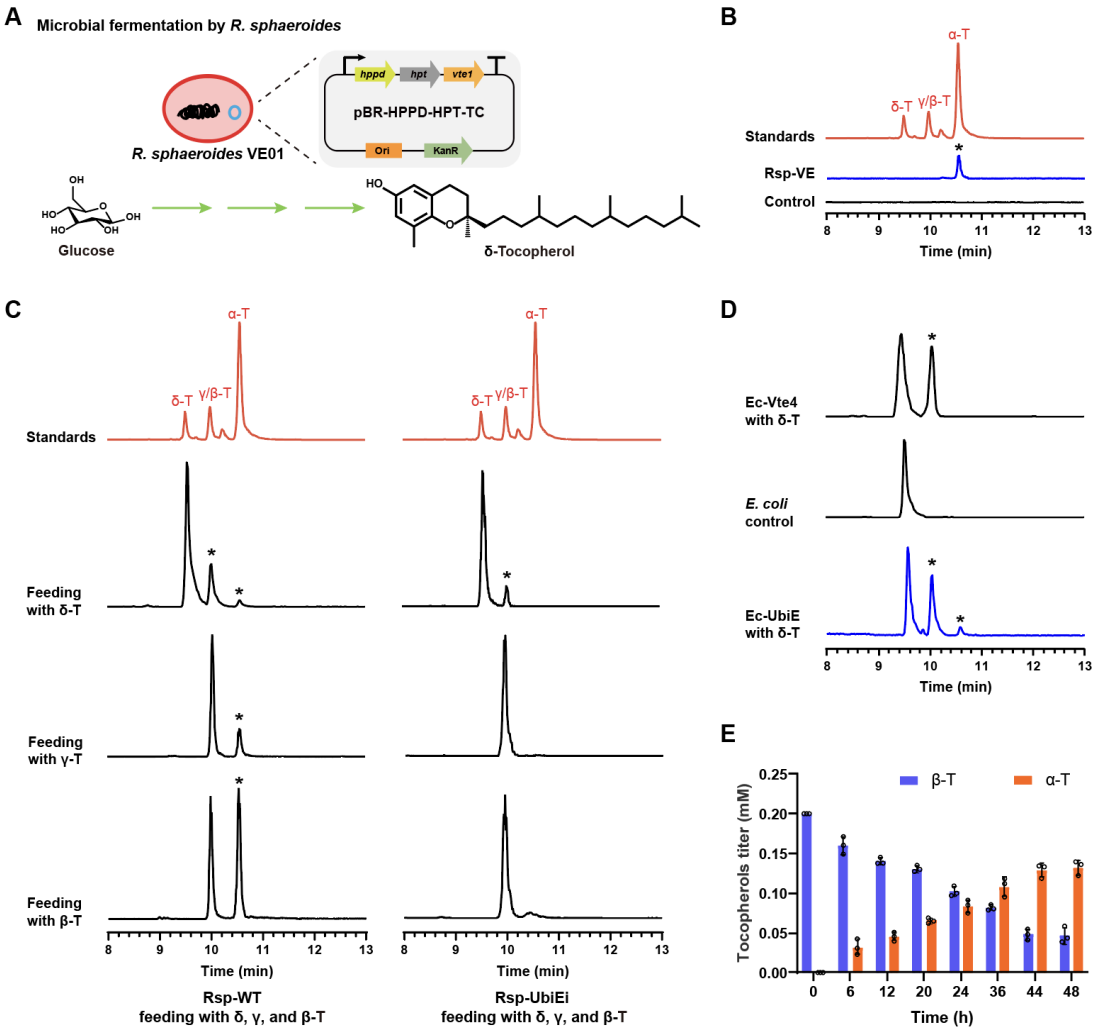

Fig. R6 Discovery of a nonclassical α-tocopherol biosynthetic pathway in *R. sphaeroides*. C, Whole cell lysate of *R. sphaeroides* results in synthesizing α-T from δ/γ/β-T. Rsp-WT, *R. sphaeroides* wild-type strain. Rsp-UbiEi indicates *R. sphaeroides* strain transformed with pBR-UbiEi for the repression of *ubiE* expression. **D,** Representative LC-MS chromatograms of heterologous characterization of RspUbiE in *E. coli*. Plasmid pRSF-Vte4 and pRSF-UbiE were transformed into *E. coli* BL21 (DE3) for preparing the whole cell lysate. Extracted ion chromatograms were acquired within the m/z range of 396-435. All experiments were carried out in three biological repeats and only representative chromatograms are presented. Asterisk (*) indicates the expected tocopherol products. **E,** Time course results of β-TMT activity of RspUbiE on converting β-tocopherol to α-tocopherol. Experiments were carried out using the cell lysate as the biocatalyst. Data represent the average ± SD from three biological repeats.

2. Reliance on computational simulations and predicted kinetic parameters is insufficient to support the manuscript's mechanistic claims. Based on our experience,

such in silico predictions frequently lack accuracy. We recommend that the authors conduct the following: (1) protein crystallography or cryo-electron microscopy to elucidate the three-dimensional structure of UbiE; (2) determination of kinetic parameters using purified recombinant enzyme; and (3) substrate specificity assays to validate the proposed bifunctional activity.

Response: We sincerely thank you for this suggestion.

(1) We agree with the view that resolving the three-dimensional structure of UbiE by protein crystallography or cryo-electron microscopy will facilitate a deeper elucidation of its catalytic mechanism. At the current stage, the discovery of a nonclassical biosynthetic route for tocopherol synthesis in *Rhodobacter* species that bypasses the classical α-tocopherol pathway requiring the 2,3-dimethyl-5-phytyl-l,4-benzoquinone (DMPBQ) as an intermediate already represents a big breakthrough as it is the first time to report β-TMT activity for tocopherol biosynthesis. Furthermore, we discovered that geranylgeranyl pyrophosphate reductase (GR) plays an important role in reducing tocotrienol into tocopherol. Protein crystallography or cryo-electron microscopy techniques are beyond our capability at this moment; we will seek collaborations to study the protein structure and employ mutagenesis approach to identify enzymes with improved biocatalytic activity and specificity in the future. To promptly share these new findings, we did not carry out protein crystallography or cryo-electron microscopy at this moment.

We have added related-content in the main text "Notably, elucidating the exact mechanism of enzyme selectivity towards different substrates will require efforts to determine high-resolution co-crystal structures, coupled with systematic site-directed mutagenesis and functional characterization.", which is expected to attract future research direction in this field.

(2) The UbiE purify and the related biochemical results are seen in the above question 1. We sought to purify *Rhodobacter* UbiE from *E. coli* for kinetics characterization. However, repeated attempts to use his-tag for purifying UbiE could not give high-purity UbiE. The poor purity of UbiE prevented us kinetics characterization.

3. To eliminate the possibility that other methyltransferases in the host chromosome

contribute to α-tocopherol synthesis, targeted knockout of UbiE should be performed. This genetic confirmation is essential to establish UbiE as the sole enzyme responsible for the observed tocopherol production phenotype.

Response: We appreciate this suggestion. However, UbiE is involved in the biosynthesis of coenzyme Q, which is essential for cell survival. Our attempts to knockout the *ubiE* gene were unsuccessful. Therefore, we have employed CRISPR interference (CRISPRi) to repress the expression of UbiE for the functional validation.

"Since UbiE is involved in coenzyme Q synthesis which is an essential step for cell survival, attempt to knockout *ubiE* was difficult. We next constructed engineered *R. sphaeroides* with UbiE repression for biocatalysis analysis. The cell lysate of wild-type *R. sphaeroides* could convert δ/β/γ-tocopherols to α-tocopherol, whereas its repression reduces α-tocopherol formation (Figure 2C). Therefore, we confirmed that UbiE from *R. sphaeroides* has the uncharacterized promiscuous γ/β-TMT activities involving in α-tocopherol biosynthesis, albeit the possibility of other MTs besides UbiE could not be completely ruled out as UbiE repression still produced a small amount of β/γ-tocopherols." (Line 169-177)

4. There is debate on the major source of PDP in photosynthetic organisms, whether primarily derived from chlorophyll degradation or from GGPP reduction. This distinction is critical, as heterologous overexpression of geranylgeranyl reductase (GR) in non-photosynthetic hosts has consistently failed in previous studies. Therefore, the authors must address whether GR overexpression also enhances tocopherol production—independent of and in parallel with GGPP supply enhancement. This experiment would clarify the contribution of the GR-mediated pathway.

Response: We appreciate this insightful comment, and we agree with the GR is important for tocopherols biosynthesis. Based on our results, tocotrienols were not in a detectable amount during LC-MS analysis in the engineered strain. We think that GR (encoded by *bchP*) might also catalyze the conversion of tocotrienols to tocopherols. As shown in Figure 4A, when δ-tocotrienol was fed to the whole cell lysate of *R. sphaeroides*, we did observe the production of different forms of tocopherols. We also examined the heterologous expression of *R. sphaeroides bchP* in *E. coli*. As shown in

Figure 4C, we found *bchP* overexpression alone could not convert δ-tocotrienol to δ-tocopherol. However, when the partner enzymes of FdxA and Fdr were co-expressed, we observed the formation of δ-tocopherol. These findings are of particular interest as it shed light on future tocopherol production in heterologous hosts like *E. coli* and yeast. We did not further test the effect of *bchP* overexpression on tocopherol production as it seems obvious to further improve the product titer.

We have revised the main text as follows:

"Based on the LC-MS results, we found that all forms of δ/β/γ/α-tocotrienols were not detected, indicating that tocotrienols might be converted to tocopherols by endogenous *R. sphaeroides* metabolism. As shown in Fig. 4A, we found that the wild-type *R. sphaeroides* transformed δ-tocotrienol to different forms of tocopherols, suggesting that its endogenous metabolism is capable of tocotrienol reduction. Since GR was reported to not only catalyze GGPP to PDP, but also reduce GGPP-derived later intermediates [27,35], we suspected that BchP (the GR from *R. sphaeroides* involved in bacteriochlorophyll synthesis) [27] might participate in converting tocotrienols to tocopherols (Fig. 4B)." (Line 229-237)

"To provide biochemical evidence of BchP's engagement in tocotrienol reduction, we further attempted to heterologously validate the BchP from *R. sphaeroide* in *E. coli*. It was found that the whole cell lysate of *E. coli* expressing *bchP* alone could not produce δ-tocopherol from δ-tocotrienol feeding (Fig. 4C). Additional expression of its coenzymes such as FdxA (ferredoxin II) and Fdr (NADPH-ferredoxin reductase) substantially enhanced the BchP-mediated reduction of δ-tocotrienol to δ-tocopherol (Fig. 4C). These findings suggested the failure of previous efforts towards functional expression of GR homologs might be attributed to the lack of compatible coenzymes in the heterologous hosts, whereas additional supplementation of their native FdxA and Fdr is expected to address this issue [35]. Our results on successful validation of BchP-mediated tocotrienol reduction shed light on future biomanufacturing of tocopherols in other microorganisms such as *E. coli* and yeast." (Line 238-249)

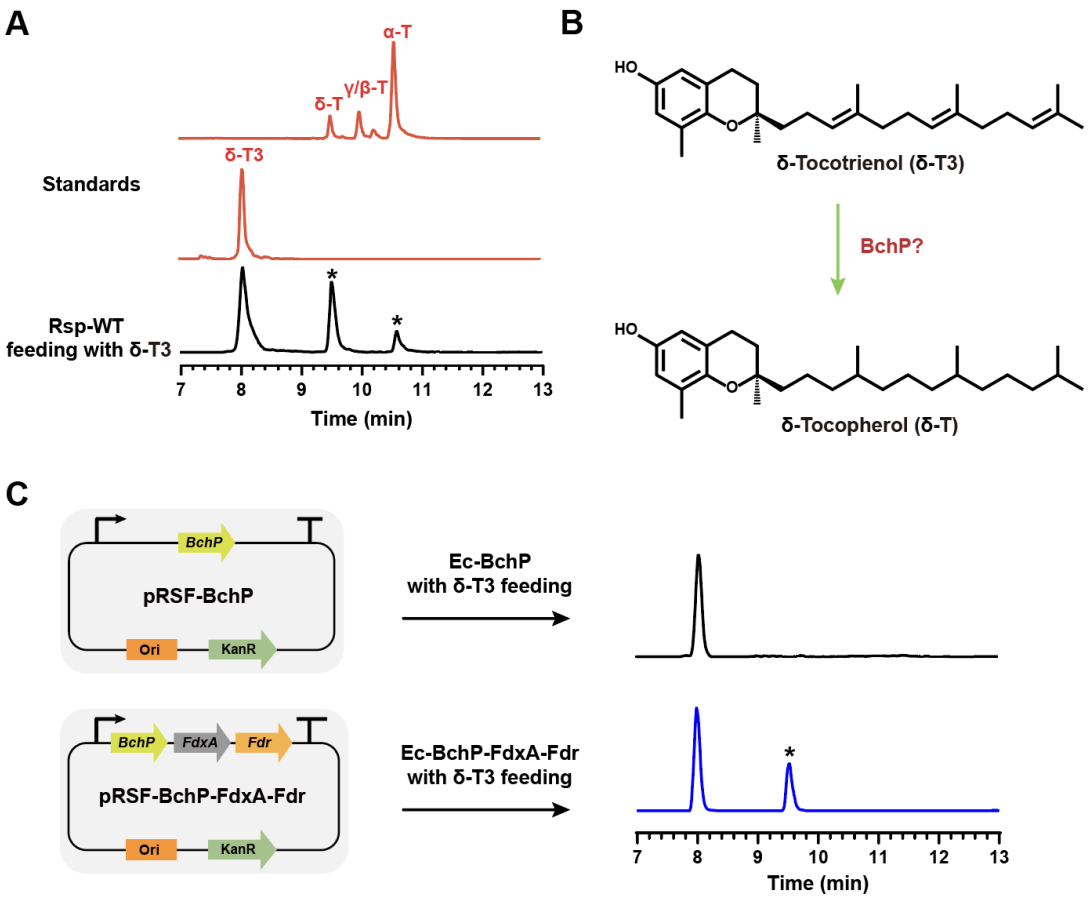

Fig. R7 Identification of BchP-mediated tocotrienol reduction. A, The whole cell lysate of *R. sphaeroides* for the synthesis of tocopherols from δ-tocotrienol. δ-T3, δ-tocotrienol. Representative LC-MS chromatograms are presented from triplicate of biological repeats. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected tocopherol products. **B,** Proposed mechanism of BchP-mediated tocotrienol reduction. **C,** Representative LC-MS results of δ-tocotrienol as substrate for δ-tocopherol production by the biotransformation of *E. coli* expressing BchP and BchP-FdxA-Fdr. Experiments were carried out using the cell lysate as the biocatalyst with 0.1 mM δ-T3 for 20 h. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected δ-tocopherol product.

5. The substantially improved tocopherol titer resulting from random chromosomal integration of the synthetic biosynthetic module suggests significant position-dependent effects. The authors should investigate the genomic context of integration sites and identify the underlying factors—such as proximity to transcriptional hotspots, effects on local chromatin structure, or influence from nearby genomic elements—that

enhance production.

Response: Thanks for your constructive suggestions. Tn5-mediated genome integration is a random process based on the recent findings (ACS Synth Biol 2025,14:2753-2763) [1], and also well documented by many previous studies. This study mainly focused on the discovery of a nonclassical biosynthetic route for tocopherol synthesis in *Rhodobacter* species that bypasses the classical α-tocopherol pathway requiring the 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ). We did not carry out further genome sequencing of various mutants to correlate the genotype with tocopherol productivity, which might be reported in a sperate study by employing multi-omics data.

6. The in vivo evaluation using Caenorhabditis elegans is informative; however, the current analysis is incomplete. While the authors emphasize the importance of feeding ratios between tocopherol-producing and wild-type bacteria, they overlook a critical variable: the impact of tocopherol composition. Since α-tocopherol is widely recognized as the most biologically active form, experiments specifically comparing batches with higher α-tocopherol content to those with lower α-tocopherol proportions would be expected to demonstrate enhanced bioactivity. This compositional effect warrants explicit investigation.

Response: Thank you for this suggestion. In *C. elegans*, α-tocopherol is the most extensively studied, with roles primarily in antioxidation, stress protection, and lifespan regulation, and exhibits a dose-dependent effect (Mech Ageing Dev 2007, 128:222-226; Indian J Biochem Biophys 2011, 48:191-196; Int J Food Sci Nutr 2019, 70:172-181)[2-4]. γ-Tocopherol shows stronger potential for lifespan extension and antioxidation than α-tocopherol, with no obvious toxicity at high doses (Mech Ageing Dev 2007, 128:222-226)[2]. Studies focusing individually on β-tocopherol or δ-tocopherol are scarce, and they are usually investigated together with mixed vitamin E preparations (J Gerontol A Biol Sci Med Sci 2000, 55:B280-B285; PLoS One 2013, 8:e71180)[5,6]. We agree with that the tocopherol compositional effect is worth studying for therapeutic applications. However, the *in vivo* evaluation using *Caenorhabditis elegans* is for the purpose of safety evaluation of *Rhodobacter*-based single cell proteins for animal feeds, rather than to investigate the effects of tocopherols on therapeutic applications in *C. elegans*.

Considering the space limit after adding additional experiments on characterization of UbiE and BchP, we decided to remove this section to avoid misleading information to the readers.

Minor issue:

1. The claim that "a new methyltransferase … is identified" is not strictly accurate. UbiE itself is not a novel methyltransferase; rather, a previously uncharacterized bifunctional activity of this enzyme has been newly demonstrated. The authors should revise this statement to reflect that the novel finding concerns the γ/β-tocopherol methyltransferase activity of UbiE, not the identification of an entirely new enzyme.

Response: Revised as recommended. We have revised the statement for more accurate expression.

Reviewer #2 (Remarks to the Author):

The manuscript by Zhang et al. describes the characterization and engineering of Rhodobacter sphaeroides as a production platform for tocopherols. The authors demonstrate the ability of native ubiquinone/menaquinone methyltransferase UbiE to convert heterologous δ-tocopherol into α-tocopherol via γ/β-tocopherols, thereby simplifying tocopherol biosynthesis in R. sphaeroides by bypassing the 2,3-dimethyl-5-phytyl-l-4-benzoquinone step of the classical pathway. Based on molecular docking and -dynamics simulations, they propose catalytically competent binding conformations which rationalize the identified enzyme activities.

In addition to reconstructing tocopherol biosynthesis in R. sphaeroides, the authors employ established engineering strategies to improve the supply of the central precursors, geranylgeranyl diphosphate and homogentisic acid. Further media optimization efforts showcase the benefit of supplementing methyl donors and antioxidants in the fermentation process, ultimately enabling gram-scale tocopherol production in a fed-batch bioreactor setup. Finally, the authors explore the dietary effects of the tocopherol-producing bacteria on the fitness of C. elegans. While the results largely suggested the equivalence of engineered R. sphaeroides to both the wild

type and commonly-fed E. coli, a slight benefit to reproductive fitness was observed at moderate tocopherol levels.

1. The experiments were well-executed, and the data were well-presented and convincing. The study not only establishes R. sphaeroides as a platform for microbial tocopherol production, but also makes UbiE available as a potential tool for synthetic biology. In this regard, an immediate question arising from this work is whether the geranylgeranyl diphosphate reductase from R. sphaeroides could facilitate the heterologous production of phytyl diphosphate in more common hosts. As the authors state in lines 103-105, insufficient PDP amounts are thought to be a major bottleneck in other systems — and the main reason the search for suitable hosts was expanded here. It would be very valuable if the authors could look into the possibility of identifying GGR homologs in the transcriptome at hand. At least though, I suggest that the authors revisit this GGR topic in the discussion.

Response: We appreciate this insightful suggestion. The geranylgeranyl pyrophosphate reductase encoded by *bchP* has been identified in *R. sphaeroides* (J Bacteriol 1999, 181:7248-7255)[7], and the relevant information was supplemented in the revised manuscript.

Since tocotrienol biosynthesis was not detected in the engineered strain, we hypothesized that GR not only converts GGPP to PDP but also catalyzes the conversion of tocotrienols to tocopherols. Accordingly, we have examined the effect of *bchP* on tocotrienol conversion in *R. sphaeroides* and further verified its catalytic activity in *Escherichia coli*. The main results are as follows:

"Based on the LC-MS results, we found that all forms of δ/β/γ/α-tocotrienols were not detected, indicating that tocotrienols might be converted to tocopherols by endogenous *R. sphaeroides* metabolism. As shown in Fig. 4A, we found that the wild-type *R. sphaeroides* transformed δ-tocotrienol to different forms of tocopherols, suggesting that its endogenous metabolism is capable of tocotrienol reduction. Since GR was reported to not only catalyze GGPP to PDP, but also reduce GGPP-derived later intermediates [27,35], we suspected that BchP (the GR from *R. sphaeroides* involved in bacteriochlorophyll synthesis) [27] might participate in converting tocotrienols to

tocopherols (Fig. 4B).” (Line 229-237)

“To provide biochemical evidence of BchP’s engagement in tocotrienol reduction, we further attempted to heterologously validate the BchP from *R. sphaeroide* in *E. coli*. It was found that the whole cell lysate of *E. coli* expressing *bchP* alone could not produce δ-tocopherol from δ-tocotrienol feeding (Fig. 4C). Additional expression of its coenzymes such as FdxA (ferredoxin II) and Fdr (NADPH-ferredoxin reductase) substantially enhanced the BchP-mediated reduction of δ-tocotrienol to δ-tocopherol (Fig. 4C). These findings suggested the failure of previous efforts towards functional expression of GR homologs might be attributed to the lack of compatible coenzymes in the heterologous hosts, whereas additional supplementation of their native FdxA and Fdr is expected to address this issue [35]. Our results on successful validation of BchP-mediated tocotrienol reduction shed light on future biomanufacturing of tocopherols in other microorganisms such as *E. coli* and yeast.” (Line 238-249)

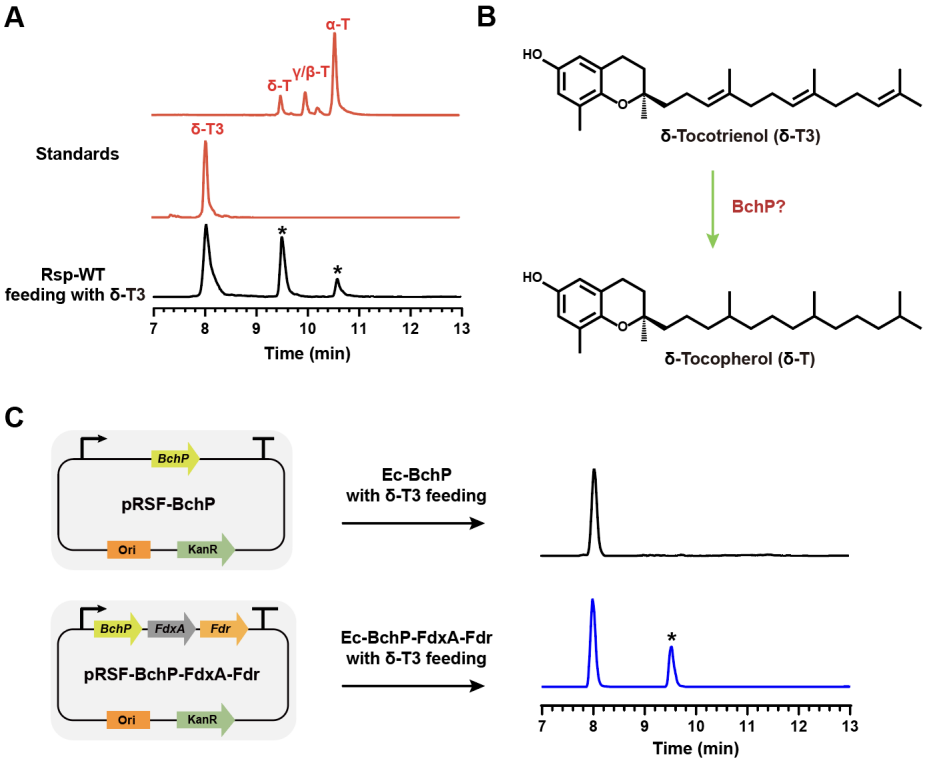

Fig. R8 Identification of BchP-mediated tocotrienol reduction. A, The whole cell lysate of *R. sphaeroides* for the synthesis of tocopherols from δ-tocotrienol. δ-T3, δ-tocotrienol. Representative LC-MS chromatograms are presented from triplicate of biological repeats. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected

tocopherol products. **B,** Proposed mechanism of BchP-mediated tocotrienol reduction. **C,** Representative LC-MS results of δ-tocotrienol as substrate for δ-tocopherol production by the biotransformation of *E. coli* expressing BchP and BchP-FdxA-Fdr. Experiments were carried out using the cell lysate as the biocatalyst with 0.1 mM δ-T3 for 20 h. LC chromatograms were extracted at the ranges of 396-435 m/z. Asterisk (*) indicates the expected δ-tocopherol product.

2. Another concern is that, especially throughout the conclusion and the abstract, some of the claims made should better reflect the experimental outcomes. For example, the phrasing of "novel biosynthetic pathway" and "new bifunctional methyltransferase" is overstated considering the final product of α-tophecorol exists, the enzymes are known, but the primary discovery is UbiE's novel activity. Similarly, comparison of feedstocks between the wildtype and modified R. sphaeroides for C. elegans suggests little difference to the benefit of the growth of the organism outside of the lengthening of the reproductive periods (though with less viable eggs) and may detract from the outcome. The observation that the feedstock mixture conferred an increase to the reproductive fitness of C. elegans is interesting but requires more testing to show the significance of this preliminary experiment. If you were to add α-tophecorol directly to the wildtype feedstock would it have a similar effect on the C. elegans as the biosynthesized α-tophecorol?

Response: Thanks for your constructive suggestions.

(1) We have revised the statement as recommended.

(2) According to the literature, α-tocopherol is the most extensively studied, with roles primarily in antioxidation, stress protection, and lifespan regulation, and exhibits a dose-dependent effect (Mech Ageing Dev 2007, 128:222-226; Indian J Biochem Biophys 2011, 48:191-196; Int J Food Sci Nutr 2019, 70:172-181)[2-4]. γ-Tocopherol shows stronger potential for lifespan extension and antioxidation than α-tocopherol, with no obvious toxicity at high doses (Mech Ageing Dev 2007, 128:222-226)[2]. Studies focusing individually on β-tocopherol or δ-tocopherol are scarce, and they are usually investigated together with mixed vitamin E preparations (J Gerontol A Biol Sci Med Sci 2000, 55:B280-B285; PLoS One 2013, 8:e71180)[5,6]. The nematode feeding experiment was designed to evaluate the safety of *Rhodobacter*-based single cell

proteins for animal feeds, rather than to investigate the effects of tocopherols on therapeutic applications in *C. elegans*. Considering the manuscript length is exceeding the space limit after adding additional experiments on characterization of UbiE and BchP, we decided to remove this section to avoid misleading information to the readers.

Minor Issues:

Phrasing around anti-cardiac benefits (line 39) suggests negative effects towards the cardiovascular system.

Response: We have revised the description as "cardiovascular prevention".

Inconsistent writing style for casing and italicization of gene names (for example line 166 and line 270) can be remedied.

Response: Revised as recommended. We have unified the writing style.

The sentence in line 163 "... indicating that the nonclassical biosynthetic route might be widespread in other bacteria" seems confusing given that these bacteria do not produce tocopherols. What was found to be widespread is the capability of ubiE to convert tocopherol substrates.

Response: In the revised manuscript, this sentence has been removed.

Correcting the vocabulary around line 306, "To access the the impact of SCPs…" can better inform the reader on the aim of the experiment.

Response: This section was removed to avoid misleading information to the readers as mentioned above.

Reviewer #3 (Remarks to the Author):

Zhang et al. report a nonclassical biosynthetic route for tocopherol production in Rhodobacter species and describe the identification of a putative bifunctional γ/β-tocopherol methyltransferase that may enable an alternative α-tocopherol synthesis route bypassing the classical DMPBQ intermediate. This topic is interesting and potentially relevant for microbial production of high-value tocopherols. The authors further combine enzyme characterization, metabolic engineering, and fermentation optimization to improve product titers. Overall, the study presents a potentially useful

direction for tocopherol biosynthesis research. However, some key experimental data and supporting validations are currently insufficient, and additional evidence is required to further strengthen the main conclusions. The following concerns should be addressed before publication.

Major Comment:

1. The authors state that vitamin E is classified into eight compounds, namely α, β, γ, δ-tocopherols and tocotrienols. According to established biosynthetic pathways, homogentisic acid (HGA) can condense not only with phytyl diphosphate (PDP) but also with geranylgeranyl diphosphate (GGPP) via HPT to form intermediates leading to tocotrienols. However, throughout the manuscript, only tocopherols are discussed, and no data are provided regarding the possible formation or exclusion of tocotrienols. It is therefore important to clarify whether tocotrienols are produced in the engineered strains. The authors should provide corresponding analytical evidence and explicitly distinguish tocopherols from tocotrienols. In particular, please clarify whether the chromatographic and mass spectrometric profiles of tocopherols and tocotrienols were resolved and how compound identities were assigned. In addition, chromatograms and mass spectra of authentic standards for the reported products should be provided and directly compared with the fermentation samples to support compound identification.

Response: We sincerely thank you for this suggestion.

(1) The LC-MS method described in the original manuscript allows for the separation and identification of tocopherols versus tocotrienols (LC chromatogram of authentic standards is shown in the figure below). Moreover, upon extracting the molecular ion peaks of target compounds from the fermentation samples, no characteristic ions corresponding to δ/β/γ/α-tocotrienols were detected in the mass spectra. We have supplemented relevant content regarding the absence of tocotrienol biosynthesis, as follows:

"Based on the LC-MS results, we found that all forms of δ/β/γ/α-tocotrienols were not detected, indicating that tocotrienols might be converted to tocopherols by endogenous *R. sphaeroides* metabolism. As shown in Fig. 4A, we found that the wild-type *R. sphaeroides* transformed δ-tocotrienol to different forms of tocopherols,

suggesting that its endogenous metabolism is capable of tocotrienol reduction. Since GR was reported to not only catalyze GGPP to PDP, but also reduce GGPP-derived later intermediates [27,35], we suspected that BchP (the GR from *R. sphaeroides* involved in bacteriochlorophyll synthesis) [27] might participate in converting tocotrienols to tocopherols (Fig. 4B)." (Line 229-237)

"To provide biochemical evidence of BchP's engagement in tocotrienol reduction, we further attempted to heterologously validate the BchP from *R. sphaeroide* in *E. coli*. It was found that the whole cell lysate of *E. coli* expressing *bchP* alone could not produce δ-tocopherol from δ-tocotrienol feeding (Fig. 4C). Additional expression of its coenzymes such as FdxA (ferredoxin II) and Fdr (NADPH-ferredoxin reductase) substantially enhanced the BchP-mediated reduction of δ-tocotrienol to δ-tocopherol (Fig. 4C). These findings suggested the failure of previous efforts towards functional expression of GR homologs might be attributed to the lack of compatible coenzymes in the heterologous hosts, whereas additional supplementation of their native FdxA and Fdr is expected to address this issue [35]. Our results on successful validation of BchP-mediated tocotrienol reduction shed light on future biomanufacturing of tocopherols in other microorganisms such as *E. coli* and yeast." (Line 237-249)

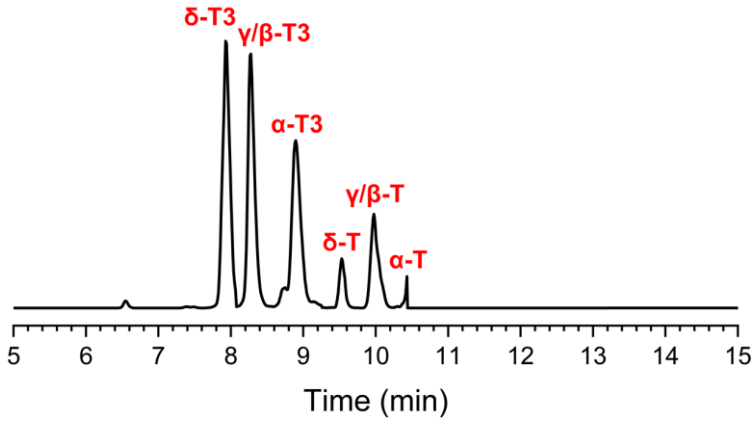

Fig. R9 Representative LC-MS chromatogram of different forms of tocopherols and tocotrienols. δ/β/γ/α-tocotrienols (δ/β/γ/α-T3); δ/β/γ/α-tocopherols (δ/β/γ/α-T).

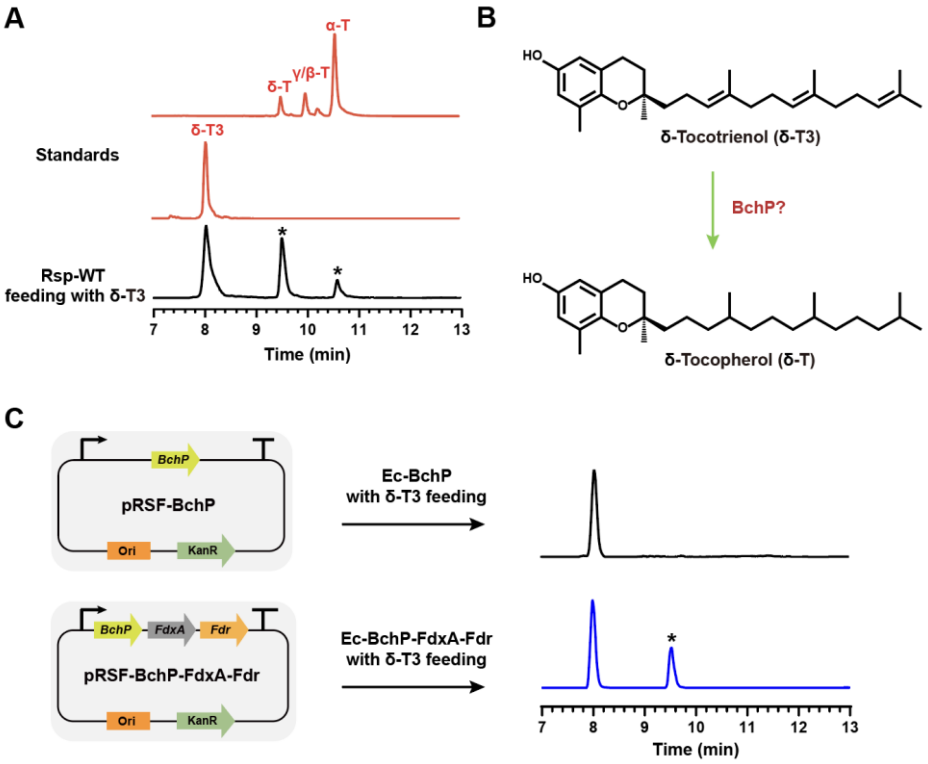

Fig. R10 Identification of BchP-mediated tocotrienol reduction. A, The whole cell lysate of *R. sphaeroides* for the synthesis of tocopherols from δ-tocotrienol. δ-T3, δ-tocotrienol. Representative LC-MS chromatograms are presented from triplicate of biological repeats. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected tocopherol products. **B,** Proposed mechanism of BchP-mediated tocotrienol reduction. **C,** Representative LC-MS results of δ-tocotrienol as substrate for δ-tocopherol production by the biotransformation of *E. coli* expressing BchP and BchP-FdxA-Fdr. Experiments were carried out using the cell lysate as the biocatalyst with 0.1 mM δ-T3 for 20 h. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected δ-tocopherol product.

(2) We have updated the MS spectra of α-tocopherol and γ/β-tocopherol. The characteristic ion peaks of the microbial fermentation extract were identical to that of the authentic α-tocopherol or γ/β-tocopherol standard, verifying the structural identity.

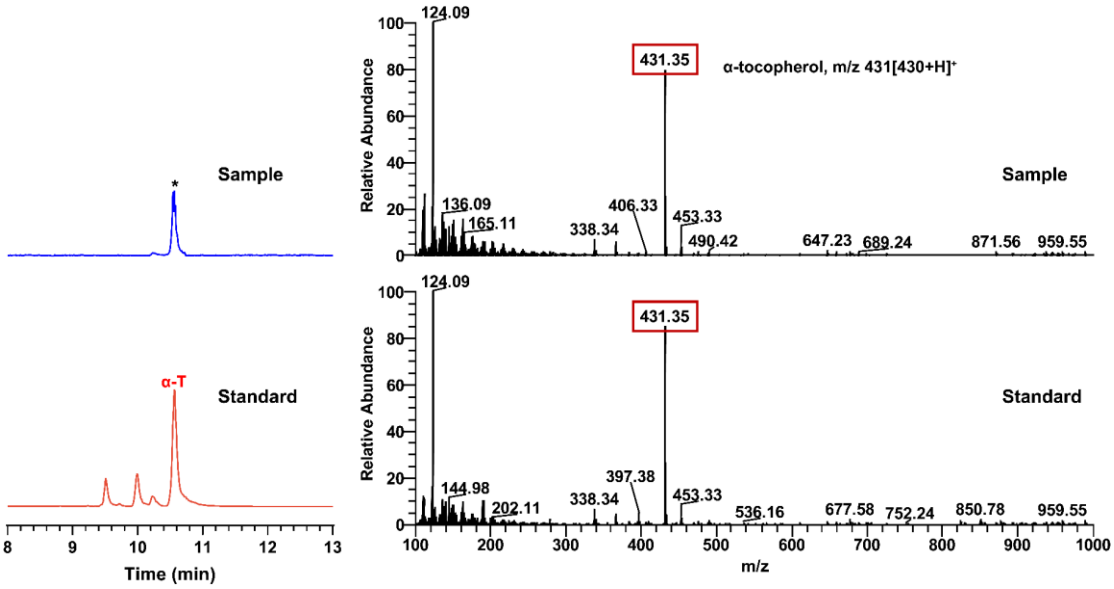

Fig. R11 Representative LC-MS results of mixed tocopherol standard and sample from strain Rsp-VE. The engineered *R. sphaeroides* Rsp-VE with the plasmid-based expression of *hppd-hpt-vte1* module was used. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected α-tocopherol product.

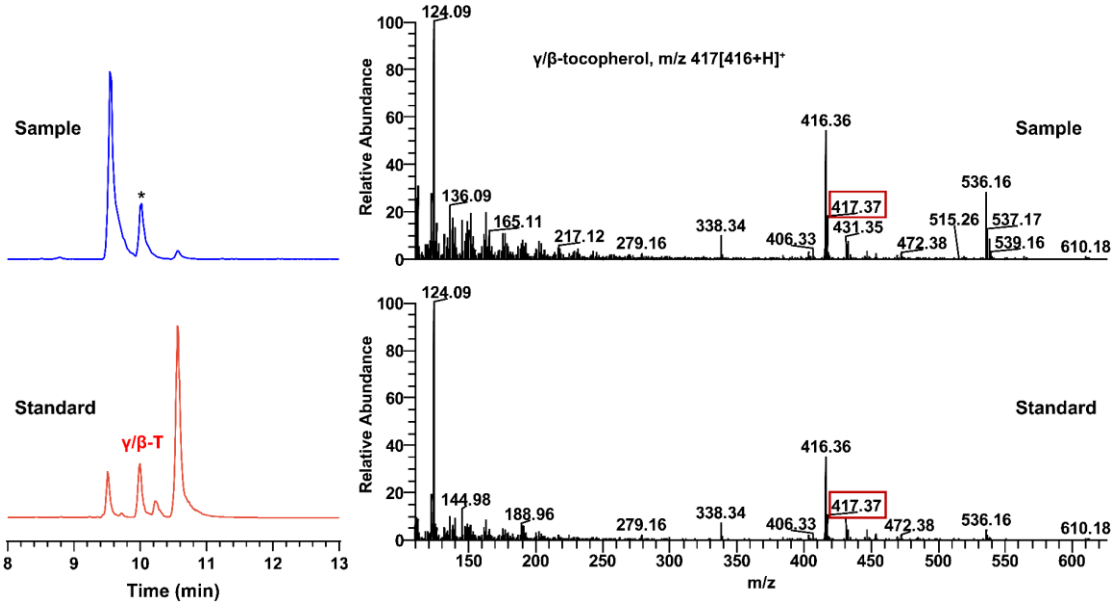

Fig. R12 Representative LC-MS results of mixed tocopherol standard and sample produced by the biotransformation of *R. sphaeroides*. The cell lysate of *R. sphaeroides* wild-type was reacted with 0.2 mM δ-tocopherol. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected γ/β-tocopherol product.

2. More detailed descriptions are also needed regarding data processing of the MS spectra shown in Figure S1 and Figure S4, including whether background subtraction

or signal correction was performed. In addition, beyond LC-MS analysis of the fermentation products, further structural confirmation is recommended. The authors are requested to provide NMR characterization data (e.g., ^1H and/or ^13C NMR) of the key products to support compound identification.

Response: Thanks for your constructive suggestions.

(1) For the mass spectrometry data presented in Figures S1 and S4 (New version Figures S1 and S3 see above), the full mass spectra were generated by directly exporting the base peak within the m/z range of 396-435 in positive ion mode using the scan mode described in the Methods section, without additional correction processing.

(2) We have updated the MS spectra of α-tocopherol and γ/β-tocopherol. The characteristic ion peaks of the microbial fermentation extract were identical to that of the authentic α-tocopherol or γ/β-tocopherol standard, verifying the structural identity.

(3) The structures of key products including δ-, γ-, β-, α-tocopherols are well established. Given the consistent retention time and mass spectra between our samples and the commercial standards, the available data are fully adequate for product identification. Therefore, we hold the opinion that no further structural characterization is required at this stage.

3. Regarding the identification of the putative γ-TMT and MPBQMT homologues in R. sphaeroides, more comprehensive bioinformatic evidence is required. The authors should provide detailed sequence analyses in the Supplementary Information, including BLAST outputs, sequence alignments, coverage and identity metrics, conserved motif comparison, and phylogenetic analysis with characterized γ-TMT, MPBQMT, and related methyltransferases. The criteria for selecting the seed sequences used in the homology search should also be clearly described and justified.

Response: We appreciate this suggestion.

(1) We display the detailed analyses including BLAST outputs, sequence alignments, coverage and identity metrics (see graph below). Because the vitamin E biosynthetic pathway and the related key enzymes in *Arabidopsis thaliana* have been fully elucidated (Trends Plant Sci 2019, 24:1040-1051)[8], and its γ-TMT and MPBQMT have been functionally validated in yeast (Table S1), we first used γ-TMT and MPBQMT

from the well-studied *A. thaliana* as the seed sequences to identify the potential γ-TMT and MPBQMT homologues from *R. sphaeroides*. It's found that only UbiE displayed the low homology (30% similarity) with MPBQMT, while no significant similarity was found for the γ-TMT. Hence, we used γ-TMT from the well-studied prokaryotic *Synechocystis* sp. PCC6803 as the seed sequence, since the tocopherol biosynthesis in *Synechocystis* sp. PCC 6803 and the key enzymes are relatively well characterized and some enzymes have been functionally validated in *Escherichia coli* and yeast (Table S1).

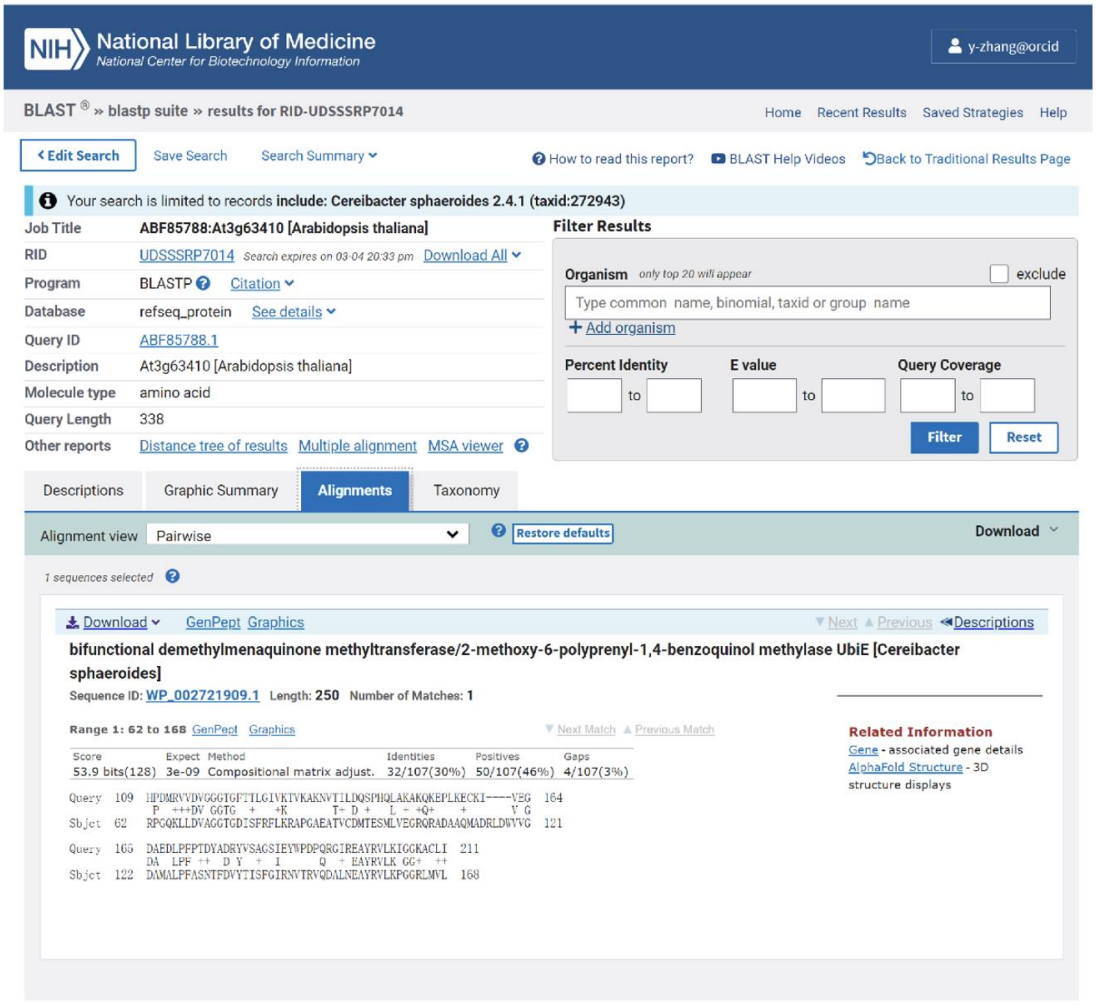

Fig. R13 A protein BLAST analysis to identify the potential γ-TMT homologues from *R. sphaeroides*. The γ-TMT (Protein ID: NP_176677.1) from the well-studied *A. thaliana* MTs was employed as the seed sequences using reference proteins as the database in NCBI.

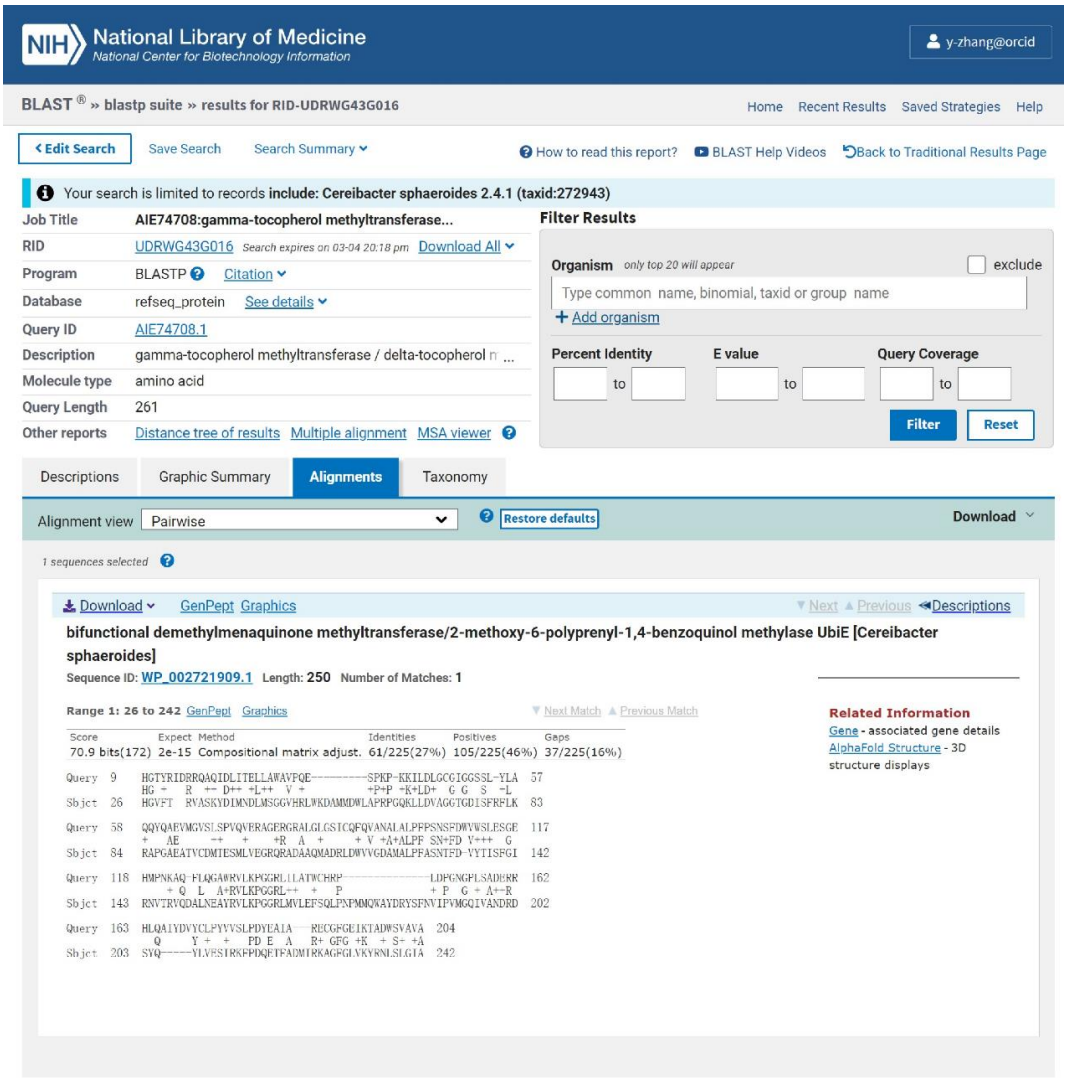

Fig. R14 A protein BLAST analysis to identify the potential γ-TMT homologues from *R. sphaeroides*. The γ-TMT (Protein ID: AIE74708.1) from *Synechocystis* sp. PCC6803 was employed as the seed sequences using reference proteins as the database in NCBI.

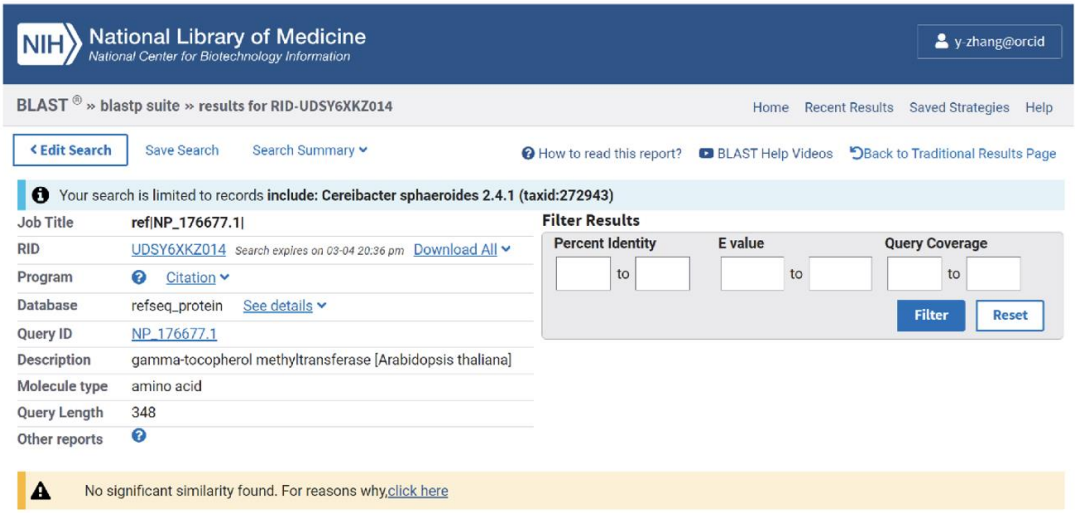

Fig. R15 A protein BLAST analysis to identify the potential MPBQMT homologues from *R.*

***sphaeroides*.** The MPBQMT (Protein ID: ABF85788.1) from the well-studied *A. thaliana* MTs was employed as the seed sequences using reference proteins as the database in NCBI.

(2) Multiple sequence alignment of RspUbiE with other homologs (Supplementary Fig. S6) revealed that RspUbiE shares the conserved catalytic amino acid residues (Y36, R143, N144). Further mutation studies indicate that R143 serves as the essential general base for substrate activation, N144 plays an indispensable role in stabilizing the key reaction intermediate, and Y36, while not absolutely required, contributes a supporting role in substrate orientation or transition state stabilization. Since UbiE is distinct from TMT and MPBQMT families, we think the phylogenetic tree to compare UbiE with characterized methyltransferases (TMT and MPBQMT) is not necessary.

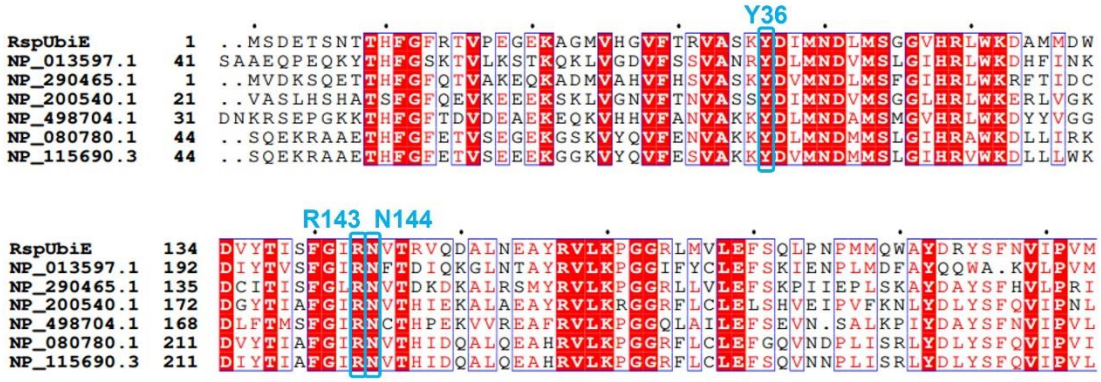

Fig. R16 Multiple sequence alignment of RspUbiE with other homologs. The putative residues responsible for substrate catalysis are indicated with blue boxes [6]. The sequences are (NCBI accession Nos. are given in parentheses) *S. cerevisiae* 2-hexaprenyl-6-methoxy-1,4-benzoquinone methyltransferase Coq5 (NP_013597.1), *E. coli* ubiquinone/menaquinone biosynthesis methyltransferase (NP_290465.1), *A. thaliana* 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase (NP_200540.1), *Caenorhabditis elegans* Coq5 (NP_498704.1), *Mus musculus* 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase (NP_080780.1) and *Homo sapiens* 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase (NP_115690.3).

4. A key claim of this work is the identification of a putative bifunctional γ/β-tocopherol methyltransferase (UbiE). However, its functional characterization could be better. The enzyme(s) from the relevant sources could be expressed and purified, and experimental kinetic parameters (Km, kcat, kcat/Km) measured. Predicted values alone by

Deepmolecules (Table S2) are not sufficient to support the claimed bifunctional activity.

Response: We sincerely thank you for this suggestion. We agree with that predicted values alone by Deepmolecules are not sufficient and we have removed these data. We further attempted to purify *Rhodobacter* UbiE from *E. coli*. However, we found that both his-tag fusion of UbiE could not give high-purity UbiE (see graph below), indicating that UbiE would interact with *E. coli* endogenous enzymes. As the β-TMT activity represents the first-time reported during tocopherol biosynthesis, we next attempted to carry out biochemical verification by monitoring β-tocopherol substrate depletion rate. Further time course analysis using β-tocopherol feeding revealed that UbiE from *R. sphaeroides* gradually converted β-tocopherol to the desired α-tocopherol (Figure 2E), and approximately 65% β-tocopherol was converted to α-tocopherol in 48 h.

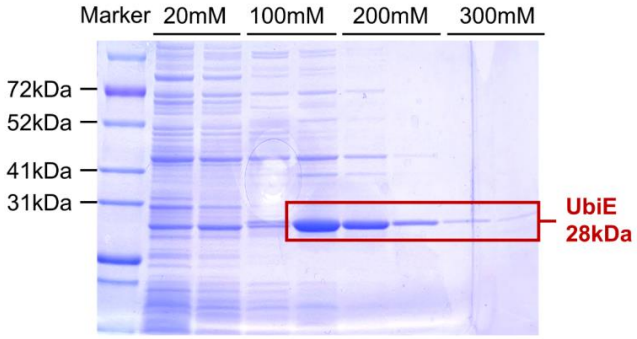

Fig. R17 SDS-PAGE gel of purified UbiE protein

5. The proposed molecular mechanism is based only on docking and MD simulations, without experimental structural validation. Experimental evidence (e.g., structural determination or site-directed mutagenesis with activity assays) is needed to support the mechanistic conclusions.

Response: We appreciate this suggestion. UibE is a methyltransferase for coenzyme Q biosynthesis, there is no mechanistic difference between coenzyme Q substrate and tocopherol substrate. The simple explanation of the substrate binding pocket of UbiE is capable of accommodating the binding of tocopherol substrate, that means the enzyme promiscuity of UbiE is relatively broad. It is unlikely that there will be another methyltransferase active site beyond coenzyme Q site. The site-directed mutagenesis of the conserved residues completely abolished the methyltransferase activity of

Rhodobacter UbiE. These results could further support the mechanism as follows:

"Furthermore, multiple sequence alignment of RspUbiE with other homologs (Supplementary Fig. S6) revealed that RspUbiE shares the conserved catalytic amino acid residues (Y36, R143, N144). The molecular docking results revealed suitable conformational proximity of the β-tocopherol substrate and SAM to the conserved active sites (Fig. 3A). Based on the common methyltransferase mechanism in UbiE homologs [34], we proposed using β-tocopherol as an example: R143 or Y36 functions as a general base to abstract a proton from a water molecule, forming a negatively charged oxygen atom; this oxygen atom subsequently interacts with the hydrogen atom at the C22 position of β-tocopherol, inducing the deprotonation of C22 before methyl transfer; α-tocopherol is then produced following the methyl transfer reaction, while N144 could stabilize the reaction intermediate (Fig. 3B). Further mutation experiments (Y36A, N144A and R143A in RspUbiE) revealed that R143A and N144A completely abolished the methyltransferase activity on β-tocopherol substrate, whereas Y36A retained partial activity (Fig. 3C). These findings indicate that R143 serves as the essential general base for substrate activation, N144 plays an indispensable role in stabilizing the key reaction intermediate, and Y36, while not absolutely required, contributes a supporting role in substrate orientation or transition state stabilization. Therefore, biochemical verification results confirmed that UbiE from *R. sphaeroides* represents the first-time reported β-TMT as depicted in Fig. 1." (Line 191-209)

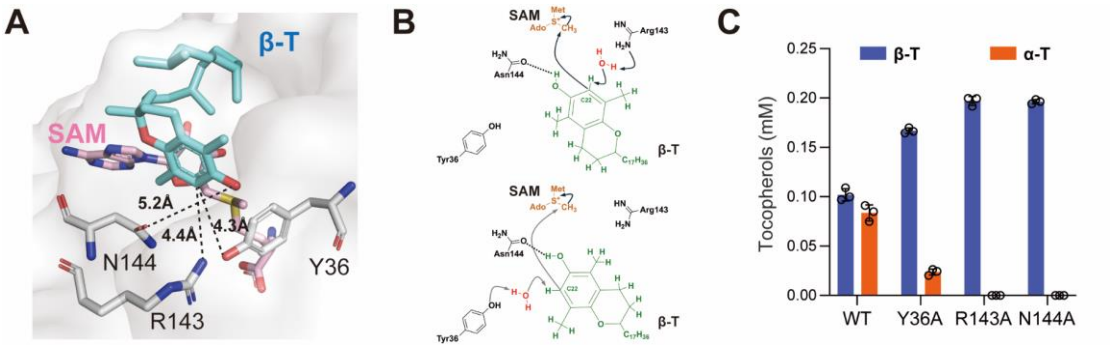

Fig. R18 Mechanism analysis of UbiE through molecular docking, mutation study and molecular dynamics (MD) simulations. A, Predicted binding pose of β-tocopherol (β-T) in the RspUbiE active site. Key residues Y36, R143 and N144 are shown as sticks, with distances (in Å) to the substrate indicated by dashed lines. **B,** Hypothesized methyltransferase mechanism using β-

tocopherol as a model based on the conserved UbiE-family mechanism and the docking pose shown in panel **A**. **C,** Mutation analysis to confirm the key amino acid residues involved in the β-TMT activity. UbiE with Y36A, N144A and R143A was tested for converting β-tocopherol to α-tocopherol at 24 h. WT, wild-type UbiE. All experiments were carried out in triplicates and data represent the average ± SD from three biological repeats.

6. Since the metabolic engineering was mainly performed via Tn5-based integration, the authors could provide experimental evidence for the copy number of the integrated gene(s) in the final selected strains (e.g., qPCR/ddPCR-based copy-number quantification or an equivalent genomic validation).

Response: Thanks for your constructive suggestions. Tn5-mediated genome integration is a random and potential multiple copy process based on the recent findings (ACS Synth Biol 2025,14:2753-2763)[1], and also well documented by many previous studies. This study mainly focused on the discovery of a nonclassical biosynthetic route for tocopherol synthesis in *Rhodobacter* species involving the UbiE with the uncharacterized γ/β-tocopherol methyltransferase activity. In addition, functional reconstitution of BchP-mediated δ-tocotrienol reduction would pave the way for future biomanufacturing of tocopherols in other microorganism such as *E. coli* and yeast. Considering the limited space of manuscript, and we did not carry out further genetic analysis of the optimal recombinant strain to correlate the genotype with tocopherol productivity. We agree that deciphering the underneath mechanism for high productivity by employing multi-omics data will be of particular interest, which might be reported in a sperate study.

7. In Figure 4A, the meaning and design of "MEV1–13" are not defined, and the corresponding construction rationale and dataset are not clearly presented. Please provide a clear description and the underlying data supporting these engineered strains. In Figure 4B, the purpose of distinguishing Library A and Library B is unclear. If hppd-hpt-vte-aroG*-tyrA* were not independently regulated, the specific role and necessity of Library A in the experimental design should be clarified.

Response: We appreciate this suggestion. We have adjusted the structure of this manuscript, and updated the description on MEV1–20 strains. The meaning and design

of MEV1–20 strains are to construct a GGPP/PDP-overproducing platform, which is one of the most important precursors to tocopherol biosynthesis, by integrating a heterologous mevalonate (MEV) pathway using transposon mutagenesis (see section "**Engineering a GGPP/PDP-overproducing platform in *R. sphaeroides***").

Considering the manuscript has too many sections after adding additional experiments on characterization of UbiE and BchP, we decided to remove the section about Library A of integrating the *hppd-hpt-vte1* module to avoid misleading information to the readers. Now, only one mutant library of integrating the *hppd-hpt-vte1* and *aroG*-tyrA** modules is shown in the revised manuscript (see section "**Engineering *R. sphaeroides* for high-level tocopherol production**").

8. In Figure 4C, the origin and genotype of strains VE22–25 are not clearly indicated, and the linkage between strain construction and the presented data lacks continuity. Please clarify where these strains are defined and how they were derived. It is recommended to provide a flowchart of strain engineering and key modification steps in the Supplementary Information, as well as a complete genotype table for all constructed strains, to improve clarity and reproducibility (see, for example, Zhu et al., Nat Catal 3, 64–74 (2020), Supplementary Fig. 14 and Supplementary Table 3).

Response: We appreciate this suggestion. In the origin manuscript, we did not construct strains VE22–25. We have updated the strains' names and provided a flowchart of strain engineering and key modification steps in the Supplementary Information, as well as a complete genotype table for all constructed strains, to improve clarity and reproducibility according to the reference (Nat Catal 2020, 3:64–74).

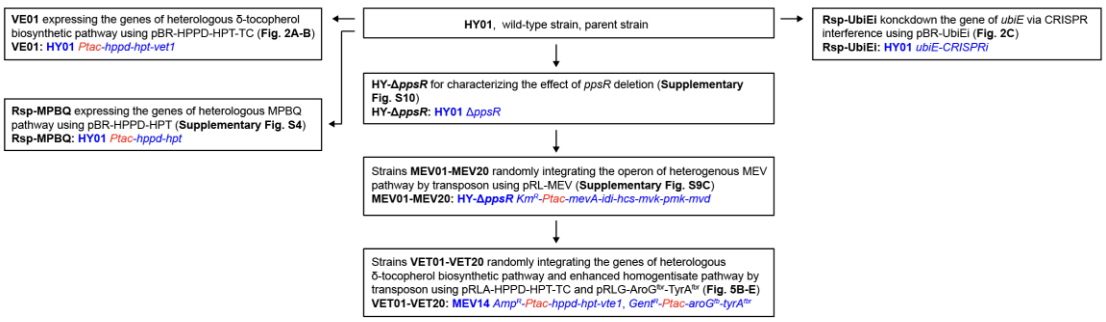

Fig. R19 The flowchart for constructing strains for tocopherol production. Names in bold and blue colour are the background strains, and genetic manipulations are highlighted in blue. Promoters

are shown in red.

9. VE01 (plasmid-based expression) reportedly produces only α-tocopherol, whereas Tn5-integrated strains generate multiple tocopherol species; this difference should be explained and supported with data (e.g., expression level and flux effects). Please also provide in vitro enzyme data showing how product distribution varies with substrate (and SAM) concentrations, and discuss whether balancing HGA, PDP, and SAM supply in vivo could drive more complete conversion to α-tocopherol.

Response: Thanks for your suggestions. In strain Rsp-VE (VE01 in the original manuscript), we only expressed the *hppd-hpt-vte1* module via the plasmid. The low metabolic flux toward the precursor synthesis (HGA and GGPP/PDP) resulted in low tocopherol production. Under these conditions, endogenous SAM synthesis was sufficient to drive the complete conversion of δ-tocopherol into α-tocopherol. However, in the high-production strains, we deleted *ppsR*, a transcriptional repressor of GGPP/PDP synthesis, and integrated the heterologous MEV pathway via transposon to construct a high-yield platform for the precursor GGPP/PDP. Based on this platform, we further integrated the *aroG*-tyrA** module via transposon to enhance the synthesis of 4-HPP (the direct substrate of HGA) as well as the *hppd-hpt-vte1* module, screening high tocopherol-producing strains through color phenotype. Under these high-flux conditions, endogenous SAM synthesis clearly became insufficient to meet the demand for α-tocopherol conversion, which is also consistent with the experiment of Met supplement. Consequently, multiple forms of tocopherols accumulated in the high production strains.

We agree with that the enzyme data facilitate the more understanding for α-tocopherols biosynthesis. At the current stage, the focus of this work lies in the discovery of a nonclassical biosynthetic route for tocopherol synthesis in *Rhodobacter* species that bypasses the classical α-tocopherol pathway requiring the DMPBQ as an intermediate already represents a big breakthrough as it is the first time to report β-TMT activity for tocopherol biosynthesis. Furthermore, we discovered that geranylgeranyl pyrophosphate reductase (GR) plays an important role in reducing tocotrienol into tocopherol. As a proof-concept, we found that a simple transposon-

based strain engineering could result in a high-level production of tocopherols to 3.5 g/L. We do agree the suggested experiments will favor in-depth mechanistic understanding on tocopherol overproduction, but it is beyond our scope for this study. We also think that, for the future commercialization of microbial synthesis of tocopherols, continuous efforts should be implemented in the following aspects: (a) utilizing the low cost of feedstocks such as lignocelluloses; (b) restricting the spheroidenone and carotenoids to divert more GGPP flux to tocopherol synthesis; (c) strengthening intracellular SAM regeneration to promote the transformation of δ- and γ/β-tocopherols into α-tocopherol, thereby reducing the cost of exogenous methyl donors; (d) introducing membrane transporters to export tocopherols and reduce intracellular stress; (e) optimizing fermentation parameters for the pilot scale production of tocopherols.

10. In Fig. 6B and 6D, the tocopherol composition clearly shifts after SAM regulation, and δ-tocopherol is no longer detected. This is intriguing and suggests that sufficient SAM supply can effectively promote further substrate conversion. However, it is unclear why the authors did not further strengthen endogenous UbiE expression to take advantage of this effect. In addition, for strain VE30, please provide the 5-L fed-batch results under MET supplementation alone (if tested), and include a more complete fermentation characterization, such as key by-product profiles, methionine and related metabolite changes, and yield calculations.

Response: We appreciate this insightful suggestion, and we agree with the UbiE overexpression will obviously improve α-tocopherol biosynthesis. However, due to lack of available selection marker for further genetic modification at this moment, we focused on culture medium optimization by supplementing methyl donor such as SAM and Met. In future study, we will further investigate the effect of additional UbiE and BchP overexpression on tocopherol production.

In addition, the fermentation by strain VET05 (VE30 in the original manuscript) under the Met supplement alone was clearly inferior to that under supplements of Met and 2-HC (Fig. 5D). Thus, in the original manuscript, we conducted the 5-L fed-batch fermentation under supplements of Met and 2-HC instead of Met supplement alone.

Given that the primary finding is the nonclassical biosynthetic route for tocopherol synthesis, we did not investigate further optimization of fermentation process on tocopherol production in this work. It is certain that further fermentation parameter adjustment such as pH, dissolved oxygen, substrate feeding rate will increase the product levels.

11. The authors should also provide complete plasmid maps for all constructs and clearly indicate their correspondence with the plasmid names and descriptions in the main text and Supplementary Information.

Response: Thank you for this suggestion. We have provided all related gene sequence for reproducible results. All plasmid maps will be provided upon request.

Minor comments

1. In the main text (Line 8) and Supplementary Information (Line 11), please remove the extra space after "2".

Response: Revised as recommended.

2. In Fig. 1, it would be helpful to label proteins together with their corresponding gene names to improve clarity and readability.

Response: We think it is more appropriate to present only enzyme names in Figure 1 as there are a number of genes from different organisms.

3. In Supplementary Table S1 and the related discussion, please include and discuss the most recent relevant literature (e.g., https://doi.org/10.1016/j.bej.2026.110079), which appears to be directly pertinent to the topic.

Response: Revised as recommended. We have added this relevant literature in the Supplementary Table S1 and the related discussion.

4. Line 96: revise to "cyclization and/or methylation".

Response: Revised as recommended.

5. The manuscript structure could be improved by presenting the complete synthetic biology and pathway engineering section first, and then the application results, to enhance clarity and narrative flow.

Response: Revised as recommended. We have revised the structure of the manuscript

by presenting the complete synthetic biology and pathway engineering section first, and then the application results.

6. In Fig. S7A, please remove the extra “v” at “mvk”.

Response: Revised as recommended.

References

1 Song, Y. *et al.* A Tn5 transposase-based system for high-efficiency genome-wide gene activation in *Escherichia coli*. *ACS Syn. Biol.* **14**, 2753-2763 (2025).

2 Zou, S. *et al.* Comparative approaches to facilitate the discovery of prolongevity interventions: Effects of tocopherols on lifespan of three invertebrate species. *Mech. Ageing Dev.* **128**, 222-226 (2007).

3 Shashikumar, S. & Rajini, P. S. Alpha-tocopherol ameliorates cypermethrin-induced toxicity and oxidative stress in the nematode *Caenorhabdtis elegans*. *Indian J. Biochem. Biophys.* **48**, 191-196 (2011).

4 Li, J. *et al.* Delivery of alpha-tocopherol through soluble dietary fibre-based nanofibres for improving the life span of *Caenorhabditis elegans*. *International J. Food Sci. Nutr.* **70**, 172-181 (2018).

5 Gilestro, G. F. *et al.* High concentration of vitamin E decreases thermosensation and thermotaxis learning and the underlying mechanisms in the nematode *Caenorhabditis elegans*. *PLoS ONE* **8**, e71180 (2013).

6 Adachi, H. & Ishii, N. Effects of tocotrienols on life span and protein carbonylation in *Caenorhabditis elegans*. *J. Gerontol. A Biol. Sci. Med. Sci.* **55**, B280-285 (2000).

7 Addlesee, H. A. & Hunter, C. N. Physical mapping and functional assignment of the geranylgeranyl-bacteriochlorophyll reductase gene, *bchP*, of *Rhodobacter sphaeroides*. *J. Bacteriol.* **181**, 7248-7255 (1999).

8 Muñoz, P. & Munné-Bosch, S. Vitamin E in plants: biosynthesis, transport, and function. *Trends Plant Sci.* **24**, 1040-1051 (2019).

Response to the Editor's comments:

1. We agree with the reviewers that it's crucial to provide compelling mechanistic insight and functional validation of the bifunctional γ/β-tocopherol methyltransferase activity of UbiE. In alignment with this, we suggest that you investigate how chromosomal integration of UbiE affects tocopherol production and exclude the contribution from other characterized methyltransferases to the production enhancement. Please follow the comments of reviewers #1 and #3 in this regard.

Response: Based on reviewers' comments about UbiE, we carried out the following experiments:

(1) We compared the reactions between the well-studied γ-tocopherol methyltransferase from *A. thaliana* and UbiE from *Rhodobacter* by expressing in the heterologous *E. coli*. As shown in Fig. 2D, we found that only *Rhodobacter* UbiE expression could result in α-tocopherol production by δ-tocopherol feeding, whereas γ-TMT from *A. thaliana* could only convert δ-tocopherol to β-tocopherol. Further molecular docking revealed that γ-TMT from *A. thaliana* could not give favorable docking of β-tocopherol (as shown in the detailed response below), which explains γ-TMT from *A. thaliana* only catalyze one methylation step.

(2) UbiE is involved in the biosynthesis of coenzyme Q, which is essential for cell survival. Our attempts to knockout the *ubiE* gene were unsuccessful. Therefore, we have employed CRISPR interference (CRISPRi) to repress the expression of UbiE for the functional validation.

"Since UbiE is involved in coenzyme Q synthesis which is an essential step for cell survival, attempt to knockout *ubiE* was difficult. We next constructed engineered *R. sphaeroides* with UbiE repression for biocatalysis analysis. The cell lysate of wild-type *R. sphaeroides* could convert δ/β/γ-tocopherols to α-tocopherol, whereas its repression reduces α-tocopherol formation (Figure 2C). Therefore, we confirmed that UbiE from *R. sphaeroides* has the uncharacterized promiscuous γ/β-TMT activities involving in α-tocopherol biosynthesis, albeit the possibility of other MTs besides UbiE could not be completely ruled out as UbiE repression still produced a small amount of β/γ-tocopherols." (Line 169-177)

(3) We employed the site-directed mutagenesis of the conserved residues in *Rhodobacter* UbiE to further elucidate the catalytic mechanism.

"Furthermore, multiple sequence alignment of RspUbiE with other homologs (Supplementary Fig. S6) revealed that RspUbiE shares the conserved catalytic amino acid residues (Y36, R143, N144). The molecular docking results revealed suitable conformational proximity of the β-tocopherol substrate and SAM to the conserved active sites (Fig. 3A). Based on the common methyltransferase mechanism in UbiE homologs [34], we proposed using β-tocopherol as an example: R143 or Y36 functions as a general base to abstract a proton from a water molecule, forming a negatively charged oxygen atom; this oxygen atom subsequently interacts with the hydrogen atom at the C22 position of β-tocopherol, inducing the deprotonation of C22 before methyl transfer; α-tocopherol is then produced following the methyl transfer reaction, while N144 could stabilize the reaction intermediate (Fig. 3B). Further mutation experiments (Y36A, N144A and R143A in RspUbiE) revealed that R143A and N144A completely abolished the methyltransferase activity on β-tocopherol substrate, whereas Y36A retained partial activity (Fig. 3C). These findings indicate that R143 serves as the essential general base for substrate activation, N144 plays an indispensable role in stabilizing the key reaction intermediate, and Y36, while not absolutely required, contributes a supporting role in substrate orientation or transition state stabilization. Therefore, biochemical verification results confirmed that UbiE from *R. sphaeroides* represents the first-time reported β-TMT as depicted in Fig. 1." (Line 191-209)

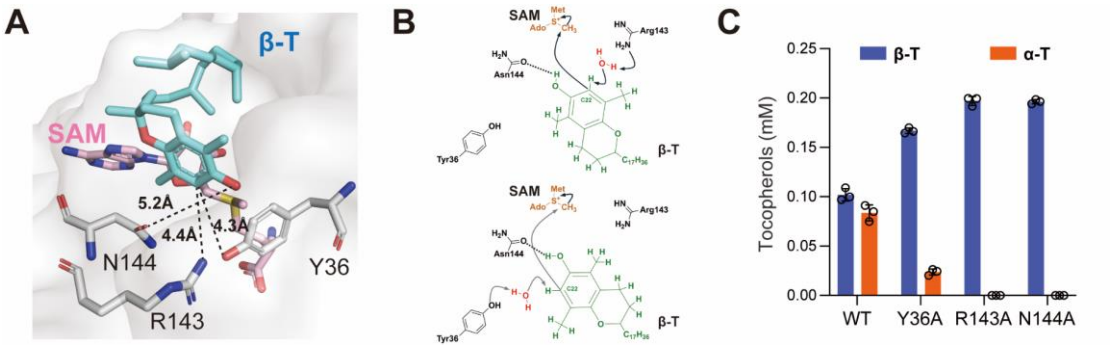

Fig. R1 Mechanism analysis of UbiE through molecular docking, mutation study and molecular dynamics (MD) simulations. A, Predicted binding pose of β-tocopherol (β-T) in the RspUbiE active site. Key residues Y36, R143 and N144 are shown as sticks, with distances (in Å)

to the substrate indicated by dashed lines. **B,** Hypothesized methyltransferase mechanism using β-tocopherol as a model based on the conserved UbiE-family mechanism and the docking pose shown in panel **A**. **C,** Mutation analysis to confirm the key amino acid residues involved in the β-TMT activity. UbiE with Y36A, N144A and R143A was tested for converting β-tocopherol to α-tocopherol at 24 h. WT, wild-type UbiE. All experiments were carried out in triplicates and data represent the average ± SD from three biological repeats.

(4) As the β-TMT activity represents the first-time reported during tocopherol biosynthesis, we next attempted to carry out biochemical verification by monitoring β -tocopherol substrate depletion rate. Further time course analysis using β-tocopherol feeding revealed that UbiE from *R. sphaeroides* gradually converted β-tocopherol to the desired α-tocopherol (Figure 2E), and approximately 65% β-tocopherol was converted to α-tocopherol in 48 h.

2. As highlighted by reviewers #2 and #3, please investigate the contribution of geranylgeranyl reductase to heterologous tocopherol production and explore its generality in other hosts.

Response: Since tocotrienol biosynthesis was not detected in the engineered strain, we hypothesized that geranylgeranyl pyrophosphate reductase (GR) not only converts GGPP to PDP but also catalyzes the conversion of tocotrienols to tocopherols. Accordingly, we have examined the effect of GR encoded by *bchP* on tocotrienol conversion in *R. sphaeroides* and further verified its catalytic activity in *E. coli*. The main results are as follows:

"Based on the LC-MS results, we found that all forms of δ/β/γ/α-tocotrienols were not detected, indicating that tocotrienols might be converted to tocopherols by endogenous *R. sphaeroides* metabolism. As shown in Fig. 4A, we found that the wild-type *R. sphaeroides* transformed δ-tocotrienol to different forms of tocopherols, suggesting that its endogenous metabolism is capable of tocotrienol reduction. Since GR was reported to not only catalyze GGPP to PDP, but also reduce GGPP-derived later intermediates [27,35], we suspected that BchP (the GR from *R. sphaeroides* involved in bacteriochlorophyll synthesis) [27] might participate in converting tocotrienols to tocopherols (Fig. 4B)." (Line 229-237)

"To provide biochemical evidence of BchP's engagement in tocotrienol reduction, we further attempted to heterologously validate the BchP from *R. sphaeroide* in *E. coli*. It was found that the whole cell lysate of *E. coli* expressing *bchP* alone could not produce δ-tocopherol from δ-tocotrienol feeding (Fig. 4C). Additional expression of its coenzymes such as FdxA (ferredoxin II) and Fdr (NADPH-ferredoxin reductase) substantially enhanced the BchP-mediated reduction of δ-tocotrienol to δ-tocopherol (Fig. 4C). These findings suggested the failure of previous efforts towards functional expression of GR homologs might be attributed to the lack of compatible coenzymes in the heterologous hosts, whereas additional supplementation of their native FdxA and Fdr is expected to address this issue [35]. Our results on successful validation of BchP-mediated tocotrienol reduction shed light on future biomanufacturing of tocopherols in other microorganisms such as *E. coli* and yeast." (Line 238-249)

3. As requested by reviewer #3, please clearly distinguish between the production of tocotrienols and tocopherols in the engineered strains.

Response: The LC-MS method described in the original manuscript allows for the separation and identification of tocopherols versus tocotrienols. Moreover, upon extracting the molecular ion peaks of target compounds from the fermentation samples, no characteristic ions corresponding to δ/β/γ/α-tocotrienols were detected in the mass spectra.

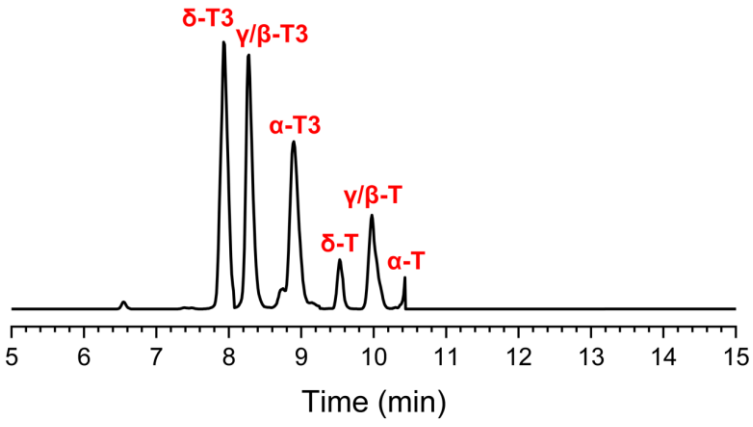

Fig. R2 Representative LC-MS chromatogram of different forms of tocopherols and tocotrienols. δ/β/γ/α-tocotrienols (δ/β/γ/α-T3); δ/β/γ/α-tocopherols (δ/β/γ/α-T).

4. As noted by reviewers #1 and #2, please investigate the tocopherol composition in

the feedstock mixture and provide further evidence to support the beneficial effect of tocopherol-producing bacteria on reproductive fitness of C. elegans.

Response: In *C. elegans*, α-tocopherol is the most extensively studied, with roles primarily in antioxidation, stress protection, and lifespan regulation, and exhibits a dose-dependent effect (Mech Ageing Dev 2007, 128:222-226; Indian J Biochem Biophys 2011, 48:191-196; Int J Food Sci Nutr 2019, 70:172-181)[2-4]. γ-Tocopherol shows stronger potential for lifespan extension and antioxidation than α-tocopherol, with no obvious toxicity at high doses (Mech Ageing Dev 2007, 128:222-226)[2]. Studies focusing individually on β-tocopherol or δ-tocopherol are scarce, and they are usually investigated together with mixed vitamin E preparations (J Gerontol A Biol Sci Med Sci 2000, 55:B280-B285; PLoS One 2013, 8:e71180)[5,6]. We agree with that the tocopherol compositional effect is worth studying for therapeutic applications. However, the *in vivo* evaluation using *Caenorhabditis elegans* is for the purpose of safety evaluation of *Rhodobacter*-based single cell proteins for animal feeds, rather than to investigate the effects of tocopherols on therapeutic applications in *C. elegans*. Considering the space limit after adding additional experiments on characterization of UbiE and BchP, we decided to remove this section to avoid misleading information to the readers.

5. As suggested by reviewer 2, please tone down the conclusion throughout the text to align more precisely with the experimental evidence.

Response: We have revised the statement for more accurate expression.

"In summary, we report a nonclassical α-tocopherol biosynthetic pathway and establish a promising approach to produce natural tocopherols by microbial fermentation. In particular, we identified UbiE as a bifunctional methyltransferase involved in transforming individual δ, γ, and β-tocopherols to α-tocopherol, which is expected to simplify α-tocopherol synthesis via a more concise route (Fig. 1). UbiE could not catalyze the MPBQ substrate, and the cyclization-mediated by TC to give δ-tocopherol is a prerequisite for the subsequent methylations to occur in *Rhodobacter* species. Functional reconstitution of BchP-mediated tocotrienol reduction in the heterologous host of *E. coli* paves the way for future biomanufacturing of tocopherols in other

microorganisms. *De novo* biosynthesis of tocopherols in *R. sphaeroides* is substantially enhanced through systematic metabolic engineering, and one of the engineered strains produced 3.51 g/L tocopherols in a fed-batch bioreactor, which represents the highest titer reported to date. Taken together, our work lays a foundation for the future commercialization of microbial synthesis of tocopherols."

6. Finally, we strongly encourage you to pay special attention to our formatting guidelines, which will be required should the manuscript be accepted for publication at a later stage. As a minimum, please prepare your figures in editable formats, present data as scatter plots, state the number of samples in the figure legends, provide source data for each figure, and provide uncropped scans of all blots and gels. Additionally, please take full advantage of our Extended figures (max. of 10) before making use of Supplementary figures (unlimited). The difference between them is that Extended figures are more easily available to readers, as they appear in the PDF and the HTML versions of the paper, whereas Supplementary figures need to be downloaded separately.

Response:

Response: We have prepared all required materials for this manuscript.

7. When preparing your revised manuscript, please be aware of our guidance on Sex and Gender reporting). Please note that:

1). If the research findings apply to only one sex or gender, that must be indicated in the title and/or abstract.

2a). For studies involving vertebrates animal and cell lines- The Reporting Summary should include whether sex was were considered in the study design.

2b). For studies involving human research participants- The Reporting Summary should include whether sex and/or gender was considered in the study design and whether sex and/or gender of participants was determined based on self-report or assigned (and methodology used).

3). Data should be reported disaggregated for sex and gender where this information has been collected and consent has been obtained for reporting and sharing individual-level data; disaggregated numbers for individual experiments must be provided in the source data as appropriate whereas overall numbers may be provided in the Nature

Portfolio Reporting Summary.

Response: We have removed the contents about the animal experiments using the hermaphrodite *Caenorhabditis elegans*, thus this study does not involve animal/gender. And we have revised this information in the Nature Portfolio Reporting Summary.

Response to Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript reports the heterologous biosynthesis of tocopherols, including alpha-tocopherol, in photosynthetic bacteria Rhodobacter. UbiE was identified as a methyltransferase with bifunctional γ/β-tocopherol methyltransferase activity. Through random chromosomal integration of the tocopherol synthetic module combined with enhanced precursor availability from MEV pathway and shikimate pathway, the authors achieved a tocopherol titer of 295 mg/L. Further process optimization via supplementation of SAM and its precursor, and antioxidants increased the total tocopherol titer to 620 mg/L. Fed-batch fermentation yielded 3.51 g/L of tocopherols. This represents the first report of de novo tocopherol biosynthesis in heterologous bacteria, and the titer achieved is impressive. However, for a manuscript intended for Nature Metabolism, the mechanistic understanding of the underlying metabolism remains insufficient.

Major concerns:

1. As the central finding of this study, the functional properties of UbiE warrant deeper investigation. The authors should provide a rigorous comparative mechanistic analysis between UbiE and other characterized methyltransferases (MTs) in the tocopherol biosynthetic pathway. Specifically, what distinguishes UbiE from MTs that catalyze only one methylation step? Current evidence of bifunctional γ/β-tocopherol methyltransferase activity requires more detailed biochemical characterization.

Response: We sincerely thank you for this valuable suggestion.

(1) We compared the reactions between the well-studied γ-tocopherol methyltransferase from *A. thaliana* and UbiE from *Rhodobacter* by expressing in the heterologous *E. coli*. As shown in Fig. 2D, we found that only *Rhodobacter* UbiE expression could result in α-tocopherol production by δ-tocopherol feeding, whereas γ-TMT from *A. thaliana* could only convert δ-tocopherol to β-tocopherol.

Further molecular docking revealed that γ-TMT from *A. thaliana* could not give favorable docking of β-tocopherol, which explains γ-TMT from *A. thaliana* only

catalyze one methylation step:

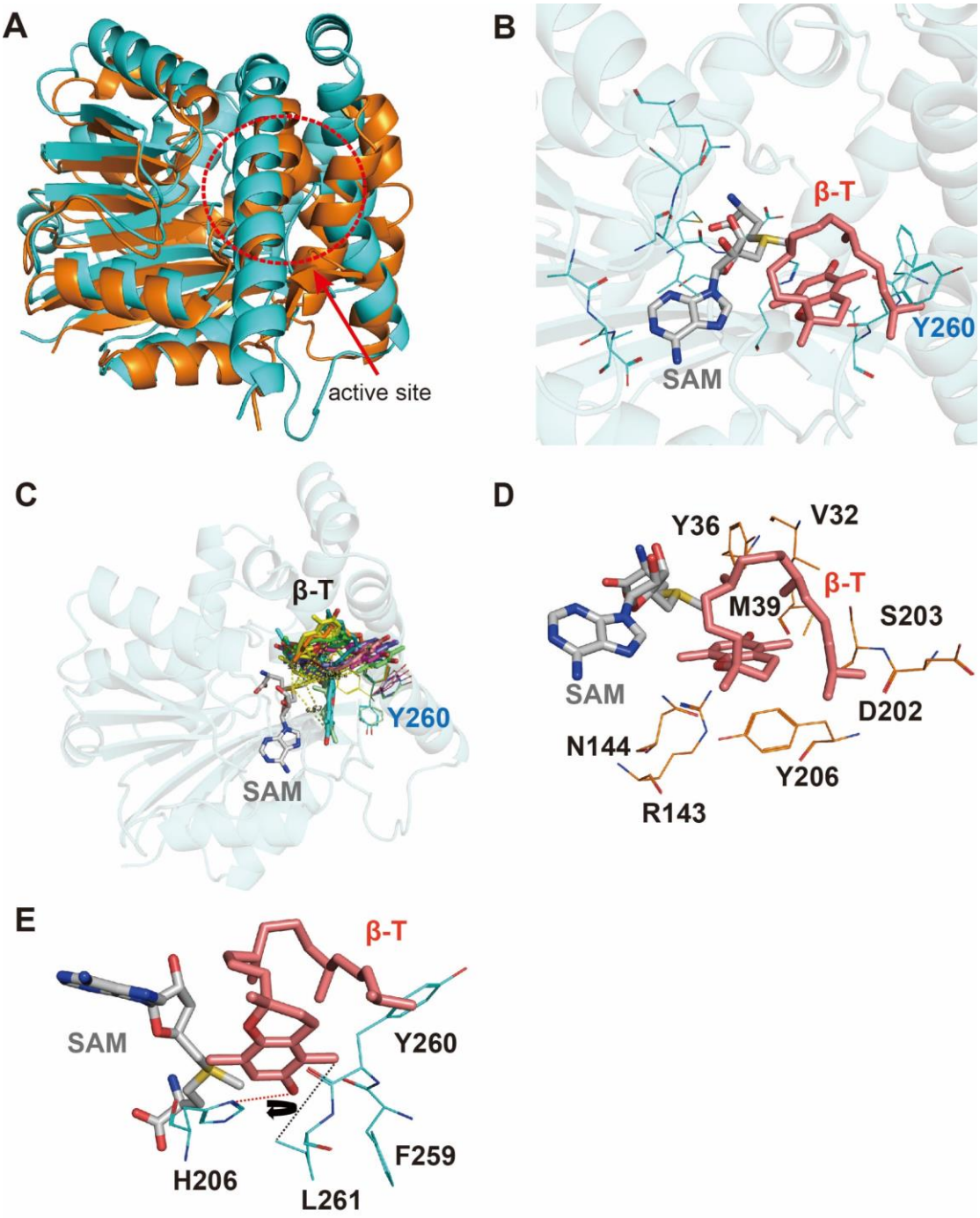

Fig. R3 Mechanistic insight into the inability of *Arabidopsis* γ-TMT to catalyze β-tocopherol. (A) Structural superposition of RspUbiE (teal) and *Arabidopsis* γ-TMT (orange). The two structures exhibit significant divergence with a backbone RMSD > 3 Å, particularly in the active site region (highlighted by a red dashed circle). (B) The docked pose of SAM and β-tocopherol from RspUbiE, when superposed onto the *Arabidopsis* γ-TMT structure, reveals a steric clash between the active site residue Y260 and β-tocopherol, indicating incompatibility of the RspUbiE binding mode in γ-

TMT. (C) Flexible docking of SAM and β-tocopherol into the Arabidopsis γ-TMT active site with residue Y260 set as flexible. Analysis of the top 10 lowest-energy conformations from 1000 docking runs shows a catalytic distance consistently greater than 5 Å, which is insufficient for methyl transfer. (D-E) Local microenvironment of the β-tocopherol binding site in (D) RspUbiE and (E) *Arabidopsis* γ-TMT. Putative hydrogen bonds and hydrophobic interactions are indicated by red and black dashed lines, respectively. The microenvironment differs significantly: the RspUbiE site is predominantly lined with polar residues, conferring binding flexibility conducive to catalysis. In contrast, in *Arabidopsis* γ-TMT, specific interactions (a hydrogen bond with H206 and hydrophobic contact with L261) rigidly fix the substrate, resulting in an unfavorable catalytic distance.

Structure superposition: The productive docking pose of SAM and β-tocopherol in RspUbiE was superposed onto the structure of mono-functional γ-TMT. This revealed severe steric clashes in the γ-TMT active sites, preventing productive binding of the intermediate (β-tocopherol) for a second methylation (Fig. R3A and B).

Flexible docking: When key γ-TMT active-site residues were allowed flexibility, the resulting optimal poses for SAM and β-tocopherol maintained a catalytic distance (>5 Å) too long for efficient methyl transfer (Fig. R3C), explaining the lack of a second catalytic step.

Active site microenvironment: Comparative analysis of the substrate-binding pockets revealed a fundamental architectural difference. The RspUbiE pocket is lined with polar residues, creating a flexible microenvironment that permits substrate repositioning between catalytic steps (Fig. R3D). In contrast, the γ-TMT pocket utilizes a rigid combination of specific hydrogen-bonding and hydrophobic interactions that "lock" the substrate (Fig. R3E), making it incompatible with the conformational changes required for the second methylation.

(2) We employed the site-directed mutagenesis of the conserved residues in *Rhodobacter* UbiE to further elucidate the catalytic mechanism. The results have added in the main text as follows:

"Furthermore, multiple sequence alignment of RspUbiE with other homologs (Supplementary Fig. S6) revealed that RspUbiE shares the conserved catalytic amino acid residues (Y36, R143, N144). The molecular docking results revealed suitable

conformational proximity of the β-tocopherol substrate and SAM to the conserved active sites (Fig. 3A). Based on the common methyltransferase mechanism in UbiE homologs [34], we proposed using β-tocopherol as an example: R143 or Y36 functions as a general base to abstract a proton from a water molecule, forming a negatively charged oxygen atom; this oxygen atom subsequently interacts with the hydrogen atom at the C22 position of β-tocopherol, inducing the deprotonation of C22 before methyl transfer; α-tocopherol is then produced following the methyl transfer reaction, while N144 could stabilize the reaction intermediate (Fig. 3B). Further mutation experiments (Y36A, N144A and R143A in RspUbiE) revealed that R143A and N144A completely abolished the methyltransferase activity on β-tocopherol substrate, whereas Y36A retained partial activity (Fig. 3C). These findings indicate that R143 serves as the essential general base for substrate activation, N144 plays an indispensable role in stabilizing the key reaction intermediate, and Y36, while not absolutely required, contributes a supporting role in substrate orientation or transition state stabilization. Therefore, biochemical verification results confirmed that UbiE from *R. sphaeroides* represents the first-time reported β-TMT as depicted in Fig. 1." (Line 191-209)

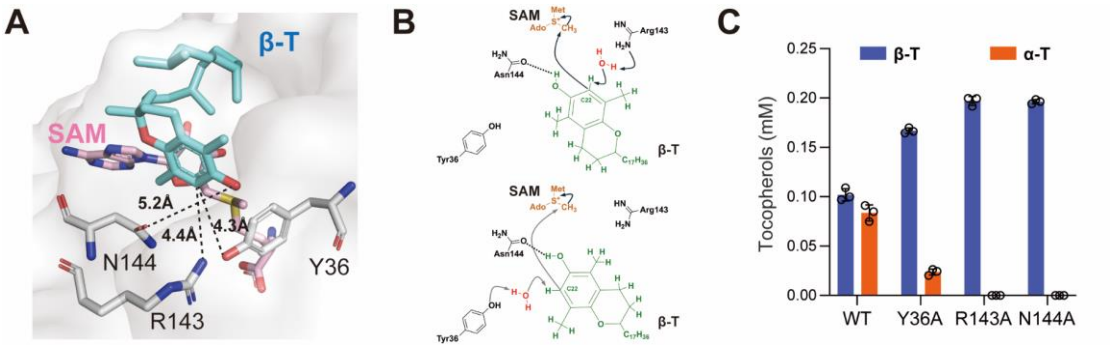

Fig. R4 Mechanism analysis of UbiE through molecular docking, mutation study and molecular dynamics (MD) simulations. A, Predicted binding pose of β-tocopherol (β-T) in the RspUbiE active site. Key residues Y36, R143 and N144 are shown as sticks, with distances (Å) to the substrate indicated by dashed lines. **B,** Hypothesized methyltransferase mechanism using β-tocopherol as a model based on the conserved UbiE-family mechanism and the docking pose shown in panel **A**. **C,** Mutation analysis to confirm the key amino acid residues involved in the β-TMT activity. UbiE with Y36A, N144A and R143A was tested for converting β-tocopherol to α-tocopherol at 24 h. WT, wild-type UbiE. All experiments were carried out in triplicates and data

represent the average ± SD from three biological repeats.

(3) We sought to purify *Rhodobacter* UbiE from *E. coli* for kinetics characterization. However, repeated attempts to use his-tag for purifying UbiE could not give high-purity UbiE (Figure below). The poor purity of UbiE prevented us kinetics characterization. As the β-TMT activity represents the first-time reported during tocopherol biosynthesis, we next attempted to carry out biochemical verification by monitoring β-tocopherol substrate depletion rate. Further time course analysis using β-tocopherol feeding revealed that UbiE from *R. sphaeroides* gradually converted β-tocopherol to the desired α-tocopherol (Figure 2E), and approximately 65% β-tocopherol was converted to α-tocopherol in 48 h. At this moment, we are confident that the novel β-TMT activity of UbiE is confirmed based on CRISPRi experiments and heterologous expression in *E. coli* via time course experiments. Meanwhile, we are still attempting to use different tagging systems and cell-free expression system to have good quality of purified UbiE, and the kinetics data will be reported in due course.

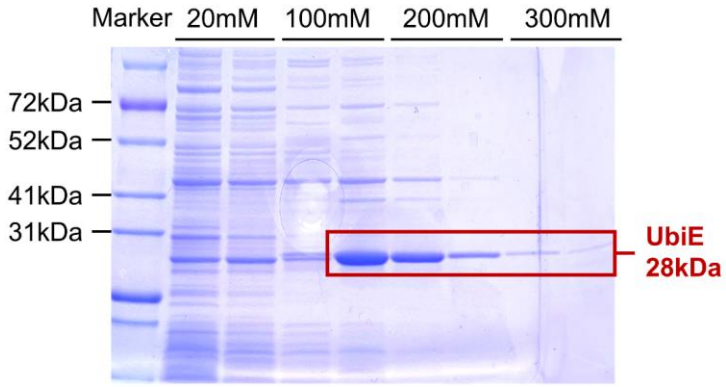

Fig. R5 SDS-PAGE gel of purified UbiE protein

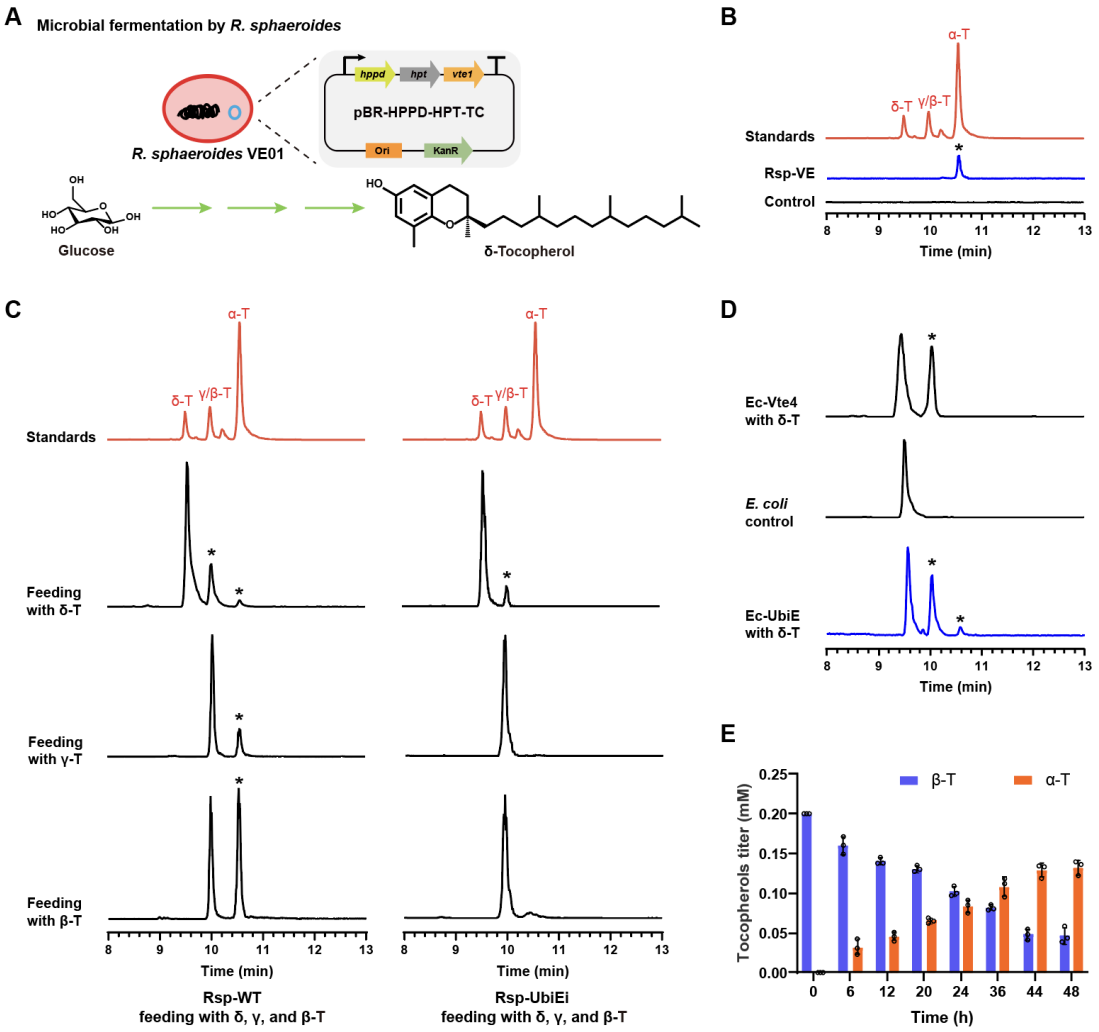

Fig. R6 Discovery of a nonclassical α-tocopherol biosynthetic pathway in *R. sphaeroides*. C, Whole cell lysate of *R. sphaeroides* results in synthesizing α-T from δ/γ/β-T. Rsp-WT, *R. sphaeroides* wild-type strain. Rsp-UbiEi indicates *R. sphaeroides* strain transformed with pBR-UbiEi for the repression of *ubiE* expression. **D,** Representative LC-MS chromatograms of heterologous characterization of RspUbiE in *E. coli*. Plasmid pRSF-Vte4 and pRSF-UbiE were transformed into *E. coli* BL21 (DE3) for preparing the whole cell lysate. Extracted ion chromatograms were acquired within the m/z range of 396-435. All experiments were carried out in three biological repeats and only representative chromatograms are presented. Asterisk (*) indicates the expected tocopherol products. **E,** Time course results of β-TMT activity of RspUbiE on converting β-tocopherol to α-tocopherol. Experiments were carried out using the cell lysate as the biocatalyst. Data represent the average ± SD from three biological repeats.

2. Reliance on computational simulations and predicted kinetic parameters is insufficient to support the manuscript's mechanistic claims. Based on our experience,

such in silico predictions frequently lack accuracy. We recommend that the authors conduct the following: (1) protein crystallography or cryo-electron microscopy to elucidate the three-dimensional structure of UbiE; (2) determination of kinetic parameters using purified recombinant enzyme; and (3) substrate specificity assays to validate the proposed bifunctional activity.

Response: We sincerely thank you for this suggestion.

(1) We agree with the view that resolving the three-dimensional structure of UbiE by protein crystallography or cryo-electron microscopy will facilitate a deeper elucidation of its catalytic mechanism. At the current stage, the discovery of a nonclassical biosynthetic route for tocopherol synthesis in *Rhodobacter* species that bypasses the classical α-tocopherol pathway requiring the 2,3-dimethyl-5-phytyl-l,4-benzoquinone (DMPBQ) as an intermediate already represents a big breakthrough as it is the first time to report β-TMT activity for tocopherol biosynthesis. Furthermore, we discovered that geranylgeranyl pyrophosphate reductase (GR) plays an important role in reducing tocotrienol into tocopherol. Protein crystallography or cryo-electron microscopy techniques are beyond our capability at this moment; we will seek collaborations to study the protein structure and employ mutagenesis approach to identify enzymes with improved biocatalytic activity and specificity in the future. To promptly share these new findings, we did not carry out protein crystallography or cryo-electron microscopy at this moment.

We have added related-content in the main text "Notably, elucidating the exact mechanism of enzyme selectivity towards different substrates will require efforts to determine high-resolution co-crystal structures, coupled with systematic site-directed mutagenesis and functional characterization.", which is expected to attract future research direction in this field.

(2) The UbiE purify and the related biochemical results are seen in the above question 1. We sought to purify *Rhodobacter* UbiE from *E. coli* for kinetics characterization. However, repeated attempts to use his-tag for purifying UbiE could not give high-purity UbiE. The poor purity of UbiE prevented us kinetics characterization.

3. To eliminate the possibility that other methyltransferases in the host chromosome

contribute to α-tocopherol synthesis, targeted knockout of UbiE should be performed. This genetic confirmation is essential to establish UbiE as the sole enzyme responsible for the observed tocopherol production phenotype.

Response: We appreciate this suggestion. However, UbiE is involved in the biosynthesis of coenzyme Q, which is essential for cell survival. Our attempts to knockout the *ubiE* gene were unsuccessful. Therefore, we have employed CRISPR interference (CRISPRi) to repress the expression of UbiE for the functional validation.

"Since UbiE is involved in coenzyme Q synthesis which is an essential step for cell survival, attempt to knockout *ubiE* was difficult. We next constructed engineered *R. sphaeroides* with UbiE repression for biocatalysis analysis. The cell lysate of wild-type *R. sphaeroides* could convert δ/β/γ-tocopherols to α-tocopherol, whereas its repression reduces α-tocopherol formation (Figure 2C). Therefore, we confirmed that UbiE from *R. sphaeroides* has the uncharacterized promiscuous γ/β-TMT activities involving in α-tocopherol biosynthesis, albeit the possibility of other MTs besides UbiE could not be completely ruled out as UbiE repression still produced a small amount of β/γ-tocopherols." (Line 169-177)

4. There is debate on the major source of PDP in photosynthetic organisms, whether primarily derived from chlorophyll degradation or from GGPP reduction. This distinction is critical, as heterologous overexpression of geranylgeranyl reductase (GR) in non-photosynthetic hosts has consistently failed in previous studies. Therefore, the authors must address whether GR overexpression also enhances tocopherol production—independent of and in parallel with GGPP supply enhancement. This experiment would clarify the contribution of the GR-mediated pathway.

Response: We appreciate this insightful comment, and we agree with the GR is important for tocopherols biosynthesis. Based on our results, tocotrienols were not in a detectable amount during LC-MS analysis in the engineered strain. We think that GR (encoded by *bchP*) might also catalyze the conversion of tocotrienols to tocopherols. As shown in Figure 4A, when δ-tocotrienol was fed to the whole cell lysate of *R. sphaeroides*, we did observe the production of different forms of tocopherols. We also examined the heterologous expression of *R. sphaeroides bchP* in *E. coli*. As shown in

Figure 4C, we found *bchP* overexpression alone could not convert δ-tocotrienol to δ-tocopherol. However, when the partner enzymes of FdxA and Fdr were co-expressed, we observed the formation of δ-tocopherol. These findings are of particular interest as it shed light on future tocopherol production in heterologous hosts like *E. coli* and yeast. We did not further test the effect of *bchP* overexpression on tocopherol production as it seems obvious to further improve the product titer.

We have revised the main text as follows:

"Based on the LC-MS results, we found that all forms of δ/β/γ/α-tocotrienols were not detected, indicating that tocotrienols might be converted to tocopherols by endogenous *R. sphaeroides* metabolism. As shown in Fig. 4A, we found that the wild-type *R. sphaeroides* transformed δ-tocotrienol to different forms of tocopherols, suggesting that its endogenous metabolism is capable of tocotrienol reduction. Since GR was reported to not only catalyze GGPP to PDP, but also reduce GGPP-derived later intermediates [27,35], we suspected that BchP (the GR from *R. sphaeroides* involved in bacteriochlorophyll synthesis) [27] might participate in converting tocotrienols to tocopherols (Fig. 4B)." (Line 229-237)

"To provide biochemical evidence of BchP's engagement in tocotrienol reduction, we further attempted to heterologously validate the BchP from *R. sphaeroide* in *E. coli*. It was found that the whole cell lysate of *E. coli* expressing *bchP* alone could not produce δ-tocopherol from δ-tocotrienol feeding (Fig. 4C). Additional expression of its coenzymes such as FdxA (ferredoxin II) and Fdr (NADPH-ferredoxin reductase) substantially enhanced the BchP-mediated reduction of δ-tocotrienol to δ-tocopherol (Fig. 4C). These findings suggested the failure of previous efforts towards functional expression of GR homologs might be attributed to the lack of compatible coenzymes in the heterologous hosts, whereas additional supplementation of their native FdxA and Fdr is expected to address this issue [35]. Our results on successful validation of BchP-mediated tocotrienol reduction shed light on future biomanufacturing of tocopherols in other microorganisms such as *E. coli* and yeast." (Line 238-249)

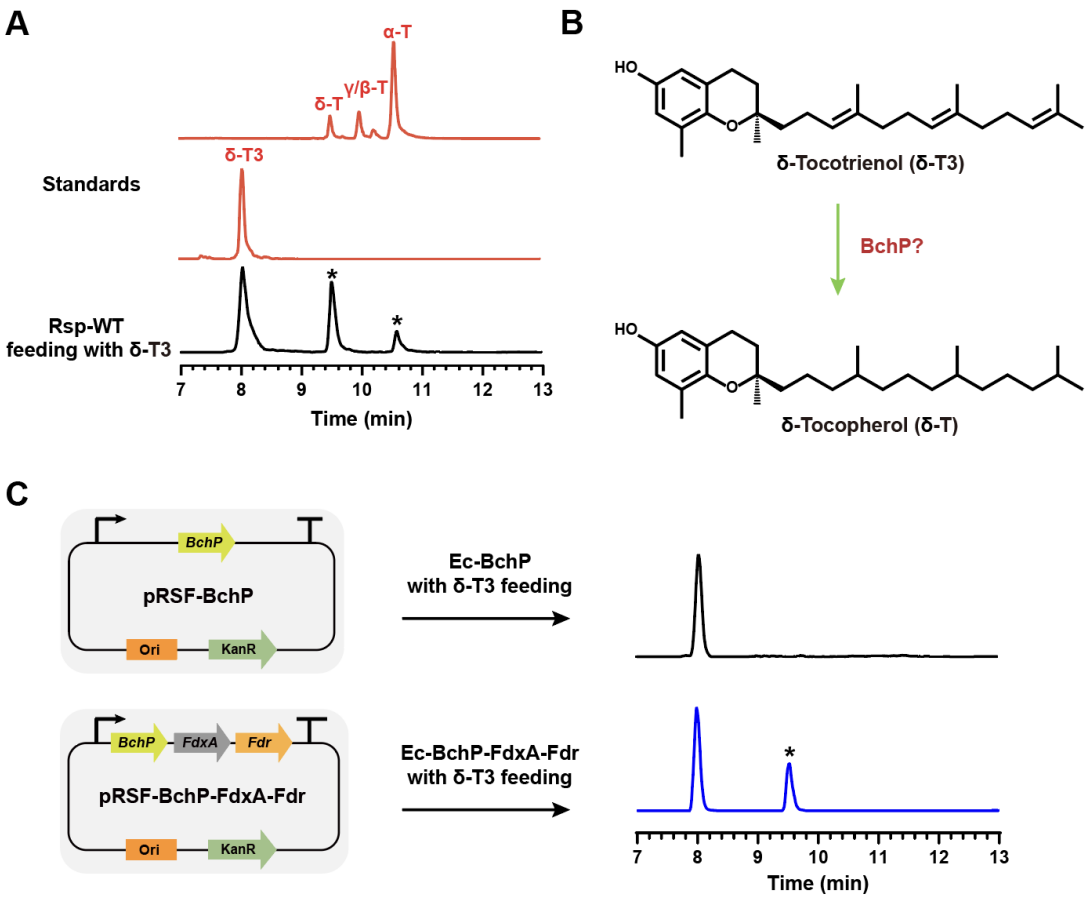

Fig. R7 Identification of BchP-mediated tocotrienol reduction. A, The whole cell lysate of *R. sphaeroides* for the synthesis of tocopherols from δ-tocotrienol. δ-T3, δ-tocotrienol. Representative LC-MS chromatograms are presented from triplicate of biological repeats. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected tocopherol products. **B,** Proposed mechanism of BchP-mediated tocotrienol reduction. **C,** Representative LC-MS results of δ-tocotrienol as substrate for δ-tocopherol production by the biotransformation of *E. coli* expressing BchP and BchP-FdxA-Fdr. Experiments were carried out using the cell lysate as the biocatalyst with 0.1 mM δ-T3 for 20 h. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected δ-tocopherol product.

5. The substantially improved tocopherol titer resulting from random chromosomal integration of the synthetic biosynthetic module suggests significant position-dependent effects. The authors should investigate the genomic context of integration sites and identify the underlying factors—such as proximity to transcriptional hotspots, effects on local chromatin structure, or influence from nearby genomic elements—that

enhance production.

Response: Thanks for your constructive suggestions. Tn5-mediated genome integration is a random process based on the recent findings (ACS Synth Biol 2025,14:2753-2763) [1], and also well documented by many previous studies. This study mainly focused on the discovery of a nonclassical biosynthetic route for tocopherol synthesis in *Rhodobacter* species that bypasses the classical α-tocopherol pathway requiring the 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ). We did not carry out further genome sequencing of various mutants to correlate the genotype with tocopherol productivity, which might be reported in a sperate study by employing multi-omics data.

6. The in vivo evaluation using Caenorhabditis elegans is informative; however, the current analysis is incomplete. While the authors emphasize the importance of feeding ratios between tocopherol-producing and wild-type bacteria, they overlook a critical variable: the impact of tocopherol composition. Since α-tocopherol is widely recognized as the most biologically active form, experiments specifically comparing batches with higher α-tocopherol content to those with lower α-tocopherol proportions would be expected to demonstrate enhanced bioactivity. This compositional effect warrants explicit investigation.

Response: Thank you for this suggestion. In *C. elegans*, α-tocopherol is the most extensively studied, with roles primarily in antioxidation, stress protection, and lifespan regulation, and exhibits a dose-dependent effect (Mech Ageing Dev 2007, 128:222-226; Indian J Biochem Biophys 2011, 48:191-196; Int J Food Sci Nutr 2019, 70:172-181)[2-4]. γ-Tocopherol shows stronger potential for lifespan extension and antioxidation than α-tocopherol, with no obvious toxicity at high doses (Mech Ageing Dev 2007, 128:222-226)[2]. Studies focusing individually on β-tocopherol or δ-tocopherol are scarce, and they are usually investigated together with mixed vitamin E preparations (J Gerontol A Biol Sci Med Sci 2000, 55:B280-B285; PLoS One 2013, 8:e71180)[5,6]. We agree with that the tocopherol compositional effect is worth studying for therapeutic applications. However, the *in vivo* evaluation using *Caenorhabditis elegans* is for the purpose of safety evaluation of *Rhodobacter*-based single cell proteins for animal feeds, rather than to investigate the effects of tocopherols on therapeutic applications in *C. elegans*.

Considering the space limit after adding additional experiments on characterization of UbiE and BchP, we decided to remove this section to avoid misleading information to the readers.

Minor issue:

1. The claim that "a new methyltransferase … is identified" is not strictly accurate. UbiE itself is not a novel methyltransferase; rather, a previously uncharacterized bifunctional activity of this enzyme has been newly demonstrated. The authors should revise this statement to reflect that the novel finding concerns the γ/β-tocopherol methyltransferase activity of UbiE, not the identification of an entirely new enzyme.

Response: Revised as recommended. We have revised the statement for more accurate expression.

Reviewer #2 (Remarks to the Author):

The manuscript by Zhang et al. describes the characterization and engineering of Rhodobacter sphaeroides as a production platform for tocopherols. The authors demonstrate the ability of native ubiquinone/menaquinone methyltransferase UbiE to convert heterologous δ-tocopherol into α-tocopherol via γ/β-tocopherols, thereby simplifying tocopherol biosynthesis in R. sphaeroides by bypassing the 2,3-dimethyl-5-phytyl-l-4-benzoquinone step of the classical pathway. Based on molecular docking and -dynamics simulations, they propose catalytically competent binding conformations which rationalize the identified enzyme activities.

In addition to reconstructing tocopherol biosynthesis in R. sphaeroides, the authors employ established engineering strategies to improve the supply of the central precursors, geranylgeranyl diphosphate and homogentisic acid. Further media optimization efforts showcase the benefit of supplementing methyl donors and antioxidants in the fermentation process, ultimately enabling gram-scale tocopherol production in a fed-batch bioreactor setup. Finally, the authors explore the dietary effects of the tocopherol-producing bacteria on the fitness of C. elegans. While the results largely suggested the equivalence of engineered R. sphaeroides to both the wild

type and commonly-fed E. coli, a slight benefit to reproductive fitness was observed at moderate tocopherol levels.

1. The experiments were well-executed, and the data were well-presented and convincing. The study not only establishes R. sphaeroides as a platform for microbial tocopherol production, but also makes UbiE available as a potential tool for synthetic biology. In this regard, an immediate question arising from this work is whether the geranylgeranyl diphosphate reductase from R. sphaeroides could facilitate the heterologous production of phytyl diphosphate in more common hosts. As the authors state in lines 103-105, insufficient PDP amounts are thought to be a major bottleneck in other systems — and the main reason the search for suitable hosts was expanded here. It would be very valuable if the authors could look into the possibility of identifying GGR homologs in the transcriptome at hand. At least though, I suggest that the authors revisit this GGR topic in the discussion.

Response: We appreciate this insightful suggestion. The geranylgeranyl pyrophosphate reductase encoded by *bchP* has been identified in *R. sphaeroides* (J Bacteriol 1999, 181:7248-7255)[7], and the relevant information was supplemented in the revised manuscript.

Since tocotrienol biosynthesis was not detected in the engineered strain, we hypothesized that GR not only converts GGPP to PDP but also catalyzes the conversion of tocotrienols to tocopherols. Accordingly, we have examined the effect of *bchP* on tocotrienol conversion in *R. sphaeroides* and further verified its catalytic activity in *Escherichia coli*. The main results are as follows:

"Based on the LC-MS results, we found that all forms of δ/β/γ/α-tocotrienols were not detected, indicating that tocotrienols might be converted to tocopherols by endogenous *R. sphaeroides* metabolism. As shown in Fig. 4A, we found that the wild-type *R. sphaeroides* transformed δ-tocotrienol to different forms of tocopherols, suggesting that its endogenous metabolism is capable of tocotrienol reduction. Since GR was reported to not only catalyze GGPP to PDP, but also reduce GGPP-derived later intermediates [27,35], we suspected that BchP (the GR from *R. sphaeroides* involved in bacteriochlorophyll synthesis) [27] might participate in converting tocotrienols to

tocopherols (Fig. 4B).” (Line 229-237)

“To provide biochemical evidence of BchP’s engagement in tocotrienol reduction, we further attempted to heterologously validate the BchP from *R. sphaeroide* in *E. coli*. It was found that the whole cell lysate of *E. coli* expressing *bchP* alone could not produce δ-tocopherol from δ-tocotrienol feeding (Fig. 4C). Additional expression of its coenzymes such as FdxA (ferredoxin II) and Fdr (NADPH-ferredoxin reductase) substantially enhanced the BchP-mediated reduction of δ-tocotrienol to δ-tocopherol (Fig. 4C). These findings suggested the failure of previous efforts towards functional expression of GR homologs might be attributed to the lack of compatible coenzymes in the heterologous hosts, whereas additional supplementation of their native FdxA and Fdr is expected to address this issue [35]. Our results on successful validation of BchP-mediated tocotrienol reduction shed light on future biomanufacturing of tocopherols in other microorganisms such as *E. coli* and yeast.” (Line 238-249)

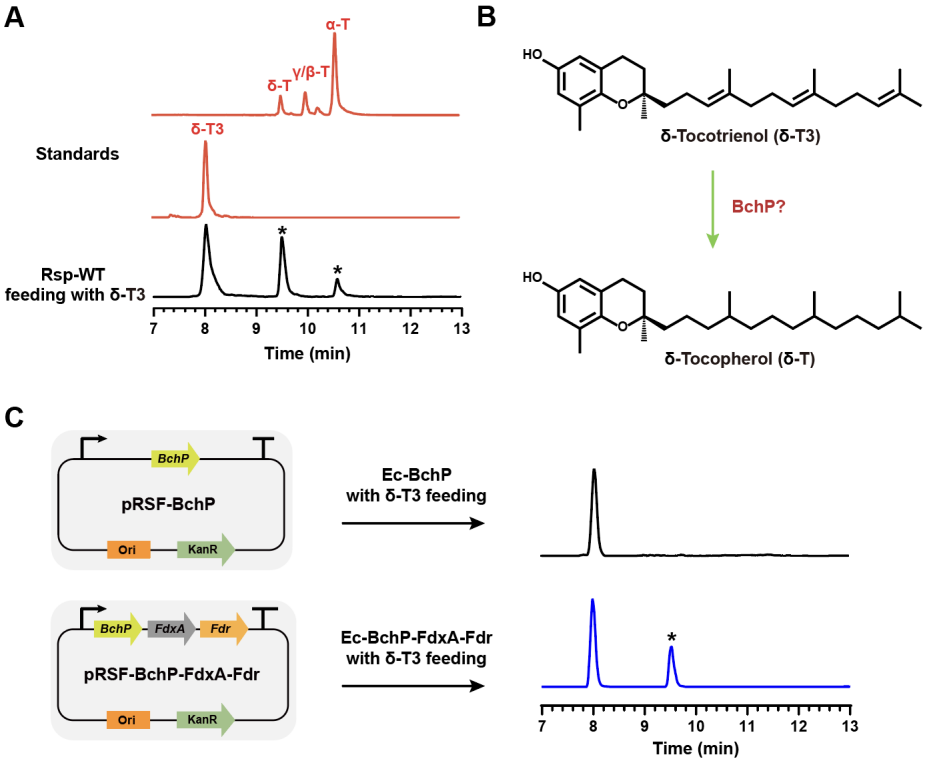

Fig. R8 Identification of BchP-mediated tocotrienol reduction. A, The whole cell lysate of *R. sphaeroides* for the synthesis of tocopherols from δ-tocotrienol. δ-T3, δ-tocotrienol. Representative LC-MS chromatograms are presented from triplicate of biological repeats. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected

tocopherol products. **B,** Proposed mechanism of BchP-mediated tocotrienol reduction. **C,** Representative LC-MS results of δ-tocotrienol as substrate for δ-tocopherol production by the biotransformation of *E. coli* expressing BchP and BchP-FdxA-Fdr. Experiments were carried out using the cell lysate as the biocatalyst with 0.1 mM δ-T3 for 20 h. LC chromatograms were extracted at the ranges of 396-435 m/z. Asterisk (*) indicates the expected δ-tocopherol product.

2. Another concern is that, especially throughout the conclusion and the abstract, some of the claims made should better reflect the experimental outcomes. For example, the phrasing of "novel biosynthetic pathway" and "new bifunctional methyltransferase" is overstated considering the final product of α-tophecorol exists, the enzymes are known, but the primary discovery is UbiE's novel activity. Similarly, comparison of feedstocks between the wildtype and modified R. sphaeroides for C. elegans suggests little difference to the benefit of the growth of the organism outside of the lengthening of the reproductive periods (though with less viable eggs) and may detract from the outcome. The observation that the feedstock mixture conferred an increase to the reproductive fitness of C. elegans is interesting but requires more testing to show the significance of this preliminary experiment. If you were to add α-tophecorol directly to the wildtype feedstock would it have a similar effect on the C. elegans as the biosynthesized α-tophecorol?

Response: Thanks for your constructive suggestions.

(1) We have revised the statement as recommended.

(2) According to the literature, α-tocopherol is the most extensively studied, with roles primarily in antioxidation, stress protection, and lifespan regulation, and exhibits a dose-dependent effect (Mech Ageing Dev 2007, 128:222-226; Indian J Biochem Biophys 2011, 48:191-196; Int J Food Sci Nutr 2019, 70:172-181)[2-4]. γ-Tocopherol shows stronger potential for lifespan extension and antioxidation than α-tocopherol, with no obvious toxicity at high doses (Mech Ageing Dev 2007, 128:222-226)[2]. Studies focusing individually on β-tocopherol or δ-tocopherol are scarce, and they are usually investigated together with mixed vitamin E preparations (J Gerontol A Biol Sci Med Sci 2000, 55:B280-B285; PLoS One 2013, 8:e71180)[5,6]. The nematode feeding experiment was designed to evaluate the safety of *Rhodobacter*-based single cell

proteins for animal feeds, rather than to investigate the effects of tocopherols on therapeutic applications in *C. elegans*. Considering the manuscript length is exceeding the space limit after adding additional experiments on characterization of UbiE and BchP, we decided to remove this section to avoid misleading information to the readers.

Minor Issues:

Phrasing around anti-cardiac benefits (line 39) suggests negative effects towards the cardiovascular system.

Response: We have revised the description as "cardiovascular prevention".

Inconsistent writing style for casing and italicization of gene names (for example line 166 and line 270) can be remedied.

Response: Revised as recommended. We have unified the writing style.

The sentence in line 163 "... indicating that the nonclassical biosynthetic route might be widespread in other bacteria" seems confusing given that these bacteria do not produce tocopherols. What was found to be widespread is the capability of ubiE to convert tocopherol substrates.

Response: In the revised manuscript, this sentence has been removed.

Correcting the vocabulary around line 306, "To access the the impact of SCPs…" can better inform the reader on the aim of the experiment.

Response: This section was removed to avoid misleading information to the readers as mentioned above.

Reviewer #3 (Remarks to the Author):

Zhang et al. report a nonclassical biosynthetic route for tocopherol production in Rhodobacter species and describe the identification of a putative bifunctional γ/β-tocopherol methyltransferase that may enable an alternative α-tocopherol synthesis route bypassing the classical DMPBQ intermediate. This topic is interesting and potentially relevant for microbial production of high-value tocopherols. The authors further combine enzyme characterization, metabolic engineering, and fermentation optimization to improve product titers. Overall, the study presents a potentially useful

direction for tocopherol biosynthesis research. However, some key experimental data and supporting validations are currently insufficient, and additional evidence is required to further strengthen the main conclusions. The following concerns should be addressed before publication.

Major Comment:

1. The authors state that vitamin E is classified into eight compounds, namely α, β, γ, δ-tocopherols and tocotrienols. According to established biosynthetic pathways, homogentisic acid (HGA) can condense not only with phytyl diphosphate (PDP) but also with geranylgeranyl diphosphate (GGPP) via HPT to form intermediates leading to tocotrienols. However, throughout the manuscript, only tocopherols are discussed, and no data are provided regarding the possible formation or exclusion of tocotrienols. It is therefore important to clarify whether tocotrienols are produced in the engineered strains. The authors should provide corresponding analytical evidence and explicitly distinguish tocopherols from tocotrienols. In particular, please clarify whether the chromatographic and mass spectrometric profiles of tocopherols and tocotrienols were resolved and how compound identities were assigned. In addition, chromatograms and mass spectra of authentic standards for the reported products should be provided and directly compared with the fermentation samples to support compound identification.

Response: We sincerely thank you for this suggestion.

(1) The LC-MS method described in the original manuscript allows for the separation and identification of tocopherols versus tocotrienols (LC chromatogram of authentic standards is shown in the figure below). Moreover, upon extracting the molecular ion peaks of target compounds from the fermentation samples, no characteristic ions corresponding to δ/β/γ/α-tocotrienols were detected in the mass spectra. We have supplemented relevant content regarding the absence of tocotrienol biosynthesis, as follows:

"Based on the LC-MS results, we found that all forms of δ/β/γ/α-tocotrienols were not detected, indicating that tocotrienols might be converted to tocopherols by endogenous *R. sphaeroides* metabolism. As shown in Fig. 4A, we found that the wild-type *R. sphaeroides* transformed δ-tocotrienol to different forms of tocopherols,

suggesting that its endogenous metabolism is capable of tocotrienol reduction. Since GR was reported to not only catalyze GGPP to PDP, but also reduce GGPP-derived later intermediates [27,35], we suspected that BchP (the GR from *R. sphaeroides* involved in bacteriochlorophyll synthesis) [27] might participate in converting tocotrienols to tocopherols (Fig. 4B)." (Line 229-237)

"To provide biochemical evidence of BchP's engagement in tocotrienol reduction, we further attempted to heterologously validate the BchP from *R. sphaeroide* in *E. coli*. It was found that the whole cell lysate of *E. coli* expressing *bchP* alone could not produce δ-tocopherol from δ-tocotrienol feeding (Fig. 4C). Additional expression of its coenzymes such as FdxA (ferredoxin II) and Fdr (NADPH-ferredoxin reductase) substantially enhanced the BchP-mediated reduction of δ-tocotrienol to δ-tocopherol (Fig. 4C). These findings suggested the failure of previous efforts towards functional expression of GR homologs might be attributed to the lack of compatible coenzymes in the heterologous hosts, whereas additional supplementation of their native FdxA and Fdr is expected to address this issue [35]. Our results on successful validation of BchP-mediated tocotrienol reduction shed light on future biomanufacturing of tocopherols in other microorganisms such as *E. coli* and yeast." (Line 237-249)

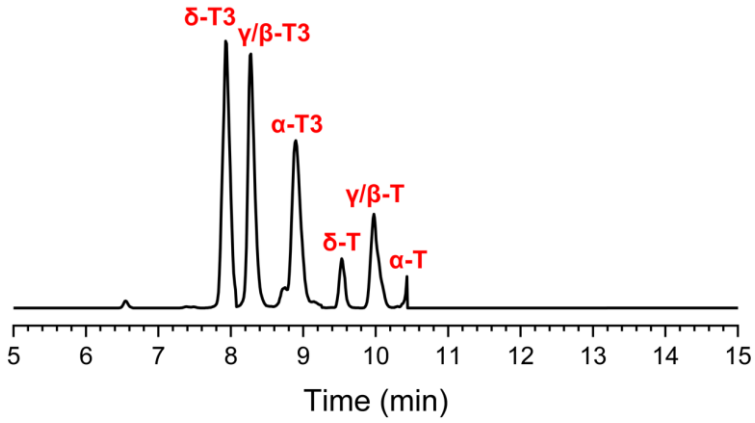

Fig. R9 Representative LC-MS chromatogram of different forms of tocopherols and tocotrienols. δ/β/γ/α-tocotrienols (δ/β/γ/α-T3); δ/β/γ/α-tocopherols (δ/β/γ/α-T).

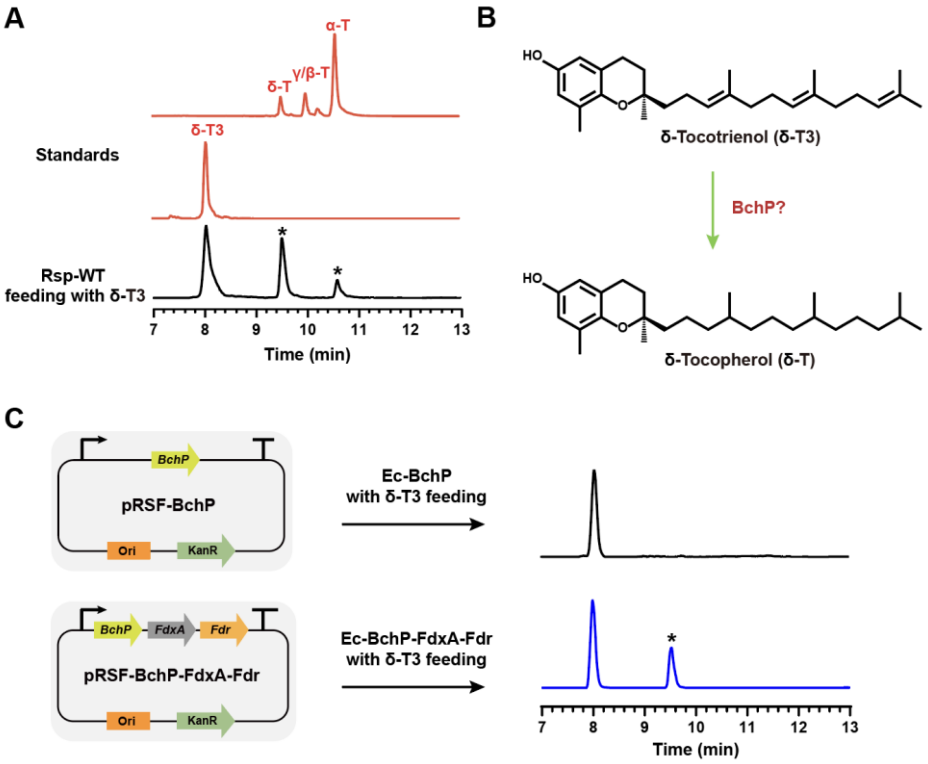

Fig. R10 Identification of BchP-mediated tocotrienol reduction. A, The whole cell lysate of *R. sphaeroides* for the synthesis of tocopherols from δ-tocotrienol. δ-T3, δ-tocotrienol. Representative LC-MS chromatograms are presented from triplicate of biological repeats. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected tocopherol products. **B,** Proposed mechanism of BchP-mediated tocotrienol reduction. **C,** Representative LC-MS results of δ-tocotrienol as substrate for δ-tocopherol production by the biotransformation of *E. coli* expressing BchP and BchP-FdxA-Fdr. Experiments were carried out using the cell lysate as the biocatalyst with 0.1 mM δ-T3 for 20 h. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected δ-tocopherol product.

(2) We have updated the MS spectra of α-tocopherol and γ/β-tocopherol. The characteristic ion peaks of the microbial fermentation extract were identical to that of the authentic α-tocopherol or γ/β-tocopherol standard, verifying the structural identity.

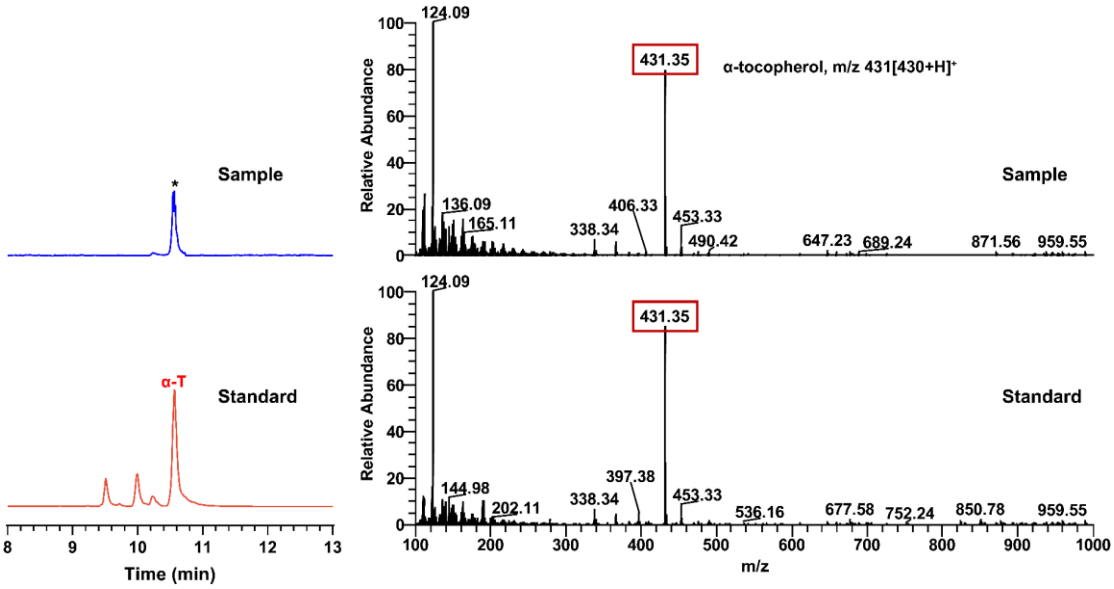

Fig. R11 Representative LC-MS results of mixed tocopherol standard and sample from strain Rsp-VE. The engineered *R. sphaeroides* Rsp-VE with the plasmid-based expression of *hppd-hpt-vte1* module was used. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected α-tocopherol product.

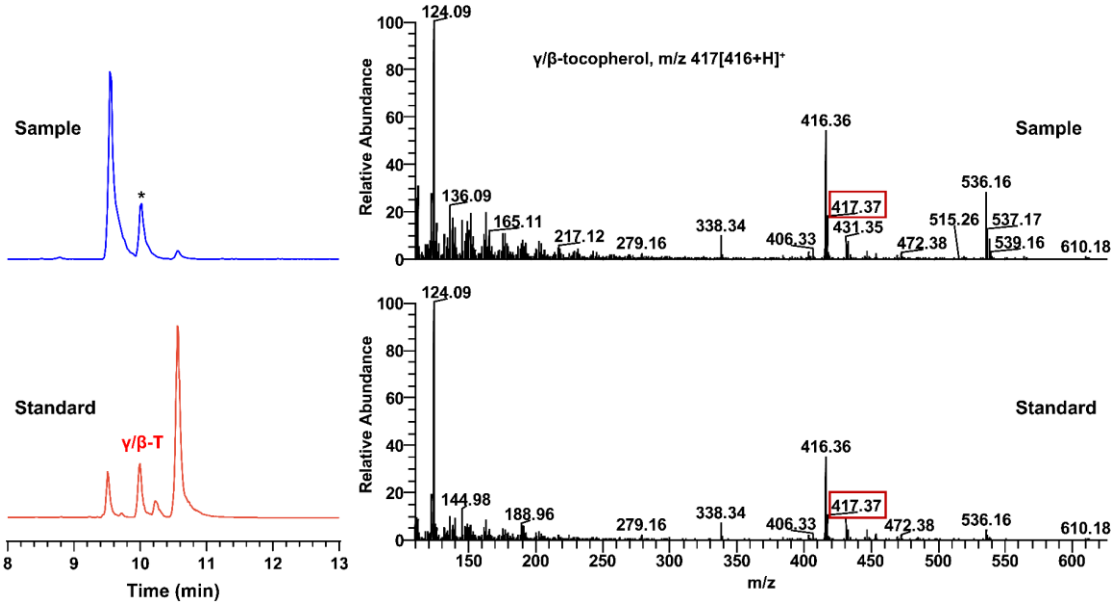

Fig. R12 Representative LC-MS results of mixed tocopherol standard and sample produced by the biotransformation of *R. sphaeroides*. The cell lysate of *R. sphaeroides* wild-type was reacted with 0.2 mM δ-tocopherol. Extracted ion chromatograms were acquired within the m/z range of 396-435. Asterisk (*) indicates the expected γ/β-tocopherol product.

2. More detailed descriptions are also needed regarding data processing of the MS spectra shown in Figure S1 and Figure S4, including whether background subtraction

or signal correction was performed. In addition, beyond LC-MS analysis of the fermentation products, further structural confirmation is recommended. The authors are requested to provide NMR characterization data (e.g., ^1H and/or ^13C NMR) of the key products to support compound identification.

Response: Thanks for your constructive suggestions.

(1) For the mass spectrometry data presented in Figures S1 and S4 (New version Figures S1 and S3 see above), the full mass spectra were generated by directly exporting the base peak within the m/z range of 396-435 in positive ion mode using the scan mode described in the Methods section, without additional correction processing.

(2) We have updated the MS spectra of α-tocopherol and γ/β-tocopherol. The characteristic ion peaks of the microbial fermentation extract were identical to that of the authentic α-tocopherol or γ/β-tocopherol standard, verifying the structural identity.

(3) The structures of key products including δ-, γ-, β-, α-tocopherols are well established. Given the consistent retention time and mass spectra between our samples and the commercial standards, the available data are fully adequate for product identification. Therefore, we hold the opinion that no further structural characterization is required at this stage.

3. Regarding the identification of the putative γ-TMT and MPBQMT homologues in R. sphaeroides, more comprehensive bioinformatic evidence is required. The authors should provide detailed sequence analyses in the Supplementary Information, including BLAST outputs, sequence alignments, coverage and identity metrics, conserved motif comparison, and phylogenetic analysis with characterized γ-TMT, MPBQMT, and related methyltransferases. The criteria for selecting the seed sequences used in the homology search should also be clearly described and justified.

Response: We appreciate this suggestion.

(1) We display the detailed analyses including BLAST outputs, sequence alignments, coverage and identity metrics (see graph below). Because the vitamin E biosynthetic pathway and the related key enzymes in *Arabidopsis thaliana* have been fully elucidated (Trends Plant Sci 2019, 24:1040-1051)[8], and its γ-TMT and MPBQMT have been functionally validated in yeast (Table S1), we first used γ-TMT and MPBQMT

from the well-studied *A. thaliana* as the seed sequences to identify the potential γ-TMT and MPBQMT homologues from *R. sphaeroides*. It's found that only UbiE displayed the low homology (30% similarity) with MPBQMT, while no significant similarity was found for the γ-TMT. Hence, we used γ-TMT from the well-studied prokaryotic *Synechocystis* sp. PCC6803 as the seed sequence, since the tocopherol biosynthesis in *Synechocystis* sp. PCC 6803 and the key enzymes are relatively well characterized and some enzymes have been functionally validated in *Escherichia coli* and yeast (Table S1).

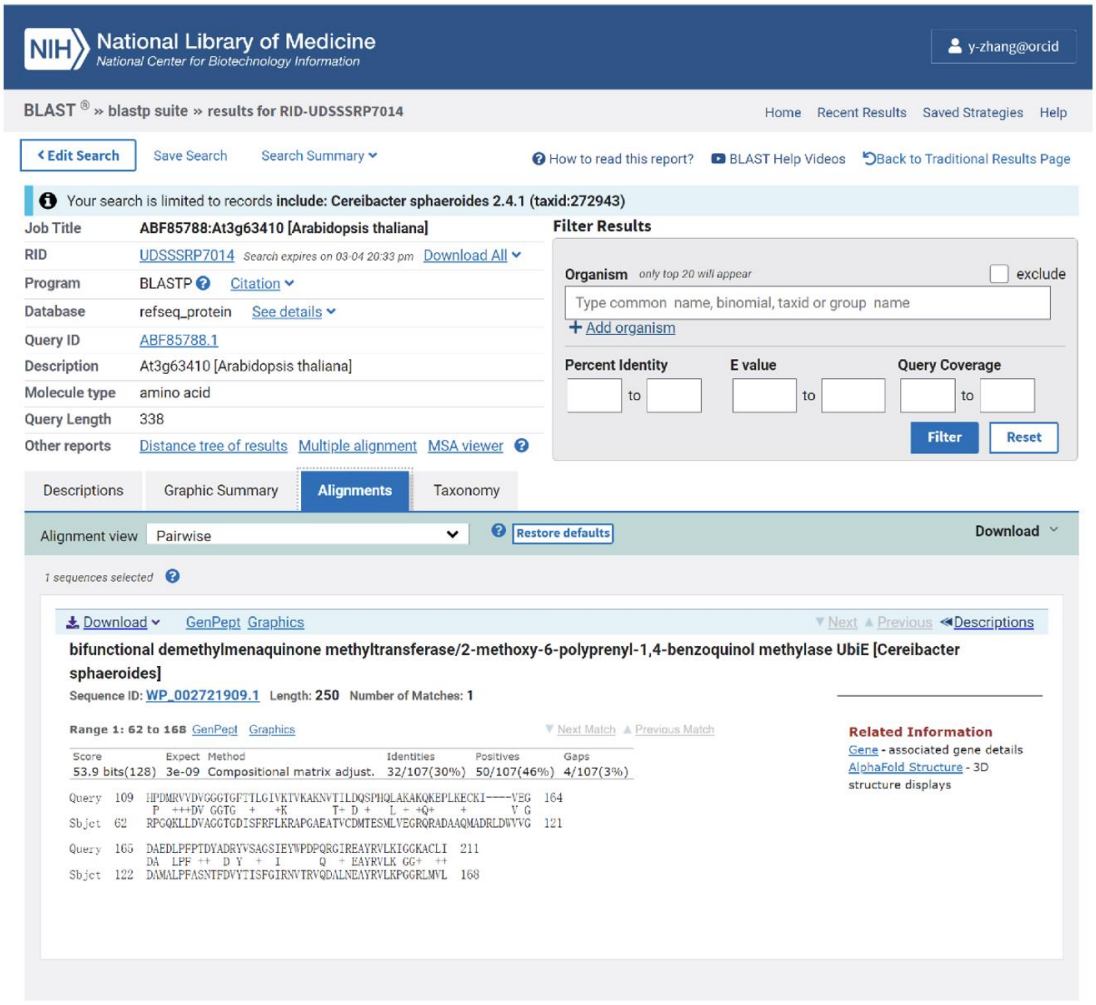

Fig. R13 A protein BLAST analysis to identify the potential γ-TMT homologues from *R. sphaeroides*. The γ-TMT (Protein ID: NP_176677.1) from the well-studied *A. thaliana* MTs was employed as the seed sequences using reference proteins as the database in NCBI.

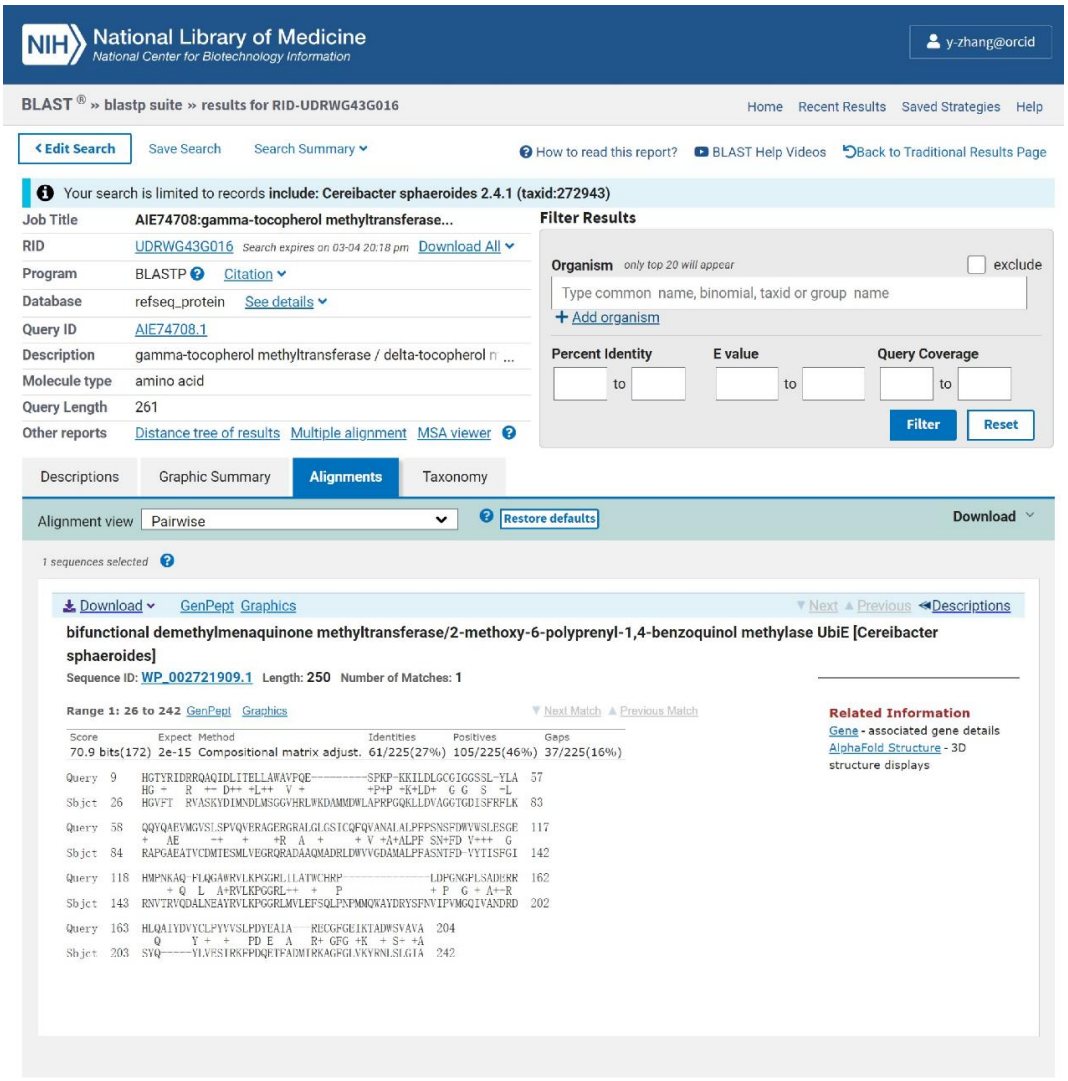

Fig. R14 A protein BLAST analysis to identify the potential γ-TMT homologues from *R. sphaeroides*. The γ-TMT (Protein ID: AIE74708.1) from *Synechocystis* sp. PCC6803 was employed as the seed sequences using reference proteins as the database in NCBI.

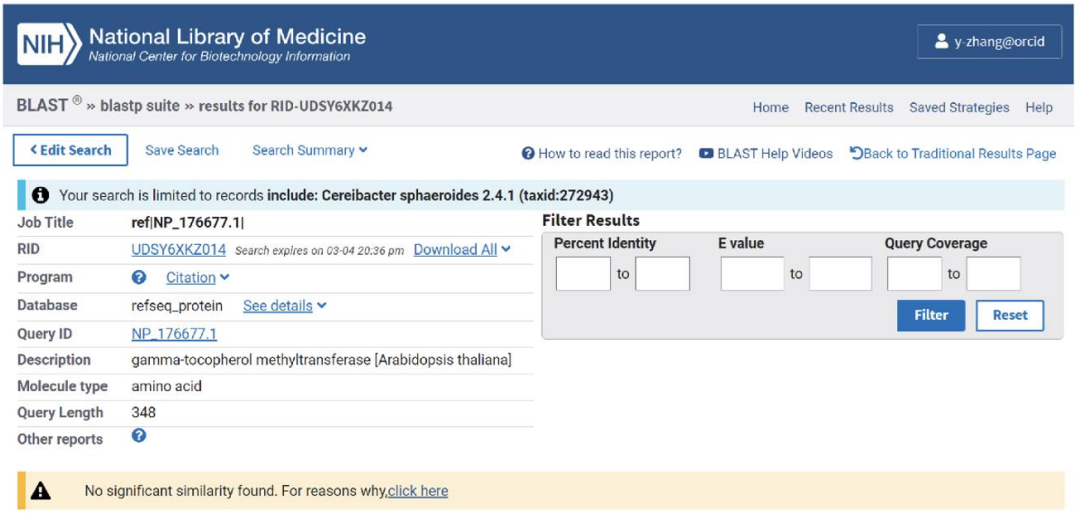

Fig. R15 A protein BLAST analysis to identify the potential MPBQMT homologues from *R.*

***sphaeroides*.** The MPBQMT (Protein ID: ABF85788.1) from the well-studied *A. thaliana* MTs was employed as the seed sequences using reference proteins as the database in NCBI.

(2) Multiple sequence alignment of RspUbiE with other homologs (Supplementary Fig. S6) revealed that RspUbiE shares the conserved catalytic amino acid residues (Y36, R143, N144). Further mutation studies indicate that R143 serves as the essential general base for substrate activation, N144 plays an indispensable role in stabilizing the key reaction intermediate, and Y36, while not absolutely required, contributes a supporting role in substrate orientation or transition state stabilization. Since UbiE is distinct from TMT and MPBQMT families, we think the phylogenetic tree to compare UbiE with characterized methyltransferases (TMT and MPBQMT) is not necessary.

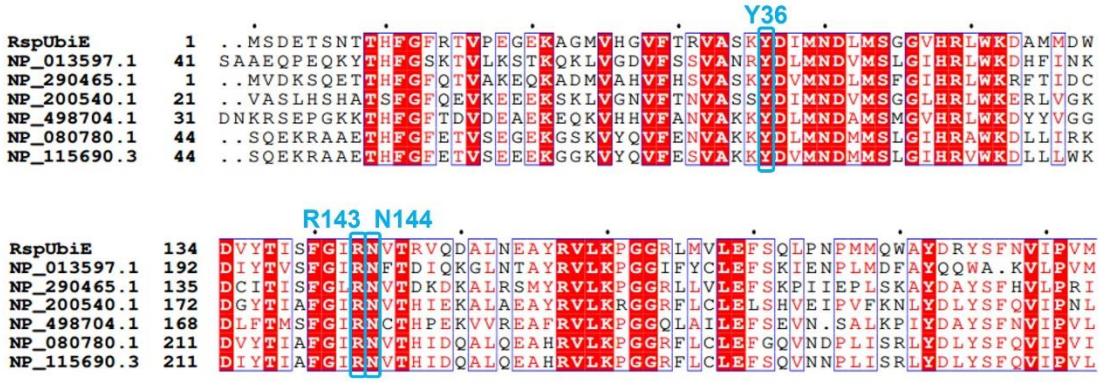

Fig. R16 Multiple sequence alignment of RspUbiE with other homologs. The putative residues responsible for substrate catalysis are indicated with blue boxes [6]. The sequences are (NCBI accession Nos. are given in parentheses) *S. cerevisiae* 2-hexaprenyl-6-methoxy-1,4-benzoquinone methyltransferase Coq5 (NP_013597.1), *E. coli* ubiquinone/menaquinone biosynthesis methyltransferase (NP_290465.1), *A. thaliana* 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase (NP_200540.1), *Caenorhabditis elegans* Coq5 (NP_498704.1), *Mus musculus* 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase (NP_080780.1) and *Homo sapiens* 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase (NP_115690.3).

4. A key claim of this work is the identification of a putative bifunctional γ/β-tocopherol methyltransferase (UbiE). However, its functional characterization could be better. The enzyme(s) from the relevant sources could be expressed and purified, and experimental kinetic parameters (Km, kcat, kcat/Km) measured. Predicted values alone by

Deepmolecules (Table S2) are not sufficient to support the claimed bifunctional activity.

Response: We sincerely thank you for this suggestion. We agree with that predicted values alone by Deepmolecules are not sufficient and we have removed these data. We further attempted to purify *Rhodobacter* UbiE from *E. coli*. However, we found that both his-tag fusion of UbiE could not give high-purity UbiE (see graph below), indicating that UbiE would interact with *E. coli* endogenous enzymes. As the β-TMT activity represents the first-time reported during tocopherol biosynthesis, we next attempted to carry out biochemical verification by monitoring β-tocopherol substrate depletion rate. Further time course analysis using β-tocopherol feeding revealed that UbiE from *R. sphaeroides* gradually converted β-tocopherol to the desired α-tocopherol (Figure 2E), and approximately 65% β-tocopherol was converted to α-tocopherol in 48 h.

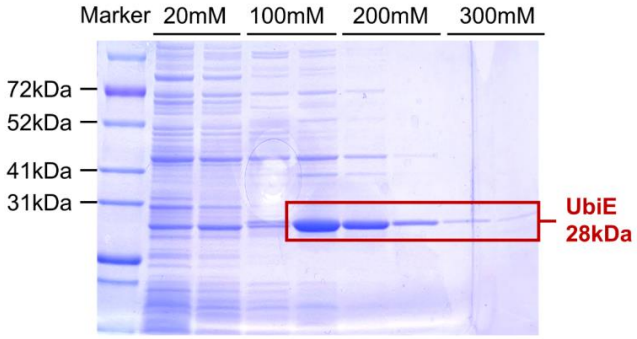

Fig. R17 SDS-PAGE gel of purified UbiE protein

5. The proposed molecular mechanism is based only on docking and MD simulations, without experimental structural validation. Experimental evidence (e.g., structural determination or site-directed mutagenesis with activity assays) is needed to support the mechanistic conclusions.

Response: We appreciate this suggestion. UibE is a methyltransferase for coenzyme Q biosynthesis, there is no mechanistic difference between coenzyme Q substrate and tocopherol substrate. The simple explanation of the substrate binding pocket of UbiE is capable of accommodating the binding of tocopherol substrate, that means the enzyme promiscuity of UbiE is relatively broad. It is unlikely that there will be another methyltransferase active site beyond coenzyme Q site. The site-directed mutagenesis of the conserved residues completely abolished the methyltransferase activity of

Rhodobacter UbiE. These results could further support the mechanism as follows:

"Furthermore, multiple sequence alignment of RspUbiE with other homologs (Supplementary Fig. S6) revealed that RspUbiE shares the conserved catalytic amino acid residues (Y36, R143, N144). The molecular docking results revealed suitable conformational proximity of the β-tocopherol substrate and SAM to the conserved active sites (Fig. 3A). Based on the common methyltransferase mechanism in UbiE homologs [34], we proposed using β-tocopherol as an example: R143 or Y36 functions as a general base to abstract a proton from a water molecule, forming a negatively charged oxygen atom; this oxygen atom subsequently interacts with the hydrogen atom at the C22 position of β-tocopherol, inducing the deprotonation of C22 before methyl transfer; α-tocopherol is then produced following the methyl transfer reaction, while N144 could stabilize the reaction intermediate (Fig. 3B). Further mutation experiments (Y36A, N144A and R143A in RspUbiE) revealed that R143A and N144A completely abolished the methyltransferase activity on β-tocopherol substrate, whereas Y36A retained partial activity (Fig. 3C). These findings indicate that R143 serves as the essential general base for substrate activation, N144 plays an indispensable role in stabilizing the key reaction intermediate, and Y36, while not absolutely required, contributes a supporting role in substrate orientation or transition state stabilization. Therefore, biochemical verification results confirmed that UbiE from *R. sphaeroides* represents the first-time reported β-TMT as depicted in Fig. 1." (Line 191-209)

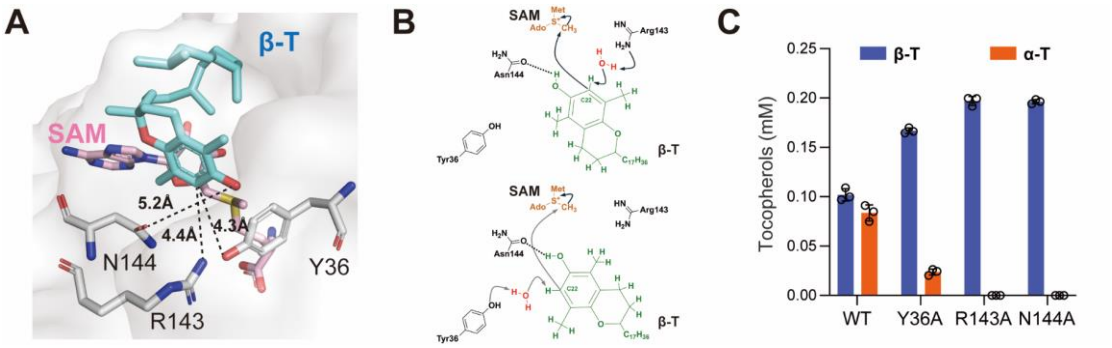

Fig. R18 Mechanism analysis of UbiE through molecular docking, mutation study and molecular dynamics (MD) simulations. A, Predicted binding pose of β-tocopherol (β-T) in the RspUbiE active site. Key residues Y36, R143 and N144 are shown as sticks, with distances (in Å) to the substrate indicated by dashed lines. **B,** Hypothesized methyltransferase mechanism using β-

tocopherol as a model based on the conserved UbiE-family mechanism and the docking pose shown in panel **A**. **C,** Mutation analysis to confirm the key amino acid residues involved in the β-TMT activity. UbiE with Y36A, N144A and R143A was tested for converting β-tocopherol to α-tocopherol at 24 h. WT, wild-type UbiE. All experiments were carried out in triplicates and data represent the average ± SD from three biological repeats.

6. Since the metabolic engineering was mainly performed via Tn5-based integration, the authors could provide experimental evidence for the copy number of the integrated gene(s) in the final selected strains (e.g., qPCR/ddPCR-based copy-number quantification or an equivalent genomic validation).

Response: Thanks for your constructive suggestions. Tn5-mediated genome integration is a random and potential multiple copy process based on the recent findings (ACS Synth Biol 2025,14:2753-2763)[1], and also well documented by many previous studies. This study mainly focused on the discovery of a nonclassical biosynthetic route for tocopherol synthesis in *Rhodobacter* species involving the UbiE with the uncharacterized γ/β-tocopherol methyltransferase activity. In addition, functional reconstitution of BchP-mediated δ-tocotrienol reduction would pave the way for future biomanufacturing of tocopherols in other microorganism such as *E. coli* and yeast. Considering the limited space of manuscript, and we did not carry out further genetic analysis of the optimal recombinant strain to correlate the genotype with tocopherol productivity. We agree that deciphering the underneath mechanism for high productivity by employing multi-omics data will be of particular interest, which might be reported in a sperate study.

7. In Figure 4A, the meaning and design of "MEV1–13" are not defined, and the corresponding construction rationale and dataset are not clearly presented. Please provide a clear description and the underlying data supporting these engineered strains. In Figure 4B, the purpose of distinguishing Library A and Library B is unclear. If hppd-hpt-vte-aroG*-tyrA* were not independently regulated, the specific role and necessity of Library A in the experimental design should be clarified.

Response: We appreciate this suggestion. We have adjusted the structure of this manuscript, and updated the description on MEV1–20 strains. The meaning and design

of MEV1–20 strains are to construct a GGPP/PDP-overproducing platform, which is one of the most important precursors to tocopherol biosynthesis, by integrating a heterologous mevalonate (MEV) pathway using transposon mutagenesis (see section "**Engineering a GGPP/PDP-overproducing platform in *R. sphaeroides***").

Considering the manuscript has too many sections after adding additional experiments on characterization of UbiE and BchP, we decided to remove the section about Library A of integrating the *hppd-hpt-vte1* module to avoid misleading information to the readers. Now, only one mutant library of integrating the *hppd-hpt-vte1* and *aroG*-tyrA** modules is shown in the revised manuscript (see section "**Engineering *R. sphaeroides* for high-level tocopherol production**").

8. In Figure 4C, the origin and genotype of strains VE22–25 are not clearly indicated, and the linkage between strain construction and the presented data lacks continuity. Please clarify where these strains are defined and how they were derived. It is recommended to provide a flowchart of strain engineering and key modification steps in the Supplementary Information, as well as a complete genotype table for all constructed strains, to improve clarity and reproducibility (see, for example, Zhu et al., Nat Catal 3, 64–74 (2020), Supplementary Fig. 14 and Supplementary Table 3).

Response: We appreciate this suggestion. In the origin manuscript, we did not construct strains VE22–25. We have updated the strains' names and provided a flowchart of strain engineering and key modification steps in the Supplementary Information, as well as a complete genotype table for all constructed strains, to improve clarity and reproducibility according to the reference (Nat Catal 2020, 3:64–74).

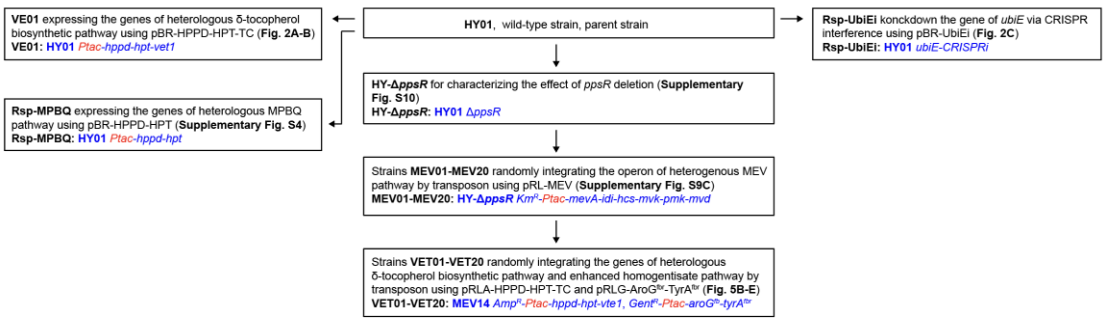

Fig. R19 The flowchart for constructing strains for tocopherol production. Names in bold and blue colour are the background strains, and genetic manipulations are highlighted in blue. Promoters

are shown in red.

9. VE01 (plasmid-based expression) reportedly produces only α-tocopherol, whereas Tn5-integrated strains generate multiple tocopherol species; this difference should be explained and supported with data (e.g., expression level and flux effects). Please also provide in vitro enzyme data showing how product distribution varies with substrate (and SAM) concentrations, and discuss whether balancing HGA, PDP, and SAM supply in vivo could drive more complete conversion to α-tocopherol.

Response: Thanks for your suggestions. In strain Rsp-VE (VE01 in the original manuscript), we only expressed the *hppd-hpt-vte1* module via the plasmid. The low metabolic flux toward the precursor synthesis (HGA and GGPP/PDP) resulted in low tocopherol production. Under these conditions, endogenous SAM synthesis was sufficient to drive the complete conversion of δ-tocopherol into α-tocopherol. However, in the high-production strains, we deleted *ppsR*, a transcriptional repressor of GGPP/PDP synthesis, and integrated the heterologous MEV pathway via transposon to construct a high-yield platform for the precursor GGPP/PDP. Based on this platform, we further integrated the *aroG*-tyrA** module via transposon to enhance the synthesis of 4-HPP (the direct substrate of HGA) as well as the *hppd-hpt-vte1* module, screening high tocopherol-producing strains through color phenotype. Under these high-flux conditions, endogenous SAM synthesis clearly became insufficient to meet the demand for α-tocopherol conversion, which is also consistent with the experiment of Met supplement. Consequently, multiple forms of tocopherols accumulated in the high production strains.

We agree with that the enzyme data facilitate the more understanding for α-tocopherols biosynthesis. At the current stage, the focus of this work lies in the discovery of a nonclassical biosynthetic route for tocopherol synthesis in *Rhodobacter* species that bypasses the classical α-tocopherol pathway requiring the DMPBQ as an intermediate already represents a big breakthrough as it is the first time to report β-TMT activity for tocopherol biosynthesis. Furthermore, we discovered that geranylgeranyl pyrophosphate reductase (GR) plays an important role in reducing tocotrienol into tocopherol. As a proof-concept, we found that a simple transposon-

based strain engineering could result in a high-level production of tocopherols to 3.5 g/L. We do agree the suggested experiments will favor in-depth mechanistic understanding on tocopherol overproduction, but it is beyond our scope for this study. We also think that, for the future commercialization of microbial synthesis of tocopherols, continuous efforts should be implemented in the following aspects: (a) utilizing the low cost of feedstocks such as lignocelluloses; (b) restricting the spheroidenone and carotenoids to divert more GGPP flux to tocopherol synthesis; (c) strengthening intracellular SAM regeneration to promote the transformation of δ- and γ/β-tocopherols into α-tocopherol, thereby reducing the cost of exogenous methyl donors; (d) introducing membrane transporters to export tocopherols and reduce intracellular stress; (e) optimizing fermentation parameters for the pilot scale production of tocopherols.

10. In Fig. 6B and 6D, the tocopherol composition clearly shifts after SAM regulation, and δ-tocopherol is no longer detected. This is intriguing and suggests that sufficient SAM supply can effectively promote further substrate conversion. However, it is unclear why the authors did not further strengthen endogenous UbiE expression to take advantage of this effect. In addition, for strain VE30, please provide the 5-L fed-batch results under MET supplementation alone (if tested), and include a more complete fermentation characterization, such as key by-product profiles, methionine and related metabolite changes, and yield calculations.

Response: We appreciate this insightful suggestion, and we agree with the UbiE overexpression will obviously improve α-tocopherol biosynthesis. However, due to lack of available selection marker for further genetic modification at this moment, we focused on culture medium optimization by supplementing methyl donor such as SAM and Met. In future study, we will further investigate the effect of additional UbiE and BchP overexpression on tocopherol production.

In addition, the fermentation by strain VET05 (VE30 in the original manuscript) under the Met supplement alone was clearly inferior to that under supplements of Met and 2-HC (Fig. 5D). Thus, in the original manuscript, we conducted the 5-L fed-batch fermentation under supplements of Met and 2-HC instead of Met supplement alone.

Given that the primary finding is the nonclassical biosynthetic route for tocopherol synthesis, we did not investigate further optimization of fermentation process on tocopherol production in this work. It is certain that further fermentation parameter adjustment such as pH, dissolved oxygen, substrate feeding rate will increase the product levels.

11. The authors should also provide complete plasmid maps for all constructs and clearly indicate their correspondence with the plasmid names and descriptions in the main text and Supplementary Information.

Response: Thank you for this suggestion. We have provided all related gene sequence for reproducible results. All plasmid maps will be provided upon request.

Minor comments

1. In the main text (Line 8) and Supplementary Information (Line 11), please remove the extra space after "2".

Response: Revised as recommended.

2. In Fig. 1, it would be helpful to label proteins together with their corresponding gene names to improve clarity and readability.

Response: We think it is more appropriate to present only enzyme names in Figure 1 as there are a number of genes from different organisms.

3. In Supplementary Table S1 and the related discussion, please include and discuss the most recent relevant literature (e.g., https://doi.org/10.1016/j.bej.2026.110079), which appears to be directly pertinent to the topic.

Response: Revised as recommended. We have added this relevant literature in the Supplementary Table S1 and the related discussion.

4. Line 96: revise to "cyclization and/or methylation".

Response: Revised as recommended.

5. The manuscript structure could be improved by presenting the complete synthetic biology and pathway engineering section first, and then the application results, to enhance clarity and narrative flow.

Response: Revised as recommended. We have revised the structure of the manuscript

by presenting the complete synthetic biology and pathway engineering section first, and then the application results.

6. In Fig. S7A, please remove the extra “v” at “mvk”.

Response: Revised as recommended.

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