

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

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Communications Chemistry.

Gene Conversion-Associated Successive Engineering of Modular Polyketide Synthases

Corresponding Author: Professor Yijun Chen

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This reviewer submits the report only in a confidential note.

Reviewer #2

(Remarks to the Author)

The authors have addressed most of my comments appropriately. However, the overall quality of the manuscript is still not at publication level, and several issues need to be addressed before manuscript acceptance.

1. Manuscript writing needs a thorough revision.

L34 "Previous notion" should be explicitly explained to non-experts.

L35 "Collectively,..." This sentence is entangled with three concepts and hard to digest.

L69 "Abe boundaries" I would suggest using "updated module boundary" as proposed by Keatinge-Clay instead of inventing a new word to avoid terminology confusion.

L77 "try-and-correct" is not a standard word. Perhaps a "try-and-error" or "test and evaluation" process?

L91 "the preservation of exquisite compatibility" This sentence does not sound accurate. The compatibility is merely a result of random gene conversion events after evolutionary screening, and is not a guaranteed characteristics. Existing PKSs are the result after natural selection, but if one performs gene conversion experiment artificially, gene conversion can also lead to collapsed assembly line.

L135 It is unclear why gene conversion can lead to the discovery of related gene clusters. Gene conversion is an event within a gene cluster after BGCs being spread into different host strains, and it is irrelevant with other BGCs.

L180 It is unclear what challenges are mentioned here.

L206 "the recommend replacement boundaries" should be "the reported boundaries"

L209 "exogenous elements" It is unclear what are exogenous elements. Also, this sentence sounds like a suggestion, not like a principle.

L216 It is unclear which homologies are compared here.

L299 "could produce" to be produced.

L300 "un unchanged ration of" the ration of 18a and 18b/c seems to be different between S5 and S5-MgmACP5.

2. Citations are not adequate.

L57 Ref.10 citation does not seem to be appropriate because it focuses on exchange boundary optimization by biosensor-guided screening but not structure-guided enzyme design.

L77 "recent studies..." citations are needed to indicate which recent studies are mentioned here. Recent works by Keatinge-Clay (<https://doi.org/10.1038/s41467-024-50844-6>) and Zhang (<https://doi.org/10.1038/s41589-025-01878-4>) can be added.

L88 Citation is missing.

3. Please check figure and table citation numbers. These must be more errors and the authors should check thoroughly.

L100 Table S1 to be S2?

L153 Table S2 to be S3?

L235 Fig. 4a does not seem to illustrate this sentence.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

All of my comments were properly addressed.

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Responses to Reviewer' s Comments

Comments from Reviewer 2

The authors have addressed most of my comments appropriately. However, the overall quality of the manuscript is still not at publication level, and several issues need to be addressed before manuscript acceptance. .

Response:

Thank you for your time and valuable comments on the careful and constructive evaluation of our manuscript. According to your comments, we have revised the manuscript for clarity and improvements.

1. Manuscript writing needs a thorough revision.

L34 "Previous notion" should be explicitly explained to non-experts.

L35 "Collectively,..." This sentence is entangled with three concepts and hard to digest.

L69 "Abe boundaries" I would suggest using "updated module boundary" as proposed by Keatinge-Clay instead of inventing a new word to avoid terminology confusion.

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L209 "exogenous elements" It is unclear what are exogenous elements.

Also, this sentence sounds like a suggestion, not like a principle.

L216 It is unclear which homologies are compared here.

L299 "could produce" to be produced.

L300 "an unchanged ratio of" the ratio of 18a and 18b/c seems to be different between S5 and S5-MgmACP5.

Responses:

In the revision, we have made corresponding changes to ensure the accuracy and scientific soundness. Detailed changes or responses are listed as follows:

L34 and L35: Moreover, the intra-module KS domain is demonstrated to associate with the proofreading of extender units. Collectively, the gene conversion-associated approach may shed a new light on modular PKS engineering.

L69: updated module boundaries

L77: try-and-error

L91: Nevertheless, although current knowledge on gene conversion is limited, emulating gene conversion events may offer a new possibility of achieving successive PKS engineering.

L135: Thanks for your comment. In our understanding, the phenomenon of gene conversion can allow to produce multiple copies of a particular element within the gene cluster, thereby increasing the likelihood of retaining this element after horizontal transfer to another strain for an independent evolutionary event. In this study, mgm gene cluster showed significant changes compared to cmm gene cluster, but mgm cluster still possessed highly homologous fragments,

which might be a result of the combination of gene conversion and other evolutionary events.

L180: By emulating the process of gene conversion and employing a rational selection of the embedded elements and their boundaries, it might be possible to expand the application scope for PKS engineering.

L206: the reported boundaries

L209: if the elements originated from other sources are selected to replace, high sequence homology to host BGCs should be a major consideration on the selection.

L216: In accordance with guideline iii), the MgmD2-AT5c region showed higher homology to CmmD2-AT4c than MgmD2-AT4c region (55.28% vs. 50.55%).

L299: produced

L300: a similar ratio

2. Citations are not adequate.

L57 Ref.10 citation does not seem to be appropriate because it focuses on exchange boundary optimization by biosensor-guided screening but not structure-guided enzyme design.

L77 "recent studies..." citations are needed to indicate which recent studies are mentioned here. Recent works by Keatinge-Clay (<https://doi.org/10.1038/s41467-024-50844-6>) and Zhang (<https://doi.org/10.1038/s41589-025-01878-4>) can be added.

L88 Citation is missing.

Responses:

L57: We apologize for misusing the reference. Ref. 10 has been replaced in the revision.

Su, L., et al. (2022). Engineering the stambomycin modular polyketide

synthase yields 37-membered mini-stambomycins. Nat. Commun. 13, 515.
<https://doi.org/10.1038/s41467-022-27955-z>.

L77: Thank you for your suggestions. These references have been added.

Ray, K. A., et al. (2024). Assessing and harnessing updated
polyketide synthase modules through combinatorial engineering. Nat.
Commun. 15, 6485. <https://doi.org/10.1038/s41467-024-50844-6>.
Huang, Z., Xie, S., Liu, R. Z., Xiang, C., Yao, S., & Zhang, L.
(2025). Plug-and-play engineering of modular polyketide
synthases. Nat. Chem. Biol. <https://doi.org/10.1038/s41589-025-01878-4>.

L88: Ref. 28-30 has been added in the revision.

3. Please check figure and table citation numbers. These must be more
errors and the authors should check thoroughly.

L100 Table S1 to be S2?

L153 Table S2 to be S3?

L235 Fig. 4a does not seem to illustrate this sentence.

Response:

We appreciate your careful evaluation to point out the errors. These
errors have been corrected in the revised version.



Response to reviewer 1

Reviewer #1 (Remarks to the Author):

The authors have carefully and to a large degree satisfyingly revised the paper. The paper can be published subject to the following suggestion:

I disagree with the statement from the authors on "Response #12: We thank the reviewer's suggestions. We have carefully examined these studies and compared them with our works. However, these studies all focus on the quenching, instead of photobleaching. Quenching would cause the decrease of fluorescence lifetime and brightness, which is not the case in our EF-G variants (Figures 1-3). Thus, we mainly focus on the mechanism of photobleaching. Nevertheless, to lay out the big picture for readers, we have cited these studies in our discussion section as shown in the response #5."

Two queries: Not all papers are only about quenching of closeby amino acids. This is incorrect and thus the relevance of the papers was not properly acknowledged. Secondly, photobleaching and quenching can be correlated.

Response #1: We sincerely appreciate the reviewer for highlighting these important points. Indeed, as correctly noted, reference #27 investigated the effects of Trp, Phe, and Tyr residues on photobleaching and brightness using single-molecule fluorescence assays. Following the reviewer's valuable suggestion, we have carefully revised the discussion section and incorporated the following paragraph on page 9:

"Previous studies have demonstrated that amino acids can quench fluorophores through either static stacking or dynamic collision mechanisms, which concomitantly reduce both fluorescence intensity and lifetime [17, 20, 27, 28]. However, our measurements reveal that photostability-reducing residues exert minimal effects on fluorescence intensity while prolonging the lifetimes of cyanine dyes. These observations suggest these residues mediate photobleaching through alternative pathways rather than conventional static or dynamic quenching mechanisms. DNA conjugation of a single Trp residue to either Cy5 or Atto647N has been shown to reduce photostability while slightly decreasing brightness, consistent with our current observations [27]. Notably, under identical experimental conditions, Phe residues were previously reported to have no significant impact on photostability, whereas our data reveal a destabilizing effect. This discrepancy suggests Phe's photostability effects may be context-dependent, potentially influenced by protein environment in our assays."



Response to reviewer 2

Reviewer #2 (Remarks to the Author):

This manuscript presents a combined experimental and theoretical study of photophysical properties of chromophores in different environments being attached to different amino acid residues. Authors measure different experimental quantities related to photobleaching. Despite large amount of experimental data, the paper lacks scientific sound, that is the interpretation of experiments is almost missing. It is not clear whether conclusions made by authors are valid for selected systems or those are transferable. At that the manuscript does not suit high standards of the COMMSCHEM.

The problem with this paper is that it has a set of experimental observations, but not their explanation. It is stated in conclusions: " Thus, we postulated that the reduced photostability may be attributed to the reactions between the highly-reactive singlet state of fluorophore and its proximate protein backbone, causing disruption of fluorophore's chemical structure " I wonder, what particular processes authors mean. The backbone is rather chemical inert and a chromophore in the excited state can hardly interact (chemically) with it. Cy5 and Cy3 are fluorophores that are widely used in biological applications and their possible interactions with the backbone should already be highlighted in literature. What is also interesting, that if the chromophore degradation happens via the excited state, than the differences in the lifetimes should likely be observed. Figure 3 depicts that for Atto488 the values are the same, and for Cy5 and Cy3 variations are not monotonous with respect to the bleaching efficiency. Even in results section, there is no clear explanation of the relashion between the observations in MD and photostability. Likely, some other processes are responsible for the photostability variations.

Response #2: We sincerely appreciate the reviewer's valuable comments. Our observation that proximal fluorophore-backbone distances (<10 Å) consistently reduce photostability—regardless of side chain properties (aromatic Phe/Trp vs. aliphatic Val/Leu)—suggests that backbone proximity itself may modulate photobleaching pathways. When disregarding side chains, the amide group emerges as the most reactive backbone moiety, despite its generally low chemical reactivity. Notably, references #29 and #30 demonstrate that amides can mediate fluorescence quenching via photoinduced electron transfer (PET). Although direct evidence remains limited, we hypothesize that PET-derived reactive intermediates could contribute to photobleaching. To prevent potential misinterpretation, we have removed the original statement and incorporated the following clarification on page 9:

“Intriguingly, molecular dynamics simulations reveal a strong inverse correlation between fluorophore-backbone distance and photostability. Specifically, proximal fluorophore-backbone distances (<10 Å) consistently reduce photostability, regardless of side chain properties (aromatic Phe/Trp and aliphatic hydrophobic Val/Leu/Met). Conversely, distal positioning (>10 Å) preserves fluorophore stability. The proximity of fluorophore to the backbone may hinder the photoisomerization of cyanine (Cy3, Cy5) [23], thereby prolonging their fluorescence lifetime (Fig. 3). Collectively, our results demonstrate that backbone proximity influences photobleaching pathways. While the amide group - an essential backbone component - is known to mediate fluorescence quenching via photoinduced electron transfer



[29, 30], its potential role in photobleaching remains unclear. The underlying mechanisms of backbone modulated photobleaching require further elucidation.”

We appreciate the reviewer's insightful comment regarding “*if the chromophore degradation happens via the excited state, then the differences in the lifetimes should likely be observed.*” The reviewer raises a theoretically valid point and the differences in the lifetimes would become experimentally detectable only if the photobleaching rate approached the fluorescence emission rate. However, our experimental data demonstrate that photobleaching events occur with a frequency 4-5 orders of magnitude lower than fluorescence emission events (Figs. 1-2, consistent with prior reports PMID: 21644785, DOI: 10.1021/ac980027p). This substantial difference in timescales explains why fluorescence lifetime measurements remain insensitive to photobleaching rate variations, even when these rates are enhanced several-fold. To ensure clarity and avoid potential misinterpretation, we have elected to remove the original statement from the revised discussion section.

In all, we sincerely appreciate the reviewer's valuable feedback and thoughtful suggestions, which have prompted us to carefully reconsider our interpretations and substantially revise the discussion section in pages 8-9. While our current study has not fully elucidated the fundamental mechanisms underlying photobleaching, this systematic work provides practical guidance for fluorophore labeling site selection and optimization. Moreover, our findings establish an important foundation for future investigations into how biomolecular microenvironments (particularly in proteins) influence fluorophore photostability, opening new avenues for understanding these complex photophysical processes.



Response to reviewer 3

Reviewer #3 (Remarks to the Author):

The authors have addressed my comments, as well as those from the other reviewers. I believe the manuscript is now ready for publication.

Response #3: We thank all the reviewers for their time and expertise. Their constructive comments have substantially enhanced the quality of our work.