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A Fluorogenic Screening Platform Enables Directed Evolution of an Alkyne Biosynthetic Tool

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Abstract

Directed evolution was used to improve the activity of JamB, a membrane-bound bifunctional desaturase/acetylenase. To quickly assess the protein engineering outcomes, we developed a new platform for quantifying extracellular alkyne-tagged metabolites through a fluorogenic click reaction. Random mutagenesis yielded the best JamB variant conferring ~20-fold increased activity in *E. coli*.

Graphical abstract

A fluorogenic screening platform enabled the engineering of a membrane-bound bifunctional desaturase/acetylenase for improved activity in *E. coli*.

The alkyne functionality has been broadly used in chemical synthesis, pharmaceuticals, and materials due to its unique reactivity^{1, 2}. In addition, alkynes, as bioorthogonal handles, can

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also be metabolically incorporated into biomolecules for various applications in chemical biology. For example, triazole-forming azide-alkyne [3+2] cycloaddition reaction (often referred to as click reaction) and Raman scattering microscopy of alkynes have enabled the imaging and mode of action study of alkyne-tagged glycans, proteins, nucleic acids, lipids, and natural products^{3–9}. However, the prevalence of the alkyne functionality in synthetic molecules and its significance in chemical biology are contrasted by the very limited number of known alkyne biosynthetic tools^{10–15}.

We recently reported the elucidation of a portable tri-gene cassette *jamABC*, which is capable of producing thio-activated 5-hexynoyl moiety^{14, 16}. JamB was demonstrated to be a weak bifunctional desaturase/acetylenase that catalyzed the insertion of a terminal double bond, then a terminal triple bond based on biochemical analysis. This gene cassette was further utilized for the *in situ* generation and incorporation of a terminal alkyne functionality into a couple of natural product scaffolds in *E. coli* without the feeding of alkynoic precursors. For example, a promiscuous type III polyketide synthase (PKS), HsPKS1, could accept a carrier protein-bound fatty acyl moiety, and consequently, a terminal alkyne-tagged polyketide **1** was produced in an *E. coli* strain (named as XZ1) upon the co-expression of *jamABC* and *hspks1* and feeding of 5-hexenoic acid¹⁴. Consistent with the *in vitro* biochemical analysis, our further *in vivo* study showed that upon the feeding of hexanoic acid, **1** was only produced at a trace amount (~50 nM), while a major polyketide metabolite **2** was produced which bears a terminal alkane functionality retained from the fed alkane. Compounds **1** and **2** were produced by XZ1 in an approximately 7:1000 ratio (Figures 1a and S1). Since HsPKS1 showed no preference towards starter units with different terminal functionalities, the relatively low efficiency in the production of **1** was probably due to the poor activity of the membrane-bound bifunctional desaturase/acetylenase JamB in *E. coli*. To obtain a more efficient enzymatic tool for alkyne biosynthesis, a protein engineering approach, in particular directed evolution, was used here to optimize the desaturase/acetylenase activity of JamB in *E. coli*.

To quickly assess the protein engineering outcomes of JamB, we constructed a novel screening method based on the quantification of alkyne-tagged metabolites directly in their producing cell cultures. The design of the quantification platform has two components: the tagging of secreted polyketides through engineered biosynthesis with an alkyne moiety, followed by a click reaction with an azido fluorogenic probe (Scheme 1). Fluorogenic probes are endowed with a functionality that suppresses fluorescence^{17–20}. Its transformation during the click reaction creates a new functionality that no longer quenches the fluorescence of the underlying system, resulting in a fluorescence enhancement^{17–20}. Such probes offer significant advantages over fluorescent probes for quick readout of a tagged metabolite when washing away unreacted probes is not feasible. In particular, the reaction of an azido fluorogenic probe with **1** is expected to result in a fluorescence enhancement that is dependent on the amount of **1**. Although fluorogenic click reactions have been applied to visualizing and studying macromolecules^{17–20}, they have not been explored for extracellular metabolite quantification due to concerns about reaction specificity, selectivity, and sensitivity in complex cell cultures.

To test the reactivity of azido fluorogenic probes with **1**, we cultured XZ1 in F1 medium fed with 5-hexenoic acid for two days¹⁴, and subsequently, cell-free supernatants were incubated with fluorogenic azidofluorescein (**3**), a Cu (I) catalyst generated *in situ* using CuSO₄, ascorbate, and the ligand 2-[4-({bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1H-1,2,3-triazol-1-yl] acetic acid (BTAA)¹⁹. The fluorescence signal of the XZ1 culture was ~2-fold higher than that of the medium-only control (Table S4), demonstrating the success of the fluorogenic reaction albeit with a limited response range. We then evaluated the performance of additional azido fluorogenic probes, including di-pegOF (**4**), CalFluors 580 (**5**), and CalFluors 647 (**6**)¹⁷ (Figure 1b). Compared to **3**, **4–6** showed significant fluorescence enhancement (>10-fold) upon the incubation with XZ1 supernatant (Table S4). Among all of the tested probes, **4** gave the most consistent and reliable response and was thus chosen for further applications. It is also notable that the presence of cells in the reaction significantly decreased the fluorescence enhancement likely due to solid interference (Table S4), and cell-free supernatants were thus used for all subsequent experiments.

To confirm that the fluorescence enhancement was due to the formation of triazole compounds, we prepared an additional control by incubating the probe **4** with the cultures of an additional *E. coli* strain in which *jamB* was omitted (named as XZ5). We have previously demonstrated that this control strain completely abolished the production of **1**¹⁴. No significant fluorescence enhancement was observed from the culture of XZ5 compared to the medium-only control (Figure 1c). High-resolution (HR) LC-MS analysis showed that the XZ1-containing reaction yielded the expected triazole compound **7** with the retention time and mass patterns matching those of the compound chemically synthesized using **1** and **4** as substrates (Figures S2 and S8). No significant amount of **7** was observed in the negative controls based on LC-HRMS, which is consistent with our fluorescence detection (Figures 1c and S2).

We then set out to determine the detection limit and linear response range for the alkynes. To mimic different alkyne titers, we added different amounts of pure alkyne to the cell-free supernatants of XZ5 and subjected the samples to the fluorogenic click reaction. Our results showed that alkynes in quantities as low as 50 nM could be detected, and a broad linear range of response could be achieved by varying the amounts of the fluorogenic probe in the assays (Figures 2a and S3). We further applied this facile quantification platform to monitor the titer of the alkynes in different cultures over the course of 40 hr after induction and the feeding of 5-hexenoic acid¹⁴ (Figure 2b). As expected, the fluorescence signal of the XZ1 culture increased steadily due to the increasing titer of alkynes. In contrast, no fluorescence enhancement was observed with XZ5 throughout the duration.

As the fluorogenic assay-based quantification of the alkynes worked in both large-scale shake flasks and small-scale 96-well culture plates, it could be used as a screening method to evolve JamB for improved activity. We first constructed a library of JamB variants by diversifying *jamB* (~1kb) randomly using error-prone PCR such that the average mutation rate was 1.5 amino acid mutations/kb. The mutant library of *jamB* was introduced into XZ5, which co-expressed *jamC*, *jamA*, and *hspks1*. After screening ~1000 clones, we isolated three clones (XZ1_1-XZ1_3) that showed higher fluorescence signals than the control strain

XZ1 expressing the wild-type *jamB*. Further LC-HRMS quantification confirmed increased titers of **1** from these mutants (Figure 3a).

Sequencing analysis of these three *jamB* mutants showed that the JamB mutants from XZ1_2 and XZ1_3 each contained one amino acid change at M5V and I23M, respectively, while the mutant from XZ1_1 contained three mutations at M5I, V189A, and K250N. We then examined the contributions of each amino acid mutation found in XZ1_1 by constructing the corresponding single mutant. M5I alone showed a large positive effect, whereas V189A and K250N had no obvious effect, consistent with the performance of the M5V mutant found in XZ1_2 (Figure 3a). To probe the effect of amino acids with different properties at these two positions, we generated additional single mutants of JamB, including M5K, M5T, M5C, M5F, M5E, M5A, I23K, I23T, I23Y, I23V, I23E, and I23C, through site-directed mutagenesis. The best mutant, JamB-M5T, increased efficiencies in the production of **1** by ~20-fold, compared to that of the wild-type upon the feeding of hexanoic acid (Figures 3a, S1, and S4). These results showed that mutations at M5 and I23 altered JamB into a more efficient bifunctional desaturase/acetylenase in *E. coli*. It is also notable that JamB mutants seem to be expressed at the levels comparable to the wild-type enzyme based on the SDS-PAGE analysis (Figure S5). A second round of directed evolution did not result in an improved alkyne production mutant.

To better understand the molecular mechanism of the beneficial mutations that led to the increased efficiency of alkyne formation, we mapped M5 and I23 to a modeled structure of JamB-JamC complex. The individual protein structures were modeled using HHpred²¹ with SCD1^{22, 23} and CurA²⁴ as templates, respectively, and then docked together using ClusPro^{25, 26} (Figures 3b and S6). The resulting structure showed that I23 is located at the interface of the two proteins, and thus I23M likely benefits the interaction between JamB and JamC (Figure 3b). M5 is located near the *N*-terminus of JamB, and interestingly, our recent characterization of JamB and its homologs CamB^{14, 27} (identity/similarity = 95%/98%), and TtuB¹⁵ (identity/similarity = 44%/60%) demonstrated that their activities in *E. coli* were affected by the amino acids at the non-homologous *N*-terminal region (Figure S7). This was further confirmed by the construction of chimeric JamB mutants with the *N*-terminal region replaced by the one from the more efficient desaturase/acetylenase TtuB or the less efficient CamB. The resulting JamB-TtuB and JamB-CamB mutants showed increased and decreased activity, respectively (Figure 3a), and these data support the sensitivity of enzymatic activity toward the *N*-terminal amino acids. We further propose that M5T may promote the binding of JamB to ferredoxin, the possible electron donor^{13, 28, 29}, since the recent crystal structure of the membrane-bound desaturase SCD1 suggested that its *N*-terminus lies along the electron donor-binding groove²³.

In summary, by taking advantage of a fluorogenic click reaction, we have developed a rapid and sensitive analytical platform that quantitatively detects extracellular alkyne-tagged metabolites directly in fermentation cultures. We further applied this platform as a screening method to optimize an alkyne biosynthetic tool through protein engineering. In particular, a membrane-bound bifunctional desaturase/acetylenase JamB was evolved, resulting in a superior mutant with ~20-fold improved desaturase/acetylenase activity in *E. coli*. This result not only promotes the mechanistic understanding of the family of membrane-bound

bifunctional desaturases/acetylenases, but it also yields an improved terminal alkyne biosynthetic tool that can be widely used in synthetic and chemical biology^{3–8}. Furthermore, this detection method for alkyne-tagged metabolites using azido fluorogenic probes can also facilitate the discovery of new alkyne-bearing natural products and the expansion of alkyne biosynthetic tools.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Detection of the alkyne in *E. coli* cultures using a fluorogenic probe. a) Schematic of the biosynthetic pathway of **1** in XZ1 and the fluorogenic reaction with **4**. b) Azido fluorogenic probes (**3–6**) tested in this study. c) Fluorescence of the culture supernatants upon reaction with **4**. Heterologous proteins expressed in each strain are shown under the corresponding strain names. One-fold fluorescence enhancement corresponds to the signal obtained from the reaction with pure medium (~500–1000 RFU). Error bars represent standard deviations from three measurements.

Figure 2.

Quantification of the alkynes in *E. coli* cultures using **4**. a) The quantification curve for the fluorescence detection of alkynes using 0.75 μM **4**. The inset shows the linear relationship between fluorescence enhancement fold change and alkyne concentrations, $R^2=0.98$. b) Fluorogenic assay-based monitoring of alkyne titers in the cultures of XZ1 and XZ5 during fermentation. One-fold fluorescence enhancement corresponds to the signal obtained from the reaction with pure medium (500~1000 RFU). Error bars represent standard deviations from three measurements.

Figure 3.

Production of **1** by *E. coli* strains expressing *jamB* or its mutants. (a) Relative efficiencies in the production of **1** compared to the wild-type strain (XZ1) when all cultures were fed with hexanoic acid. The efficiency of the wild-type JamB in the production of compound **1** in XZ1 was set to be 100%. Error bars represent standard deviations from three measurements. (b) Modeled structure of the JamB-JamC complex. JamC and JamB are shown in red and beige, respectively. M1, M5, and I23 of JamB are highlighted in yellow, green, and blue, respectively.

Scheme 1.

Illustration of the screening platform for JamB mutants based on the quantification of alkyne-tagged extracellular metabolites using a fluorogenic click reaction.