Using the inner membrane of Escherichia coli as a scaffold to anchor enzymes for metabolic flux enhancement

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June 8, 2022

Abstract

Clustering enzymes in the same metabolic pathway is a natural strategy to enhance productivity. Synthetic protein, RNA and DNA scaffolds have been designed to artificially cluster multiple enzymes in the cell, which require complex construction processes and possess limited slots for target enzymes. We utilized the Escherichia coli inner cell membrane as a native scaffold to cluster four fatty acid synthases and achieved to improve the efficiency of fatty acid synthesis in vivo. The construction strategy is as simple as fusing target enzymes to the N-terminus or C-terminus of the membrane anchor protein (Lgt), and the number of anchored enzymes is not restricted. This novel device not only presents a similar efficiency in clustering multiple enzymes to that of other artificial scaffolds but also promotes the product secretion, driving the entire metabolic flux forward and further increasing the gross yield compared with that in a cytoplasmic scaffold system.

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#You Wang and Yushu Wang contributed equally to this work and considered as the first author.

ABSTRACT: Clustering enzymes in the same metabolic pathway is a natural strategy to enhance productivity. Synthetic protein, RNA and DNA scaffolds have been designed to artificially cluster multiple enzymes in the cell, which require complex construction processes and possess limited slots for target enzymes. We utilized the *Escherichia coli* inner cell membrane as a native scaffold to cluster four fatty acid synthases and achieved to improve the efficiency of fatty acid synthesis in vivo. The construction strategy is as simple as fusing target enzymes to the N-terminus or C-terminus of the membrane anchor protein (Lgt), and the number of anchored enzymes is not restricted. This novel device not only presents a similar efficiency in clustering multiple enzymes to that of other artificial scaffolds but also promotes the product secretion,

driving the entire metabolic flux forward and further increasing the gross yield compared with that in a cytoplasmic scaffold system.

KEYWORDS: *cell membrane, scaffold, metabolic flux, fatty acid synthesis*

Introduction

In cells, many enzymes undergo energy uptake and produce materials essential for daily life processes. A number of enzymes in a metabolic flux naturally cluster as multi-enzyme "sequential" or "cascade" reactions, such as glycolysis and Krebs cycle [1, 2]. The existence of these natural "flow lines" indicates that clustering relevant enzymes can improve the efficiency of metabolic flux and thus save biological energy.

To mimic a natural multi-enzyme complex and to organize functional-related enzymes, researchers developed several approaches, such as designing an artificial protein scaffold for the generation of the desired metabolic flux [3]. Using well-characterized and widespread protein–protein interaction domains from metazoan signaling proteins (SH3-, PDZ-, and GBD-binding domains), the author constructed a modular genetically encoded scaffold system, where enzyme localization was predefined and programmable. With this system, the amount of the target product increased by 77-fold, demonstrating the advantages of artificial scaffolds[3, 4].

Nevertheless, protein and other scaffold systems with predefined artificial scaffolds are generally limited by the length of scaffolds or the number of artificially clustered modules [5, 6]. To simplify the clustering system and expand the number of enzymes that can be exerted to the system, we proposed that the inner cell membrane could be a good candidate because of its several properties. First, unlike previously synthesized scaffolds, the cell membrane is an innate organelle that has no limitation on the number of scaffolds. Second, the membrane has a much more compact space than the cytoplasm, suggesting the presence of unlimited slots for scaffolding proteins. Third, the membrane structure restricts the reaction space to a 2D plane compared with discrete scaffolds, thereby facilitating the interaction among the anchored proteins. Moreover, the enzymes can be organized in a 2D pattern on the membrane to further enhance the metabolic flux. The proposed membrane scaffold could be used to effectively increase the concentration of the final synthesized products near the membrane, thereby facilitating the transmembrane transportation of products and further simplifying the post-processing procedure. Thus, we decided to develop the potential of the cell membrane as a native scaffold for the clustering of enzyme systems.

To verify this concept, we selected key enzymes of the fatty acid metabolic pathway in *Escherichia coli* and anchored them onto the inner cell membrane. *E. coli* has nine fatty acid synthases (FAS), namely, FabA, FabB, FabD, FabF, FabG, FabH, FabI, FabZ, and ACP (Fig. 1). Besides, TesA, a periplasmic thioesterase, can release free fatty acids from acyl-ACP species [7]. Previous studies suggested that FabG, FabI, FabZ, and TesA control the rate-limiting steps in fatty acid biosynthesis in *E. coli*[7-9]. By conducting a systematic kinetic analysis on the fully reconstituted *E. coli* (FAS), X. Yu suggested that different combinations of the molar ratios of the four rate-limiting enzymes of FAS, namely, FabZ, FabG, FabI, and TesA', remarkably influenced the overproduction of fatty acids. Therefore, we determined to fuse FabG, FabI, FabZ, and TesA', which is a TesA mutant without a signal sequence peptide that redirects it to become localized in the cytoplasm and thus increase the accessibility of substrates to the active site, with phosphatidylglycerol::prolipoprotein diacylglyceryl transferase, which is a well-studied *E. coli* inner transmembrane protein [10], to confirm the viability of the membrane scaffold. By anchoring these enzymes, we observe increased final products yield and dramatically enhanced products exportation. Collectively, our results provide novel insight into the potential application of cell membrane as a scaffold for important metabolism pathway to produce valuable bioproducts.

Experimental section

Plasmids and strains

E. coli strains DH5α and BL21 (DE3) were used for cloning and protein expression, respectively. The vectors used for construction included pETDuet1, pACYCDuet1, pRSF-Duet1, and pBAD18. All of the plasmids used in this study are shown in Table 1.

The arabinose operon from pBAD18 was amplified and cloned twice into pETDuet1, pACYCDuet1, and pRSF-Duet1 to replace the original T7 operon, thereby producing pET-Ara, pACYC-Ara, and pRSF-Ara. Each vector contained two copies of arabinose operons. For the verification of membrane localization, the DNA fragment containing the N-terminal DsbA signal sequence, followed by the genes of β-lactamase, phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (Lgt), and GFP was cloned into pET-Ara (Fig. 2A). For the verification of artificial clustering, the DNA fragment comprising a DsbA signal sequence (ssDsbA), one type of interacting protein, Lgt, and a split EGFP was cloned into arabinose operon (Figs. 3A and 3B). A flexible linker FL3 was introduced between crucial protein parts to ensure their proper functioning.

Four groups of engineered fatty acid-related enzymes were used to verify our design (Fig. 3A). The genes involved in the membrane binding FAS (MBF), membrane FAS (MF), and cytoplasmic binding FAS (CBF) groups were cloned into pET-Ara, pACYC-Ara, and pRSF-Ara, respectively. The genes in the cytoplasmic FAS (CF) group were cloned into pET-Ara. The MBF group contained cytoplasmic and periplasmic protein interaction domains to cluster engineered proteins. The enzymes in the MF group were directly fused with the C-terminus of Lgt. The enzymes in the CBF group were directly fused with the protein interaction domains and expressed in the cytoplasm. The CF group comprised the enzymes expressed in the cytoplasm. In each group, the FabI and FabZ proteins or the fusion proteins were cloned into two arabinose operons in pET-Ara. The FabG and TesA' proteins or the fusion proteins were cloned into two arabinose operons in pRSF-Ara.

Cell culture conditions for fatty acid biosynthesis

Cells carrying different constructs were incubated in 5 mL of LB medium supplemented with antibiotics and cultured overnight at 37 °C. Overnight cell culture (3% [v/v]) was added to a 250 mL flask containing 50 mL of LB medium supplemented with 15 g*L-1 glucose and then cultivated at 37 degC at a frequency of 150 rpm. Antibiotics were added to maintain the plasmids in the recombinant strains: ampicillin (100 mg/L), kanamycin (50 mg/L), or chloramphenicol (12.5 mg/L). The cultures were induced by adding 0.2% arabinose at OD600 = 0.6, and the samples were collected at 20 h post-induction for fatty acid analysis.

Free fatty acid extraction and measurement

Cell culture samples (20 mL; three replicates for each sample) were centrifuged at 8000 rpm for 10 min to separate the cell-associated fatty acids from the extracellular fatty acids. Fatty acid extraction was performed as previously described [11]. The fatty acids extracted from the supernatant were analyzed through gas chromatography–mass spectrometry (GC-MS) by using a 5975 C Series MSD and Agilent 6850 equipped with an HP-5 MS column (30 m x 0.32 mm; film thickness of 0.25 mm). Helium was used as a carrier gas. The temperatures of the injector and the detector were 250 degC and 280 degC, respectively. The GC elution conditions were as follows: 100 degC as the starting temperature for 5 min, 15 min ramp to 250 degC, and 250 degC held constant for 5 min. All of the samples were spiked with pentadecanoic acid (C15) as an internal standard. The growth of the cells and the analysis of the fatty acid products were repeated thrice.

Results

Localizing target enzymes to *E.coli* inner membrane

The membrane scaffold system has two indispensable elements. The phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (Lgt), an *E. coli* transmembrane protein, is selected as the anchor module. Target enzymes can be fused with its N-terminus as a periplasmic enzyme or C-terminus as a cytoplasmic enzyme. Aside from Lgt, one short peptide named ssDsbA, the signal recognition particle (SRP)-dependent signaling

sequence of DsbA, is necessary to orient the combined proteins onto the inner membrane of *E. coli* . To verify the function of the membrane anchor part, we fused β-lactamase and EGFP with the N-terminus of Lgt and with the C-terminus, respectively (Fig. 2A)[12]. The expression plasmid containing this construct was transformed to BL21 (DE3) and induced by L-arabinose (Fig. 2B). The BL21/pETara-Anchor strain exhibited a clear green fluorescence on the cell margin under the laser confocal microscope, confirming that the membrane anchor part was correctly localized. To further characterize the membrane anchor and define the optimal induction condition, we observed the growth phenotype of the BL21/pETara-Anchor strain at different ampicillin levels (Fig. S1) and quantitatively tested its growth status under different concentration combinations of L-arabinose and ampicillin (Fig. S2). The results revealed that 0.2% L-arabinose induction was the most satisfactory condition for membrane protein expression. In generally speaking, by fusing with the membrane anchor, the target proteins performed normal functions regardless of their periplasmic or cytoplasmic locations.

Clustering target enzymes on the membrane through mammalian protein–protein interaction domains and ligands

Given that the cell membrane has a more compact space than the volume of the cytoplasm, we chose the inner cell membrane of *E. coli* as the scaffold. However, whether the proteins anchored onto the membrane can be clustered to improve the metabolic flux, as in the case of other artificial scaffolds, remains uncertain. To confirm that our membrane scaffold equipped with metazoan interacting proteins could be clustered, we conducted fluorescence complementation assay (Figs. 3A and 3B) assay[3, 13]. First, protein–protein interaction domains and ligands from metazoan cells (mouse SH3 and PDZ domains and rat GBD domain) were utilized on the basis of the combination of the protein domains and their corresponding cognate ligands to rationally assemble and arrange enzymes onto the inner membrane of *E. coli* . These three groups of interacting proteins were fused with the N- or C-terminus of Lgt to create the desired protein complexes on the membrane (Fig. 3B).

In the fluorescence complementation assay, the fluorescent protein EGFP was split into two halves (1EGFP and 2EGFP), and the proteins that were postulated to interact were fused with the unfolded complementary fragments of EGFP and expressed in *E. coli* . The interaction between protein domain and the ligand brought the fluorescent fragments within proximity, allowing the reporter protein to restore its native 3D structure and emit a fluorescent signal. Therefore, fluorescence could be observed if an interaction occurred between 1EGFP and 2EGFP. Otherwise, no fluorescence could be detected. The split EGFP parts were fused with Lgt without interacting proteins as the negative control or fused with Lgt interaction groups to test the protein clustering (Fig. 3B). In Fig. 3C, the detected green fluorescence signal implied that all of the three groups of Lgt fusion proteins successfully developed a functional EGFP on the membrane, demonstrating that the membrane proteins with interacting proteins could dimerize with one another. Thus, we could easily use the inner membrane as a scaffold to cluster target proteins by recruiting Lgt, which is the native membrane protein of *E. coli* , interacting proteins (SH3, PDZ, and GBD), and the desired enzymes.

Clustering fatty acid synthetic enzymes on the membrane

Considering the successful construction of the membrane scaffold, we next recruited FabI, FabZ, FabG, TesA', the four crucial fatty acid synthases, in our membrane scaffold system to demonstrate the applicability and efficiency of our proposed device. Four groups of fatty-acid-overproducing strains with different scaffolding patterns were developed. The MBF group (Fig. 4A) had four cascaded FAS enzymes on the inner membrane with the help of interacting proteins, whereas MF group only comprised four FAS enzymes anchored on the membrane without the aid of interacting proteins. The cells harboring these four groups were cultured, and the total fatty acids were extracted and measured through GC-MS (Fig. 4B). The total fatty acid produced by anchoring the enzymes onto the membrane in the MBF (1021.92 mg/L) and MF (1056.43 mg/L) groups was more than that obtained by simply overexpressing the enzymes in the cytoplasm (897.91 mg/L). However, the total fatty acids obtained by simply anchoring the enzymes on the membrane in MF group was similar to that produced by clustering enzymes in MBF group, which indicated that the inner membrane, in contrast to the cytoplasm, likely retained the membrane-anchored proteins in a relatively restrained zone,

resulting in effects similar to those of clustering proteins. Thus, we performed fluorescence complementation or fluorescence resonance energy transfer experiments to verify whether simply anchoring enzymes on the membrane can cluster proteins. However, no positive results were observed (Fig. 3C), which is possibly due to the enzymes anchored on the membrane were sufficiently close with one another to generate cascading effects and enhance the metabolic flux, but not close enough to be detected through fluorescence complementation or fluorescence resonance energy transfer experiments. Nonetheless, our results demonstrated that simply anchoring the target enzymes on the membrane without the introduction of interacting proteins can enhance the metabolic flux as described in other artificial scaffold systems [5, 6].

Clustering fatty acid synthesis enzymes on the membrane accelerates products exportation

In addition, we analyzed the fatty acid products and found that anchoring the enzymes on the membrane remarkably changed the ratio between the products in the cell and in the medium. To further investigate this phenomenon, we constructed the clustered enzymes in the cytoplasm (CBF group) and compared the fatty acid product yields of the four groups (Fig. 4A). We found that the amount of the fatty acid products exported by the membrane groups (MBF group produced 431.42 mg/L extracellular fatty acids while MF group produced 476.78 mg/L) was higher than that obtained by the cytoplasm groups (78.12 mg/L and 26.38mg/L in CBF and CF groups, respectively) did (Fig. 4C). Meanwhile, the total fatty acids produced by clustering the enzymes in the cytoplasm (853.9 mg/L fatty acids in CBF group) was higher than that obtained by the CF group (771.53 mg/L) (Fig. 4C), which was consistent with previous protein scaffolding studies [3]. These results confirmed that membrane scaffold enhanced the product titer in the medium, indicating that high amounts of free fatty acids were secreted outside.

Discussion

Organisms naturally cluster related enzymes to improve the efficiency of the whole metabolism pathway and save energy. Enzymes fused with membrane anchors were directed toward the membrane as expected after these enzymes were translated. Thus, the enzyme distribution was restricted to the 2D membrane rather than randomly diffused throughout the cytoplasm. Each membrane anchor is inclined to interact with another anchor because of spatial restriction, and such interaction could be stabilized by phospholipids around the transmembrane domain. Therefore, a series of enzymes involved in sequential reactions could be swiftly and orderly organized on the membrane of our proposed device. Any suitable enzyme could be localized in the periplasm and could utilize the substrates from the medium to synthesize target products. Our work has demonstrated the feasibility of the membrane scaffold to cluster multiple enzymes and further enhance the involved metabolic flux. The efficiency of fatty acid synthesis and the yield of free fatty acids were improved by artificially engineering different scaffolding patterns. To our knowledge, this report is the first to utilize the inner cell membrane of *E. coli* as a novel protein scaffold to enhance fatty acid production. Although the number of clustered enzymes in our design was limited because of the available interacting proteins, the freely anchored enzymes could produce similar amounts of fatty acids, indicating that the introduction of interacting proteins was not relevant. Our findings expanded the capacity of scaffolding proteins and reduced the complexity of device construction.

The random diffusion of small molecules through the cell membrane was slow and consistent with the concentration difference across the membrane. Our results showed that the products accumulated near the cell membrane when enzymes were anchored on the membrane, resulting in an increased local concentration. Such an increase triggered the product molecules to diffuse outside through the cell membrane, thereby remarkably increasing the product titer in the medium. The yield produced by the system with anchored enzymes on the membrane was higher than that obtained by the clustered enzymes in the cytoplasm presumably because of the continuous secretion of products. A fundamental reaction model has shown that accumulated products inhibit or reverse catalytic reactions. Our membrane scaffold device facilitated the exportation of products, promoted the metabolic flux, and simplified the post-processing work of the desired products.

Together, our design of using cell membrane as scaffold to anchor target enzymes to enhance the metabolic flux is effective. The construction process is simplified as fusing target enzymes to the N-terminal or the C-terminal of the membrane anchor protein (Lgt), and the number of the enzymes are not limited (Fig. 5). Potentially, enzymes can be anchored in the periplasm and utilize substrates from medium to make target products. Our design not only shows similar enzymes clustering effects as other artificial scaffolds, but also enhance the products exportation, driving the whole metabolism flux to the positive direction and resulting in further increased final yield compared to the cytoplasm scaffold system.

SUPPORTING INFORMATION

Figure S1: Growth phenotypes of BL21/pETara-Anchor strain; Figure S2: The optimized growth condition test for BL21/pETara-Anchor strain.

ACKNOWLEDGEMENTS

This work was supported by the National Nature Science Foundation of China (31671504, 81421061) and the National Key Technology R&D Program (2012BAI01B09). Y.W., and G.M. conceived and designed the experiments. Y.W., Y.S.W., Y.Q.W., Y.S., H.Q.G., Y.N.Y., R.N.Y., R.X., J.J.W., N.H., Y.H.Z., S.B.Z., and Z.M.J. performed the experiments. Y.W., Y.S.W., and G.M. analyzed the data. Y.S.W., L.H., and G.M. contributed reagents/materials/analysis tools. Y.W., Y.S.W. and G.M. wrote the paper.

Data availability statement: Data openly available in a public repository that issues datasets with DOIs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. Plasmids used in this study

Plasmids	Description	Source
pBAD18	Expression vector, Amp^R, P_{ara}, pBR322 ori	Beckwith
pET-28a(+)	Expression vector, Kan^R, P_{T7}, pBR322 ori	Novagen
pETDuet1	Expression vector, Amp^R, P_{T7}, pBR322 ori	Novagen
pACYCDuet1	Expression vector, Cm^R, P_{T7}, p15A ori	Novagen
pRSFDuet1	Expression vector, Km^R, P_{T7}, RSF ori	Novagen
pET-Ara	Modified expression vector containing two arabinose operons, Amp^R, P_{ara}, pBR322 ori	This study
pACYC-Ara	Modified expression vector containing two arabinose operons, Cm^R, P_{ara}, p15A ori	This study
pRSF-Ara	Modified expression vector containing two arabinose operons, Km^R, P_{ara}, RSF ori	This study
pETara-Anchor	SsDsbA-Bla-Lgt-GFP inserted into pET-28a	This study
MA1-1EGFP	SsDsbA-Lgt-GBDligand-1EGFP inserted into BglII/XhoI sites of pET-Ara	This study
MA2-2EGFP	SsDsbA-PDZdomain-Lgt-GBDdomain-2EGFP inserted into EcoRI/PstI sites of pACYC-Ara	This study
MA3-1EGFP	SsDsbA-PDZligand-Lgt-SH3ligand-1EGFP inserted into EcoRI/PstI sites of pET-Ara	This study
MA4-2EGFP	SsDsbA-Lgt-SH3domain-2EGFP inserted into EcoRI/PstI sites of pRSF-Ara	This study
M-1EGFP	SsDsbA-Lgt-1EGFP inserted into EcoRI/PstI sites of pET-Ara	This study
M-2EGFP	SsDsbA-Lgt-2EGFP inserted into EcoRI/PstI sites of pRSF-Ara	This study
MA1-FabI	SsDsbA-Lgt-GBDligand-FabI inserted into BglII/XhoI sites of pET-Ara	This study
MA2-FabZ	SsDsbA-PDZdomain-Lgt-GBDdomain-FabZ inserted into EcoRI/PstI sites of pACYC-Ara	This study
MA3-FabG	SsDsbA-PDZligand-Lgt-SH3ligand-FabG inserted into EcoRI/PstI sites of pET-Ara	This study
MA4-TesA'	SsDsbA-Lgt-SH3domain-TesA' inserted into EcoRI/PstI sites of pRSF-Ara	This study
M-FabI	SsDsbA-Lgt-FabI inserted into BglII/XhoI sites of pET-Ara	This study
M-FabZ	SsDsbA-Lgt-FabZ inserted into EcoRI/PstI sites of pACYC-Ara	This study
M-FabG	SsDsbA-Lgt-FabG inserted into EcoRI/PstI sites of pET-Ara	This study
M-TesA'	SsDsbA-Lgt-TesA' inserted into EcoRI/PstI sites of pRSF-Ara	This study
CB-FabI	GBDligand-FabI inserted into BglII/XhoI sites of pET-Ara	This study
CB-FabZ	PDZdomain-GBDdomain-FabZ inserted into EcoRI/PstI sites of pACYC-Ara	This study
CB-FabG	PDZligand-SH3ligand-FabG inserted into EcoRI/PstI sites of pET-Ara	This study
CB-TesA'	SH3domain-TesA' inserted into EcoRI/PstI sites of pRSF-Ara	This study
C-FabI	FabI inserted into BglII/XhoI sites of pET-Ara	This study
C-FabZ	FabZ inserted into EcoRI/PstI sites of pACYC-Ara	This study
C-FabG	FabG inserted into EcoRI/PstI sites of pET-Ara	This study
C-TesA'	TesA' inserted into EcoRI/PstI sites of pRSF-Ara	This study

Figure legend

Figure 1. Fatty acid metabolism pathway in *E. coli* . FabA, FabB, FabD, FabF, FabG, FabH, FabI, FabZ are the main enzymes of the fatty acid biosynthesis pathway in *E. coli* . FabB, FabF, Fab G, FabI, FabZ/FabA are mainly responsible for fatty acid elongation. TesA is the enzyme that is able to release free fatty acid

by hydrolysis of acyl-ACP species. FabG, FabZ, FabI and TesA were picked as targets for manipulation in these experiments based on previous studies.

Figure 2. Design and verification of the membrane localization of the engineered Lgt. (A) Schematic of the engineered membrane protein. Lgt is used as a scaffold to carry functional groups (β-lactamase and EGFP as examples) to the membrane. (B) Membrane localization is verified by confocal microscopy.

Figure 3. Design and verification of the artificial membrane clustering. (A) Schematic of the engineered membrane protein. Protein interaction domains are fused with the ends of Lgt. (B) Schematic of the four groups of engineered membrane protein. The latter three groups use SH3, PDZ, and GBD interactions, respectively. The No binding group has no interaction domains. Split EGFP is fused with the N-terminus of the membrane protein to verify the protein interactions. (C) The protein interactions in different groups are verified by confocal microscopy. The detected EGFP fluorescence indicates the interactions between designed proteins.

Figure 4. Clustering fatty acid metabolism enzymes on the membrane enhances the product yield and secretion. (A) Schematic of the four strategies of clustering enzymes. The MBF Group uses protein interaction domains to cluster target enzymes on the membrane. The MF Group directly localizes target enzymes on the membrane. The CBF Group utilizes protein interaction domains to cluster target enzymes in the cytoplasm. The CF Group overexpressing cytoplasmic target enzymes is taken as the control. B) Total fatty acid extracted from the cell expressing each groups of engineered enzymes. (C) The intracellular and extracellular fatty acid titer produced by different groups.

Figure 5. Summary of applications of membrane scaffold system. Potentially, unlimited number of target enzymes can be fused to Lgt and clustered on the membrane. The enzymes can be either presented in the cytoplasm site or the periplasm site. In the cytoplasm site, enzymes can utilize substrates produced in the cell, and the products could pass the cell membrane by diffusion. In the periplasm site, enzymes can utilize the substrates added in the culture and directly release products into the culture.