Toward a glycol radical enzyme containing synthetic bacterial microcompartment to produce pyruvate from formate and acetate

Henning Kirst,a,b,c, Bryan H. Ferlez,a,d, Steffen N. Lindner,a, Charles A. R. Cotton,a, Arren Bar-Even,e,1 and Cheryl A. Kerfelda,b,c,d,2

*Michigan State University-Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824; bEnvironmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720; cMolecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720; dDepartment of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824; and eSystems and Synthetic Metabolism, Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm 14476, Germany

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Formate has great potential to function as a feedstock for biorefineries because it can be sustainably produced by a variety of processes that don't compete with agricultural production. However, naturally formatotrophic organisms are unsuitable for large-scale cultivation, difficult to engineer, or have inefficient native formate assimilation pathways. Thus, metabolic engineering needs to be developed for model industrial organisms to enable efficient formatotrophic growth. Here, we build a prototype synthetic formate utilizing bacterial microcompartment (sFUT) encapsulating the oxygen-sensitive glycol radical enzyme pyruvate formate lyase and a phosphate acyltransferase to convert formate and acetylphosphate into the central biosynthetic intermediate pyruvate. This metabolic module offers a defined environment with a private cofactor coenzyme A that can cycle efficiently between the encapsulated enzymes. To facilitate initial design-build-test-refine cycles to construct an active metabolic core, we used a "wiffleball" architecture, defined as an icosahedral bacterial microcompartment (BMC) shell with unoccupied pentameric vertices to freely permit substrate and product exchange. The resulting sFUT prototype wiffleball is an active multi enzyme synthetic BMC functioning as platform technology.

Significance

The enormous complexity of metabolic pathways, in both their regulation and propensity for metabolite cross-talk, represents a major obstacle for metabolic engineering. Self-assembling, catalytically programmable and genetically transferable bacterial microcompartments (BMCs) offer solutions to decrease this complexity through compartmentalization of enzymes within a selectively permeable protein shell. Synthetic BMCs can operate as autonomous metabolic modules decoupled from the cell's regulatory network, only interfacing with the cell's metabolism via the highly engineered proteooseaceous shell. Here, we build a synthetic, modular, multienzyme BMC. It functions not only as a proof-of-concept for next-generation metabolic engineering, but also provides the foundation for subsequent tuning, with the goal to create a microanaerobic environment protecting an oxygen-sensitive reaction in aerobic growth conditions that could be deployed.

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1Deceased September 18, 2020.

2To whom correspondence may be addressed. Email: ckerfeld@lbl.gov.

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(GRMs) encapsulate propanediol dehydratase (17, 18) or choline trimethylamine-lyases (19); however, no GRM has been identified that naturally encapsulates the GRE PFL. By mimicking the naturally evolved BMCs and using them as blueprints, we aim to engineer a BMC encapsulating PFL that can function as a metabolic module for further engineering efforts. This platform can potentially be broadly used in production strains by addition of product biosynthesis pathway enzymes to be grown on the abundant feedstocks formate and acetate.

In contrast to eukaryotic organelles, BMCs are not enclosed by a phospholipid membrane but by a protein shell acting as a semipermeable barrier (10, 20). The BMC shell is formed by conserved families of proteins (10, 20) that assemble into three distinct oligomeric building blocks: BMC-T proteins, which assemble into hexagons (21); BMC-T proteins, which also form hexagons (22); and BMC-P proteins, forming pentagons (23). The BMC-Ts subdivide into two types: single-layer BMC-T and double-layer BMC-T (10, 20). These components tile together to form an icosahedral shell (24) that serves as a selectively permeable interface with the cytosol. Synthetic BMC shells can form without the presence of the BMC-P proteins (25–27) at the vertices, an architecture we define as “wiffleball.” The 12 6-nm-diameter gaps of wiffleballs permit free exchange of substrate and product across the shell, and therefore we chose this shell architecture for prototyping synthetic enzymatic cores.

Recently developed methods have made it possible to specifically load a BMC shell with a desired nonnative cargo (25, 28–30). One method adopted SpyTag/SpyCatcher bacterial split adhesin domains (31) to covalently bind cargo to the inside of the shell. SpyTag is a short peptide (13 amino acids) that forms an isopeptide bond upon encountering its protein partner, SpyCatcher (31). By incorporating SpyTag into a lumen-facing loop of the shell protein BMC-T and tagging the synthetic cargo with SpyCatcher, it is possible to precisely encapsulate proteins into a synthetic BMC (25). Here we expanded this approach to the SnoopTag/SnoopCatcher system (32), a molecular adhesin that has no cross-talk with SpyTag/SpyCatcher (32). With the ability to specifically target two distinct cargo proteins to the lumen, we built a synthetic GRE-containing wiffleball. It encapsulates active PFL and an active phosphotransacetylase (PTA) to utilize formate and acetyl phosphate as substrates to produce pyruvate, a versatile bioisosteric precursor. It has potential to incorporate SpyT ag into a lumen-facing loop of the BMC-T component, there are 60 contact points to attach cargo across the shell, and therefore we chose this shell architecture for prototyping synthetic enzymatic cores.

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### Results

**Designing a Synthetic Formate and Acetate Utilizing BMC.** We used the model shell system derived from the BMC of the myxobacterium *Halothiobacillus neapolitanus* (HO-shell) for our synthetic formate utilizing BMC (sFUT) designs (Fig. 1A). This BMC shell can self-assemble without cargo (27) and has been characterized in detail structurally (24). Moreover, the pentamers at the vertices are not essential for shell assembly (25, 27) (Fig. 1B). We define this BMC architecture with unoccupied pentameric vertices as wiffleballs. The wiffleballs leave 6-nm-wide gaps in the shell to permit unrestricted substrate and product exchange, which facilitated testing of iterative sFUT designs. In the HO-shell facets there are three BMC-T proteins: single-layer T1, and double-layer T2 and T3 (Fig. 1). Loading the HO-shell with enzymes can be done using the previously developed method (25) that uses SpyCatcher/SpyTag (31) inserted into T1. When a BMC shell architecture is used that contains only T1 as the BMC-T component, there are 60 contact points to attach cargo into the lumen of the shell (referred to as a minimal shell (25)) (Fig. 1C). T1 can be diluted out of the shell by coexpression of T2 and T3 (Fig. 1B). This mitigates potential steric hindrance when assembling the shell with cargo (25).

The designed sFUT function is based on utilizing the reverse direction of the PFL for the conversion of formate and acetate to pyruvate (Fig. 1D). This requires three enzymatic reactions: an acetate kinase (ACK) converting acetate into acetyl phosphate, a PTA to produce acetyl-CoA from acetyl phosphate, and the PFL condensing formate and acetyl-CoA to pyruvate (Fig. 1D). Additionally, an activating enzyme for the PFL is required (PFL-AE) to generate the glycolyl radical in the active site of the PFL. PFL-AE is theoretically only needed once to activate the enzyme, which can occur before/during sFUT BMC assembly. Thus, only two enzymes are strictly required to be encapsulated, PFL and the PTA, in order to cycle CoA between them (Fig. 1D). For the PTA, the endogenous E. coli PTA could be used; however, it has been reported to be a homohexamer (34). This oligomeric state could be problematic when encapsulation tags are added to the enzyme, because all six subunits would then function as contact points with the shell proteins and this might not necessarily match the geometry needed to form a complete shell. To decrease the chance of oligomerization interfering with the sFUT BMC assembly, we used the homodimeric E. coli PFL-EutD (35). This enzyme has been reported to be a bidirectional PTA (35) and has in fact higher acetyl-CoA–forming activity than the PTA (35), making it the preferred candidate for building the sFUT BMC, structurally and enzymatically.

For our goal to build an active sFUT prototype platform, we used the wiffleball architecture. This simplifies activity testing of the iterate design-build-test-refine cycles to yield an active, context-independent prototype. Future work can build on this platform by engineering permeability, which will include closing the pentamer gaps, and metabolic contextualization into industrial relevant strains with potential to create a microaerobic environment for the PFL inside the synthetic BMC. This concept of streamlining the building process of complex synthetic “organelles” using a wiffleball to shorten development periods can be applied broadly to BMC synthetic biology.

**Specific Encapsulation of Two Cargo Proteins.** Initial versions of sFUT designs aimed to encapsulate a fusion protein of PFL-EutD, an approach that has been successful before encapsulating a synthetic three-domain fusion protein into a carboxysome (36). To identify functional PFL-EutD-adaptor domain fusion proteins for incorporation into a BMC shell, we tested variations of which enzyme carried the adaptor domain, the order in which the proteins were fused, the length of the glycine-serine linker (GS; 5x[GS]) between the fusion proteins, as well as which shell proteins were coexpressed with the cargo. However, disappointingly low expression of the PFL-EutD fusion protein and, presumably, steric hinderance obstructed SpyCatcher/SpyTag conjugation, resulting in no observed encapsulation.

Because of the complications associated with the PFL-EutD fusion protein, we chose to modify the T1 shell protein to encapsulate two different enzymes simultaneously. To achieve this, we added a SnoopTag (32) to the lumen facing loop (between G84 and G86) in T1 that also displays SpyTag (KLGIDIEFIKVNK), resulting in a double-tagged shell protein building block (T1-6xHis_Spyt-Snpt) (Fig. 2A). There are three linkers within the double-tagged T1-6xHis_Spyt-Snpt (Fig. 2A): the first is between the N-terminal region of T1 and SpyTag, the second between SpyTag and SnoopTag, and the third connecting SnoopTag to the C-terminal sequence of T1. We tested two different linkers in the second position, between SpyTag and SnoopTag: a flexible glycine-serine linker (2x[GSGG]) and a stiff proline-lysinine linker with a flexible GS part (GP[KPKPPKK][GSGG][GGGGG]). The other two linker positions were kept invariable as short, stiff, proline-lysine peptides (5x and 4x [PK]). Encapsulation

Kirst et al. Towards a glycyl radical enzyme containing synthetic bacterial microcompartment to produce pyruvate from formate and acetate

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was tested with two distinct cargo proteins, mVenus (yellow) and mTurquoise2 (blue), fused to SpyCatcher and SnoopCatcher, respectively (Fig. 2 B and C). Both linker variants successfully encapsulated mTurquoise2-SnoopCatcher (mTurquoise2-SnpC) and SpyCatcher-mVenus (scmVenus).

Wiffleball NiNTA purifications are shown in Fig. 2D using a 6xHis tag on T1-spyt-snpt-6xHis. Wiffleballs were coexpressed with the cargo proteins mTurquoise2-SnpC and scmVenus, minimal wiffleballs (BMC-H and T1) with wild-type T1 (HT1), a minimal wiffleball containing the modified T1-spyt-snpt-6xHis (HT1-spyt-snpt-6xHis), and their respective full wiffleballs equivalents (HT1T2T3 and HT1T2T3+PFL+PTA), in which not only T1 is present but also T2 and T3. The different plasmids expressing the shell proteins and their resulting shell architecture are summarized in Table 1 and operon schematics are shown in SI Appendix, Fig. S1. The protein bands of T1-spyt-snpt-6xHis conjugated to either one of the fluorescent proteins (~80 kDa) can be identified on the Coomassie-stained SDS/PAGE gel for both the H T1-spyt-snpt-6xHis and H T1-spyt-snpt-6xHisT2T3 wiffleballs, and was verified by an αxHis Western blot against the C-terminal 6xHis Tag of T1 and T1-spyt-snpt-6xHis. Additionally, tandem conjugations of SpyTag and SnoopTag with the respective fluorophores were also detected (Fig. 2D). The protein bands containing T1 (unconjugated T1-spyt-snpt-6xHis, T1-spyt-snpt-6xHis-scMvenus, or T1-spyt-snpt-6xHis-mTurquoise2-SnpC) are much fainter for the H T1-spyt-snpt-6xHisT2T3 compared to the H T1-spyt-snpt-6xHis wiffleballs because the amount of T1-spyt-snpt-6xHis is lower in the full wiffleball architecture due to the presence of T2 and T3. Nevertheless, these results indicate that in minimal and full shell designs both fluorescent proteins are simultaneously encapsulated and, although less common, sometimes attached to the same T1-spyt-snpt-6xHis protein.

Further evidence for the coencapsulation of both fluorescent proteins was provided by fluorescence emission spectra using excitation wavelengths specific for mVenus (490 nm) (Fig. 2F) and mTurquoise2 (425 nm) (Fig. 2F). For the HT1-spyt-snpt-6xHis as well as the HT1-spyt-snpt-6xHisT2T3 wiffleball sample, we noticed that the mTurquoise2 emission spectrum contains mVenus emission at around 540 nm, which is not present when both fluorescent proteins are mixed together without being loaded into wiffleballs (mVenus+mTurquoise2 sample) (Fig. 2F). This suggests FRET between mTurquoise2 and mVenus when loaded into the wiffleballs, consistent with both fluorophores being coencapsulated.

To investigate if complete wiffleballs formed incorporating T1-spyt-snpt-6xHis conjugated to both mTurquoise2-SnpC and scmVenus, we used transmission electron microscopy (TEM) (Fig. 2 G and H). The images revealed structures of about 40 nm in diameter, similar to the published HO-shell TEM images (24, 25) for the minimal (Fig. 2G) as well as the full wiffleballs (Fig. 2H). These results suggest that BMC wiffleballs can be specifically loaded with SpyCatcher- and SnoopCatcher-tagged cargo after addition of SpyTag and SnoopTag to T1 without impairing wiffleball formation.

**Addition of Encapsulation Tags to sFUT Cargo.** PFL is a member of the GRE family, some of which are encapsulated in GRMs (15, 16). Most of the GREs in BMCs have an encapsulation peptide, which is not present in cytosolic GREs (15–17). The majority of the encapsulation peptides for these GREs are not found at the N or C terminus, but are located in an internal loop (15–17). Therefore, we tested if we could use the corresponding loop in the PFL to integrate the SpyCatcher domain into the PFL (between E551 and D552). Additionally, we created N- and C-terminal fusions of SpyCatcher to the PFL (SI Appendix, Fig. S2). We tested the expression level of the three different PFL variants: SpyCatcher loop insertion (PFL-spcy), N-terminal SpyCatcher fusion (PFL-Nspcy), and C-terminal SpyCatcher fusion (PFL-Cspcy) (SI Appendix, Fig. S4A). Although the N-terminal fusion of the SpyCatcher to the PFL (PFL-Nspcy) did not express well, the two other variants and the unmodified PFL control expressed well and are visible on a Coomassie-stained gel (SI Appendix, Fig. S4A). However, most of the proteins are found in the insoluble fraction; only the wild-type PFL and PFL-Cspcy are found in significant amounts in the soluble fraction (SI Appendix, Fig. S4A), indicating that the PFL-
Fig. 2. Specific encapsulation of two cargo proteins. (A) Schematic of T1-spyt-snpt-6xHis depicting the position of the linkers 1 to 3, the SpyTag, and the SnoopTag. (B) Schematic of a HO-shell wiffleball with T1-spyt-snpt-6xHis, T2 and T3 and conjugated cargo mTurquoise2, and sCMVenus (yellow). (C) Schematic of a HO-shell minimal wiffleball with only T1-spyt-snpt-6xHis and conjugated cargo mTurquoise2, and sCMVenus (yellow). (D) NiNTA purification of wiffleballs coexpressed with mTurquoise2 and sCMVenus. Lane 1: BMC-H and T1; lane 2: BMC-H and T1-spyt-snpt-6xHis; lane 3: BMC-H, T1, T2 and T3; lane 4: BMC-H, BMC-T1-spyt-snpt-6xHis, T2 and T3. (Left) Coomassie stained SDS/PAGE gel. H T1-spyt-snpt-6xHis and H T1-spyt-snpt-6xHis T2T3 architectures show conjugations between T1-spyt-snpt-6xHis, mTurquoise2, and sCMVenus, either individually (∼80 kDa) or together (∼120 kDa). (Right) α6xHis Western blot. T1-spyt-snpt-6xHis contains a 6xHis tag used for detection. Conjugation bands between T1-spyt-snpt-6xHis and the fluorescent proteins can be observed. (E) Fluorescence emission spectrum after mVenus excitation at 490 nm. The emission spectra were normalized to mVenus emission intensity. (F) Fluorescence emission spectrum using mTurquoise2 excitation at 425 nm avoiding mVenus excitation. mVenus emission can be observed in the loaded wiffleballs due to FRET, while this isn’t present when only mVenus or mTurquoise2 emission intensity. (G) TEM image of H T1-spyt-snpt-6xHisT2T3 minimal wiffleballs. (Scale bar, 200 nm.) (H) TEM image H T1-spyt-snpt-6xHisT2T3 full wiffleballs. (Scale bar, 200 nm.)
Csycp was the most promising variant to be used in the sFUT prototype. Additionally, we created and tested an N-terminal and C-terminal fusion of SnoopCatcher to EutD (EutD-NsnpC and EutD-Csnpc, respectively) (SI Appendix, Fig. S3). Both variants expressed equally well (SI Appendix, Fig. S4B) and we proceeded to use the EutD-Csnpc for the sFUT construction.

Activity Assays of SpyCatcher Fusion Variants to the PFL. We tested enzymatic activity of our PFL fusion proteins in forward and reverse directions by expressing them from a plasmid in anaerobically grown E. coli lacking the endogenous PFL and its activating enzyme (ΔaceAΔpflAB) under different carbon sources (Fig. 3). The plasmids included genes for the PFL-AE as well as METK to synthesize the substrate S-Adenosyl methionine for the PFL-AE (SI Appendix, Fig. S2). Fig. 3A shows a positive control experiment where strains were grown anaerobically on glycerol using nitrate as an electron acceptor, a condition that doesn’t require PFL activity for growth, as the cells can use pyruvate dehydrogenase in the presence of an electron acceptor. Under these conditions, all strains were able to grow, demonstrating that none of the constructs caused a lethal phenotype when expressed. Anaerobic growth on pyruvate (Fig. 3B) and glucose (Fig. 3D) is only possible when the forward direction of the PFL is functioning. Under these conditions, strains with N- and C-terminal PFL fusions showed similar growth compared to the strain with unmodified PFL. However, the negative control (no PFL) and the PFL variant with the SpyCatcher insertion into the loop did not show any growth in these conditions, indicating the SpyCatcher insertion abolishes PFL activity in the forward direction. PFL activity in the reverse direction is tested by growth on acetate and formate (Fig. 3C). The N-terminal fusion shows lagging growth in this condition when compared to the strain expressing the wild-type PFL, which could be caused by lower expression, insolubility (SI Appendix, Fig. S4A), and partial inhibition of the enzyme by the presence of SpyCatcher. The internal loop insertion of SpyCatcher into the PFL again showed no growth, demonstrating complete loss of activity of this PFL variant in both directions. However, this experiment revealed that the strain expressing PFL-Csycp has comparable growth kinetics to a strain expressing unmodified PFL, suggesting that this PFL variant is active to a similar degree as wild type PFL. Furthermore, we employed electron paramagnetic resonance (EPR) spectroscopy to confirm the PFL-Csycp is successfully converted to its active glycyl radical-containing form by PFL-AE in vivo (Fig. 3E). Whole cells expressing either the PFL-Csycp, or our unmodified PFL-Csycp control, exhibited identical EPR spectra with a principal doublet splitting of ~1.5 mT characteristic of the PFL glycyl radical (37). As expected, this radical signal was not detected in the PFL deletion strain (ΔaceAΔpflAB). These experiments strongly suggest that PFL-AE is able to interact with PFL-Csycp and generate the glycyl radical necessary for PFL activity, making this variant the best candidate to be used in the sFUT.

Assembly of Active sFUT Synthetic BMCs. To build the complete sFUT wiffleballs, we coexpressed three synthetic operons: one containing the shell proteins (SI Appendix, Fig. S1), another containing the PFL-Csycp as well as the PFL-AE and the METK (SI Appendix, Fig. S2), and the third expressing EutD-Csnpc and ACK (SI Appendix, Fig. S3). For the shell operon, we expressed two different versions: the minimal wiffleball (HT1-spyt-snpt and a full wiffleball (HT1-spyt-snpt-6xHisT2T3); synthetic BMCs produced using both wiffleball versions were purified using the shell purification method described in the Materials and Methods. Although no sFUT BMCs based on the minimal HT1-spyt-snpt wiffleball architecture could be isolated, we were able to obtain a pure fraction of sFUT BMCs using full H T1-spyt-snpt-6xHisT2T3 wiffleballs (Fig. 4A). Estimated molecular weights of the bands on the Coomassie-stained SDS/PAGE match the predicted sizes for HT1-spyt-snpt-6xHis in conjunction with PFL-Csycp and EutD-Csnpc (~170 kDa), T1-spyt-snpt-6xHis conjugated to PFL-Csycp (~120 kDa), PFL-Csycp (~95 kDa), EutD-Csnpc conjugated to T1-spyt-snpt-6xHis (~80 kDa), EutD-Csnpc (~50 kDa), T1-spyt-snpt-6xHis (~40kD), T2 and T3 (22 and 23 kDa, respectively), as well as the BMC-H (10 kDa). We confirmed the presence of the proteins by mass spectrometry. To investigate if we indeed built complete sFUT BMCs, we used negative staining with TEM to image the purified assemblies (Fig. 4B).

To investigate if the isolated sFUT wiffleballs are active, we measured the forward reaction of the enzymes producing formate in the presence of 1 μmol pyruvate. sFUT wiffleballs were able to produce about 0.5 to 0.6 μmol of formate, while an inactivated control showed only little formate present (Fig. 4D). 14 μmol sFUT wiffleballs were used in the reactions, which were loaded with an estimated 40 copies of the PFL and EutD enzymes based on the intensity of the protein bands in the SDS/PAGE gel (Fig. 4A). Considering that 500 to 600 nmol formate was produced, each enzyme carried out, on average at least 1,000 turnovers until activity was lost. This provides evidence that both enzymes are actively encapsulated and that CoA, which is presumably coencapsulated with the enzymes, can cycle between the enzymes. When the cofactor CoA was added to the sample, the activity improved slightly, by about 20%. Similarly, the activity could also be improved with the addition of acetyl-CoA, giving another indication that EutD is active in the sFUT wiffleballs, because acetyl-CoA would need to be converted first to acetyl phosphate and CoA in order for the PFL to produce formate. The reported kcat for the PFL is between 105–770 s⁻¹, assuming that the enzymes kinetics do not change significantly after addition of the encapsulation tag and incorporation into a wiffleball, it means that the reaction ended within seconds. This is consistent with our observation that we were not able to time-resolve this reaction with our experimental setup, which only allowed us to measure on a minute time scale.

Based on these results, we built a model of our assembled sFUT wiffleball (Fig. 4C) depicting a potential arrangement of the enzyme cargo inside a shell. It should be noted that the PFL as well as the EutD form homodimers, hence the presence of the unconjugated versions of these enzymes in the SDS/PAGE. Considering the SDS/PAGE and the TEM images, we therefore conclude that we successfully assembled active sFUT wiffleballs encapsulating the PFL-Csycp as well as EutD-Csnpc.

Discussion

The overarching goal of the sFUT BMC design is to create a platform metabolic module that can integrate oxygen-sensitive metabolism into an aerobic host to produce, from a cheap
feedstock, a central biosynthetic intermediate for the production of high-value compounds. We produced a prototype synthetic microcompartment core for the oxygen-sensitive enzyme PFL based on the HO-shell because of the availability of molecular tools to load the synthetic HO-shell with cargo and its potential to form wiffleball architectures. We modified this PFL...
architecture with a purification tag that facilitates rapid isolation and the testing of enzymatic core designs. This decouples the design-test-refine cycle for the catalytic core, from optimization of shell permeability, by allowing unrestricted substrates and product exchange for activity measurements. However, for future designs, the BMC-P protein can be added to the shell system to form complete HO-shells, and therefore complete sFUT BMCs. This concept can be applied broadly, streamlining other engineering efforts aiming to design and optimize synthetic BMCs.

Core design and assembly constitute the first phase toward constructing a completely functional synthetic “organelle.” This required expanding the technique to specifically encapsulate cargo by covalent linkage developed by Hagen et al. (25) to include the addition of a new adaptor system (SnoopTag/SnoopCatcher). Heterologous enzymes have been targeted to the lumen of the BMC before (38, 39) by using encapsulation peptides (40, 41) on the cargo; however, this approach suffers from low efficiency, is hampered by aggregation, and it is unknown how the encapsulation peptides associate with the shell, making this method nonquantitative (27, 42–45). Our strategy enabled us to reliably, specifically, and independently target two different cargo proteins into the lumen of a BMC. Additionally, the expansion of the adaptor system effectively

Fig. 4. Purification of complete assembled active sFUTs. (A) Coomassie-stained SDS/PAGE gel. T₁-spyt-snpt-6xHis in conjugation with PFL-Cspyc and EutD-Csnpc (∼170 kDa), T₁-spyt-snpt-6xHis conjugated to PFL-Cspyc (∼120 kDa), PFL-Cspyc (∼95 kDa), EutD-Csnpc conjugated to T₁-spyt-snpt-6xHis (∼80 kDa), EutD-Csnpc (∼50 kDa), T₂ and T₃ (∼40 kDa), T₂ and T₃ (22 kDa and 23 kDa, respectively) as well as the BMC-H (10 kDa). Proteins have been identified by mass spectrometry. (B) TEM images of the sFUT compartments. Structures observed at ∼40 nm in diameter. (C) Model of a complete assembled sFUT wibbleball. PFL and EutD form dimers. Not all T₁-spyt-snpt-6xHis conjugation points are occupied, probably due to steric hindrance. (D) Activity of sFUT wibbleballs. Lane 1: no sFUT control; lane 2: inactivated sFUT by exposure to oxygen after isolation; lane 3: sFUT; lane 4: sFUT + CoA; lane 5 sFUT + acetyl-CoA. Formation of formate is shown per sFUT wibbleball over a 15-min time frame when provided with 1 μmol pyruvate. Addition of 1 mM CoA boosts activity, as well as addition of 1 mM acetyl-CoA. Acetyl-CoA can only be used by the PFL if EutD converts it to acetyl phosphate and CoA, thus indicating EutD activity.
doubled the number of proteins that can be encapsulated from 60 to 120 per BMC, compared to the previously described method (25). This can increase the overall efficiency of the synthetic BMC by increasing the metabolite flux and permitting faster substrate channeling due to a greater enzyme density.

This first-generation sFUT prototypes aimed to encapsulate the minimal number of proteins needed (PFL and EutD) to cycle CoA (Fig. 1C), which is a natural property of BMCs (46). Tagging the PFL with SpyCatcher for encapsulation was challenging because of low expression level, and if expressed it produced mostly inactive (Fig. 3) and insoluble protein (SI Appendix, Fig. S4A). However, we succeeded in creating an active version of the PFL with a C-terminal encapsulation adapter (PFL-Cspyc). EPR revealed the presence of a glycy1 radical signal consistent with activated PFL, suggesting that PFL-AE was able to bind PFL-Cspyc and successfully activate the enzyme. Furthermore, our E. coli growth-based activity assays show that the PFL-Cspyc retains activity comparable to the unmodified PFL in both forward and reverse direction, making it suitable for constructing the sFUT wiffleball. It should be noted that because of the versatility of the SpyCatcher/SpyTag system, this C-terminal–tagged version of the PFL can potentially be used in other scaffolding or engineering efforts.

Considering that both PFL and EutD form homodimers (35, 47–49), and the relatively large size of the PFL-Cspyc (calculated molecular mass of 95 kDa), we anticipated that sFUT wiffleballs cannot fully assemble when all 120 contact points are loaded with PFL and EutD. Our activity measurements of the forward reaction show that the enzymes are active and can undergo multiple turnovers cycling CoA between the enzymes. However, we don’t recruit cargo into the lumen. Using this approach, we were able to isolate completely assembled sFUT wiffleballs loaded with PFL and EutD. Our activity measurements of the forward reaction show that the enzymes are active and can undergo multiple turnovers cycling CoA between the enzymes. Although CoA is known to associate with cargo proteins before encapsulation in BMCs (45), we note that the sFUT wiffleball architecture can be improved by adding CoA or acetyl-CoA to the reaction. Considering that isolation of sFUT wiffleballs is a lengthy process, including washes of the streptavidin column, we hypothesize that some CoA was released from the shell in this process; thus, its addition was able to slightly increase sFUT wiffleball activity. We have focused on the forward reaction for the activity assay because this is readily measurable, only requiring the addition of pyruvate to the sample, and we could follow the formation of formate. It should be noted that our in vivo experiments show that the modified enzymes have activity in both forward and reverse directions. However, we note that the conversion of the provided pyruvate to formate does not reach the published equilibrium of the enzymatic reaction (50), suggesting that over time the enzymes get inactivated and there is not sufficient PFL-AE or Ada-Met available to re-activate the PFL. A \( k_{cat} \) for the encapsulated PFL can be calculated roughly from the activity assay; using the estimated 40 PFL enzymes per sFUT wiffleball, it yields about 1.2 M\(-1\) s\(^{-1}\). However, given that we could not time-resolve the enzymatic reaction because it presumably ends within seconds, and we could only measure on a minute timescale with our experimental setup, the \( k_{cat} \) of the encapsulated PFL could be much closer to the published PFL \( k_{cat} \) of 105–770 M\(^{-1}\) s\(^{-1}\) (51, 52).

In summary, we have built a synthetic BMC, directly targeting two enzymes to be encapsulated, one of which is extremely oxygen sensitive, and expressing three auxiliary enzymes (PFL-AE, METK, ACK), to enable its function in the cell. In contrast to the synthetic BMCs first pioneered (38, 39, 53), which were used for ethanol production, polyphosphate storage and hydrogen production, the sFUT prototype can be used as a platform in ambitious engineering projects to compartmentalize entire metabolic pathway for the production of a biomolecule of interest starting at the easily accessible feedstocks acetate and formate. Moreover, our prototype metabolic module is poised for shell permeability engineering to address the grand challenge of constructing devices for to compartmentalize oxygen-sensitive reactions for use in aerobic growth conditions.

Materials and Methods

Cloning Procedure. The operon encoding for the foxA (Uniprot accession no.: P0AC23), pfbB (Uniprot accession no.: P09373), pFLA (Uniprot accession no.: P0AA9N4), and metK (Uniprot accession no.: P0A8171) genes were PCR-amplified from E. coli K12 genomic DNA and cloning into pBB2AC (54) using a Gibson assembly (55). The pfbB gene was then modified with additions of SpyCatcher, which was PCR-amplified using plasmids from Hagen et al. (25) and assembled into pBB2AC (54). The gene EutD (Uniprot accession no.: P77218) was ordered on a gBlock (IDT), PCR-amplified, and cloned along with a snooopCatcher PCR product (32), as well as the ackA gene (Uniprot accession no.: P09373), in a pBBK2 PCR-clamped plasmid to make K12 genomic DNA into pBB2AC (54) using a Gibson assembly (55). The intergenic region between cpcB and cpcA was PCR-amplified from the Synechocystis sp. PCC 6803 genome and used as an intergenic region between EutD and Ack in a synthetic operon via a Gibson assembly (55). Synthetic shell operons were modified by Gibson assembly (55) using plasmids from Hagen et al. (25) as templates to add a C-terminal 6xHis tag and an inserted snoopTag (32) to BMC-T1.

Protein Expression. BL21(DE3) strains harboring the shell and cargo plasmids were grown to OD600 0.6 to 0.8 in Luria-Bertani broth at 37°C in the presence of 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, and/or 50 μg/mL kanamycin, depending on the selectable marker in the plasmids and cold-shocked on ice for 5 min. The expression of the proteins was induced with 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and/or 5 mM anhydrotracycline, depending on the induction system of the plasmids, and then incubated at 18°C for 16 to 20 h.

BMC Shell and Wiffleball Purifications. Shells/wiffleballs were isolated according to the method described by Sutter et al. (24) with slight modifications. Briefly, the cell pellet from a 1-L culture expressing the HO-shell/wiffleballs and cargo plasmids was resuspended in phosphate-buffered saline (50 mM phosphate pH 7.5, 100 mM NaCl, referred to as 50/100 PBS), lysed by French-press and centrifuged at 12,000 × g for 10 min to pellet unbroken cells. The supernatant was loaded on a sucrose cushion (20% sucrose [w/vol]) and centrifuged at 26,000 rpm in a Beckman SW28 rotor. The pellet was resuspended in 50/100 PBS and then purified on an Ni-IDA affinity column, and in case of the purification of the complete sFUT applied onto a sucrose gradient 20 to 60% (w/vol) after Ni-IDA purification. At around 55% sucrose, a clean sFUT fraction could be obtained. Wiffleball samples were buffer-exchanged to 50/100 PBS and concentrated with a 15 mL 10 kDa MWCO filter (Amicon); for storage 0.02 M sodium azide as a preservative was added.

SDS/PAGE and Western Blot Analysis of Protein Preparations. Protein preparations were separated on 10%–12% gel, denaturated in reducing sample buffer and loaded on 4 to 20% polyacrylamide gradient gel. Gels were washed and stained with Coomassie blue. Proteins were transferred to a nitrocellulose membrane via a tank transfer system and blocked in PBS + 5% (w/vol) nonfat dry milk, 0.1% (w/vol) Triton X-100. Cross-reactions with a monoclonal antibody against a His tag and an intergenic region of the BMC were detected using a 1:10,000 diluted primary antibody and a 1:10,000 secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). The secondary antibody was horseradish peroxidase–conjugated, and the substrate was visualized by chemiluminescence using Pierce SuperSignal West Pico Chemiluminiscence substrate detection system (Thermo Scientific) and imaged with ChemiDoc XR+ System (Bio-Rad).

Fluorescence Measurements. An M1000 or Spark plate reader (Tecan) was used to collect the fluorescence spectra. Samples were collected from the fluorescence spectra. Samples were collected to total protein, 2.5 mg/mL and 100 μL of the samples were loaded into 96-well microplates to collect fluorescence intensity readings. Excitation and emission bandwidths were set to 5 nm; 425 nm was used for mTurquoise2 excitation and 490 nm for mVenus. The gain was kept at 100. The fluorescence intensity of sample containing the free fluorophores mTurquoise2 and mVenus without a shell was normalized to the HT1 spnypt-snt sample for comparison.
Transmission Electron Microscopy. Purified shells were imaged by negative staining. A JEM-1400Flash microscope (accelerating voltage was 100 kV and images were taken with a “Matataki Flash” S/CMOS camera. Purified shells were diluted 10-fold in HPLC-grade water and 5 μL of each sample was applied to 150-mesh carbon-coated copper grids (Electron Microscopy Sciences) for 30 s, wicked dry, stained for 15 s with 1% uranyl acetate, and again wicked dry before imaging.

PFL Activity-Dependent Growth Experiments. ΔaceAΔflAB E. coli cells (8) transformed with plasmids expressing PFL, PFL-spyc, PFL-spnc, and PFL-cspyc were grown in M9 minimal medium (47.8 mM Na2HPO4, 22 mM KH2PO4, 8.6 mM NaCl, 18.7 mM NH4Cl, 2 mM MgSO4 and 100 mM CaCl2), supplemented with trace elements (134 μM EDTA, 31 μM FeCl3·6H2O, 6.2 μM ZnCl2, 0.76 μM CuCl2·2H2O, 0.42 μM CoCl2·6H2O, 1.62 μM H2BO3, 0.081 μM MnCl2·4H2O). Precultures for growth experiments were incubated in aerobic conditions overnight in a 4 mL M9 medium containing 10 mM glucose and 2 mM hydroxytrate-cycline. Prior to inoculation cells were harvested by centrifugation (6,000 × g, 3 min, room temperature), washed three times in M9, and inoculated in M9 containing 10 mM glucose to a starting optical density (OD600 nm) of 0.01. The plasmids used the native PFL promoter, no inducer was needed, as cultures for growth experiments were incubated in aerobic conditions over mM NaCl, 18.7 mM NH4Cl, 2 mM MgSO4 and 100 OD600 measurements were followed by cycles consisting of three repeats of four shaking phases, 1 min of each; linear shaking, orbital shaking at amplitude of 3 mm, linear shaking, and orbital shaking at amplitude of 2 mm. Raw data from the plate reader were calibrated to cuvette values according to ODcuvette = ODplate/0.23.

EP Spectroscopy. ΔaceAΔflAB E. coli cells (8) transformed with plasmids expressing wild-type PFLI or C-terminally fused Spycatcher (PFL-spyc) from a plasmid along with wild-type activating enzyme PFLA were grown anaerobi- cally and prepared for EP analysis in an anaerobic chamber (Coy Laboratory Products). The PFLA flow measurements were followed by cycles consisting of three repeats of four shaking phases, 1 min of each; linear shaking, orbital shaking at amplitude of 3 mm, linear shaking, and orbital shaking at amplitude of 2 mm. The sample temperature was maintained at 253 K using a Bruker liquid nitrogen temperature control system.

Formate Quantification. Reactions were mixed together in an anaerobic atmosphere containing 1 μmol Pyruvate, 14 pmol isolated sFUT wiffleballs with or without the addition of 1 mM CoA or acetyl-CoA in 100 μL final volume in 50/100 PBS pH 7.5. The inactivated controls were mixed together in aerobic conditions. Samples were incubated for 15 min in a vinyl anaerobic chamber (N2 with 2.5% H2, model B, Coy Laboratory Products). Formate was then determined in aerobic conditions using the Formate assay kit (Sigma-Aldrich, MAK059) according to the manufacturer's protocol.

Liquid Chromatography-Tandem Mass Spectrometry Analysis of SD5/PAGE Protein Bands. Gel bands were digested in-gel as described in Shevchenko et al. (56) with modifications. Briefly, gel bands were dehydrated using 100% acetone and then incubated in 10 mM dithiothreitol, 100 mM ammonium bicarbonate, pH ~8, at 56 °C for 45 min. The gel bands were dehydrated again and incubated in the dark in 50 mM iodoacetamide, 100 mM ammonium bicarbonate for 20 min. Gel bands were washed with ammonium bicarbonate fol- lowed by dehydration. Gel bands were resuspended in ~100 μL of a 0.01 μM sequencing grade modified trypsin solution in 50 mM ammonium bicarbonate and incubated at 37 °C overnight. Peptides were extracted from the gel pieces in a solution of 60% acetonitrile (ACN)/1% trifluoroacetic acid (TFA) using water bath sonication and then vacuum dried to ~2 μL. Dried samples were resuspended to 20 μL in 2% ACN/0.1% TFA. 5 μL were automatically injected onto a Thermo Acclaim PepMap RSLC 0.1-mm × 20-mm C18 trapping column using a Thermo EASYLC 1000 (https://www.thermofisher.com.cn/zh/home/brand/thermo-scientific.html). The column was washed for ~5 min with buffer A (Buffer A = 99.9% water/0.1% formic acid). Bound peptides were then eluted over 35 min onto a Thermo Acclaim PepMap RSLC 0.075-mm × 250-mm resolving column with a gradient of 5% B to 40% B in 24 min, ramp- ing to 90% B at 25 min and held at 90% B for the rest of the run (Buffer B = 98% acetonitrile/1% formic acid/0.1% water) at a constant flow rate of 300 nL/min. Column temperature was maintained at 50 °C using an integrated column oven (PRSO-V1, Sonation). Eluted peptides were sprayed into a ThermoScientific Q-Exactive mass spectrometer (https://www. thermofisher.com/us/en/home/brand/thermo-scientific.html) using a Flex- Spray ion spray source. The resulting MS/MS spectra scan processed using Mascot Distiller, v2.7.1 (www.matrixscience.com), and searched against a database containing all E. coli K12 protein sequences with addition of common laboura- tory contaminants (downloaded from https://www.uniprot.org/ and https://www.thegpm.org/, respectively) using the Mascot searching algorithm, v2.7. The Mascot output was then analyzed using Scaffold, v5.0 (https://www.proteomesoftware.com/) to validate protein identifications.

Data Availability. All study data are included in the main text and SI Appendix.

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