Localization of Proteins to the 1,2-Propanediol Utilization Microcompartment by Non-native Signal Sequences Is Mediated by a Common Hydrophobic Motif

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Background: N-terminal signal sequences localize enzymes to bacterial microcompartments.

Results: Signal sequences from various microcompartments localize proteins to the 1,2-propanediol utilization (Pdu) microcompartment.

Conclusion: Encapsulation of cargo in Pdu microcompartments by N-terminal signal sequences is mediated by a common motif.

Significance: This motif will inform the design of a suite of signal sequences de novo, and promiscuous localization may be metabolically relevant in vivo.

Various bacteria localize metabolic pathways to proteinaceous organelles known as bacterial microcompartments (MCPs), enabling the metabolism of carbon sources to enhance survival and pathogenicity in the gut. There is considerable interest in exploiting bacterial MCPs for metabolic engineering applications, but little is known about the interactions between MCP signal sequences and the protein shells of different MCP systems. We found that the N-terminal sequences from the ethanolamine utilization (Eut) and glycyl radical-generating protein (Grp) MCPs are able to target reporter proteins to the 1,2-propanediol utilization (Pdu) MCP, and that this localization is mediated by a conserved hydrophobic residue motif. Recapitulation of this motif by the addition of a single amino acid conferred targeting function on an N-terminal sequence from the ethanol utilization MCP system that previously did not act as a Pdu signal sequence. Moreover, the Pdu-localized signal sequences competed with native Pdu targeting sequences for encapsulation in the Pdu MCP. *Salmonella enterica* natively possesses both the Pdu and Eut operons, and our results suggest that Eut proteins might be localized to the Pdu MCP in vivo. We further demonstrate that *S. enterica* LT2 retained the ability to grow on 1,2-propanediol as the sole carbon source when a Pdu enzyme was replaced with its Eut homolog. Although the relevance of this finding to the native system remains to be explored, we show that the Pdu-localized signal sequences described herein allow control over the ratio of heterologous proteins encapsulated within Pdu MCPs.

Bacterial microcompartments (MCPs) are proteinaceous bacterial organelles that function to localize metabolic pathways to sequester toxic intermediates and contain private pools of cofactors (1, 2). There is increasing interest in applying these organelles to the encapsulation of engineered enzymatic pathways to enhance pathway flux (3). Heterologous proteins can be directed to the 1,2-propanediol utilization (Pdu) MCPs, for example, by fusion of the N-terminal signal sequences from the natively encapsulated PduP and PduD proteins to the proteins of interest (4, 5). Interactions between the N termini of encapsulated enzymes and the structural proteins of the associated MCP shell are a general mode of enzyme localization to diverse MCP systems, including the Pdu MCP (6, 7), the ethanolamine utilization (Eut) MCP (8), and the smaller MCP of *Haliangium ochraceum* (9).

One of these interactions, between the N-terminal signal sequence of the Pdu enzyme PduP and the C terminus of the Pdu shell protein PduA, is modeled to be mediated by interactions between residues Glu7, Ile10, and Leu14 presented on the α-helical N terminus of PduP and His83, Val85, and Leu88 on the C terminus of PduA (6). Further investigation by NMR confirmed that the PduP N-terminal signal sequence adopts an α-helical conformation, providing support for this model of signal sequence-shell protein interaction (3). Only two signal sequences (both from natively encapsulated Pdu enzymes) have been identified and shown experimentally to localize proteins to the Pdu MCP, however, and a more diverse set of signal sequences with a variety of encapsulation levels is desirable for the encapsulation of heterologous pathways to allow tuning of the loading and stoichiometry of multiple heterologous enzymes in the Pdu MCP. Also desirable is a system of orthogonal compartments in which certain proteins are localized

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* This work was supported by National Science Foundation Grant 1150567 (to C. M. J., E. Y. K., M. F. S., and D. T.-E.), Army Research Office Grant W911NF-15-1-0144 (to D. T.-E.), and a University of California Berkeley fellowship (to C. M. J.). The authors declare that they have no conflicts of interest with the contents of this article.

*† This article contains supplemental Table S1.

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The abbreviations used are: MCP, microcompartment; Pdu, 1,2-propanediol utilization; Eut, ethanolamine utilization; Grp, glycyl radical-generating protein; Etu, ethanol utilization; PD, propanediol; NCE, no-carbon E; aTc, anhydrous tetracycline; Ald, aldehyde dehydrogenase; Hoch, *H. ochraceum*. 

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TABLE 1

Bacterial strains used in this study

| Strain   | Organism              | Genotype               |
|----------|-----------------------|------------------------|
| CMJS271  | S. enterica serovar Typhimurium LT2 | Wild type              |
| CMJS256  | S. enterica serovar Typhimurium LT2 | ΔeutR::FRT             |
| CMJS273  | S. enterica serovar Typhimurium LT2 | ΔpocR::FRT             |
| CMJS374  | S. enterica serovar Typhimurium LT2 | ΔpduP::cat/sacB        |
| CMJS377  | S. enterica serovar Typhimurium LT2 | ΔpduP::eutE            |
| EYK3003  | E. coli DH10B          | Wild type              |
| TUC01 (11) | E. coli DH10B       | cat/sacB               |
| EYK3006  | E. coli DH10B          | FRT                    |
| EYK3007  | E. coli DH10B          | pocR::FRT              |
| EYK3008  | E. coli DH10B          | ΔpocR::FRT             |
| EYK3009  | E. coli DH10B          | ΔpocR::FRT             |
| EYK3010  | E. coli DH10B          | ΔpocR::FRT             |

Common Hydrophobic Motif in Pdu-localized Signal Sequences

The sequences from several other systems, including the Eut system from the same organism, and that at high expression levels these sequences compete with the native targeting sequences for encapsulation. Interestingly, the ratio of encapsulated proteins can be controlled by modulating the expression levels of these individual signal sequence-cargo protein fusions. These results are useful as a guide to engineering the Pdu MCP to house biosynthetic pathways and raise questions as to whether the native Pdu and Eut systems maintain unique cargo protein localization exclusively to one type of compartment and other proteins are localized exclusively to another compartment.

Here, we show that heterologous proteins are encapsulated within the Pdu MCP when fused to N-terminal targeting sequences from several other systems, including the Eut system from the same organism, and that at high expression levels these sequences compete with the native targeting sequences for encapsulation. Interestingly, the ratio of encapsulated proteins can be controlled by modulating the expression levels of these individual signal sequence-cargo protein fusions. These results are useful as a guide to engineering the Pdu MCP to house biosynthetic pathways and raise questions as to whether the native Pdu and Eut systems maintain unique cargo protein content when natively co-expressed.

Experimental Procedures

Bacterial Strains, Media, and Growth Conditions—Salmonella enterica serovar Typhimurium LT2 was used in this study along with Escherichia coli DH10B. S. enterica LT2 ΔpocR::FRT and ΔeutR::FRT strains were constructed by the λ-red-based method described previously (10). The kanamycin resistance cassette was amplified from pKD13 with primers EYKP616 and EYKP617 to yield the amplicon used to create the ΔpocR::FRT strain and with primers CMJP132 and CMJP133 to yield the amplicon used to create the ΔeutR::FRT strain. The S. enterica LT2 ΔpduP::cat/sacB and S. enterica LT2 ΔpduP::eutE strains were constructed by the λ-red-based method of Court and co-workers (11). The cat/sacB cassette was amplified from the TUC01 genome with primers CMJP228 and CMJP229 to yield the amplicon used to create the ΔpduP::cat/sacB strain and the eutE allele was amplified from the S. enterica genome with primers CMJP257 and CMJP258 to yield the amplicon used to create the ΔpduP::eutE strain. The genotype of these strains was confirmed by Sanger sequencing of DNA amplified by PCR from the appropriate regions of the S. enterica genome (see Table 1).

S. enterica strains were grown in no-carbon E (NCE) medium (23 mM monobasic potassium phosphate (Fisher), 48 mM dibasic potassium phosphate (Fisher), 17 mM sodium magnesium sulfate (Fisher), and 50 μM ferric citrate (Sigma)) with 42 mM succinate (Sigma) and either 55 mM 1,2-propanediol (Sigma) or 30 mM ethanolamine (Alfa) and 150 mM vitamin B12 (Sigma) as indicated (12, 13). Growth on 1,2-propanediol as the sole carbon source was assayed in the absence of succinate and with the addition of 150 mM vitamin B12. E. coli was grown in lysogeny broth (LB)-Miller medium (10 g/liter NaCl; EMD Chemicals). For LB cultures, kanamycin (50 μg/ml; Fisher), chloramphenicol (34 μg/ml; Fisher), and carbenicillin (50 μg/ml; Fisher) were added when necessary for plasmid maintenance. For NCE cultures, kanamycin (25 μg/ml), chloramphenicol (17 μg/ml), and carbenicillin (25 μg/ml) were added when necessary. When indicated, anhydrous tetracycline (aTc; Fisher) and arabinose (Calbiochem) were added at the concentrations indicated when cultures reached an A600 of ~0.4. Cultures were grown at 37 °C with 225 rpm orbital shaking unless otherwise indicated.

MCP Expression and Purification—5-ml cultures were grown in LB-Miller medium from a single colony for 24 h at 30 °C, then subcultured 1:1000 into 400 ml of NCE medium with 55 mM 1,2-PD, and grown for 13–15 h at 37 °C. At an A600 of ~0.4, appropriate inducer was added at the concentrations indicated, and the cultures were grown for a further 5.5 h. MCPs were purified by sedimentation as described previously (14) with the following modification: in the place of BPER-II bacterial lysis solution (Thermo), a solution of 1% (w/v) octyl thioglucoside (Santa Cruz Biotechnology) in 20 mM Tris (Fisher), pH 7.5 in water was used for cell lysis.

Transcriptional Regulation Analysis—5-ml cultures were grown in LB-Miller medium from a single colony for 24 h at 30 °C, then subcultured 1:1000 into 5 ml of NCE medium, and grown for 14–15 h at 37 °C. At an A600 of ~0.4, inducing molecules were added (50 mM 1,2-PD, 30 mM ethanolamine, and 150 nM vitamin B12 as appropriate), and the cultures were grown for the time indicated with time points being collected every hour for analysis by flow cytometry as described below.

Competition Analysis—5-ml cultures were grown in LB-Miller medium from a single colony for 24 h at 30 °C, then subcultured 1:1000 into 5 ml of NCE medium with 55 mM 1,2-PD, and grown for 14–15 h at 37 °C. At an A600 of ~0.4, appropriate inducers were added (aTc and arabinose) at the concentrations indicated, and the cultures were grown for a further 5.5 h before samples were collected for analysis by flow cytometry as described below.

Reference Genomes for Glycol Radical-generating Protein (Grp) and Eut MCPs—Genes of the Grp MCP operon of Clostridium beijerinckii and the Etu MCP operon of Clostridium kluyveri were identified in a previous bioinformatics study (15). Gene sequences from the putative Grp MCP operon were retrieved from the NCBI reference genome NC_009617 of C. beijerinckii. The gene encoding the aldehyde dehydrogenase of the Grp operon, here denoted GrpAld, has accession number Cbi_4045 in the genome NC_009617. Gene sequences from the Etu operon were retrieved from the NCBI reference genome NC_011837 of C. kluyveri. The first aldehyde dehydrogenase encoded in the Etu operon, denoted EtuAld, has accession number Cbi_4045 in the genome NC_009617. Gene sequences from the Etu operon were retrieved from the NCBI reference genome NC_011837 of C. kluyveri. The first aldehyde dehydrogenase encoded in the Etu operon, denoted EtuAld here, has accession number Ckr_0979 in this genome. Its first 60 nucleotide bases are identical to those of the second aldehyde dehydrogenase, Ckr_0979, of the Etu operon in the genome NC_011837. Multiple sequences alignments were conducted using the Clustal Omega tool (16).

SDS-PAGE and Western Blotting—Polyacrylamide gel electrophoresis was carried out by standard procedures (17) with 12.5 or 15% acrylamide gels in a denaturing buffer system. Whole culture lysate sample loading was normalized by culture A600 at time of sample collection. Purified MCP sample loading was normalized by total protein concentration as judged by bicinchoninic acid (BCA) assay performed according to the
manufacturer’s instructions (Thermo). Proteins were transferred to a PVDF membrane for Western blotting. Samples were probed with a Clontech mouse anti-GFP primary antibody, a Life Technologies rat anti-mCherry primary antibody, or a Sigma mouse anti-FLAG primary antibody diluted 1:2000 in 50 mM Tris, 150 mM NaCl, pH 7.6 with 0.05% Tween 20 (TBST) with 1% (w/v) dry milk and then with a Thermo HRP-conjugated goat anti-mouse or a Santa Cruz Biotechnology HRP-conjugated goat anti-rat secondary antibody diluted 1:1000 in TBST. Labeling was visualized with Thermo West Pico Chemiluminescent substrate using a Bio-Rad ChemiDoc XRS+ system.

Fluorescence Microscopy—Bacteria were viewed using a Nikon Ni-U upright microscope with a 100×, 1.45 numerical aperture plan apochromat oil immersion objective. Images were captured using an Andor Clara-Lite digital camera. Fluorescence images were collected using a C-FL Endow GFP HYQ band pass filter. Images were captured using Nikon NIS Elements software. All images intended for direct comparison (e.g., images of the same strain in Pdu MCP-inducing and -non-inducing conditions) were captured using the same exposure and aperture at room temperature and were adjusted identically in the Adobe Photoshop software for contrast.

Transmission Electron Microscopy of Purified Pdu MCPs—MCPs were purified from S. enterica cultures as described above. 10 μl of purified MCP samples at a concentration of 100 μg/ml were placed on 400 mesh Formvar-coated copper grids with a carbon film (Electron Microscopy Sciences) for 2 min. The grids were washed three times with deionized water and then stained with 2% aqueous uranyl acetate for 2 min. Samples were observed and photographed with a Gatan Ultrascan 1000 camera (Gatan, Inc., Pleasanton, CA) on an FEI Tecnai T12 transmission electron microscope.

Plasmid Construction—Plasmids bearing genes encoding fluorescent reporters and competitor fusion proteins were prepared by the golden gate assembly method (18). Briefly, for the construction of fluorescent reporters, a gfpmut2 gene was generated with a silent mutation, GAC to GAT, at residue 237 to abrogate a BsaI restriction site and enhance assembly efficiency. Once appropriate PCR products were prepared, pTET or pBAD inducible plasmids were assembled by a standard golden gate temperature cycling protocol (25 × 2 min at 37 °C, 5 min at 16 °C, 5 min at 50 °C, 5 min at 80 °C) using T4 DNA ligase and BsaI restriction endonuclease and transformed into E. coli DH10B. The sequences of assembled constructs were confirmed by Sanger sequencing of the reporter- or competitor-encoding regions. Enzymes and other molecular biology reagents were obtained from New England Biolabs. Sequences of primers used can be found in supplemental Table S1.

The Pcut transcriptional reporter was synthesized by the same method described previously for the reporter of Ppdu transcription (19). Briefly, golden gate assembly was used to assemble the promoter region of interest; a fluorescent reporter gene, gfpmut2; and pPROTET vector backbone to form the reporter plasmid. Correct assembly was once again confirmed by Sanger sequencing. Sequences for each plasmid (Table 2) are available for download at the AddGene database.

Flow Cytometry— Cultures were grown as described above, and at the indicated time points, aliquots of the samples were diluted to an A600 of ~0.01 in phosphate-buffered saline (PBS) supplemented with 2 g/liter kanamycin (to halt translation) and stored at 4 °C (20). Samples were then diluted 1:40 into PBS supplemented with 2 g/liter kanamycin in 96-well plates for flow cytometry. The GFP fluorophore was allowed to mature by gating on the forward and side scatter channels using FlowJo software. Reported fluorescence values are the arithmetic mean of the geometric mean green fluorescence of three independent samples acquired on three different days. Unless otherwise noted, error bars represent one S.D.

Two-color Competition Assays— Cultures were grown as described with appropriate inducing molecules added when the A600 reached ~0.4. At 5.5 h postinduction, cells were diluted 1:4 into PBS with 2 g/liter kanamycin, and we measured A600 as well as bulk fluorescence in the GFP and mCherry channels for each sample. Measurements were collected using flat bottomed UV transparent 96-well plates (Corning, Inc.) in a BioTek Synergy HTX multimode plate reader. Reported fluorescence values were normalized to A600 and subsequently normalized to the fluorescence of a control sample without 1,2-PD and with the same concentrations of inducing molecules. Values reported are the arithmetic mean of three biological replicates.

| Plasmid | Genotype | Antibiotic resistance |
|---------|----------|-----------------------|
| EYK054  | pBAD pduD21–224-gfpmut2-ssrA | Chloramphenicol |
| EYK345  | pTET pduD21–224 | Kanamycin |
| EYK346  | pTET pduD1–20 | Kanamycin |
| CMI038  | pBAD pduD21–20-gfpmut2-ssrA | Chloramphenicol |
| CMI116  | pBAD eutC1–20-gfpmut2-ssrA | Chloramphenicol |
| CMI119  | pBAD eutC1–20-ssrA | Chloramphenicol |
| CMI129  | pBAD eutAld1–20-ssrA | Chloramphenicol |
| CMI130  | pBAD grpAld1–20-ssrA | Chloramphenicol |
| CMI131  | pBAD HochAld1–20-ssrA | Chloramphenicol |
| CMI137  | pBAD eutAld1–20-ssrA | Chloramphenicol |
| CMI156  | pTET eutC1–20-pduD21–224-FLAG | Kanamycin |
| CMI157  | pTET eutE1–20-pduD21–224-FLAG | Kanamycin |
| CMI158  | pTET gfpmut2-ssrA | Kanamycin |
| CMI159  | pTET HochAld1–20-pduD21–224-FLAG | Kanamycin |
| CMI161  | pTET eutAld1–20-pduD21–224-FLAG | Kanamycin |
| CMI162  | pTET eutAld1–20-ssrA | Chloramphenicol |
| CMI163  | pTET eutAld1–20-mCherry-ssrA | Kanamycin |
| CMI206  | pTET pduP1–18-mCherry-ssrA | Kanamycin |
| pKD13(10) | FRT-Kanamycin^−/FRT^− | Carbenicillin |
| pKD46(10) | pBAD λ-red, TS (30 °C) | Carbenicillin |
| pSIM6(11) | λ-red, TS (30 °C) | Carbenicillin |
| PFC220(10) | fbl recombinase | Carbenicillin |
Results

*S. enterica* ΔeutR::FRT Does Not Form Eut MCPs but Can Form Pdu MCPs—We hypothesized that because the known or putative N-terminal signal sequences from other MCP systems bear similar hydrophobic residue motifs (Fig. 1) these N-terminal signal sequences would localize heterologous cargo to the Pdu MCPs. *S. enterica* LT2, however, can express both Pdu and Eut MCPs, and we wished only to observe Pdu MCP formation and cargo encapsulation. The transcriptional regulator PocR is necessary for Pdu MCP formation (19, 21–23). We confirmed that the analogous transcriptional regulator EutR is necessary for Eut MCP formation by two methods: first using a fluorescent reporter of Peut transcription (24, 25) and then using a fluorescent reporter of encapsulation in the Eut or Pdu MCP (8). We observed activation of the Peut promoter by 30 mM ethanolamine and 150 nM vitamin B₁₂ as indicated by increased GFP fluorescence in an *S. enterica* strain containing the Peut-gfpmut2 transcriptional reporter as compared with a control culture to which no ethanolamine and vitamin B₁₂ were added. This activation was abrogated in an *S. enterica* ΔeutR::FRT strain (Fig. 2). Activation could be complemented in the *S. enterica* ΔeutR::FRT strain by expression of EutR from a secondary plasmid (Fig. 2). Episomal maltose-binding protein was expressed as a negative control for these experiments. To observe Eut MCP formation, we used a fluorescent reporter of encapsulation in which EutC₁–₂⁰ is fused to GFP followed by a C-terminal ssrA tag, which mediates degradation of the fluorophore by the ClpXP protease in the cytosol (encapsulation in an MCP thus rescues the reporter from proteolysis). When expression of the encapsulation reporter construct pBAD-eutC₁–₂⁰-gfp-ssrA was induced by the addition of 0.02% arabinose, fluorescent puncta were not observed in a *S. enterica* ΔeutR::FRT strain upon the addition of 30 mM ethanolamine and 150 nM vitamin B₁₂, but puncta were observed in the ΔpocR::FRT strain under the same conditions (Figs. 2 and 3). We therefore conducted our subsequent experiments in the *S. enterica* ΔeutR::FRT strain to ensure that we observed only Pdu MCP formation and encapsulation.

EutC₁–₂⁰ and EutE₁–₂⁰ Direct Heterologous Proteins to the Pdu MCP—We next explored whether two signals sequences from the Eut MCP are promiscuous in their localization of heterologous cargo, that is, whether the N-terminal signal sequences EutC₁–₂⁰ and EutE₁–₂⁰ localize proteins to the Pdu MCP. When modeled as α-helices, the Eut signal sequences present hydrophobic motifs similar to those of the Pdu signal sequences, leading us to suspect that they would interact with the Pdu MCP shell proteins in a similar manner (Fig. 1). We therefore tested whether the EutC and EutE signal sequences

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**Common Hydrophobic Motif in Pdu-localized Signal Sequences**

### A. N-terminal MSA

| Protein   | Sequence                  | Pdu localized? |
|-----------|---------------------------|---------------|
| PduP      | MNTSELETIRTESEQL          | Y             |
| PduD      | MEINEKLLROIIEDVLRDMK      | Y             |
| EutC      | MDQKQIEEIVRSMASMG         | Y             |
| EutE      | MNQQDIQEVKVALLKMK         | Y             |
| GrpAld    | MDVVVEKLRQRSEEVK          | Y             |
| EtuAld    | MDKDLQISQEEVRTLIAKAK      | N             |
| EtuAld+Ile| MDKDLQISQEEIVRTLIAKAK     | Y             |
| H.ochraceum| MALREDRAEIEVRLARLD       | N             |
| GFPmut2   | MSKGEELTFGVPIWELD         | N             |

### B. C-terminal MSA

| Protein   | Sequence                  |
|-----------|---------------------------|
| PduJ      | SCVHRPSDVEAIPKSA          |
| Grp (Cbei_4058)| SIHVPRPHDTEKIPLKFV   |
| PduA      | AVHVPRPHDTEKIPLGISQ      |
| EutM      | SVHVPRPHGDELVEFPISFKGDSNI|
| Etu (Ckr_0975)| SVHVPRPHDTEKIPLNIG  |
| H.ochraceum| AVHVPRPHNVDAALPLGTRPGMDKSA|

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**FIGURE 1. Sequence alignment of N-terminal sequences of encapsulated enzymes and C-terminal sequences of structural proteins.** A, a multiple sequence alignment (MSA) of the N-terminal amino acid residues of the Pdu enzymes PduD and PduP, the Eut enzymes EutC and EutE, the Grp aldehyde dehydrogenase, the Etu aldehyde dehydrogenase, the *H. ochraceum* compartment signal sequence, the Etu aldehyde dehydrogenase with Ile added at residue 11, and the first 20 amino acids of the GFP protein for comparison. B, a multiple sequence alignment of the C-terminal amino acid residues of the Pdu structural proteins PduJ and PduA, the Eut structural protein EutM, the putative Grp structural protein from *C. beijerinckii* denoted Cbei_4058, the putative Etu structural protein from *C. kluyveri* denoted Ckr_0975, and the hexagonal *H. ochraceum* structural protein. Y, yes; N, no.
function to mediate the encapsulation of the GFP-ssrA reporter in the Pdu MCP. Upon the induction of either pBAD-eutC1–20-gfp-ssrA or pBAD-eutE1–20-gfp-ssrA plasmids with 0.02% arabinose in S. enterica LT2/ΔeutR::FRT, punctate fluorescence was observed when 55 mM 1,2-propanediol was added to the culture medium to induce the formation of Pdu MCPs. Cells grown in ethanolamine MCP-inducing conditions (in the presence of 0.02% arabinose, 30 mM ethanolamine, and 150 nM vitamin B12) showed no punctate fluorescence (Fig. 3). A Western blot against GFP of purified Pdu MCPs from S. enterica LT2 ΔeutR::FRT expressing EutC1–20-GFP-ssrA and EutE1–20-GFP-ssrA confirmed that these two Eut signal sequences target GFP to the Pdu MCP (Fig. 4). Transmission electron micrographs of purified Pdu MCPs confirmed that MCPs are morphologically normal when EutC1–20-GFP-ssrA and EutE1–20-GFP-ssrA are encapsulated (Fig. 4).

**GrpAld1–20 from the Grp MCP Directs Heterologous Proteins to the Pdu MCP**—The aldehyde dehydrogenase enzyme from the computationally predicted Grp MCP (15) contains a putative N-terminal signal sequence as identified by comparison with the known Pdu and Eut signal sequences (Fig. 1).
The putative signal sequence is referred to here as GrpAld1–20. We hypothesized that this signal sequence would also mediate encapsulation of GFP in the Pdu MCP. The induction of a pBAD-grpAld1–20-gfp-ssrA plasmid with 0.02% arabinose in S. enterica LT2/H9004 eutR::FRT yielded punctate fluorescence when 1,2-propanediol was added to the culture medium (Fig. 3). An anti-GFP Western blot of purified Pdu MCPs from S. enterica LT2 ΔeutR::FRT expressing GrpAld1–20-GFP-ssrA confirmed that the putative Grp signal sequence localizes protein to the Pdu MCP (Fig. 4) as we observed for the EutC1–20-GFP-ssrA and EutE1–20-GFP-ssrA constructs. Transmission electron micrographs again confirmed that Pdu MCPs are morphologically normal when GrpAld1–20-GFP-ssrA is encapsulated (Fig. 4). S. enterica LT2 does not possess the Grp MCP genes so the use of the S. enterica LT2 ΔeutR::FRT strain was sufficient to ensure that the puncta observed arose from Pdu MCPs alone.

Recapitulation of the Hydrophobic Motif Confers Signal Sequence Function on a Peptide That Did Not Previously Mediate Encapsulation—The aldehyde dehydrogenase enzyme Ald1 from the predicted ethanol utilization (Etu) MCP (15, 26) also

![FIGURE 3. Several non-native targeting sequences appear to localize GFP to Pdu MCPs in S. enterica. Phase-contrast and fluorescence microscopy of S. enterica LT2 ΔeutR expressing PduD1–20-GFP-ssrA (A), PduP1–18-GFP-ssrA (B), EutC1–20-GFP-ssrA (C), EutE1–20-GFP-ssrA (D), GrpAld1–20-GFP-ssrA (E), or EtuAld1–21-GFP-ssrA (F) in the presence of 55 mM 1,2-PD (left columns) or 30 mM ethanolamine (EA) and 150 nM vitamin B12 (right columns). Scale bars represent 1 μm.](image-url)
bears a putative N-terminal signal sequence, here referred to as EtuAld1–20. We found that this peptide does not mediate detectable encapsulation of the heterologous fluorescent reporter protein EtuAld1–20-GFP-ssrA in the Pdu MCP. We noted that the Etu signal sequence had an apparent gap that disrupted the hydrophobic motif, so we inserted an Ile residue at position 11 of the putative EtuAld sequence (denoted EtuAld/H11001Ile1–21) to recapitulate the putative signal sequence motif discussed above (Fig. 1). Remarkably, this engineered peptide functions as a Pdu signal sequence, as indicated by the appearance of punctate fluorescence when we express the peptide as a genetic fusion to GFP under Pdu MCP-forming conditions (Fig. 3). Localization was confirmed by an anti-GFP Western blot of purified Pdu MCPs from S. enterica LT2 ΔeutR cultures expressing PduD1–20-GFP-ssrA, EutC1–20-GFP-ssrA, EutE1–20-GFP-ssrA, GrpAld1–20-GFP-ssrA, or untagged GFP-ssrA and from a strain bearing no plasmid. B, Coomassie stain and Western blot against GFP of an SDS-PAGE separation of purified Pdu MCPs from S. enterica LT2 ΔeutR cultures expressing PduD1–20-GFP-ssrA, EutC1–20-GFP-ssrA, EutE1–20-GFP-ssrA, GrpAld1–20-GFP-ssrA, or untagged GFP-ssrA and from a strain bearing no plasmid. The Western blots for the two sets of purified MCP samples were conducted separately due to large variations in expression and encapsulation between different GFP fusion constructs. C, transmission electron micrographs of Pdu MCPs purified from S. enterica LT2 ΔeutR cultures expressing EutC1–20-GFP-ssrA, EutE1–20-GFP-ssrA, GrpAld1–20-GFP-ssrA, and EutAld/H11001Ile1–21-GFP-ssrA as indicated. Samples were stained with uranyl acetate. Scale bars represent 100 nm.

A Signal Peptide from a Smaller MCP Does Not Direct Heterologous Proteins to the Pdu MCP—A smaller MCP (~40 nm in diameter) of unknown function from H. ochraceum has been heterologously expressed in E. coli, and its N-terminal signal peptide has been characterized (9). The first 20 amino acids of the H. ochraceum N-terminal signal peptide (HochAld1–20) also include the characteristic hydrophobic motif. We tested this signal peptide for the ability to target GFP to the Pdu MCP but found no evidence of GFP encapsulation as assessed by fluorescence microscopy (data not shown).

Non-native Pdu-localized Signal Sequences Compete with the Native Pdu Signal Sequences for Encapsulation—To investigate whether the localization of the various signal sequences tested above to the Pdu MCP is mediated by the same interaction with the Pdu shell proteins as mediates the encapsulation of PduD1–20- and PduP1–18-tagged proteins, we devised a competition assay for Pdu targeting. We constructed translational fusions of each signal peptide to PduD21–224-FLAG on an aTc-inducible pTET plasmid and then co-expressed these proteins at varying expression levels with a constant level of PduD1–20- and PduP1–18-tagged GFP-ssrA expression from an arabinose-inducible pBAD plasmid. We measured the associated levels of PduD1–20-GFP-ssrA or PduP1–18-GFP-ssrA encapsulation by flow cytometry (27). Decreases in the GFP fluorescence with increasing levels of competitor expression indicate competition between the two proteins for encapsula-

FIGURE 4. Characterization of purified Pdu MCPs containing encapsulation reporters. A, Coomassie stain and Western blot against GFP of an SDS-PAGE separation of purified Pdu MCPs (with loading normalized by total protein concentration as judged by BCA assay) from S. enterica LT2 ΔeutR cultures expressing PduD1–20-GFP-ssrA, EutC1–20-GFP-ssrA, EutE1–20-GFP-ssrA, GrpAld1–20-GFP-ssrA, or untagged GFP-ssrA and from a strain bearing no plasmid. B, Coomassie stain and Western blot against GFP of an SDS-PAGE separation of purified Pdu MCPs (with loading normalized by total protein concentration as judged by BCA assay) from S. enterica LT2 ΔeutR cultures expressing PduP1–18-GFP-ssrA, EutAld/H11001Ile1–21-GFP-ssrA, or untagged GFP-ssrA and from a strain bearing no plasmid. The Western blots for the two sets of purified MCP samples were conducted separately due to large variations in expression and encapsulation between different GFP fusion constructs. C, transmission electron micrographs of Pdu MCPs purified from S. enterica LT2 ΔeutR cultures expressing EutC1–20-GFP-ssrA, EutE1–20-GFP-ssrA, GrpAld1–20-GFP-ssrA, and EutAld/H11001Ile1–21-GFP-ssrA as indicated. Samples were stained with uranyl acetate. Scale bars represent 100 nm.
tion and suggest that the proteins may be encapsulated via the same mechanism or interaction.

We found that EutC1–20 and EutE1–20 compete significantly \((p < 0.05)\) with PduD1–20-GFP-ssrA for encapsulation as compared with a negative control, PduD 21–224-FLAG, as did GrpAld1–20, EtuAld1–21, and the positive control PduD1–224-FLAG (Fig. 5). As expected, EtuAld 1–20 and HochAld1–20 exhibited no competition by this assay because they do not function as Pdu signal sequences (Fig. 5). The assay using PduP1–20-GFP-ssrA was somewhat less sensitive likely due to lower expression of the PduP1–20-tagged construct, but significant competition \((p < 0.05)\) was still observed for EutC1–20 and for the positive control PduD1–224-FLAG. Flow cytometry results were confirmed for EutC1–20-PduD21–224-FLAG by a Western blot of purified Pdu MCPs and cell lysates against GFP and against the FLAG epitope, which shows the same trends as the flow cytometry data (Fig. 6). We therefore conclude that all of the Pdu-localized sequences compete with the native Pdu signal sequences for encapsulation.

*S. enterica* LT2 \(\Delta pduP::eutE\) Retains the Ability to Grow on 1,2-PD as the Sole Carbon Source—We speculated that, because EutE1–20-GFP-ssrA is localized to the Pdu MCP, EutE might...
likewise be encapsulated in the Pdu MCP in vivo if the two MCP systems were expressed contemporaneously in a cell. Furthermore, a multiple sequence alignment reveals that the full-length pduP and eutE aldehyde dehydrogenase genes are homologs with 45% sequence identity. We therefore tested whether S. enterica LT2 ΔpduP::eutE retained the ability to grow on 1,2-PD as the sole carbon source presumably with EutE encapsulated in the Pdu MCP and carrying out the metabolic function of PduP. We found that S. enterica LT2 ΔpduP::eutE retains the ability to grow on 1,2-PD as the sole carbon source in NCE medium, exhibiting significantly greater growth than a control strain, S. enterica LT2 ΔpduP::cat/sacB, although growth was somewhat slower than that of the wild type (Fig. 7). A ΔpduP::cat/sacB strain retained some growth as reported previously, but growth of the knock-out was significantly slower than that of the wild type and the ΔpduP::eutE strain (28). The observed residual growth in the ΔpduP::cat/sacB strain as compared with a ΔpocR::FRT strain (which forms no Pdu MCPs) is most likely due to cytosolic aldehyde dehydrogenases acting on propionaldehyde that escaped the Pdu MCP.

The Ppdu Promoter Is Activated by 1,2-PD in the Presence of Ethanolamine and Vitamin B12 and the Peut Promoter Is Activated by Ethanolamine and Vitamin B12 in the Presence of 1,2-Propanediol—Given the apparent interaction of various signal sequences with the Pdu MCP shell proteins, we wondered whether cellular mechanisms might be in place to avoid cross-localization of compartmentalized enzymes such as those observed in the ΔpduP::eutE strain. We hypothesized that transcriptional regulation may limit expression to only one compartment system in each cell when conditions would otherwise induce the expression of multiple types of MCPs. To test this idea, we made use of a set of reporters for promoter activity. We previously constructed a fluorescent reporter of Ppdu promoter activation (19), and in this study, we coupled that reporter with an analogous fluorescent reporter of Peut transcriptional activation to compare the induction of Ppdu and Peut in response to 1,2-propanediol and to ethanolamine and vitamin B12. The Ppdu:gfpmut2 reporter is identical in sequence to that reported previously, consisting of the 373 bases immediately 5′ of the pduA open reading frame transcriptionally fused to gfpmut2 (19). The Peut:gfpmut2 reporter contains a 300-base portion of the promoter of the S. enterica Eut operon immediately 5′ of the eutS open reading frame transcriptionally fused to gfpmut2. We measured the response of each reporter to the presence of 1,2-propanediol, to the presence of ethanolamine and vitamin B12, and to neither or both conditions by sampling cultures grown in appropriate media hourly and interrogating the samples by flow cytometry (20). The Ppdu reporter showed activation in response to 55 mM 1,2-propanediol but not in response to 30 mM ethanolamine and 150 nM vitamin B12. AΔpocR was subsequently measured at the time points indicated. Values shown are the arithmetic mean of three independent replicates; error bars indicate one S.D. A.U., absorbance units.
observed that both promoters retain activation over the course of the experiment upon the addition of both sets of inducing molecules (1,2-PD and ethanolamine and vitamin B₁₂), and we conclude that there are no readily apparent mechanisms to abrogate the transcription of one MCP polycistron when the other is expressed (Fig. 8).

Manipulating Signal Sequence and Induction Level Allows the Tuning of Cargo Protein Ratios—We next explored whether combinations of cargo proteins bearing different N-terminal signal sequences cause different ratios of cargo proteins to be loaded to the Pdu MCP. To make these measurements, we used the same fluorescent encapsulation reporter scheme described above but in this case used two fluorophores (GFPmut2 and mCherry) simultaneously, each bearing an N-terminal signal sequence and a C-terminal ssrA degradation tag. This approach allowed us to quantify encapsulation of both proteins simultaneously using measurements of cellular fluorescence. We tested several combinations of cargo proteins (pduD¹⁻¹⁸-mCherry-ssrA and pduD¹⁻¹⁰,gfp-ssrA, pduD¹⁻¹⁰,gfp-ssrA, pduD¹⁻¹⁰,gfp-ssrA, or eutE¹⁻¹⁰,gfp-ssrA) with varying levels of transcriptional activation for each cargo protein as modulated by varying concentrations of inducing molecules. As above, increasing ratios of fluorescence in the case with Pdu MCPs as compared with the case without Pdu MCPs indicate increased cargo encapsulation. We further characterized the relative ratio of the two cargo fluorophores by taking the quotient of these two independent fluorescence ratios. This measurement is not an absolute measurement of the stoichiometric ratio of the cargo proteins but is indicative of the stoichiometric ratio and can be made in high throughput to characterize many signal-sequence and induction-level combinations.

The encapsulation of tagged GFP-ssrA protein under the control of the pBAD promoter as indicated by the fluorescence ratio increased with increasing concentrations of arabinose as did the encapsulation of tagged mCherry-ssrA under the control of the pTET promoter with increasing concentrations of anhydrous tetracycline (Fig. 10). Notably, the fluorescence ratios observed for each reporter do not vary significantly with respect to the induction of the other reporter protein (Fig. 10). We calculated the relative ratio of the fluorescence ratios for

FIGURE 8. Pₚₚ₅ and Pₚₑᵤₜ are transcriptionally activated when both 1,2-PD and ethanolamine are present. Flow cytometry of S. enterica LT2 cultures bearing plasmids encoding the Pₚₑᵤₜ-gfpmut2 (A) or the Pₚₚ₅-gfpmut2 reporter constructs (B) was carried out. Inducing molecules (55 mM 1,2-PD, 30 mM ethanolamine (EA), and 150 nm vitamin B₁₂ as indicated) were added at an A₆₀₀ of 0.4. Values shown are the arithmetic mean of the geometric mean fluorescence (Fluor.) of three independent replicates measured at 1-h intervals after induction as indicated; error bars indicate one S.D. Representative histograms can be found in Fig. 9. A.U., arbitrary units.

FIGURE 9. Flow cytometry histograms of S. enterica expressing fluorescent reporters of Pₚₚ₅ and Pₑᵤₜ transcriptional activation. Flow cytometry of S. enterica LT2 cultures bearing plasmids encoding (A) the Pₑᵤₜ-gfpmut2 or (B) the Pₚ₅-gfpmut2 reporter constructs was carried out. Inducing molecules (55 mM 1,2-PD, 30 mM ethanolamine (EA), and 150 nm vitamin B₁₂ as indicated) were added at an A₆₀₀ of 0.4 (0 h). Representative histograms are shown from the 0-, 4-, 8-, 12-, and 16-h time points. The gain at the 0-h time point is increased relative to later time points to ensure that samples are identical prior to induction. A.U., arbitrary units.
these various combinations of GFP and mCherry reporters as a proxy for the stoichiometric ratio of the two cargo proteins and found that a wide range of relative cargo fluorescence ratios can be achieved using the native Pdu signal sequences and the EutC1–20 and EutE1–20 sequences (Fig. 11). The observations made by fluorescence for the PduD1–20-GFP-ssrA/EutE1–20-mCherry-ssrA reporter pair were confirmed by MCP purification and Western blotting against GFP and mCherry. As observed using fluorescence measurements, encapsulation of the PduD1–20-GFP-ssrA or EutE1–20-mCherry-ssrA protein was not significantly decreased by encapsulation of the other reporter protein (Fig. 12). Colocalization of PduD1–20-GFP-ssrA and EutE1–20-mCherry-ssrA to Pdu MCPs in S. enterica cells expressing both reporters was confirmed by fluorescence microscopy (Fig. 12).

**Discussion**

We demonstrated that heterologous proteins tagged with several known or putative N-terminal signal sequences from the Eut and Grp bacterial MCP systems can be localized to the Pdu MCP. These signal sequences include EutC1–20, a known signal sequence for the Eut MCP, and EutE1–20, a peptide that has not previously been shown to localize heterologous proteins to the Eut MCP. The fact that the aldehyde dehydrogenase EutE has an N-terminal signal sequence is not surprising considering its homology and functional similarity to the encapsulated Pdu MCP aldehyde dehydrogenase PduP. Rather than the Ile10/Leu14 motif of the PduP N terminus, however, the Eut signal sequences bear the motifs Val10/Met14 and Val10/Leu14 at the amino acid positions previously shown to be most impor-
tant for encapsulation (Fig. 1) (6, 27). We further tested the putative N-terminal signal sequence from the aldehyde dehydrogenase of the Grp MCP and found that it too mediates the encapsulation of proteins in the Pdu MCP. This finding aligns well with the hypothesis that MCP systems are evolutionarily centered around “signature” enzymes, such as aldehyde dehydrogenases, which must be encapsulated and form the basis of many evolutionarily related MCP operons (29–31). The N-terminal signal sequence from the smaller \textit{H. ochraceum} MCP did not mediate Pdu MCP encapsulation as judged by fluorescence microscopy perhaps due to structural differences between Pdu shell proteins and the shell proteins of its smaller compartment, particularly at the C termini of the putative binding partners.

The EtuAld + Ile\textsuperscript{1–21} peptide, which recapitulated the appropriate hydrophobic motif upon insertion of Ile at position 11, also localized GFP to the Pdu MCP. This hydrophobic motif alone, however, is apparently not sufficient for a peptide to function as a Pdu MCP signal sequence. Alignment of the first 20 amino acids of GFP to the signal peptides (Fig. 1) demonstrates that the N terminus of a non-localizing protein can strongly resemble the motif in question but not confer encapsulation. This suggests that $\alpha$-helical structure as has been shown by NMR for the PduP1–18 signal sequence (3) in addition to primary amino acid sequence is important to the signal sequence–shell protein interaction that mediates encapsulation. The signal sequences that we show here to be localized to the Pdu MCP are all predicted to form $\alpha$-helices by the JPred4 secondary structure prediction tool, whereas the first 20 amino acids of GFP are not (32). Interestingly, the \textit{H. ochraceum} N-terminal signal sequence is predicted to adopt an $\alpha$-helical conformation and includes the appropriate hydrophobic motif but does not mediate localization. Together, this evidence suggests that $\alpha$-helical structure and the appropriate hydrophobic residue motif are necessary but not sufficient for Pdu MCP encapsulation by N-terminal signal sequences. Recent computational studies confirm that an N-terminal amphipathic, $\alpha$-helical motif is widespread among encapsulated protein genes in \textit{Pdu} and \textit{Eut} loci in various organisms (33).

To investigate whether the various Pdu-localized signal sequences interact with the Pdu MCP by the same mechanism as the native Pdu N-terminal signal sequences, we tested whether these signal sequences competed with the Pdu signal

\begin{figure}
\centering
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\caption{Relative fluorescence measurements of \textit{S. enterica} expressing two fluorescent reporters of encapsulation reveal that a range of cargo ratios can be achieved. Plate reader fluorescence measurements of \textit{S. enterica} LT2 cultures containing plasmids encoding fluorescent reporters of encapsulation as indicated were recorded. Inducing molecules (55 mM 1,2-PD, arabinose (\textit{ara}), and aTc as indicated) were added at an $A_{600}$ of 0.4. Values shown are the quotients of the normalized (Norm.) green and red fluorescence of each culture (see Fig. 9). Higher values indicate a greater ratio of green to red normalized fluorescence; lower values indicate a greater ratio of red to green normalized fluorescence. Values are the arithmetic mean of measurements from three independent replicates. The S.D. of all samples was less than 0.10 apart from samples labeled with the * symbol for which the S.D. was less than 0.20. A.U., arbitrary units.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure12}
\caption{Characterization of purified MCPs from \textit{S. enterica} expressing two fluorescent encapsulation reporters. A, top, Coomassie stain of an SDS-PAGE separation of purified MCPs purified from \textit{S. enterica} LT2 bearing an arabinose (\textit{ara})-inducible plasmid encoding PduD\textsuperscript{1–20}-GFP-ssrA and bearing a secondary aTc-inducible plasmid encoding EutE\textsuperscript{1–20}-mCherry-ssrA. Inducing molecules (55 mM 1,2-PD, arabinose, and aTc as indicated) were added at an $A_{600}$ of 0.4. Loading was normalized by total protein concentration as judged by BCA assay. Middle, Western blot of the same samples against mCherry. Bottom, Western blot of the same samples against GFP. B, phase-contrast and fluorescence microscopy of \textit{S. enterica} LT2 expressing PduD\textsuperscript{1–20}-GFP-ssrA and EutE\textsuperscript{1–20}-mCherry-ssrA. Inducing molecules (55 mM 1,2-PD, 0.005% arabinose, and 1 ng/ml aTc) were added at an $A_{600}$ of 0.4. Scale bars represent 1 \textmu m.}
\end{figure}
sequences PduD<sup>1–20</sup> and Pdub<sup>1–18</sup> for encapsulation. We found that they do compete for encapsulation, suggesting that the encapsulation of proteins bearing non-native signal sequences in Pdu MCPs is mediated by the same signal sequence-shell protein interaction as native Pdu enzyme encapsulation. It has previously been demonstrated computationally that multiple Pdu cargo enzymes may interact with PduA or Pdj (7). The localization of Eut and Grp signal sequences to the Pdu MCP is perhaps not surprising considering the similarity in C-terminal amino acid sequence among the PduP binding partner PduA, the Pdu shell protein Pdj, the Eut shell protein EutM, and the Grp MCP shell protein homolog denoted Cbei_4058 in the <i>C. beijerinckii</i> genome (Fig. 1). If the C termini of the shell proteins of these various compartments all interact with the N termini of their respective cargo in the same manner as the Pdu shell proteins interact with their cargo enzymes, then the similarities in C-terminal amino acid sequences of the shell proteins dictate the similar patterns of residues at the N termini of cargo proteins. For this reason, we suspect that the Pdu-localized signal sequences in this study interact with PduA, Pdj, or both proteins. It remains possible that non-native signal sequence-tagged proteins are encapsulated via another interaction but still compete with native Pdu signal sequences due to effects of steric exclusion during Pdu MCP loading; the assays described here cannot discriminate between these two possibilities.

The localization of proteins to the Pdu MCPs by the N-terminal signal sequences from other MCP systems raises important microbiological and engineering questions with respect to the function and use of bacterial MCPs. From a microbiological perspective, we speculated that a regulatory mechanism may dictate that each <i>S. enterica</i> cell expresses only one type of MCP (Pdu or Eut) at a time, avoiding the potential for mislocalization. Using fluorescence-based reporters of transcriptional activation, we found no evidence of such a mechanism at the transcriptional level as both the <i>P<sub>pdu</sub></i> and <i>P<sub>eut</sub></i> promoters were activated similarly by their cognate inducing molecules in the presence and absence of the inducing molecule of the other operon. We therefore conclude that it is possible for an <i>S. enterica</i> cell in the gut endothelium to encounter 1,2-propanediol and ethanolamine simultaneously and form both the Pdu and Eut MCPs. Both of these metabolites have been shown to contribute to bacterial proliferation in models of <i>Salmonella</i> and <i>E. coli</i> infection so it may be advantageous for a cell to metabolize them both concurrently (44, 45). Furthermore, because the Pdu operon is coregulated with the Cob vitamin B<sub>12</sub> synthesis operon by PocR and the Eut operon is not, the co-expression of both MCP systems along with the Cob biosynthetic operon would allow the Eut MCP access to its requisite vitamin B<sub>12</sub> cofactor.

In the absence of regulation to prevent MCP system co-expression in an individual cell, it is important to explore whether the catalytic enzymes associated with the Pdu and Eut MCPs are exclusively localized to their cognate MCP shells as previously assumed or whether upon co-expression of both the Pdu and Eut MCPs enzymes from each operon are localized to both types of MCPs. Although we do not directly demonstrate here that the N-terminal signal sequences from Pdu enzymes localize heterologous proteins to the Eut MCPs, we believe that further investigation will confirm this phenomenon. Promiscuous localization of Eut proteins to the Pdu MCP may be avoided in vivo, however, because the heterologous fusions tested above are expressed at levels significantly higher than those of native cargo proteins. Conversely, we demonstrated that <i>S. enterica</i> LT2 Δ<i>pduP::eute</i> can grow on 1,2-PD as its sole carbon source, indicating that EutE can serve the same metabolic function as PdUP in the Pdu MCP and suggesting that a natively encapsulated protein such as EutE can be encapsulated in a non-native compartment such as the Pdu MCP when expressed at near-native levels. If this behavior extends to the other cargo enzymes and to Pdu enzymes targeted to the Eut MCP, then this could indicate that selective transport of certain metabolites attributable to the characteristics of shell protein pores, e.g. of 1,2-propanediol over propionaldehyde (34–36), is not as important for MCP function as general retardation of diffusion (37), sequestration of cofactor pools (38, 39), or simple colocalization of the relevant metabolic enzymes (40–43).

It is important to note that the replacement of <i>pduP</i> in favor of <i>eute</i> relied on a non-native genomic context. It is possible that the genomic context of the cargo proteins is important to localization, that is, that enzymes are encapsulated within the MCPs forming from shell proteins expressed from the same genetic locus. If cargo proteins quickly and strongly associate with their cognate shell protein binding partners upon expression, then Pdu enzymes may be confined to the Pdu MCP simply by virtue of being translated from the same mRNA. The cross-talk observed here for cargo encoded on plasmids would then be an informative and interesting engineering observation but not a biologically relevant phenomenon.

We have demonstrated an engineering application of these signal sequences by characterizing the ratio of two fluorescent cargo proteins encapsulated in the Pdu MCP when they were encapsulated using various signal sequences and expressed at various levels. Different combinations of signal sequences allow the loading of the Pdu MCPs with different absolute and relative amounts of each cargo protein. We have shown that the stoichiometric ratio of two cargo proteins in the Pdu MCPs can be controlled by altering N-terminal signal sequences and expression levels. No previous study has demonstrated systematic measurement or control of these ratios, and we anticipate that future studies will reveal that the performance of heterologous enzymatic pathways is dependent on the tuning of this cargo-protein ratio using the approaches described here. Interestingly, we did not observe competition between the tagged fluorophores for localization; we therefore suspect that competition is not a significant factor in cargo localization at the moderate expression levels required for our dual fluorophore assay (which requires that all non-encapsulated reporter proteins be degraded by a limited number of ClpXP proteases). In contrast, expression levels of the signal sequence-tagged Pdu<sup>21–224</sup> protein in our competition assay could be increased without regard for ClpXP degradation capacity until competition was observed.

From an engineering perspective, the ability of non-native signal sequences to target cargo to the Pdu MCP presents challenges to the development of a system with multiple, orthogo-
The discovery of a wide array of functional signal sequences by observed for the various Pdu-localized sequences will inform than that which mediates Pdu MCP encapsulation. We also signal sequence is indeed mediated by a different interaction such an orthogonal system if the interaction of its N-terminal

Author Contributions—C. M. J., E. Y. K., M. F. S., and D. T.-E. conceived and designed the experiments. C. M. J., E. Y. K., M. F. S., and A. C. performed the experiments. All authors contributed to analysis of the results. C. M. J., E. Y. K., M. F. S., and D. T.-E. wrote the manuscript.

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