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Directed Evolution of Membrane Transport Using Synthetic Selections

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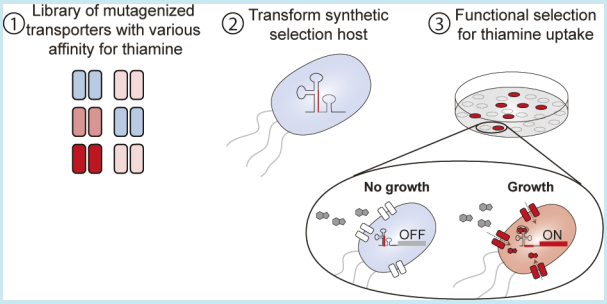
Supporting Information

ABSTRACT: Understanding and engineering solute transporters is important for metabolic engineering and the development of therapeutics. However, limited available experimental data on membrane transporters makes sequence-function relationships complex to predict. Here we apply ligand-responsive biosensor systems that enable selective growth of *E. coli* cells only if they functionally express an importer that is specific to the biosensor ligand. Using this system in a directed evolution framework, we successfully engineer the specificity of nicotinamide riboside transporters, PnuC, to accept thiamine as a substrate. Our results provide insight into the molecular determinants of substrate recognition of the PnuC transporter family and demonstrate how synthetic biology can be deployed to engineer the substrate spectrum of small molecule transporters.



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The *in vivo* characterization of solute membrane transporters typically involves labor-intensive experimental protocols and complex radiolabeled substrates.[1] Unfortunately, *in silico* predictions of transporter function, in particular, substrate specificity, are also challenging, and this is, in part, due to a lack of experimental data.[1,2] Consequently, functional data and accurate annotations of membrane transporters and their activities are lagging behind that of enzymes. This gap in our knowledge of transporters challenges the engineering of microbial cell factories and limits the development of therapeutics that target transporters. We previously developed a synthetic selection system for transporters using ligand-specific RNA biosensors that control the expression of selectable markers (Figure 1A). Using this system, we robustly coupled ligand uptake to bacterial growth enabling the functional mining of novel vitamin transporters from the metagenome.[3] Here, we extend this method and show that synthetic selections can be applied to probe the sequence-function relationship of small molecule transporters and engineer functionalities, such as substrate acceptance. Using this approach we functionally identified key residues that control the substrate recognition of the nicotinamide riboside transporter (PnuC) and expand its substrate recognition to include thiamine (Figure 1B).

The Pnu family of transporters is broadly distributed among bacteria and facilitates the uptake of structurally diverse B-vitamins, as experimentally demonstrated for riboflavin (PnuX),[4] nicotinamide riboside (PnuC),[5] and thiamine (PnuT).[3,6] Similarly to PnuX and PnuC it was recently shown that PnuT mediates thiamine uptake by facilitated diffusion.[6] Directionality is achieved by metabolic trapping as thiamine is immediately phosphorylated in the cell and as a consequence can no longer serve as substrate for PnuT. The pnu transporters provide an important route to bypass the biosynthesis of essential B-vitamins through direct import to save energy and resources. Furthermore, Pnu transporters are essential for many bacteria and higher organisms, in which a complete *denovo* pathway does not exist. For example does PnuT appear to be the only thiamine transporter in several pathogens, including *Bacteroides fragilis and Helicobacter pylori,* of which the latter is an experimentally validated thiamine auxotroph.[3] Similarly, PnuC is essential for *Haemophilus influenzae* growth and virulence, as *H. influenzae* does not have the enzymes necessary for *denovo* synthesis of NAD^+.[7] For biological production of B-vitamins, the Pnu-family of transporters has also shown to be important: overexpression of PnuX in an industrial production strain of *Bacillus subtilis* facilitated riboflavin export and led to increased riboflavin titers during fermentation.[8]

The crystal structure of PnuC from *Neisseria mucosa* provides structural insight into this important class of transporters.[9] Guided by this, a recent study by Jaehme et al. experimentally identified residues involved in substrate binding and gating of PnuT.[6] To extend this analysis, we performed a sequence and structural comparison of 5 experimentally validated PnuC transporters and 26 experimentally validated PnuT transporters (Figure 2A,B). The structural homology modeling in Figure 2A

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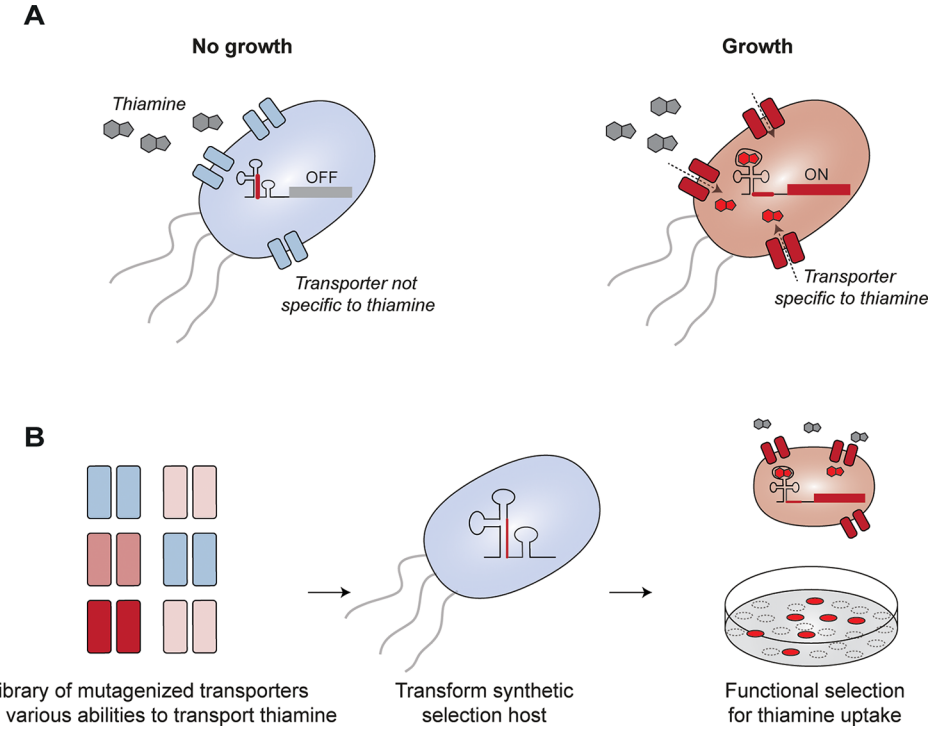

Figure 1. Synthetic selections for elucidating the sequence-function relationship of solute transporters. (A) The principle for the selection system of thiamine transport. A thiamine pyrophosphate (TPP) dependent riboswitch controls the expression of an antibiotic marker gene. When thiamine cannot be transported into the cell (left), the ribosome binding site (RBS) (red line) is inaccessible for the ribosome, and the strain will not be resistant toward antibiotic selection, resulting in no growth for strains lacking thiamine transport (blue transporter and blue cells). When the cell is capable of transporting (red transporter), thiamine will be phosphorylated to TPP (red structure) inside the cell and bind to the riboswitch. The binding triggers a conformational change making the RBS accessible for the rioboswitch, leading to expression of the antibiotic resistance gene and allowing growth under antibiotic selection conditions (red cell). (B) Selection strategy based on the functional selection for thiamine uptake among established libraries of mutagenized PnuC transporters.

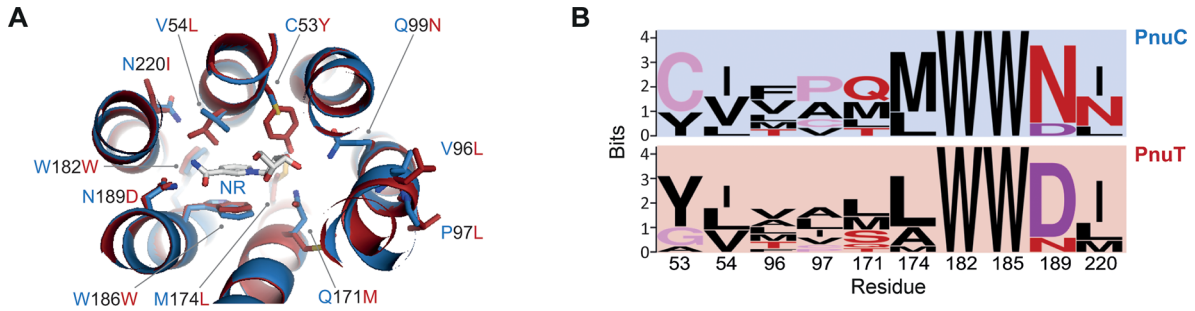

Figure 2. Structural characterization of Pnu transporters. As a foundation for choosing a mutagenesis strategy to change transporter specificity, structural examinations of various Pnu-transporters were carried out. On the basis of the investigations, the amino acid compositions of key residues in the different Pnu-transporters were compared to direct rational mutagenesis and understand the determinants of substrate specificity. (A) The crystal structure of the PnuC nicotinamide transporter substrate-binding site with nicotinamide riboside (NR) from *N. mucosa* (blue) aligned with the homology model of the PnuT thiamine transporter from *B. fragilis* (red). (B) Logo-plots of residues of the PnuC substrate-binding site. The logo-plot is based on a protein alignment of functionally validated PnuCs and PnuTs. The color codes of the amino acids indicate the properties as follows: charged side chains (dark purple); polar uncharged side chains (red); hydrophobic side chains (black); and special cases (light purple). PnuT from *B. fragilis* was chosen as a representative sequence for homology modeling, based on its phylogenetic localization in a clade of validated PnuT transporters.[3]

was based on the sequence of the experimentally validated PnuT transporter from the pathogenic bacteria *B. fragilis*[3] guided by the crystal structure of *N. mucosa* PnuC[10] (see Methods), to enable visual inspection of amino acids of interest. However, a lack of conserved sequence motifs in specific subtypes of the Pnu transporters (Figure 2B) meant that the molecular basis of the differential substrate specificities remained elusive.[11]

In this study, we used random mutagenesis combined with synthetic selections to investigate residues important for the substrate specificity of Pnu transporters. For our analysis, we chose the nicotinamide riboside transporter, PnuC, from *Streptococcus pneumoniae,* which does not exhibit thiamine transport activity *in vivo*.[3] We generated a PnuC mutant library by error-prone PCR and transformed this into an *Escherichia coli* strain harboring a riboswitch-based thiamine selection system which only allows growth of mutant variants that enable

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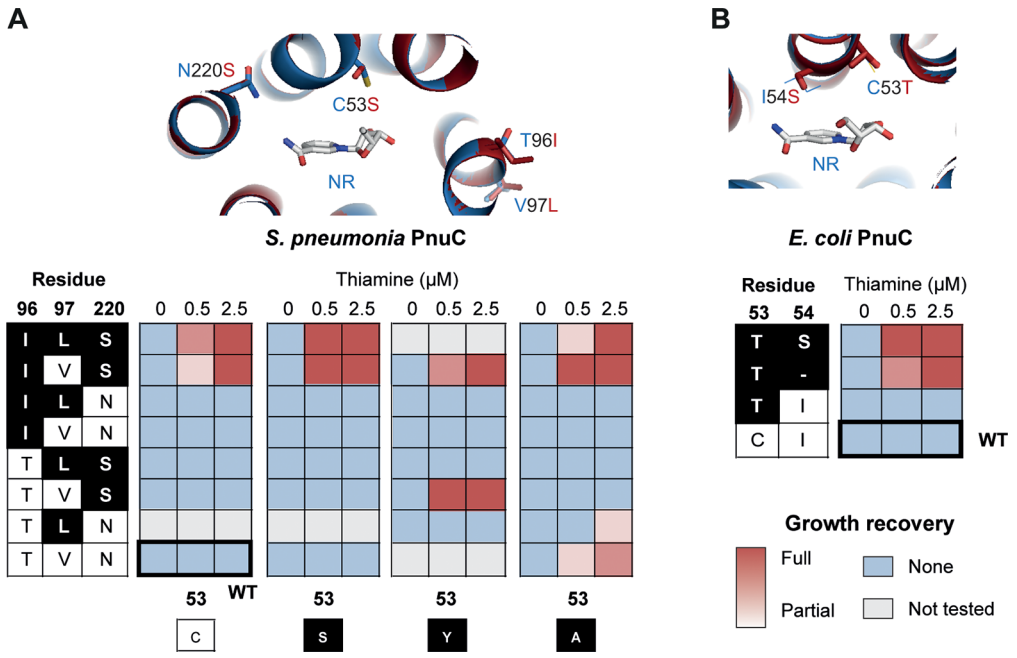

Figure 3. Elucidating the determinants of thiamine substrate specificity by directed evolution. To investigate the sequence determinants of thiamine transport we performed random and site-directed mutagenesis of PnuC nicotinamide riboside transporters from *S. pneumonia* and *E. coli*, respectively. Using thiamine functional selections, the mutant libraries were assayed for variants that enable thiamine uptake when expressed in *E. coli*. (A) Functional analysis of the PnuC mutants from *S. pneumonia* (*N. mucosa* numbering) with mutations at residues 53, 96, 97, and 220. "WT" indicates the wild type PnuC. The mutant proteins were expressed in *E. coli* harboring the selection system, and the thiamine transport of each strain was assayed by spotting 10× serial dilutions of an overnight culture on selective growth medium supplemented with 0.0, 0.5, or 2.5 μM thiamine. The figure above the heatmap represents a comparison of the homology models of an *S. pneumonia* wild type (blue) and a mutant (red) PnuC. (B) *E. coli* PnuC variants with mutations at residues 53 and 54 (*N. mucosa* numbering). The dash (-) indicates a deletion. The figure above the heatmap represents a comparison of the homology models of an *E. coli* wild type (blue) and a mutant (red) PnuC.

thiamine uptake (Figure 1B).[3] This enabled the rapid functional interrogation for the acquired ability to take up thiamine among 6×10^5 PnuC variants (see Methods).

Interestingly, several colonies that are able to import thiamine were selected, and among these, one showed a particularly strong growth phenotype. The sequencing of *pnuC* isolated from this clone revealed four mutated residues, comprising Cys53Ser, Thr96Ile, Val97Leu, and Asn220Ser (*N. mucosa* numbering). Of these, only Cys53 is predicted to directly interact with the substrate by forming a hydrogen bond to the 2′-OH group of the ribose unit of nicotinamide riboside in PnuC from *N. mucosa*.[10] This is likely to be true for *S. pneumoniae* PnuC as well, based on the structural homology modeling (Figure 3A, top). Our comparative analysis of Pnu sequences showed that Cys53 is furthermore highly conserved among the Pnu transporters specific to nicotinamide riboside, while the Pnu transporters specific to thiamine display variability, with the majority of sequences containing a Tyr at position 53 (Figure 2B). Asn220 is predicted to be proximal to the substrate-binding site but is not conserved, whereas Thr96 and Val97 are positioned at the exterior of the protein facing the lipid bilayer for both PnuC and PnuT and are also not conserved (Figure 2A,B). Whereas the mutation of Asn220 and in particular Cys53 was expected to impact substrate binding due to the proximity of these residues to the binding pocket, mutation of the more distant Thr96 and Val97 was highly surprising.

To dissect which of the four residues (Cys53, Thr96, Val97, and Asn220) are required for thiamine acceptance by *S. pneumonia* PnuC, we constructed several mutant transporters with combinations of wild-type and mutant residues. In addition to Ser, Cys53 was mutated to Tyr and Ala, as these are found in Pnu transporters specific to thiamine (Figure 2B). In total, we successfully generated 28 of 32 possible PnuC mutant variants (Figure 3A). The ability of each variant to facilitate thiamine uptake was evaluated by thiamine-dependent uptake assays using the thiamine selection assay strain (Figure 3A).

The thiamine-dependent growth selections showed that the Asn220Ser and surprisingly the Thr96Ile combination is critical for thiamine transport (Figure 3A and Supplementary Figure 1B). When combined with the Asn220Ser and Thr96Ile mutations, both Cys53Ala and Cys53Tyr generated strong thiamine uptake phenotypes, similar to the Cys53Ser combinatorial mutant (Figure 3A and Supplementary Figure 1B). Strikingly, the introduction of Cys53Ala alone enabled thiamine transport. Although the resulting phenotype of this variant is not as strong as that of the combinatorial mutants, it demonstrates that even a single residue mutation is capable of changing the substrate specificity of a Pnu transporter.

To further explore the effect on substrate specificity by changing the conserved Cys53 (Figure 2B), we generated a site saturation mutational library of *E. coli* PnuC at Cys53. *E. coli* PnuC is distantly related to *S. pneumoniae* PnuC and shows less than 20% sequence identity (and less than 30% sequence similarity) on the amino acid level. The resulting PnuC mutant library was transformed into an *E. coli* thiamine selection strain and was assayed for thiamine uptake by thiamine-dependent growth selections as described previously. An analysis of the selected PnuC sequences revealed two unique variants (Supplementary Figure 2). One variant encoded a Cys53Thr substitution with a concomitant Ile54 deletion, and another

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clone encoded a Cys53Thr and an Ile54Ser substitution. Since mutagenic PCR primers were designed for Cys53 mutagenesis only, the Ile54 mutations were likely introduced as an error during PCR but were identified due to the high-throughput capacity of the selection system. On the basis of the growth assays at selective conditions with and without thiamine, the functionally selected PnuC variants displayed a thiamine uptake performance that was similar to the positive control (*H. pylori* PnuT expressed in *E. coli* (Figure 3B and Supplementary Figure 1A)). No change in the phenotype was observed when Cys53 was mutated alone (Figure 3B and Supplementary Figure 1A).

In summary, we have demonstrated how synthetic biology can effectively be deployed to solve complex biological challenges related to substrate specificity of small molecule transporters. Using synthetic selections our investigations led to the identification of several PnuC variants with an expanded substrate range that now includes thiamine. Whether the ability to import nicotinamide riboside is maintained or is reduced was not analyzed as part of this study, but additional biochemical analyses will likely uncover such properties and further expand our understanding of the sequence-function relationship of the Pnu transporter family. Similarly, further mutational and structural studies could be conducted to mechanistically explain the change in substrate specificity of the selected mutant variants. The majority of previous efforts toward engineering substrate recognition of membrane transporters have achieved changes in specificity to molecules that are structurally related, such as purines[12] and hexose and pentose sugars.[13−17] In contrast, thiamine and nicotinamide riboside are structurally highly diverse molecules with distinct chemical properties. Our finding that even a single residue enables such a change in substrate specificity demonstrates an impressive degree of sequence-function flexibility of the Pnu transporter family. While our functional investigation confirmed residue 53 as being central to substrate specificity as suggested by the structural analysis,[10] our broad analysis illustrates that multiple combinations of residue substitutions even distant to the binding pocket are important to substrate specificity. Such nonintuitive results would have been challenging to obtain without the robust selection system used in this study, highlighting that synthetic selections represent a powerful approach to elucidate complex genotypic−phenotypic relations of membrane transporters. By tailoring synthetic selection systems to new compounds of interest through the replacement of the biosensor module, the approach is expandable to other important compound and transporter families.

METHODS

Materials and General Considerations. Plasmid DNA and PCR products were purified using the QIAprep Spin Miniprep Kit and QIAquick PCR Purification Kit, respectively (Qiagen). Gel extractions were performed using NucleoSpin Gel and PCR clean-up protocol (Macherey−Nagel). Thiamine−HCl, thiamine monophosphate−HCl, thiamine pyrophosphate−HCl, and all antibiotics used herein were purchased from Sigma-Aldrich. Synthetic oligonucleotides were purchased from Integrated DNA Technologies. *Escherichia coli* DH10B was used for all the experiments. For all the incubations in liquid media, the cells were grown with shaking at 250 rpm at 37 °C. The strains were stored at −80 °C in a 15% v/v glycerol solution. The plasmid manipulations were performed using USER cloning.[18,19] The sequences of all the vectors constructed were verified by DNA sequencing (Beckman Coulter). The cells were cultured in Luria Broth (LB), or modified rich MOPS medium without thiamine (mrMOPS) (Supporting Information Note 2).

Structural Comparisons of the PnuC and PnuT Proteins. The PnuC and PnuT homology models were computed using the HHPred prediction server[20] with the default settings. The *Neisseria mucosa* PnuC crystal structure (PDB entry 4qtn)[10] was used as a template for generating all the homology models, and the structures were visualized and analyzed using PyMOL v1.7.[21] To identify the conserved residues and motifs, a multiple sequence alignment of 26 functionally verified PnuTs[3] in addition to PnuT from *Helicobacter pylori*, as well as the five functionally validated PnuCs (*E. coli, Haemophilus influenzae, N. mucosa, Salmonella enterica, Streptococcus pneumoniae*), was computed using CLC Main Workbench 6. Ten positions, of which eight are predicted to interact with nicotinamide riboside in *N. mucosa* PnuC,[10] were selected and presented as a sequence logo plot generated using WebLogo.[22] For the logo plot visualization, representative sequences from 11 phylogenetically different groups of the 26 validated PnuT sequences[3] were used in order avoid biasing the logo plot.

Error-Prone PCR Mutagenesis. Random mutagenesis of *S. pneumoniae pnuC* by error-prone PCR was performed with the GeneMorphII Random Mutagenesis Kit (Agilent Technologies, No. 200550) using the primers oGEN292/oGEN291. pGEN64 encoding *S. pneumoniae pnuC* was used as the template for the backbone amplification with the PCR primers oGEN308/oGEN320. The generated *pnuC* mutational PCR fragment library was assembled with the pGEN64 backbone by Gibson Assembly following the manufacturer's instructions. The ligation mix was desalinated using 0.025 μM CSWP membrane filters (Merck Millipore Ltd.) and was subsequently transformed into electrocompetent *E. coli* DH10B cells already harboring the pGEN37 thiamine selection plasmid. The resulting cell libraries were washed twice in mrMOPS and assayed for mutated *S. pneumoniae* PnuC variants capable of transporting thiamine through plating on mrMOPS agar plates with 0 to 5 μM thiamine. EcGEN186 (encoding the *S. pneumoniae* wild type *pnuC*) was used as a negative control.

Construction of *S. pneumoniae* PnuC Combinatorial Variants by Multiple Site-Directed Mutagenesis. To explore the combinatorial effects of the Cys53Ser, Thr96Ile, Val97Leu, and Asn220Ser mutations identified in the isolated *S. pneumoniae* PnuC variant capable of transporting thiamine, we used the USER cloning strategy for multiple-site directed mutagenesis.[19] Using the AMUSER Web server,[19] a set of three semidegenerate primer sets were designed for the combinatorial introduction of Ala/Cys/Ser/Tyr at position 53 (primers oGEN306/308), Thr/Ille at position 96, Val/Leu at position 97 (primers oGEN323/324), and Asn/Ser at position 220 (primers oGEN309/310). Following PCR amplification, the three fragments were assembled to generate a library that covered all 32 combinatorial variants. Following ligation, the library was transformed into electrocompetent *E. coli* DH10B harboring the pGEN37 selection plasmid. We sequenced 264 randomly selected transformants and identified 28 unique combinatorial PnuC mutants. The capability of the individual PnuC mutants to transport thiamine was assayed by spot assays as described above under "Characterization of specific Pnu transporters" using 0.0, 0.5, and 2.5 μM thiamine in the agar plates.

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Site-Saturation Mutagenesis. Site-saturation mutagenesis of Cys53 (*N. mucosa* numbering) of *E. coli* and *S. pneumoniae* PnuC was performed using the USER cloning strategy for site-directed mutagenesis.[19] Mutagenic primers containing NNN degenerate nucleotides at the Cys53 codon were designed using the AMUSER-Web server.[19] pGEN62, encoding *E. coli pnuC*, and pGEN64, encoding *S. pneumoniae pnuC*, were used as the templates for whole plasmid amplifications with the PCR primers oGEN321/oGEN322 and oGEN308/oGEN320 for the two plasmids, respectively. Following USER assembly and ligation,[19] the ligation mixes were desalinated using 0.025 μm CSWP membrane filters (Merck Millipore Ltd.) and were subsequently transformed into electrocompetent *E. coli* DH10B cells already harboring the pGEN37 selection plasmid. The resulting cell libraries were grown, stored, and assayed for that mutated PnuC variants capable of transporting thiamine as performed for the metagenomic libraries described above. EcGEN184 (encoding the *E. coli* wild-type *pnuC*) and EcGEN186 (encoding the *S. pneumoniae* wild-type *pnuC*) were used as negative controls for all the selections. The capability of the individual PnuC mutants to transport thiamine was assayed by the spot assays as described above under "Characterization of specific Pnu transporters," using 0.0, 0.5, and 2.5 μM thiamine in the agar plates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00407.

Additional figures and notes as described in the text (PDF)

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Author Contributions

#A.P.B. and H.J.G. contributed equally. A.P.B. designed and performed experiments and wrote the paper. H.J.G. designed and performed experiments, supervised and wrote the paper. M.S. supervised and wrote the paper.

Notes

The authors declare no competing financial interest.

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