

A semisynthetic organism engineered for the stable expansion of the genetic alphabet

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All natural organisms store genetic information in a four-letter, two-base-pair genetic alphabet. The expansion of the genetic alphabet with two synthetic unnatural nucleotides that selectively pair to form an unnatural base pair (UBP) would increase the information storage potential of DNA, and semisynthetic organisms (SSOs) that stably harbor this expanded alphabet would thereby have the potential to store and retrieve increased information. Toward this goal, we previously reported that *Escherichia coli* grown in the presence of the unnatural nucleoside triphosphates dNaMTP and d5SICSTP, and provided with the means to import them via expression of a plasmid-borne nucleoside triphosphate transporter, replicates DNA containing a single dNaM-d5SICS UBP. Although this represented an important proof-of-concept, the nascent SSO grew poorly and, more problematically, required growth under controlled conditions and even then was unable to indefinitely store the unnatural information, which is clearly a prerequisite for true semisynthetic life. Here, to fortify and vivify the nascent SSO, we engineered the transporter, used a more chemically optimized UBP, and harnessed the power of the bacterial immune response by using Cas9 to eliminate DNA that had lost the UBP. The optimized SSO grows robustly, constitutively imports the unnatural triphosphates, and is able to indefinitely retain multiple UBPs in virtually any sequence context. This SSO is thus a form of life that can stably store genetic information using a six-letter, three-base-pair alphabet.

unnatural base pair | CRISPR | Cas9 | DNA replication | nucleotide transporter

The natural genetic alphabet is composed of four letters whose selective pairing to form two base pairs underlies the storage and retrieval of virtually all biological information. This alphabet is essentially conserved throughout nature, and has been since the last common ancestor of all life on Earth. Significant effort has been directed toward the development of an unnatural base pair (UBP), formed between two synthetic nucleotides, that functions alongside its natural counterparts (1–3), which would represent a remarkable integration of a man-made, synthetic component into one of life's most central processes. Moreover, semisynthetic organisms (SSOs) that stably harbor such a UBP in their DNA could store and potentially retrieve the increased information, and thereby lay the foundation for achieving the central goal of synthetic biology: the creation of new life forms and functions (4).

For over 15 years, we have sought to develop such a UBP (1), and these efforts eventually yielded a family of predominantly hydrophobic UBPs, with that formed between dNaM and d5SICS (dNaM-d5SICS; Fig. 1A) being a particularly promising example (5–7). Despite lacking complementary hydrogen bonding, we demonstrated that the dNaM-d5SICS UBP is well replicated by a variety of DNA polymerases in vitro (7–10), and that this efficient replication is mediated by a unique mechanism that draws upon interbase hydrophobic and packing interactions (11, 12). These efforts then culminated in the first progress toward the creation of an SSO in 2014, when we reported that *Escherichia coli* grown in the presence of the corresponding unnatural nucleoside triphosphates

(dNaMTP and d5SICSTP), and provided with a plasmid-encoded nucleoside triphosphate transporter (NTT2) from *Phaeodactylum tricorutum* (which we denote as PtNTT2) (13), is able to import the unnatural triphosphates and replicate a single dNaM-d5SICS UBP on a second plasmid (14).

Although this first SSO represented an important proof-of-concept, the generality of the expanded genetic alphabet remained unclear, as retention of the UBP was explored at only a single locus and in only a single sequence context. True expansion of the genetic alphabet requires the unrestricted retention of multiple UBPs at any loci and in any sequence context. Moreover, several limitations were already apparent with the nascent SSO (14). First, although expression of the nucleoside triphosphate transporter enabled *E. coli* to import dNaMTP and d5SICSTP, its expression caused the SSO to grow poorly, with doubling times twice that of the parental strain. Second, the UBP was not well retained during high-density liquid growth or during growth on solid media, presumably due to the secretion of phosphatases that degrade the unnatural triphosphates. Finally, even under optimal conditions, the nascent SSO was unable to retain the UBP with extended growth. Clearly, the ability to robustly grow under the standard repertoire of culture conditions and indefinitely retain the UBP is a prerequisite for true semisynthetic life. Here, we used genetic and chemical approaches to optimize different components of the SSO, ultimately resulting in a simplified and optimized SSO that grows robustly and is capable of the virtually unrestricted storage of increased information.

Significance

The genetic alphabet encodes all biological information, but it is limited to four letters that form two base pairs. To expand the alphabet, we developed synthetic nucleotides that pair to form an unnatural base pair (UBP), and used it as the basis of a semisynthetic organism (SSO) that stores increased information. However, the SSO grew poorly and lost the UBP under a variety of standard growth conditions. Here, using chemical and genetic approaches, we report the optimization of the SSO so that it is healthy, more autonomous, and able to store the increased information indefinitely. This SSO constitutes a stable form of semisynthetic life and lays the foundation for efforts to impart life with new forms and functions.

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Conflict of interest statement: Y.Z., B.M.L., and F.E.R. have filed a patent application based on the use of Cas9 for enforced retention of the UBP. Y.Z. and F.E.R. have filed a patent application for the truncated transporter. F.E.R. has a financial interest (shares) in Synthorx Inc., a company that has commercial interests in the UBP. The other authors declare no other competing financial interests.

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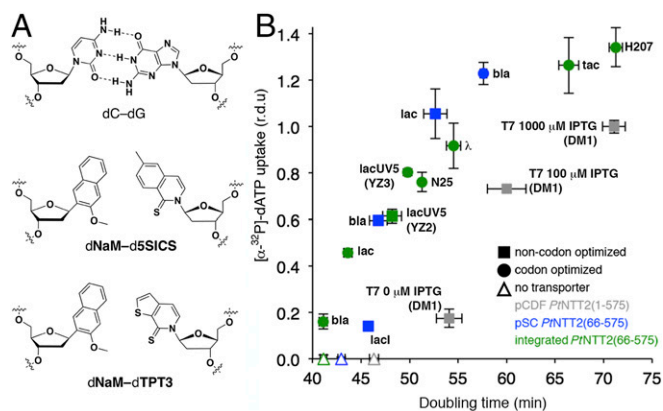


Fig. 1. UBPs and transporter optimization. (A) Chemical structure of the dNaM-d5SICS and dNaM-dTPT3 UBPs with a natural dC-dG base pair included for comparison. (B) Comparison of fitness and [α - 32 P]-dATP uptake in DM1 and the various constructed strains: pCDF and inducible *PtNTT2*(1-575) (gray); pSC and constitutive *PtNTT2*(66-575) (blue); and chromosomally integrated and constitutive *PtNTT2*(66-575) (green). The promoters from which *PtNTT2* is expressed are indicated by the labels next to their corresponding markers. Open triangles denote corresponding control strains without *PtNTT2*. The pCDF plasmids are in *E. coli* C41(DE3); pSC plasmids and integrants are in *E. coli* BL21(DE3). All *PtNTT2* strains are non-codon-optimized for plasmid-based expression and codon-optimized for chromosomal expression unless otherwise indicated; r.d.u. is relative decay units, which corresponds to the total number of radioactive counts per minute normalized to the average OD₆₀₀ across a 1-h window of uptake, with the uptake in DM1 induced with 1,000 μ M IPTG set to 1 (see *Materials and Methods* for additional details). Error bars represent SD of the mean, $n = 3$ cultures grown and assayed in parallel; the error bars on some data points are smaller than their marker.

Results and Discussion

In our previously reported SSO (hereafter referred to as DM1), the transporter was expressed from a T7 promoter on a multi-copy plasmid (pCDF-1b) in *E. coli* C41(DE3), and its toxicity mandated carefully controlled induction (14). In its native algal cell, *PtNTT2*'s N-terminal signal sequences direct its subcellular localization and are ultimately removed by proteolysis. However, in *E. coli*, they are likely retained, and could potentially contribute to the observed toxicity. Using the cellular uptake of [α - 32 P]-dATP as a measure of functional transporter expression and as a proxy for the uptake of the unnatural triphosphates, we found that removal of amino acids 1 to 65 and expression of the resulting N-terminally truncated variant *PtNTT2*(66-575) in *E. coli* C41(DE3) resulted in significantly lower toxicity, but also significantly reduced uptake (Fig. S1 A and B), possibly due to reduced expression (15). Expression of *PtNTT2*(66-575) in *E. coli* BL21 (DE3) resulted in significant levels of [α - 32 P]-dATP uptake with little increase in toxicity relative to an empty vector control, but the higher level of T7 RNAP in this strain (16) was itself toxic (Fig. S1 A and C).

We next explored constitutive expression of *PtNTT2*(66-575) from a low-copy plasmid or a chromosomal locus, which we anticipated would not only eliminate the need to use T7 RNAP but would also impart the SSO with greater autonomy (eliminating the need to induce transporter production), and, importantly, would result in more homogeneous transporter expression and triphosphate uptake across a population of cells, which we reasoned might improve UBP retention. We explored expression of *PtNTT2*(66-575) in *E. coli* BL21(DE3) with the promoters P_{lacI} , P_{bla} , and P_{lac} from a pSC plasmid, and with P_{bla} , P_{lac} , P_{lacUV5} , P_{H207} , P_{λ} , P_{tac} , and P_{N25} from the chromosomal *lacZYA* locus (Dataset S1). We also explored the use of a codon-optimized variant of the truncated transporter (see Dataset S1). Although increasing expression of *PtNTT2*(66-575) (as measured by uptake of [α - 32 P]-dATP) was

correlated with increasing doubling time, indicating that expression of *PtNTT2*(66-575) still exhibited some toxicity (uptake of [α - 32 P]-dATP is itself not toxic), each strain exhibited an improved ratio of uptake to fitness compared with DM1 (Fig. 1B). Strain YZ3, which expresses the codon-optimized, chromosomally integrated *PtNTT2*(66-575) from the P_{lacUV5} promoter, exhibited an optimal compromise of robust growth (<20% increased doubling time relative to the isogenic strain without the transporter), and [α - 32 P]-dATP uptake, and was thus selected for further characterization.

To determine whether the optimized transporter system of YZ3 facilitates high UBP retention, we constructed three plasmids that position the UBP within the 75-nt TK1 sequence (14) [with a local sequence context of d(A-NaM-T)]. These include two high-copy pUC19-derived plasmids, pUCX1 [referred to in previous work as pINF (14)] and pUCX2, as well as one low-copy pBR322-derived plasmid, pBRX2 (Fig. S2). In addition to allowing us to examine the effect of copy number on UBP retention, these plasmids position the UBP at proximal (pUCX1) and distal (pUCX2 and pBRX2) positions relative to the origin of replication, which we previously speculated might be important (14). Strains YZ3 and DM1 were transformed with pUCX1, pUCX2, or pBRX2 and directly cultured in liquid growth media supplemented with dNaMTP and d5SICSTP [and isopropyl β -D-1-thiogalactopyranoside (IPTG) for DM1 to induce the transporter], and growth and UBP retention were characterized at an OD₆₀₀ of \sim 1 (Fig. 2A and Fig. S3A; see also *Materials and Methods*). Although DM1 showed variable levels of retention and reduced growth, especially with the high-copy plasmids, YZ3 showed uniformly high levels of UBP retention and robust growth with all three UBP-containing plasmids (Fig. 2B and Fig. S3A).

Given that no plasmid locus or copy number biases on UBP retention were observed in YZ3, we chose pUCX2 as a representative UBP-containing plasmid to explore the effect of local sequence context on UBP retention, and we constructed 16 pUCX2 variants in which the UBP was flanked by each possible combination of natural base pairs within a fragment of the GFP gene (see Dataset S1). Under the same growth conditions as above, we observed a wide range of UBP retentions, with some sequence contexts showing complete loss of the UBP (Fig. 2C). However, since the development of DM1 with the dNaM-d5SICS UBP, we have determined that ring contraction and sulfur derivatization of d5SICS, yielding the dNaM-dTPT3 UBP (Fig. 1A), results in more efficient replication in vitro (17). To explore the in vivo use of dNaM-dTPT3, we repeated the experiments with YZ3 and each of the 16 pUCX2 plasmids, but with growth in media supplemented with dNaMTP and dTPT3TP. UBP retentions were clearly higher with dNaM-dTPT3 than with dNaM-d5SICS (Fig. 2C).

Although dNaM-dTPT3 is clearly a more optimal UBP for the SSO than dNaM-d5SICS, its retention is still moderate to poor in some sequence contexts (Fig. 2C). Moreover, several sequences that show good retention in YZ3 cultured in liquid media show poor retention when growth includes culturing on solid media (Fig. S3B). To further increase UBP retention with even these challenging sequences and/or growth conditions, we sought to selectively eliminate plasmids that lose the UBP. In prokaryotes, the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) system provides adaptive immunity against viruses and foreign plasmids (18–20). In type II CRISPR-Cas systems, such as that from *Streptococcus pyogenes* (21, 22), the endonuclease Cas9 uses encoded RNAs [or their artificial mimics known as single-guide RNAs (sgRNAs) (23)] to introduce double-strand breaks in cDNA upstream of a 5'-NGG-3' protospacer adjacent motif (PAM) (24), which then results in DNA degradation by exonucleases (25) (Fig. 3A). In vitro, we found that the presence of a UBP in the target DNA generally reduces Cas9-mediated cleavage relative to sequences that are fully complementary to the provided sgRNA (Fig. S4). We thus

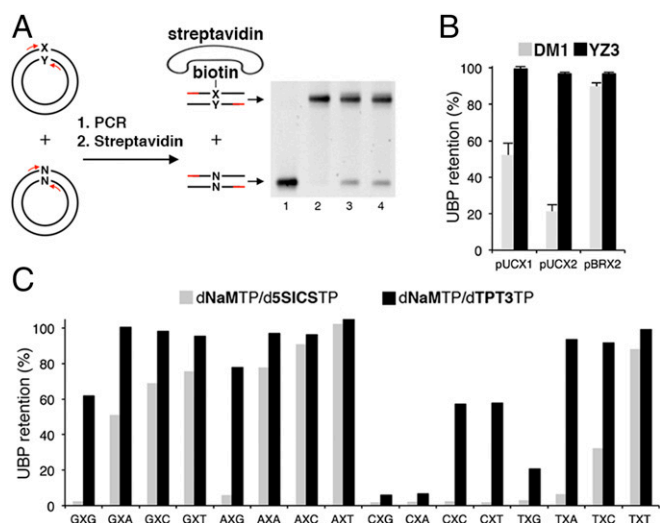


Fig. 2. UBPs retention assay and the effects of transporter and UBPs optimization. (A) Schematic representation of the biotin shift assay used to determine UBPs retention. The plasmid DNA to be analyzed is first amplified in a PCR supplemented with the unnatural triphosphates, and the resulting products are then incubated with streptavidin and subjected to PAGE analysis. X = dNaM, or, in the PCR, its biotinylated analog dMMO₂^{biotin}. Y = d5SICs in the PCR, whereas Y = dTPT3 or d5SICs in the plasmid DNA, depending on the experimental conditions. Lane 1 is the product from the oligonucleotide analogous to that used to introduce the UBPs during plasmid assembly, but with the UBPs replaced by a natural base pair (negative control). This band serves as a marker for DNA that has lost the UBPs. Lane 2 is the product from the synthetic oligonucleotide containing the UBPs that was used for plasmid assembly. The shift of this band serves as a marker for the shift of DNA containing the UBPs. Lane 3 is the product from the in vitro-assembled plasmid before SSO transformation (positive control). The unshifted band results from DNA that has lost the UBPs during in vitro plasmid assembly. Lane 4 is the product from an in vivo replication experiment. (B) UBPs retentions of plasmids pUCX1, pUCX2, and pBRX2 in strains DM1 and YZ3. Error bars represent SD of the mean, *n* = 4 transformations for pUCX1 and pUCX2, *n* = 3 for DM1 pBRX2, and *n* = 5 for YZ3 pBRX2. (C) UBPs retentions of pUCX2 variants, wherein the UBPs is flanked by all possible combinations of natural nucleotides (NXN, where N = d(G, C, A, or T) and X = dNaM), in strain YZ3 grown in media supplemented with either dNaMTP and d5SICSTP (gray bars) or dNaMTP and dTPT3TP (black bars).

hypothesized that, within a cell, Cas9 programmed with sgRNA(s) complementary to natural sequences that arise from UBPs loss would enforce retention in a population of plasmids (by eliminating those that lose the UBPs), which we refer to as immunity to UBPs loss. To test this, we used a p15A plasmid to construct pCas9, which expresses Cas9 via an IPTG-inducible *lacO* promoter, as well as an 18-nt sgRNA that is complementary to the TK1 sequence containing the most common dNaM-dTPT3 mutation (dT-dA) via the constitutive *proK* promoter (Figs. S2 and S5A). Initial exploratory experiments were carried out with strain YZ2 (a forerunner of YZ3 with slightly less optimal transporter performance, but which was available before strain YZ3; Fig. 1B and Fig. S1 D and E). Strain YZ2 was transformed with corresponding pairs of pCas9 and pUCX2 plasmids (i.e., the pUCX2 variant with the UBPs embedded within the TK1 sequence such that loss of the UBPs produces a sequence targeted by the sgRNA encoded on pCas9), grown to an OD₆₀₀ of ~4, diluted 250-fold, and regrown to the same OD₆₀₀. UBPs retention in control experiments with a nontarget sgRNA dropped to 17% after the second outgrowth; in contrast, UBPs retention in the presence of the correct sgRNA was 70% after the second outgrowth (Fig. 3B). Sequencing revealed that the majority of plasmids lacking a UBPs when the correct sgRNA was provided contained a single nucleotide deletion in its place, which was not observed with the

nontarget sgRNA (Fig. S5 B and C). With a pCas9 plasmid that expresses two sgRNAs, one targeting the most common substitution mutation and one targeting the single nucleotide deletion mutation (Fig. S5A), and the same growth and regrowth assay, loss of the UBPs was undetectable (Fig. 3B).

To more broadly explore Cas9-mediated immunity to UBPs loss, we examined retention using 16 pUCX2 variants with sequences that flank the UBPs with each possible combination of natural base pairs but also vary its position relative to the PAM, and vary which unnatural nucleotide is present in the strand recognized by the sgRNAs (Table S1). We also constructed a corresponding set of 16 pCas9 plasmids that express two sgRNAs, one targeting a substitution mutation and one targeting the single nucleotide deletion mutation, for each pUCX2 variant. Strain YZ2 carrying a pCas9 plasmid was transformed with its corresponding pUCX2 variant and grown in the presence of the unnatural triphosphates and IPTG (to induce Cas9), and UBPs retention was assessed after cells reached an OD₆₀₀ of ~1. As a control, the 16 pUCX2 plasmids were also propagated in YZ2 carrying a pCas9 plasmid with a nontarget sgRNA. For 4 of the 16 sequences explored, UBPs loss was already minimal without immunity (nontarget sgRNA), but was undetectable with expression of the correct sgRNA (Fig. 4A). The remaining sequences showed moderate to no retention without immunity, and significantly higher retention with it, including at positions up to 15 nts from the PAM.

To further simplify and streamline the SSO, we next constructed strain YZ4 by integrating an IPTG-inducible Cas9 gene at the *arsB* locus of the YZ3 chromosome, which allows for the use of a single plasmid that both carries a UBPs and expresses the sgRNAs that enforce its retention. Sixteen such “all-in-one” plasmids (pAIO) were constructed by replacing the Cas9 gene in each of the pCas9 variants with a UBPs sequence from the corresponding pUCX2 variant (Fig. S2 and Table S1). YZ4 and YZ3 (included as a no-Cas9 control due to leaky expression of Cas9 in YZ4) were transformed with a single pAIO plasmid and cultured on solid growth media supplemented with the unnatural triphosphates and with or without IPTG to induce Cas9. Single colonies were used to inoculate liquid media of the same composition, and UBPs retention was assessed after cells reached an

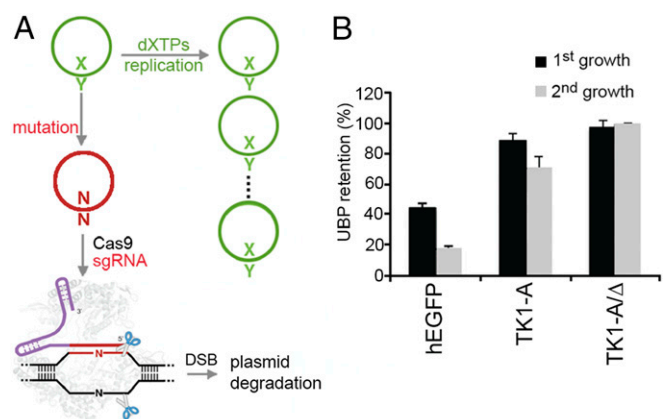


Fig. 3. Cas9-based editing system. (A) Model for Cas9-mediated immunity to UBPs loss. (B) UBPs retention for pUCX2 TK1 is enhanced by targeting Cas9 cleavage to plasmids that have lost the UBPs. The bars labeled hEGFP correspond to UBPs retention with growth (black) and regrowth (gray) with an sgRNA that has a sequence taken from the hEGFP gene and thus does not target DNA containing the TK1 sequence (negative control). The bars labeled TK1-A or TK1-A/Δ correspond, respectively, to growth and regrowth with an sgRNA that targets the dNaM to dT mutation or two sgRNAs that individually target the dNaM to dT mutation and a single nucleotide deletion of the UBPs.

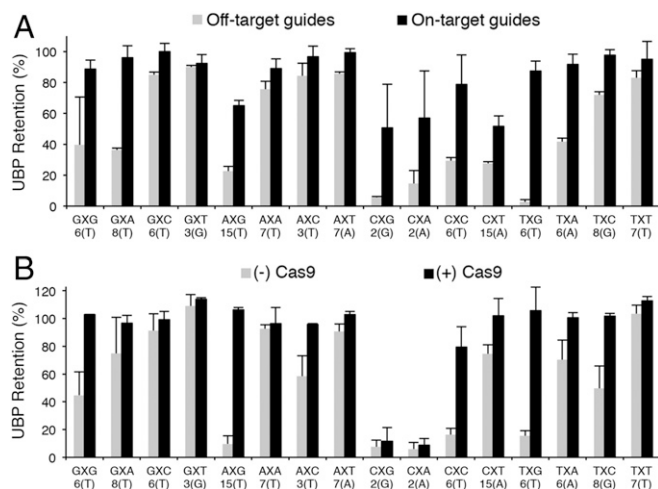


Fig. 4. Variation in Cas9-mediated immunity with sequence context. (A) UBPs retentions of pUCX2 variants in strain YZ2 with a pCas9 plasmid that expresses a nontarget sgRNA (gray) or an on-target sgRNA (black). Error bars represent SD of the mean, and $n = 3$ transformations for all sequences except on-target CXA and CXG, where $n = 5$. (B) UBPs retentions of pAIO plasmids in strain YZ3 (gray), which does not express Cas9, or in strain YZ4 (black) with expression of Cas9. In A and B, the nucleotides immediately flanking X = dNaM are indicated, as is distance to the PAM. “(N)” denotes the nucleotide N in the sgRNA that targets a substitution mutation of the UBPs; all pCas9 and pAIO plasmids also express a sgRNA that targets a single nucleotide deletion of the UBPs. Error bars represent SD of the mean, and $n \geq 3$ colonies; see Table S1 for exact values of n , sequences, and IPTG concentrations used to induce Cas9 in YZ4. See Materials and Methods for additional experimental details.

OD₆₀₀ of ~1 to 2 (Fig. 4B). Despite variable levels of retention in the absence of Cas9 (YZ3), with induction of Cas9 expression in YZ4, loss was minimal to undetectable in 13 of the 16 sequences. Although retention with the three problematic sequences—d(C-NaM-C), d(C-NaM-A), and d(C-NaM-G)—might be optimized, for example, through alterations in Cas9 or sgRNA expression, the undetectable loss of the UBPs with the majority of the sequences after a regimen that included growth both on solid and in liquid media, which was not possible with our previous SSO DM1, attests to the vitality of YZ4.

Finally, we constructed a pAIO plasmid, pAIO2X, containing two UBPs: dNaM paired opposite dTPT3 at position 453 of the sense strand of the GFP gene and dTPT3 paired opposite dNaM at position 36 of the sense strand of the SerT tRNA gene, as well as encoding the sgRNAs targeting the most common substitution mutation expected in each sequence (Fig. S2). YZ4 and YZ3 (again used as a control) were transformed with pAIO2X and subjected to the challenging growth regime depicted in Fig. 5, which included extensive high-density growth on solid and in liquid growth media. Plasmids were recovered and analyzed for UBPs retention (Fig. S6) when the OD₆₀₀ reached ~1 to 2 during each liquid outgrowth. In YZ3, which does not express Cas9, or in the absence of Cas9 induction (no IPTG) in YZ4, UBPs retention steadily declined with extended growth (Fig. 5). With induction of immunity (20 or 40 μM IPTG), we observed only a marginal reduction in growth rate (less than 17% increase in doubling time; Fig. S7), and, remarkably, virtually 100% UBPs retention (no detectable loss) in both genes.

Conclusion

Since the last common ancestor of all life on Earth, biological information has been stored with the same four-letter, two-base-pair genetic alphabet. By combining chemical optimization with genetic and immunological engineering, we have created an SSO

that is more autonomous (it is naturally competent to import the unnatural triphosphates) and which stores increased information with a fidelity approaching that of natural information. However, unlike any natural organism, the SSO includes an inanimate, man-made component: a UBPs that allows it to store information with a six-letter, three-base-pair genetic alphabet. With the virtually unrestricted ability to maintain increased information, the optimized SSO now provides a suitable platform for efforts to retrieve the increased information and create organisms with wholly unnatural attributes and traits not found elsewhere in nature.

Materials and Methods

Strains, Plasmids, and Oligonucleotides. A complete list of strains and plasmids and the sequences of oligonucleotides used in this work can be found in Dataset S2; for information regarding strain construction and plasmid cloning, as well as additional experimental details, see SI Materials and Methods. Unless otherwise stated, liquid bacterial cultures were grown in 2xYT (casein peptone 16 g/L, yeast extract 10 g/L, NaCl 5 g/L) supplemented with potassium phosphate (50 mM, pH 7), referred to hereafter as “media,” and incubated at 37 °C in a 48-well flat-bottomed plate (CELLSTAR; Greiner Bio-One) with shaking at 200 rpm. Solid growth media was prepared with

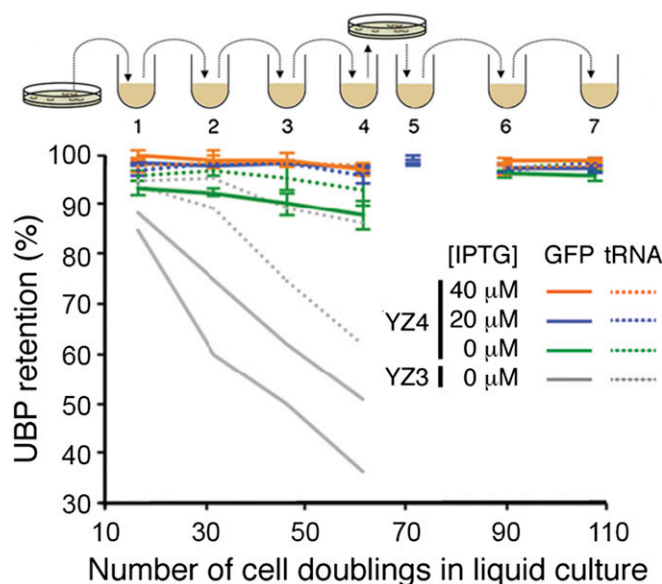


Fig. 5. Simultaneous retention of two UBPs during extended growth. Strains YZ3 and YZ4 were transformed with pAIO2X and plated on solid media containing dNaMTp and dTPT3TP, with or without IPTG to induce Cas9. Single colonies were inoculated into liquid media of the same composition, and cultures were grown to an OD₆₀₀ of ~2 (point 1). Cultures were subsequently diluted 30,000-fold and regrown to an OD₆₀₀ of ~2 (point 2), and this dilution-regrowth process was then repeated two more times (points 3 and 4). As a no-immunity control, strain YZ3 was grown in the absence of IPTG, and two representative cultures are indicated in gray. Strain YZ4 was grown in the presence of varying amounts of IPTG, and averages of cultures are indicated in green (0 μM, $n = 5$), blue (20 μM, $n = 5$), and orange (40 μM, $n = 4$). Retentions of the UBPs in *gfp* and *serT* are indicated with solid or dotted lines, respectively. After the fourth outgrowth, two of the YZ4 cultures grown with 20 μM IPTG were subcultured on solid media of the same composition. Three randomly selected colonies from each plate ($n = 6$ total) were inoculated into liquid media of the same composition, and each of the six cultures was grown to an OD₆₀₀ of ~1 (point 5), diluted 30,000-fold into media containing 0, 20, and 40 μM IPTG, and regrown to an OD₆₀₀ of ~1 (point 6). This dilution-regrowth process was subsequently repeated (point 7). The pAIO2X plasmids were isolated at each of the numbered points and analyzed for UBPs retention (Fig. S6). Cell doublings are estimated from OD₆₀₀ (Materials and Methods) and did not account for growth on solid media, thus making them an underestimate of actual cell doublings. Error bars represent SD of the mean.

2% (wt/vol) agar. Antibiotics were used, as appropriate, at the following concentrations: carbenicillin, 100 $\mu\text{g}/\text{mL}$; streptomycin, 50 $\mu\text{g}/\text{mL}$; kanamycin, 50 $\mu\text{g}/\text{mL}$; zeocin, 50 $\mu\text{g}/\text{mL}$; and chloramphenicol, 33 $\mu\text{g}/\text{mL}$ for plasmids, 5 $\mu\text{g}/\text{mL}$ for chromosomal integrants. All selective agents were purchased commercially. Cell growth, indicated as OD_{600} , was measured using a PerkinElmer Envision 2103 Multilabel Reader with a 590/20-nm filter.

Natural oligonucleotides were purchased from IDT with standard purification and desalting. Gene synthesis of the codon-optimized *PtNTT2* and GFP gene sequences was performed by GeneArt Gene Synthesis (Thermo Fisher) and GenScript, respectively, and kindly provided by Synthorx. Sequencing was performed by Eton Biosciences or Geneviz. Plasmids were isolated using commercial miniprep kits (QIAprep; Qiagen, or ZR Plasmid Miniprep Classic; Zymo Research).

The [α - ^{32}P]-dATP (3,000 Ci/mmol, 10 mCi/mL) was purchased from PerkinElmer. Triphosphates of dNaM, d5SICS, dTPT3, and dMMO2^{bio} were synthesized as described previously (5, 7, 10, 17) or kindly provided by Synthorx. The dNaM-containing TK1 oligonucleotide was described previously (14). All other unnatural oligonucleotides containing dNaM were synthesized by Biosearch Technologies with purification by reverse phase cartridge and kindly provided by Synthorx.

dATP Uptake Assay. Radioactive uptake assays were conducted as described (26), with the following modifications: C41(DE3) and BL21(DE3) strains carrying plasmid-based transporters and their appropriate empty plasmid controls, as well as BL21(DE3) chromosomal transporter integrants and their appropriate isogenic transporterless control, were grown overnight with appropriate antibiotics (streptomycin for pCDF plasmids and chloramphenicol for pSC plasmids and integrants) in 500 μL of media. Cultures were diluted to an OD_{600} of 0.02 in 500 μL of fresh media, grown for 2.5 h, induced with IPTG (0 mM to 1 mM, pCDF strains only) or grown (all other strains) for 1 h, and incubated with dATP spiked with [α - ^{32}P]-dATP [final concentration = 250 μM (0.5 $\mu\text{Ci}/\text{mL}$)] for \sim 1 h. This experimental scheme is analogous to the protocol used to prepare cells for transformation with UBP-containing plasmids, with the 1 h of dATP incubation simulating the 1 h of recovery in the presence of unnatural triphosphates following electroporation. A duplicate 48-well plate without [α - ^{32}P]-dATP was grown in parallel to monitor growth.

Following incubation with dATP, 200 μL of each culture was collected through a 96-well 0.65- μm glass fiber filter plate (MultiScreen; EMD Millipore) under vacuum and washed with cold potassium phosphate (3 \times 200 μL , 50 mM, pH 7) and cold ddH₂O (1 \times 200 μL). Filters were removed from the plate and exposed overnight to a storage phosphor screen (BAS-IP MS; GE Healthcare Life Sciences), which was subsequently imaged using a flatbed laser scanner (Typhoon 9410; GE Healthcare Life Sciences). The resulting image was quantified by densitometric analysis using Image Studio Lite (LI-COR). Raw image intensities of each sample were normalized to the length of time and average OD_{600} during dATP incubation (i.e., normalized to an estimate of the area under the growth curve corresponding to the window of uptake), followed by subtracting the normalized signals of the appropriate negative, with no transporter controls.

Doubling times for strains grown in the dATP uptake assay were calculated by doubling time as $(t_2 - t_1) / \log_2(\text{OD}_{600,2} / \text{OD}_{600,1})$, averaging across three \sim 30-min time intervals roughly corresponding to 30 min before dATP uptake and 60 min during dATP uptake.

Golden Gate Assembly of UBP-Containing Plasmids. Plasmids containing UBP(s) were generated by Golden Gate Assembly. Inserts containing the UBP were generated by PCR of chemically synthesized oligonucleotides containing dNaM, using dTPT3TP and dNaMTP, and primers that introduce terminal BsaI recognition sites that, when digested, produce overhangs compatible with an appropriate destination plasmid; see [Dataset S2](#) for a full list of primers, templates, and their corresponding Golden Gate destination plasmids. Template oligonucleotides (0.025 ng per 50 μL reaction) were PCR-amplified using reagent concentrations and equipment as described previously (14), under the following thermocycling conditions (times denoted as mm:ss): [96 $^{\circ}\text{C}$ 1:00 | 20 \times (96 $^{\circ}\text{C}$ 0:15 | 60 $^{\circ}\text{C}$ 0:15 | 68 $^{\circ}\text{C}$ 4:00)].

To assemble the UBP-containing plasmids, destination plasmid (200 ng to 400 ng), PCR insert(s) (3:1 insert:plasmid molar ratio), T4 DNA ligase (200 U), BsaI-HF (20 U), and ATP (1 mM) were combined in 1 \times New England Biolabs (NEB) CutSmart buffer (final volume 30 μL) and thermocycled under the following conditions: [37 $^{\circ}\text{C}$ 20:00 | 40 \times (37 $^{\circ}\text{C}$ 5:00 | 16 $^{\circ}\text{C}$ 5:00 | 22 $^{\circ}\text{C}$ 2:30) 37 $^{\circ}\text{C}$ 20:00 | 55 $^{\circ}\text{C}$ 15:00 | 80 $^{\circ}\text{C}$ 30:00]. Following the Golden Gate reaction, T5 exonuclease (10 U) and additional BsaI-HF (20 U) were added, and the reaction was incubated (37 $^{\circ}\text{C}$, 1 h) to digest unincorporated plasmid and insert fragments.

In Vivo Plasmid Replication Experiments. Electrocompetent YZ3 cells were prepared by overnight growth in \sim 5 mL of media supplemented with chloramphenicol, dilution to OD_{600} of 0.02 in the same media (variable

volumes, \sim 10 mL of media per transformation), and growth to OD_{600} of \sim 0.3 to 0.4. Cells were then rapidly chilled in an ice water bath with shaking, pelleted (2,500 \times g, 10 min), and washed twice with one culture volume of ice-cold ddH₂O. Electrocompetent cells were then resuspended in ice-cold ddH₂O (50 μL per transformation), mixed with a Golden Gate assembled plasmid (\sim 1 μL , \sim 1 ng) containing the UBP, and transferred to a prechilled 0.2-cm-gap electroporation cuvette. Cells were electroporated (Gene Pulser II; Bio-Rad) according to the manufacturer's recommendations (voltage 25 kV, capacitor 2.5 μF , resistor 200 Ω), then immediately diluted with 950 μL of prewarmed media supplemented with chloramphenicol. An aliquot (10 μL to 40 μL) of this dilution was then immediately diluted fivefold with the same prewarmed media, but additionally supplemented with dNaMTP (250 μM) and d5SICSTP (250 μM). The samples were incubated (37 $^{\circ}\text{C}$, 1 h), and then \sim 15% (vol/vol) of the sample was used to inoculate media (final volume 250 μL to 300 μL) supplemented with chloramphenicol, carbenicillin, dNaMTP (250 μM), and d5SICSTP (250 μM). Cells were then monitored for growth, collected at the density (OD_{600}) indicated in *Results and Discussion*, and subjected to plasmid isolation. Dilutions of the recovery mixture were also spread onto solid media with chloramphenicol and carbenicillin to ascertain transformation efficiencies. Experiments with dNaMTP (150 μM) and dTPT3TP (37.5 μM) were performed analogously.

Experiments with DM1 were performed analogously using media supplemented with streptomycin, with the additional step of inducing transporter expression with IPTG (1 mM, 1 h) before pelleting the cells. All media following electrocompetent cell preparation was also supplemented with streptomycin and IPTG (1 mM) to maintain expression of the transporter.

In Vivo Plasmid Replication Experiments with Cas9 (Liquid Culture Only). Electrocompetent YZ2 cells were transformed with various pCas9 guide plasmids, and single clones were used to inoculate overnight cultures. Cells were then grown, prepared, and electroporated as described above for YZ3, with the following modifications: All media was additionally supplemented with zeocin (to select for pCas9) and 0.2% glucose, electrocompetent cells were stored in 10% (vol/vol ddH₂O) DMSO at -80 $^{\circ}\text{C}$ until use, and recovery and growth media were supplemented with dNaMTP (250 μM) and dTPT3TP (75 μM). Varying concentrations of IPTG (0 μM to 100 μM) were added to the growth media (but not the recovery media) to induce Cas9 expression. The sgRNAs corresponding to the d(AXT) sequence are the nontarget guides for all sequences except for the d(AXT)-containing sequence itself, the nontarget guides for which correspond to the d(GXT) sequence, and all experiments with nontarget sgRNAs were conducted with the addition of IPTG (10 μM) to the growth media. For growth and regrowth experiments, cells were grown to an OD_{600} of 3.5 to 4.0, then diluted 1:250 and regrown to an OD_{600} of 3.5 to 4.0, after which plasmids were isolated.

In Vivo Plasmid Replication Experiments with Cas9 (Plating and Liquid Culture). Electrocompetent YZ4 cells were grown, prepared, and electroporated as described in *In Vivo Plasmid Replication Experiments with Cas9 (Liquid Culture Only)* for YZ2, with the following modifications: Media for growing cells before electroporation only contained chloramphenicol (i.e., no zeocin), zeocin was used to select for pAIO (i.e., no carbenicillin), and recovery and growth media were supplemented with dNaMTP (150 μM) and dTPT3TP (37.5 μM). Following transformation with pAIO, dilutions of the recovery mixture were spread onto solid media containing chloramphenicol, zeocin, dNaMTP (150 μM), dTPT3TP (37.5 μM), 0.2% glucose, and various concentrations of IPTG (0–50 μM). Following overnight growth (37 $^{\circ}\text{C}$, \sim 14 h), individual colonies were used to inoculate liquid media of the same composition as the solid media. Experiments performed with pAIO2X were conducted as described above for YZ4 without using frozen electrocompetent cells or glucose. The second plating depicted in Fig. 5 was performed by streaking cells from liquid culture onto solid media of the same composition as the liquid media, and growth at 37 $^{\circ}\text{C}$ (\sim 14 h). Six random colonies were selected to continue propagation in liquid culture.

Cell Doubling Calculation. Cell doublings for liquid culture growth–dilution–regrowth experiments were calculated by \log_2 of the dilution factor (30,000 or 300,000) between growths, except for growths inoculated from a plated colony, the cell doublings for which were calculated by averaging, for each individual clone, the time from inoculation to target OD_{600} (9.4 ± 1.1 h (1 SD) for the first plating inoculation, 10.2 ± 3 h for the second plating inoculation) and dividing these averages by an estimated doubling time of 40 min. Growth times vary for each clone because colonies were isolated when they were barely visible to the naked eye, and thus we did not attempt to control for variability in the number of cells inoculated into the liquid cultures. Note that the reported number of cell doublings is only an estimate of doublings in liquid culture, which underreports the total number of cell doublings, as we did not attempt to estimate the number of cell doublings that occur during each of the growths on solid media.

Biotin Shift Assay. The retention of the UBP(s) in isolated plasmids was determined as previously described and validated (14), with the following modifications: Plasmid minipreps or Golden Gate assembled plasmids (0.5 μ L, 0.5 ng/ μ L to 5 ng/ μ L), or dNaM-containing oligonucleotides (0.5 fmol), were PCR-amplified with dNTPs (400 μ M), 1 \times SYBR Green, MgSO₄ (2.2 mM), primers (500 nM each), d5SICSTP (65 μ M), dMMO2^{Bio}TP (65 μ M), OneTaq DNA polymerase (0.018 U/ μ L), and DeepVent DNA polymerase (0.007 U/ μ L) in 1 \times OneTaq standard reaction buffer (final volume 15 μ L), under the following thermocycling conditions: [20 \times (95 $^{\circ}$ C 0:15 | x° C 0:15 | 68 $^{\circ}$ C 4:00)]; see Dataset S2 for a list of primers and their corresponding annealing temperatures (x° C) used in this assay. After amplification, 1 μ L of each reaction was mixed with streptavidin (2.5 μ L, 2 μ g/ μ L; Promega) and briefly incubated at 37 $^{\circ}$ C. After incubation, samples were mixed with loading buffer and run on a 6% (wt/vol) polyacrylamide (29:1 acrylamide:bis-acrylamide) Tris/borate/EDTA (TBE) gel, at 120 V for \sim 30 min. Gels were then stained with 1 \times SYBR Gold dye (Thermo Fisher) and imaged using a Molecular Imager Gel Doc XR+ (Bio-Rad) equipped with a 520DF30 filter (Bio-Rad).

Calculation of UBP Retention. UBP retention was assessed by densitometric analysis of the gels (ImageJ or Image Studio Lite; LICOR) from the biotin shift assay and calculation of a percent raw shift, which equals the intensity of the streptavidin-shifted band divided by the sum of the intensities of the shifted and unshifted bands. See Fig. 2A for representative gels. Reported UBP retentions are normalized values.

Unless otherwise indicated, for experiments not involving plating on solid media, UBP retention was normalized by dividing the percent raw shift of each propagated plasmid sample by the percent raw shift of the Golden Gate assembled input plasmid. We assume that the starting UBP content of the cellular plasmid population is equivalent to the UBP content of the input plasmid, which is a valid assumption given direct inoculation of the transformation into liquid culture. Thus, in these experiments, normalized UBP retention is a relative value that relates the UBP content of the propagated

plasmid population to the UBP content of the starting population, which is not 100%, due to loss during the PCR used to generate the insert for input plasmid assembly (Fig. 2A).

For experiments involving plating on solid media, UBP retention was normalized by dividing the percent raw shift of each propagated plasmid sample by the percent raw shift of the dNaM-containing oligonucleotide template used in the assembly of the input plasmid. Plating enables clonal isolation of UBP-containing plasmids from fully natural plasmids that arose during plasmid construction [some of which may contain sequences that are not recognized by the sgRNA(s) used]. Because there is no PCR-mediated loss of the UBP in the oligonucleotide template, normalization to the oligonucleotide template is a better indicator of absolute UBP retention than normalization to the input plasmid. Under the conditions used in the biotin shift assay, most oligonucleotide templates and sequence contexts give >90% raw shift, with <2% shift for a cognate fully natural template (i.e., UBP misincorporation during the biotin shift assay is negligible).

Plating allows for the differentiation between UBP loss that occurs in vivo from loss that occurs in vitro, with the exception of clonally derived samples that give <2% shift, for which we are unable to differentiate between whether the UBP was completely lost in vivo or if the sample comes from a transformant that originally received a fully natural plasmid. Such samples are excluded from reported average values when other samples from the same transformation give higher shifts.

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