A semi-synthetic organism with an expanded genetic alphabet

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Organisms are defined by the information encoded in their genomes, and since the origin of life this information has been encoded using a two-base-pair genetic alphabet (A–T and G–C). In vitro, the alphabet has been expanded to include several unnatural base pairs (UBPs)1–4. We have developed a class of UBPs formed between nucleotides bearing hydrophobic nucleobases, exemplified by the pair formed between d5SICS and dNaM (d5SICS–dNaM), which is efficiently PCR-amplified1 and transcribed2 in vitro, and whose unique mechanism of replication has been characterized3,4. However, expansion of an organism’s genetic alphabet presents new and unprecedented challenges: the unnatural nucleoside triphosphates must be available inside the cell; endogenous polymerases must be able to use the unnatural triphosphates to faithfully replicate DNA containing the UBP within the complex cellular milieu; and finally, the UBP must be stable in the presence of pathways that maintain the integrity of DNA. Here we show that an exogenously expressed algal nucleotide triphosphate transporter efficiently imports the triphosphates of both d5SICS and dNaM (d5SICSTP and dNaMTP) into Escherichia coli, and that the endogenous replication machinery uses them to accurately replicate a plasmid containing d5SICS–dNaM. Neither the presence of the unnatural triphosphates nor the replication of the UBP introduces a notable growth burden. Lastly, we find that the UBP is not efficiently excised by DNA repair pathways. Thus, the resulting bacterium is the first organism to propagate stably an expanded genetic alphabet.

To make the unnatural triphosphates available inside the cell, we previously suggested using passive diffusion of the free nucleosides into the cytoplasm followed by their conversion to the corresponding triphosphate via the nucleoside salvage pathway5. Although we have shown that analogues of d5SICS and dNaM are phosphorylated by the nucleoside kinase from Drosophila melanogaster6, monophosphate kinases are more specific7, and in E. coli we found that overexpression of the endogenous nucleoside diphosphate kinase results in poor growth. As an alternative, we focused on the nucleotide triphosphate transporters (NTTs) of obligate intracellular bacteria and algal plastids8,9,10. We expressed eight different NTTs in E. coli C41(DE3)11–15 and measured the uptake of [α-32P]-dATP as a surrogate for the unnatural triphosphates (Extended Data Fig. 1). We confirmed that [α-32P]-dATP is efficiently transported into cells by the NTTs from Phaeodactylum tricornutum (PtNTT2)16 and Thalassiosira pseudonana (TpNTT2)17. Although NTTs from Protoclamydia amoebophila (PamNTT2 and PamNTT5)18 also import [α-32P]-dATP, PtNTT2 showed the most activity, and both it and TpNTT2 are known to have broad specificity19, making them the most promising NTTs for further characterization.

Transport via an NTT requires that the unnatural triphosphates are sufficiently stable in culture media; however, preliminary characterization of d5SICSTP and dNaMTP indicated that decomposition occurs in the presence of actively growing E. coli (Extended Data Fig. 2). Similar behaviour was observed with [α-32P]-dATP, and the dephosphorylation products detected by thin-layer chromatography (TLC) for [α-32P]-dATP, or by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization (MALDI) for d5SICSTP and dNaMTP, suggest that decomposition is mediated by phoshatases. As no degradation was observed upon incubation in spent media, decomposition seems to occur within the periplasm. No increase in stability was observed in cultures of single-gene-deletion mutants of E. coli BW25113 lacking a specific periplasmic phosphatase18 (as identified by the presence of a Sec-type amino-terminal leader sequence), including phoA, ushA, appA, apnA, yjjX, surE, yfbR, yjjG, yfoO, mutT, nagD, yggV, yrfG or ynmF, suggesting that decomposition results from the activity of multiple phosphatases. However, the extracellular stability of [α-32P]-dATP was significantly greater when 50 mM potassium phosphate (KPi) was added to the growth medium (Extended Data Fig. 3). Thus, we measured [α-32P]-dATP uptake from media containing 50 mM KPi after induction of the transporter with isopropyl-β-d-thiogalactoside (IPTG) (Extended Data Fig. 4). Although induction with 1 mM IPTG resulted in slower growth, consistent with the previously reported toxicity of NTTs18, it also resulted in maximal [α-32P]-dATP uptake. Thus, after addition of 1 mM IPTG, we analysed the extracellular and intracellular stability of [α-32P]-dATP as a function of time (Extended Data Fig. 5). Cells expressing PtNTT2 were found to have the highest levels of intracellular [α-32P]-dATP, and although both extra- and intracellular dephosphorylation was still observed, the ratio of triphosphate to dephosphorylation products inside the cell remained roughly constant, indicating that the extracellular concentrations and PtNTT2-mediated influx are sufficient to compensate for intracellular decomposition.

Likewise, we found that the addition of KPi increased the extracellular stability of d5SICSTP and dNaMTP (Extended Data Fig. 2), and

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Figure 1 | Nucleoside triphosphate stability and import. a, Chemical structure of the d5SICS–dNaM UBP compared to the natural dG–dC base pair. b, Composition analysis of d5SICS and dNaM in the media (top) and cytoplasmic (bottom) fractions of cells expressing PtNTT2 after 30 min incubation; dα shown for comparison. 3P, 2P, 1P and 0P correspond to triphosphate, diphosphate, monophosphate and nucleoside, respectively; [3P] is the intracellular concentration of triphosphate. Error bars represent s.d. of the mean, n = 3.
when a stationary phase culture was diluted 100-fold into fresh media, the half-lives of both unnatural triphosphates (initial concentrations of 0.25 mM) were found to be approximately 9 h, which seemed sufficient when a stationary phase culture was diluted 100-fold into fresh media, although intracellular decomposition was still apparent, the intracellular concentrations of intact triphosphate are significantly above the sub-micromolar K_M values of the unnatural triphosphates for DNA polymerases, setting the stage for replication of the UBP in a living bacterial cell.

The replication of DNA containing dSICS-dNaM has been validated in vitro with different polymerases, primarily family A polymerases, such as the Klenow fragment of E. coli DNA polymerase I (pol I)^20,21. As the majority of the E. coli genome is replicated by pol III, we engineered a plasmid to focus replication of the UBP to pol I. Plasmid pINF (the information plasmid) was constructed from pUC19 using solid-phase DNA synthesis and circular-extension PCR to replace the da–dt pair at position 505 with dNaM paired opposite an analogue of dSICS (dTPT3^22) (Fig. 2a, b). This positions the UBP 362 bp downstream of the ColE1 origin of replication where leading-strand replication is mediated by pol I, and within the TK-1 Okazaki processing site^24, where lagging-strand synthesis is also expected to be mediated by pol I. Synthetic pINF was constructed using the dSICS analogue because it should be efficiently replaced by dSSICS if replication occurs in vivo, making it possible to differentiate in vivo replicated pINF from synthetic pINF.

To determine whether E. coli can use the imported unnatural triphosphates to stably propagate plINF, C41(DE3) cells were first transformed with a pCDF-1b plasmid encoding PNTT2 (hereafter referred to as pACS, for accessory plasmid, Fig. 2a) and grown in media containing 0.25 mM of both unnatural triphosphates, 50 mM KPi and 1 mM IPTG to induce transporter production. Cells were then transformed with plINF, and after a 1-h recovery period, cultures were diluted tenfold with the same media supplemented with ampicillin, and growth was monitored via culture turbidity (Extended Data Table 1). As controls, cells were also transformed with pUC19, or grown without either IPTG or without the unnatural triphosphates. Again, growth was significantly slower in the presence of IPTG, but the addition of dSICS-dTPT and dNaM-TTP resulted in only a slight further decrease in growth in the absence of pINF, and interestingly, it eliminated a growth lag in the presence of pINF (Fig. 2c), suggesting that the unnatural triphosphates are not toxic and are required for the efficient replication of plINF.

To demonstrate the replication of plINF, we recovered the plasmid from cells after 15 h of growth. The introduction of the UBP resulted in the synthesis of DNA containing dSICS-dNaM. The structure of plINF was confirmed by sequencing analysis (Extended Data Table 1). The addition of dSICS-dTPT and dNaM-TTP eliminates a growth lag of cells harboring pINF. The dNaM-TTP mode of replication was confirmed by LC-MS/MS analysis of the global nucleoside content in cells expressing pINF. The presence of dNaM-TTP was confirmed by LC-MS/MS analysis of the global nucleoside content in cells expressing pINF and pUC19 recorded in dynamic multiple reaction monitoring (DMRM) mode. pINF and pUC19 (control) were propagated in E. coli JK53 and sequenced using the Klenow fragment of E. coli DNA polymerase I (pol I), the DNA was amplified and biotinylated. Biotinylation only occurs in the presence of the UBP, the unnatural triphosphates and transporter induction. After growth, plINF was recovered, and a 194-nucleotide region containing the site of UBP incorporation (nucleotides 437–630) was amplified and biotinylated. The natural pUC19 control plasmid was prepared identically to plINF. A 50-bp DNA ladder is shown to the left. Sequencing analysis demonstrates retention of the UBP. An abrupt termination in the Sanger sequencing reaction indicates the presence of UBP incorporation (site indicated with arrow).
a small (approximately twofold) reduction in the copy number of pINF, as gauged by its ratio to pACS (Extended Data Table 1); we determined that the plasmid was amplified 2 × 10^7-fold during growth (approximately 24 doublings) based on the amount of recovered plasmid and the transformation efficiency. To determine the level of UBP retention, the recovered plasmid was digested, dephosphorylated to single nucleosides, and analysed by liquid chromatography–tandem mass spectrometry (LC-MS/MS)25. Although the detection and quantification of dNaM were precluded by its poor fragmentation efficiency and low product ion counts over background, signal for dSICS was clearly observable (Fig. 2d). External calibration curves were constructed using the unnatural nucleoside and validated by determining its ratio to dA in synthetic oligonucleotides (Extended Data Table 2). Using the resulting calibration curve, we determined the ratio of dA to d5SICS in recovered pINF was 1.106 ± 0.1, which when compared to the expected ratio of 1.25 to 1, suggests the presence of approximately one UBP per plasmid. No dSICS was detected in control experiments in which the transporter was not induced, or when the unnatural triphosphates were not added to the media, or when pUC19 was used instead of pINF (Fig. 2d, inset), demonstrating that its presence results from the replication of the UBP and not from misinsertion of the unnatural triphosphates opposite a natural nucleotide. Importantly, as the synthetic pINF contained an analogue of dSICS, and dSICS was only provided as a triphosphate added to the media, its presence in pINF confirms in vivo replication.

To independently confirm and quantify the retention of the UBP in the recovered plasmid, the relevant region was amplified by PCR in the presence of d5SICSTP and a biotinylated dNaMTP analogue (Fig. 2e). Analysis by streptavidin gel shift showed that 67% of the amplified DNA contained biotin. No shift was observed in control experiments where the transporter was not induced, or when unnatural triphosphates were not added, or when pUC19 was used instead of pINF, demonstrating that the shift results from the presence of the UBP. Based on a calibration curve constructed from the shifts observed with the amplification products of controlled mixtures of DNA containing dNaM or its fully natural counterpart (Methods and Extended Data Fig. 6), the observed gel shift corresponds to a UBP retention of 86%. Similarly, when the amplification product obtained with dSICSTP and dNaMTP was analysed by Sanger sequencing in the absence of the unnatural triphosphates1,26,27, the sequencing chromatogram showed complete termination at the position of UBP incorporation, which with an estimated lower limit of read-through detection of 5%, suggests a level of UBP retention in excess of 95% (Fig. 2f). In contrast, amplification products obtained from pINF recovered from cultures grown without PNTT2 induction, without added unnatural triphosphates, or obtained from pUC19 propagated under identical conditions, showed no termination. Overall, the data unambiguously demonstrate that DNA containing the UBP was replicated in vivo and allow us to estimate that replication occurred with fidelity (retention per doubling) of at least 99.4% (24 doublings; 86% retention; 0.994^24 = 0.86). This fidelity corresponds to an error rate of approximately 10^-3, which is comparable to the intrinsic error rate of some polymerases with natural DNA28.

The high retention of the UBP over a 15-h period of growth (approximately 24 doublings) strongly suggests that it is not efficiently excised by DNA repair pathways. To test further this hypothesis and to examine retention during prolonged stationary phase growth, we repeated the experiments, but monitored UBP retention, cell growth and unnatural triphosphate decomposition for up to 6 days without providing any additional unnatural triphosphates (Fig. 3 and Extended Data Fig. 7). At 15 and 19 h of growth, the cultures reached an optical density at 600 nm (OD600) of approximately 0.9 and 1.2, respectively, and both dSICSTP and dNaMTP decomposed to 17–20% and 10–16% of their initial 0.25–mM concentrations (Extended Data Fig. 7a). In agreement with the experiments described above, retention of the UBP after 15 h was 97 ± 5% and >95%, as determined by gel shift and sequencing, respectively, and after 19 h it was 91 ± 3% and >95%. As the cultures entered stationary phase and the triphosphates decomposed completely, plasmid loss began to compete with replication (Extended Data Fig. 7b, c, d), but even then, retention of the UBP remained at approximately 45% and 15%, at days 3 and 6 respectively. Moreover, when dSICS-dNaM was lost, it was replaced by dA–dT, which is consistent with the mutational spectrum of DNA pol F29. Finally, the shape of the retention versus time curve mirrors that of the growth versus time curve. Taken together, these data suggest that in the absence of unnatural triphosphates, the UBP is eventually lost by replication-mediated mispairing, and not from the activity of DNA repair pathways.

We have demonstrated that PNTT2 efficiently imports dSICSTP and dNaMTP into E. coli and that an endogenous polymerase, possibly pol I, efficiently uses the unnatural triphosphates to replicate DNA containing the UBP within the cellular environment with reasonable efficiency and fidelity. Moreover, the UBP appears stable during both exponential and stationary phase growth despite the presence of all DNA repair mechanisms. Remarkably, although expression of PNTT2 results in a somewhat reduced growth rate, neither the unnatural triphosphates nor replication of the UBP results in significant further reduction in growth. The resulting bacterium is the first organism that stably harbours DNA containing three base pairs. In the future, this organism, or a variant with the UBP incorporated at other episomal or chromosomal loci, should provide a synthetic biology platform to orthogonally re-engineer cells, with applications ranging from site-specific labelling of nucleic acids in living cells to the construction of orthogonal transcription networks and eventually the production and evolution of proteins with multiple, different unnatural amino acids.

**METHODS SUMMARY**

To prepare electrocompetent C41(DE3) pACS cells, freshly transformed E. coli C41(DE3) pACS was grown overnight in 2 × YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) supplemented with streptomycin and KPi. After 100-fold dilution into the same medium and outgrowth at 37 °C to OD600 = 0.20, IPTG was added to induce expression of PNTT2. After 40 min, cultures were rapidly cooled, washed with sterile water and resuspended in 10% glycerol. An aliquot of electrocompetent cells was mixed with pINF and electroporated. Pre-warmed 2 × YT medium containing streptomycin, IPTG and KPi was added, and an aliquot was diluted 3.3-fold in the same media supplemented with 0.25 mM each of dNaMTP and dSICSTP. The resulting mixture was allowed to recover at 37 °C with shaking. After recovery, cultures were centrifuged. Spent media was analysed for nucleotide contamination by HPLC (Extended Data Fig. 7a); cells were resuspended in fresh medium containing streptomycin, ampicillin, IPTG, KPi and 0.25 mM each of dNaMTP and dSICSTP, and grown with shaking. At defined time points, OD600 was determined and aliquots were removed and centrifuged. Spent media were analysed for nucleotide
composition, and pINF was recovered by spin column purification. UBP retention was characterized by LC-MS/MS, PCR amplification and gel electrophoresis, or sequencing, as described in the Methods.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 27 November 2013; accepted 8 April 2014.
Published online 7 May 2014.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank I. Haferkamp and J. Audia for kindly providing the NTT plasmids and helpful discussions, and P. Orduoukhian for providing access to the Center for Protein and Nucleic Acid Research at TSRI. This work was supported by the US National Institutes of Health (NIH) (GM060005).

Author Contributions D.A.M., K.D., T.C. and F.E.R. designed the experiments. D.A.M., K.D., T.C. and F.E.R. performed the experiments. N.D., J.M.F. and I.R.C.J. performed LC-MS/MS data analysis. D.A.M., K.D. and F.E.R. performed the experiments. N.D., J.M.F. and I.R.C.J. performed LC-MS/MS analysis. D.A.M., K.D. and F.E.R. analysed data and D.A.M. and F.E.R. wrote the manuscript with assistance from the other authors.

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Preparation of cytoplasmic fraction for nucleotide of diphosphate analysis. To analyse the intracellular desphosphorylation of the unnatural nucleoside triphosphate, cell pellets were subjected to 3 × 10⁶ u l of ice-cold KPi (50 mM). Pellets were then resuspended in 250 μl of ice cold KPi (50 mM) and lysed with 250 μl of lysis buffer B7 of the PureLink Quick Plasmid DNA MiniPrep Kit (200 mM NaOH, 1% w/v SDS), after which the resulting solution was incubated at 22 °C for 5 min. Precipitation buffer N4 (350 μl, 3.1 M potassium acetate, pH 5.5) was added, and the sample was mixed to homogeneity. Following centrifugation (>12,000 r.c.f. for 10 min, at 22 °C) the supernatant containing the unnatural nucleotides was applied to a Hypersil C18 solid phase extraction column (Thermo Scientific) pre-washed with acetonitrile (1 ml) and buffer A (1 ml, see HPLC protocol for buffer composition). The column was then washed with buffer A and nucleotides were eluted with 1 ml of 50% acetonitrile:50% triethylammonium bicarbonate (TEAB) 0.1 M (pH 7.5). The eluent was reduced to approximately 50 μl in a SpeedVac and its volume was adjusted to 100 μl with buffer B before HPLC analysis.

HPLC protocol and nucleotide triphosphate quantification. Samples were applied to a Phenomenex Jupiter LC column (3 μm C18 300 Å, 250 × 4.6 mm) and subjected to a linear gradient of 0–40% B over 40 min at a flow rate of 1 ml min⁻¹. Buffer A: 95% 0.1 M TEAB, pH 7.5, 5% acetonitrile. Buffer B: 20% 0.1 M TEAB, pH 7.5, 80% acetonitrile. Absorbance was monitored at 230, 273, 288, 326 and 365 nm.

Optimization of nucleotide extraction from cells for HPLC injection. To minimize the effect of the lysis and triphosphate extraction protocols on the decomposition of nucleotide triphosphate within the cell, the extraction procedure was optimized for the highest recovery with the lowest extent of decomposition (Extended Data Table 3). To test different extraction methods, cells were grown as described above, washed, and then 5 μmol of either dNAMP or dSSCSTP was added to the pellets, which were then subjected to different extraction protocols including boiling water, hot ethanol, cold methanol, freeze and thaw, lysozyme, glass beads, NaOH, trichloroacetic acid (TCA) with Freon, and perchloric acid (PCA) with KOH³⁰. The recovery and composition of the control was quantified by HPLC as described above to determine the most effective procedure. Method 3—that is, cell lysis with a freeze-thaw cycle (Extended Data Table 3)—was found to be most effective and reproducible, thus we further optimized it by resuspension of the pellets in ice-cold KPi (50 mM, 10 μl) to remove excess radiolabeled substrate, lysed with NaOH (0.2 M, 100 μl) and centrifuged (10,000 r.c.f. for 5 min at 22 °C) to remove cell debris; supernatant was analysed by TLC.

TLC analysis. Samples (1 μl) were applied on a 0.5 mm polyethyleneimine cellulose TLC plate and developed with sodium formate pH 3.0 (0.5 M, 30 × 2.5 M, 2.5 min; 4.0 M, 40 min). Plates were dried using a heat gun and quantified by phosphorimaging (Storm Imager, Molecular Dynamics) and Quantity One software.

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with ultra-mild DNA synthesis phosphoramidites on CPG ultramild supports (1 μmol, Glen Research, Sterling, Virginia, USA) and an ABI Expedite 8905 synthesizer. After the synthesis, the DTM-ON oligonucleotide was cleaved from the solid support, deprotected and purified by Glen-Pak cartridge according to the manufacturer’s recommendation (Glen Research), and then subjected to 8 M urea 8% PAGE. The gel was visualized by ultraviolet shadowing, the band corresponding to the 75-mer was excised, and the DNA was recovered by crush and soak extraction, filtration (0.45 μm), and final desalting over Sephadex G-25 (NAP-25 Columns, GE Healthcare). The sequence of the Watson-Crick strand was determined by ultraviolet absorption at 260 nm assuming that the extinction coefficient of dNaM at 260 nm is equal to that of dA. TK-1-dNaM (4 ng) was next amplified by PCR under the following conditions: 1 × OneTaq reaction buffer, MgSO4 adjusted to 3.0 mM, 0.2 mM of dNTP, 1 μM of each of the d5SICSTP analogue dTPT3TP, 1 μM of each of the primers pUC19-linearization forward and pUC19-linearization reverse, and 0.02 μM of OneTaq DNA Polymerase (in a total of 4 × 50 μl reactions) under the following thermal cycling conditions: initial denaturation (96 °C, 1 min) followed by 12 cycles of denaturation (96 °C, 10 s), annealing (60 °C, 15 s), and extension (68 °C, 2 min); and final extension (72 °C, 5 min). The desired PCR product (2,611 bp) was purified by a 4% agarose gel.

**pUC19 linearization for pINF construction.** pUC19 (20 ng) was amplified by PCR under the following conditions: 1 × Q5 reaction buffer, MgSO4 adjusted to 3.0 mM, 0.2 mM of dNTP, 1 μM of each primers pUC19-lin-forward and pUC19-lin-reverse, and 0.02 U μl−1 of Q5 Hot Start High-Fidelity DNA Polymerase (in a total of 4 × 50 μl reactions with one reaction containing 0.5 × Sybr Green I) under the following thermal cycling conditions: initial denaturation (98 °C, 30 s); 20 cycles of denaturation (98 °C, 10 s), annealing (60 °C, 15 s), and extension (72 °C, 2 min); and final extension (72 °C, 5 min). The desired PCR product (2,611 bp) was purified by a 4% agarose gel.

**PCR assembly of pINF and the natural control plasmid.** A linear fragment was amplified from pUC19 using primers pUC19-lin-forward and pUC19-lin-reverse. The resulting product (800 ng, 4.6 × 1013 mol) was combined with either the natural or unnatural insert (see above) (56 ng, 7.0 × 1012 mol) and assembled by circular overlap extension PCR under the following conditions: 1 × OneTaq reaction buffer, MgSO4 adjusted to 3.0 mM, 0.2 mM of dNTP, 1 μM of each primers pUC19-lin-forward and pUC19-lin-reverse, and 0.02 U μl−1 of Q5 Hot Start High-Fidelity DNA Polymerase (in a total of 4 × 50 μl reactions with one reaction containing 0.5 × Sybr Green I) under the following thermal cycling conditions: initial denaturation (98 °C, 30 s); 20 cycles of denaturation (98 °C, 10 s), annealing (60 °C, 30 s); and extension (72 °C, 2 min); and final extension (72 °C, 5 min). The desired PCR product (2,611 bp) was purified by a 4% agarose gel.

**Streptavidin shift calibration for gel shift mobility assay.** We have already reported Streptavidin shift calibration for gel shift mobility assay.

**Electroporation and recovery for pINF replication in E. coli.** The aliquot of cells was mixed with 2 μl of plasmid (400 ng), transferred to 0.2 cm gap electroporation cuvette and electroporated using a Bio-Rad Gene Pulser according to the manufacturer’s recommendations (voltage 25 kV, capacitor 2.5 μF, resistor 200 Ω, time constant 4.8 ms). Pre-warmed 2 × YT media (0.95 μmol, streptomycin, 1 mM IPTG, 50 mM KPi) was added, and after mixing, 45 μl was removed and combined with 105 μl of the same media (3.3-fold dilution) supplemented with 0.25 mM of dNaMTP and d5SICSTP. The resulting mixture was allowed to recover for 1 h at 37 °C with shaking (210 revolutions per min (r.p.m.)). The original transformation media (10 μl) was spread onto 2 × YT agar containing streptomycin with 10- and 50-fold dilutions for the determination of viable colony forming units after overnight growth at 37 °C to calculate the number of the transformed pINF molecules (see the section on calculation of the plasmid amplification). Transformation, recovery and growth were carried out identically for the natural control plasmid. In addition, a negative control was run and treated identically to pINF transformation except that it was not subjected to electroporation (Extended Data Fig. 7b). No growth in the untransformed negative control samples was observed even after 6 days. No PCR amplification of the negative control was detected, which confirms that unamplified pINF plasmid is not carried through cell growth and later detected erroneously as the propagated plasmid.

**Analysis of pINF replication in E. coli.** After recovery, the cells were centrifuged (4,000 r.c.f. for 5 min, 4 °C), and spent media (0.15 ml) was removed and analysed for nucleotide composition by HPLC (Extended Data Fig. 7a). The cells were resuspended in fresh 2 × YT media (1.5 mM, streptomycin, ampicillin, 1 mM IPTG, 50 mM KPi, 0.25 mM dNaMTP, 0.25 mM d5SICSTP) and grown overnight at 37 °C while shaking (250 r.p.m.), resulting in tenfold dilution compared to recovery media or 33.3-fold dilution compared to the originally transformed cells. Aliquots (100 μl) were taken after 15, 19, 24, 32, 43, 53, 77 and 146 h, OD600 was determined, and the cells were centrifuged (8,000 r.c.f. for 5 min, 4 °C). Spent media were analysed for nucleotide composition by HPLC (Extended Data Fig. 7a), and the pINF and pACS plasmid mixtures were recovered and linearized with NdeI restriction endonuclease; pINF plasmid was purified by 1% agarose gel electrophoresis (Extended Data Fig. 7b) and analysed by LC-MS/MS. The retention of the UBp on the pINF plasmid was quantified by biotin gel shift mobility assay and sequencing as described below.

**Mass spectrometry of pINF.** Linearized pINF was digested to nucleosides by treatment with a mixture of nucleases P1 (Sigma-Aldrich), shrimp alkaline phosphatase (NEB), and DNase I (NEB), overnight at 37 °C, following a previously reported protocol26. LC-MS/MS analysis was performed in duplicate by injecting 15 ng of digested DNA on an Agilent 1290 UHPLC equipped with a G4212A diode array detector and a 6490A Triple Quadrupole Mass Detector operating in the positive electrospray ionization mode (+ESI). UHPLC was carried out using Waters XSelect HSS T3 XP column (21.0 × 100 mm, 2.5 μm) with the gradient mobile phase consisting of methanol and 10 mM aqueous ammonium formate (pH 4.4). MS data acquisition was performed in Dynamic Multiple Reaction Monitoring (DMRM) mode. Each nucleoside was identified in the extracted chromatogram associated with its specific MS/MS transition: dA at m/z 252 + 136, dSICS at m/z 292 + 176, and dNaM at m/z 275 + 171. External calibration curves with known amounts of the natural and unnatural nucleosides were used to calculate the ratios of individual nucleosides within the samples analysed. LC-MS/MS quantitation was validated using synthetic oligonucleotides containing unnatural dSICS and dNaM (Extended Data Table 2).

**DNA biotinylation by PCR to measure fidelity by gel shift mobility assay.** Purified mixtures of pINF and pACS plasmids (1 ng) from growth experiments were amplified by PCR under the following conditions: 1 × OneTaq reaction buffer, MgSO4 adjusted to 3.0 mM, 0.3 mM of dNTP, 0.1 mM of the biotinylated d5SICSTP analogue dMOMO255526, 0.1 mM of d5SICSTP, 1 μM of each of the primers pUC19-seq-forward and pUC19-seq-reverse, 0.02 U μl−1 of OneTaq DNA Polymerase and 0.0025 μl μ−1 of DeepVent DNA Polymerase in a total volume of 25 μl in an CFX Connect Real-Time PCR Detection System (Bio-Rad) under the following thermal cycling conditions: initial denaturation (96 °C, 1 min); 10 cycles of denaturation (96 °C, 30 s), annealing (64 °C, 30 s), and extension (68 °C, 4 min). PCR products were purified, and the resulting biotinylated DNA duplexes (5 μl, 25–50 ng) were mixed with streptavidin (1 μl, 1 μg μl−1, Promega), Promega, then buffer phosphate (50 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1 mM EDTA), incubated for 30 min at 37 °C, mixed with 5 × non-denaturing loading buffer (Quagen), and loaded onto 6% non-denaturing PAGE. After running at 110 V for 30 min, the gel was visualized and quantified. The resulting fragment (194 bp) with primer regions, including the unnatural nucleotide in bold (X represents dNaM or its biotinylated analogue dMOMO2555). LC-MS/MS acquisition was performed in Dynamic Multiple Reaction Monitoring (DMRM) mode. Each nucleoside was identified in the extracted chromatogram associated with its specific MS/MS transition: dA at m/z 252 + 136, dSICS at m/z 292 + 176, and dNaM at m/z 275 + 171. External calibration curves with known amounts of the natural and unnatural nucleosides were used to calculate the ratios of individual nucleosides within the samples analysed. LC-MS/MS quantitation was validated using synthetic oligonucleotides containing unnatural dSICS and dNaM (Extended Data Table 2).
TK-1-dNaM template and its fully natural counterpart with a known ratio of unnatural and natural templates (0.04 ng) were amplified under the same conditions to observe 10 cycles of PCR with pUC19-fusional primers and analysed identically to samples from the growth experiment (see the section on DNA biotinylation by PCR). Each experiment was run in triplicate (a representative gel assay is shown in Extended Data Fig. 6b), and the streptavidin shift (SAS, %) was plotted as function of the UBP (UBP, %). The data was then fit to a linear equation, \( SAS = 0.77 \times UBP + 2.0 (R^2 = 0.999) \), where UBP corresponds to the retention of the unnatural nucleotide in the analysed samples after cellular replication and was calculated from the SAS shift using the equation above.

**Calculation of plasmid amplification.** The cells were plated on 2 \( \times \) 2YT agar containing ampicillin and streptavidin directly after transformation with pINF, and the colonies were counted after overnight growth at 37°C. Assuming each cell is only transformed with one molecule of plasmid, colony counts correspond to the original amount of plasmid that was taken up by the cells. After overnight growth, the plasmids were purified from a specific volume of the cell culture and quantified. As purified plasmid DNA represents a mixture of the pINF and pACS plasmids, digestion restriction analysis with NdeI exonuclease was performed to linearize both plasmids, followed by 1% agarose gel electrophoresis (Extended Data Fig. 7b).

An example of calculations for the 19-h time point with one of three triplicates is provided in Supplementary Information.

**Fragment generation for Sanger sequencing to measure fidelity.** Purified mixtures of pINF and pACS plasmids (1 ng) after the overnight growth were amplified by PCR under the following conditions: 1 \( \times \) OneTaq reaction buffer, MgSO\(_4\) adjusted to 3.0 mM, 0.2 mM of dNTP, 0.1 mM of dNaMTP, 0.1 mM of the 5'-dSSICSTP analogue dTPT3TP, 1 mM of each of the primers pUC19-seq2-forward and pUC19-seq-reverse (see below), and 0.02 U of OneTaq DNA Polymerase in a total volume of 25 \( \mu \)l under the following thermal cycling conditions: initial denaturation (96°C, 1 min); and 10 cycles of denaturation (96°C, 30 s), annealing (64°C, 30 s), and extension (68°C, 2 min). Products were purified by spin column, quantified to measure DNA concentration and then sequenced as described below. The sequenced fragment (304 bp) with primer regions underlined and the unnatural nucleotide in bold (X, dNaM) is

\[
\begin{align*}
\text{5'-GCTGCAAGGCAGTGTGAAACGACGGCGAGTGAATTGCGAGCTCG} \\
\text{GTACCCGGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCG} \\
\text{TTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCG} \\
\end{align*}
\]

The cycle sequencing reactions (10 \( \mu \)l under the following thermal cycling conditions: initial denaturation (96°C, 1 min); and 25 cycles of denaturation (96°C, 10 s), annealing (60°C, 15 s), and extension (68°C, 2.5 min). Upon completion, the residual dye terminators were removed from the reaction with Agencourt CleanSEQ (Beckman-Coulter, Danvers, Massachusetts, USA). Products were eluted off the beads with deionized water and sequenced directly on a 3730 DNA Analyzer (Applied Biosystems). Sequencing traces were collected using Applied Biosystems Data Collection software v3.0 and analysed with the Applied Biosystems Sequencing Analysis v5.2 software.

**Analysis of Sanger sequencing traces.** Sanger sequencing traces were analysed as described previously to determine the retention of the unnatural base pair. In brief, the extent of a base read-through was observed in the direction of the pUC19-seq2-forward primer even with the control plasmid (synthetic pINF); sequencing of only the opposite direction (pUC19-seq-reverse) was used to gauge fidelity. Raw sequencing traces are shown in Fig. 2f and provided as Supplementary Data.

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Extended Data Figure 1 | Natural triphosphate uptake by NTTs. a, Survey of reported substrate specificity (K_{\text{M}}, \mu M) of the NTTs assayed in this study. 

b, PtNTT2 is significantly more active in the uptake of [α-^{32}P]-dATP compared to other nucleotide transporters. Raw (left) and processed (right) data are shown. Relative radioactivity corresponds to the total number of counts produced by each sample. Interestingly, both PamNTT2 and PamNTT5 exhibit a measurable uptake of dATP although this activity was not reported before. This can possibly be explained by the fact that substrate specificity was only characterized using competition experiments, and assay sensitivity might not have been adequate to detect this activity \(^{15}\). References 35, 36 are cited in this figure.

| Transporter | Deoxyribotriphosphates | Ribotriphosphates | Reference |
|-------------|------------------------|-------------------|-----------|
|             | dG | dA | dC | T | G | A | C | U |       |
| PtNTT2      | 82 | 271 | 31 | 428 | 78 | 197 | 49 | 86 | 18    |
| TpNTT2      | 74 | 665 | 60 | 251 | 59 | 49 | 33 | 80 | 18    |
| PamNTT2     | 156 | 437 | 570 | 676 | 1320 | 15 |
| PamNTT3     |       | 22 | 360 | 15 |
| PamNTT5     | 121 | 22 | 360 | 15 |
| SnNTT2      | 170 | 654 | 35 |
| SnNTT3      | 42 | 407 | 375 | 9 | 34 | 35 |
| RpNTT2      | substrate is unknown | 36 |
Extended Data Figure 2 | Degradation of unnatural triphosphates in growth media. Unnatural triphosphates (3P) of dNaM and d5SICS are degraded to diphosphates (2P), monophosphates (1P) and nucleosides (0P) in the growing bacterial culture. Potassium phosphate (KPi) significantly slows down the dephosphorylation of both unnatural triphosphates. a, Representative HPLC traces (for the region between ~20 and 24 min). dNaM and d5SICS nucleosides are eluted at approximately 40 min and not shown. b, Composition profiles.
Extended Data Figure 3 | Effect of potassium phosphate on dATP uptake and stability in growth media. 

**a**, KPi inhibits the uptake of $[^{32}P]d$-ATP at concentrations above 100 mM. Raw (left) and processed (right) data are shown. The NTT from *Rickettsia prowazekii* (*R*NTT2) does not mediate the uptake of any of the dNTPs and was used as a negative control: its background signal was subtracted from those of *P*NTT2 (black bars) and *T*NTT2 (white bars). Relative radioactivity corresponds to the total number of counts produced by each sample.

**b**, KPi (50 mM) significantly stabilizes $[^{32}P]d$-ATP in the media. Triphosphate stability in the media is not significantly affected by the nature of the NTT expressed. 3P, 2P and 1P correspond to triphosphate, diphosphate and monophosphate states, respectively. Error bars represent s.d. of the mean, $n = 3$. 

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Extended Data Figure 4 | dATP uptake and growth of cells expressing PtNTT2 as a function of inducer (IPTG) concentration. Growth curves and $[^{32}\text{P}]-$dATP uptake by bacterial cells transformed with pCDF-1b-PtNTT2 (pACS) plasmid as a function of IPTG concentration. a, Total uptake of radioactive substrate (left) and total intracellular triphosphate content (right) are shown at two different time points. Relative radioactivity corresponds to the total number of counts produced by each sample. b, A stationary phase culture of C41(DE3) pACS cells was diluted 100-fold into fresh 2 × YT media containing 50 mM KPi, streptomycin, and IPTG at the indicated concentrations and were grown at 37°C. Error bars represent s.d. of the mean, n = 3.
Extended Data Figure 5 | Stability and uptake of dATP in the presence of 50 mM KPi and 1 mM IPTG. Composition of [α-32P]-dATP in the media (left) and cytoplasmic fraction (right) as a function of time. TLC images and their quantifications are shown at the bottom and the top of each of the panels, respectively. 3P, 2P and 1P correspond to nucleoside triphosphate, diphosphate and monophosphate, respectively. M refers to a mixture of all three compounds that was used as a TLC standard. The position labelled 'Start' corresponds to the position of sample spotting on the TLC plate.
Extended Data Figure 6  | Calibration of the streptavidin shift (SAS). a. The SAS is plotted as a function of the fraction of template containing the UBP. Error bars represent s.d. of the mean, n = 3. b, Representative data. SA, streptavidin.
Extended Data Figure 7 | Decomposition of unnatural triphosphates, pINF quantification, and retention of the UBP with extended cell growth.

**a** Dephosphorylation of the unnatural nucleoside triphosphate. 3P, 2P, 1P and 0P correspond to triphosphate, diphosphate, monophosphate and nucleoside states, respectively. The composition at the end of the 1 h recovery is shown at the right.

**b** Restriction analysis of pINF and pACS plasmids purified from *E. coli*, linearized with NdeI restriction endonuclease and separated on a 1% agarose gel (assembled from independent gel images). Molar ratios of pINF/pACS plasmids are shown at the top of each lane. For each time point, triplicate data are shown in three lanes with the untransformed control shown in the fourth, rightmost lane (see Methods).

**c** Number of pINF doublings as a function of time. The decrease starting at approximately 50 h is due to the loss of the pINF plasmid that also results in increased error. See the section on pINF replication in *E. coli* in the Methods for details.

**d** UBP retention (%) as a function of growth as determined by gel shift (data shown in Fig. 3) and Sanger sequencing (sequencing traces are available as Supplementary Data). In a, c and d, error shown is the s.d. of mean, *n* = 3.
Extended Data Table 1 | OD$_{600}$ of *E. coli* cultures and relative copy number of plasmid (pINF or control pUC19) as determined by its molar ratio to pACS after 19 h of growth

| Plasmid | IPTG | αXTP/αYTP | Relative Copy Number | OD$_{600}$ (15 h) | OD$_{600}$ (19 h) |
|---------|------|-----------|---------------------|------------------|------------------|
| pINF    | +    | +         | 1.8                 | 0.24             | 0.75             |
|         | +    | –         | 4.6                 | 0.15             | 0.75             |
|         | –    | +         | 8.9                 | 3.13             | 3.98             |
| pUC19   | +    | +         | 2.8                 | 0.54             | 1.25             |
|         | +    | –         | 2.6                 | 0.73             | 1.39             |

X, NaM; Y, SSICS.
Extended Data Table 2 | Relative quantification by LC-MS/MS using synthetic oligonucleotides containing d5SICS and dNaM

| Oligonucleotide (ssDNA) | Size (nt) | Sequence                                                                 | dA/d5SICS Exp (calcd.) | dA/dNaM Exp (calcd.) |
|------------------------|----------|--------------------------------------------------------------------------|------------------------|-----------------------|
| D6-NaM                 | 82       | CAC ACA GGA AAC AGC TAT GAC CCG GGT TAT TAC ATG                          | 22.5 (23.5)*           |                       |
|                        |          | CCG TAG CAC TTG GAA TTC ACC AG ACG NNN NaM NNN                           |                        |                       |
|                        |          | CGG GAC CCA TAG T                                                        |                        |                       |
|                        |          | GAA ATT AAT ACG ACT CAC TAT AGG GTT AAG CTT AAC                            |                        |                       |
| D6-5SICS               | 87       | TTT AAG AAG GAG ATT TAC TAG GGG GCC NNN NaM SSICS                         | 23.4 (25.5)*           |                       |
|                        |          | INN CGG CTG GCC GTC AAT TCC                                               |                        |                       |
|                        |          | CAC ACA GGA AAC AGC TAT GAC CCG GGT TAT TAC ATG                          |                        |                       |
|                        |          | CCG TAG CAC TTG GAA TTC ACT ACC NaM AGT CAC                               |                        |                       |
| D13-NaMx2              | 130      | AAG AGT AAT CCA TAG TAA ACC TCC TTG TTA AGC TTA                           | 16.1 (19.5)            |                       |
|                        |          | ACC CTA TAG TGA GCC GTA ATT ATT TCT                                      |                        |                       |

* dA/d5SICS and dA/dNaM ratios were calculated assuming that randomized nucleotides (N) around the unnatural base are distributed equally.
Extended Data Table 3 | Summary of the most successful extraction methods

| Method | Protocol summary | Total recovery (%)\* | Triphosphate stability (%)\† | Ref. |
|--------|------------------|----------------------|-----------------------------|------|
|        |                  | dNaM | dSSICS | dNaM | dSSICS |                  |
| 1. TCA with Freon | Lyse with cold TCA | 38  | 23    | 92   | 99    | Adapted from Ref. 37 |
|        | Extract aqueous phase using Freon with trioctylamine solution |                  |                  |      |        |                  |
| 2. PCA w/KOH | Lyse with cold PCA | 36  | 21    | 98   | 77    | Adapted from Ref. 38 |
|        | Precipitate proteins with KOH and KPi |                  |                  |      |        |                  |
| 3. NaOH w/ KOAc | Lyse with NaOH and SDS | 21  | 26    | 86   | 100   | see footnotes    |
|        | Precipitate proteins with potassium acetate |                  |                  |      |        |                  |
| 4. NaOH w/ KOAc supplemented w/ KPi (50 mM) | Suspend cells in KPi | 38  | 30    | 99   | 100   | see footnotes    |
|        | Lyse with NaOH and SDS |                  |                  |      |        |                  |
|        | Precipitate proteins with potassium acetate |                  |                  |      |        |                  |

\* Recovery of all nucleotides (3P, 2P, 1P and nucleoside).
\† Calculated as a ratio of 3P composition (%) before and after the extraction.

References 37, 38 are cited in this figure. Details of methods 3 and 4 can be found online (http://2013.igem.org/wiki/images/e/ed/BGU_purelink_quick_plasmid_qrc.pdf).