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Short communication

Adaptive laboratory evolution of a fully re-coded *Escherichia coli* for single-molecule tracking

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Introduction

Genomically recoded organisms (GROs) are emerging chassis in synthetic biology that enable systematic codon reassignment for the site-specific incorporation of non-canonical amino acids (ncAAs) (Lajoie et al., 2013). This expanded chemical functionality allows fluorescent labelling of intracellular proteins for single-molecule tracking (SMT), a method that captures the in vivo kinetics of dynamically delicate proteins intolerant of bulky self-labelling tags.

Recent engineering of the C321.ΔA lineage produced the GRE6 strain, which grows at 37 °C in rich-defined medium (RDM) used for live-cell imaging, yet retains a ≈50 % longer doubling time than wild-type MG1655 (Ilievski et al., 2025). Since rational engineering alone proved insufficient, the present work applies adaptive laboratory evolution (ALE), previously successful on recoded *E. coli* (Wannier et al., 2018), to recover fitness through compensatory mutations. ALE-derived GRE6 derivatives (GRE*) are characterised and their utility for single-molecule tracking (SMT).

Materials and methods

For ALE, three independent GRE6 clones were inoculated into 3 mL of RDM (0.2 % glucose) and incubated at 37 °C with shaking; every 20 h, 1 µL was transferred to 3 mL of fresh medium for nine consecutive passages. After the final passage, dilutions were plated and seven random colonies per lineage were assayed for growth. For the ncAA reporter assay, cells were induced at $OD_{600} \approx 0.3$ with 1 mM IPTG and 1 mM BCN-exoK; ncAA was omitted in negative controls. Mother-machine microfluidic phenotypic analysis and SMT of FLORENCE-labelled EF-G (301UAG), EF-Tu (181UAG) and the GTPase-deficient controls (EF-G H92A, EF-Tu H84A) were performed as previously described (Ilievski et al., 2025), with IPTG omitted during protein production so that ncAA-dependent leaky expression alone supplied the labelled construct. Imaging used 1 ms camera exposure with a 0.5 ms laser pulse; step-length and hidden Markov model (HMM) analyses followed published pipelines. Genomic DNA was extracted from two evolved clones (GRE*1 and GRE*2) and short-read sequenced, with the parental GRE6 genome used as reference.

Results and discussion

After nine days of serial propagation in RDM at 37 °C, seven colonies from each evolved lineage showed substantial improvement, with doubling times of ≈30 min — indistinguishable from MG1655

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and ≈2-fold faster than parental GRE6 (≈60 min). As an independent control, the *prfB* E170K mutation, previously suggested to relieve the absence of release factor 1 (Abdalaal et al., 2020; Wannier et al., 2018), was introduced into GRE6 but produced no detectable improvement, indicating that the evolved phenotype reflects mutations outside the release-factor machinery. The best-growing isolate from each lineage (GRE*1, GRE*2 and GRE*3) was retained for further characterisation.

Whole-genome sequencing of GRE*1 and GRE*2 revealed deletion of the *ratA* gene, encoding the RatA translation-initiation toxin that blocks 70S complex assembly (Zhang and Inouye, 2011). Its convergent loss in two independently evolved lineages suggests translation initiation as a tractable bottleneck in the C321 background.

In minimal media, none of GRE*1–3 improved in M9 with 0.2 % or 1 % glucose, but supplementing with 1× or 2× RPMI amino-acid mixtures reduced doubling times to 30–40 min for all evolved strains and MG1655, whereas parental GRE6 was unaffected — indicating a preference for amino acids over glucose, consistent with re-wired catabolism in the C321 lineage (Yi et al., 2021). With an SCFP3a-UAG reporter, the fluorescence lag was ≈1 h for MG1655 and GRE*1–3, far shorter than the ≈3 h previously reported for GRE6 in LB (Ilievski et al., 2025); no signal was observed without ncAA, confirming UAG-specific incorporation. GRE*1 was evaluated as an SMT chassis with FLORENCE-labelled EF-G (301UAG) and EF-Tu (181UAG). Step-length analysis identified a slow-diffusing population ($D \approx 0.2–0.5\ \mu m^2\ s^{-1}$) corresponding to ≈30 % of EF-G molecules; the catalytic H92A mutant, expected to trap EF-G on the ribosome, increased this fraction to ≈65 %, consistent with ribosome binding. The 181UAG-H84A control further increased its occupancy and dwell time. However, HMM dwell times (≈100 ms) exceed the in vitro ≈50 ms elongation timescale, and non-mutant constructs displayed diffusion behaviour reminiscent of free tRNA (Volkov et al., 2018), most likely because labelled factors are outcompeted by endogenous untagged elongation factors under leaky expression — identifying expression-level tuning as the critical next step (Metelev and Johansson, 2025).

Conclusion

This work establishes GRE*1 as an ALE-derived recoded *E. coli* with MG1655-like growth in optically transparent imaging media; deletion of *ratA* identifies translation-initiation relief as a productive evolutionary trajectory in heavily recoded genomes. GRE*1 supports FLORENCE labelling of essential elongation factors, providing a platform for studying proteins of pharmaceutical relevance, although finely tuned expression vectors are still needed to fully decipher their *in vivo* kinetics.

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