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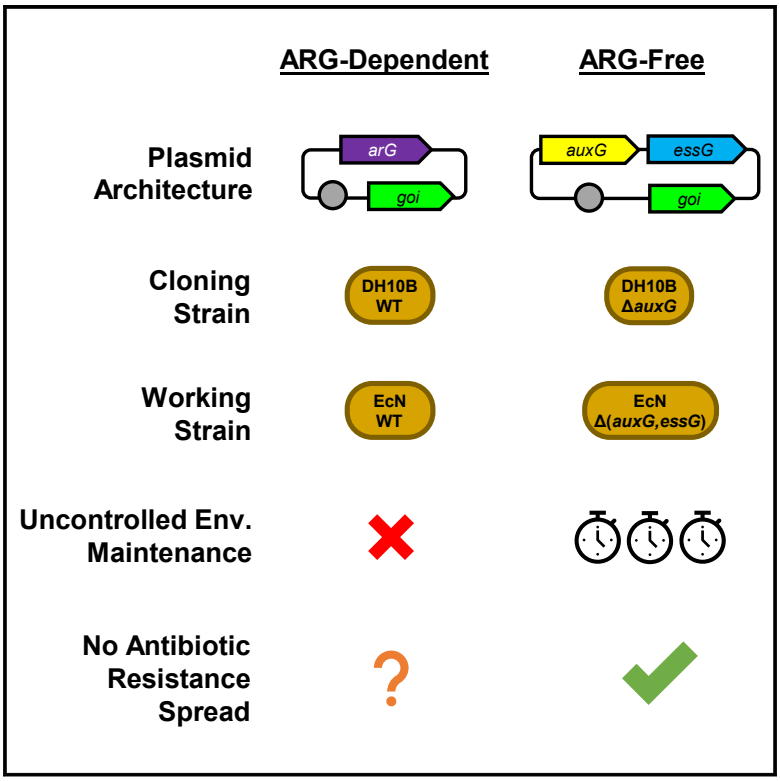
Engineering *E. coli* strains using antibiotic-resistance-gene-free plasmids

Graphical abstract



Highlights

- We develop an antibiotic-resistance-gene-free plasmid (ARGFP)-based cloning method
- ARGFPs are maintained without selection pressure for a month *in vitro*
- ARGFPs show long-term maintenance in the mouse gut
- We apply the ARGFP method to multiple strains, showing its generalizability

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In brief

Widespread use and incomplete disposal of antibiotic resistance genes (ARGs) can increase antibiotic resistance spread via horizontal gene transfer. Amrofell et al. develop an ARG-free, plasmid-based cloning method, demonstrate the long-term maintenance of such plasmids *in vitro* and *in vivo*, and show its generalizability using *E. coli*.

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Engineering *E. coli* strains using antibiotic-resistance-gene-free plasmids

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MOTIVATION Plasmids are essential tools of genetic engineering. However, the current use of antibiotic resistance genes to maintain plasmids in cellular populations risks the spread of antibiotic resistance, particularly in uncontrolled environments. Here, we present antibiotic-resistance-gene-free plasmids (ARGFPs) that can be used for cloning and maintained in engineered strains of *E. coli* that are undergoing extended subculturing or that have colonized the murine gut.

SUMMARY

We created a generalizable pipeline for antibiotic-resistance-gene-free plasmid (ARGFP)-based cloning using a dual auxotrophic- and essential-gene-based selection strategy. We use auxotrophic selection to construct plasmids in engineered *E. coli* DH10B cloning strains and both auxotrophic- and essential-gene-based selection to (1) select for recombinant strains and (2) maintain a plasmid in *E. coli* Nissle 1917, a common chassis for engineered probiotic applications, and *E. coli* MG1655, the laboratory "wild-type" *E. coli* strain. We show that our approach has comparable efficiency to that of antibiotic-resistance-gene-based cloning. We also show that the double-knockout Nissle and MG1655 strains are simple to transform with plasmids of interest. Notably, we show that the engineered Nissle strains are amenable to long-term plasmid maintenance in repeated culturing as well as in the mouse gut, demonstrating the potential for broad applications while minimizing the risk of antibiotic resistance spread via horizontal gene transfer.

INTRODUCTION

Bacterial plasmids are an indispensable tool of biology. Their small size, various copy numbers, and streamlined purification protocols have made them a convenient way to genetically engineer cells for almost 70 years.[1–3] For example, plasmids have been used to create complex genetic circuits[4–6] and remain the simplest way to overexpress heterologous proteins for biotechnological applications.[7] Plasmids require a marker to ensure both the initial selection of cells that contain it, which is often an antibiotic resistance gene (ARG), and the continued propagation of plasmid-containing, antibiotic-resistant cells. This works well in a controlled laboratory environment; however, antibiotics are (1) too expensive to use in large-volume bioreactors[8] and (2) impractical to use in non-controlled environments (e.g., engineered microbes used for environmental remediation or therapeutic interventions).[9,10] Even in laboratory settings, the disposal of antibiotics or organisms containing ARGs are potential vectors for the propagation of harmful antibiotic resistance.[11–14] Persistent exposure to sublethal antibiotic concentrations derived from laboratory sources can result in the spontaneous evolution of resistance in aquatic bacteria.[15] Even more concerning, ARGs in engineered laboratory strains can be spread via horizontal gene transfer (HGT) to human and agricultural pathogens, even if the engineered strains have been killed prior to disposal.[14,16,17] Bacterium-derived plasmids

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can persist and be used to transform native microbes in non-sterile soil, freshwater, and salt water environments for up to 8 days.[17] Furthermore, translation-inhibiting antibiotics may promote HGT even in plasmids that do not contain a resistance cassette for those antibiotics.[16] There is a substantial need for alternatives to the use of ARGs for plasmid selection and maintenance inside and outside of the laboratory.

This need may be met in two ways: genomic incorporation[18–22] or a custom non-antibiotic-based plasmid selection system.[23–27] In genomic incorporation, genetic payloads are introduced into the genome via site-specific recombination.[28,29] Since the genome must be replicated during bacterial cell division, the incorporation is stably maintained for generations. The change in copy number from plasmid to genome may require retuning of expression levels via expression element engineering and multiple integrations. Furthermore, iteratively modifying genomic loci as part of the design-build-test-learn cycle is more laborious than doing the same with plasmids.[29] Custom non-ARG-based plasmid selection systems may overcome those limitations by maintaining multi-copy plasmids within the cell, relying on the expression of a marker gene (e.g., an essential gene, an antitoxin gene, or an auxotrophic marker) instead of an antibiotic resistance cassette, or a repurposed strain-specific native plasmid.[30] Such a system has drawbacks: auxotrophic-based strategies, where the lack of a specific gene is lethal if a certain exogenous compound is absent, may be susceptible to cross-feeding and premature loss of selection pressure in uncontrolled environments[31–33] or require suppression of amber stop codon tRNA that could interfere with translational termination in other endogenous genes.[34,35] An essential gene-based system requires a simultaneous knockout of the genomic copy of the gene and transformation of the new plasmid containing the same gene, with which the strain is transformed,[36] every time, while another requires a recombination event to remove an original ARG marker.[37] Finally, although antibiotics are not needed in the final use case, many of these plasmids still contain the antibiotic resistance cassettes from the original cloning workflow.[25–27,30,38–41] These cassettes may be horizontally transferred to other bacteria in the environment, leading to further spread of pathogenic antibiotic-resistant strains.[42–45] Otherwise, the plasmids must undergo an additional step to remove the resistance marker.[24,37,46]

We made several innovations toward a streamlined, generalizable ARG-free cloning and maintenance strategy. We engineered plasmids with dual auxotrophic and essential gene markers and developed a generalizable pipeline in which auxotrophic *E. coli* DH10B cloning strains were used to construct those plasmids without the need for antibiotics. As an example of a working strain, we used *E. coli* Nissle 1917 (EcN), a common chassis for live engineered microbial therapeutics and diagnostics,[47–51] and *E. coli* K12 MG1655, the canonical "wild-type" laboratory *E. coli* strain, missing both essential and auxotrophic gene. Specifically, we show that the engineered EcN and MG1655 may be transformed with the engineered plasmids; the auxotrophic marker is used for initial selection in both strains, while the essential gene makes the cell depend on the plasmid for long-term maintenance. Unlike previously reported methods, our system never requires ARGs for plasmid construction and specific cloning, working strains require genomic modification only once, and no additional steps are added to the typical cloning workflow.

In this work, we present comprehensive measurements of plasmid transformation efficiency using auxotrophic selection and demonstrate that this method is convenient for most laboratory transformation applications. We constructed EcN and MG1655 double-knockout strains and showed that a recombineering plasmid used to knock out an essential gene while maintaining cellular viability may be swapped for our plasmids of interest during a single transformation step. Furthermore, we show that EcN double knockouts containing these plasmids do not have a growth defect and can maintain the plasmids for up to a month of continuous subculturing *in vitro* as well as more stably *in vivo* at least for 12 days compared to control strains. Our ARG-free plasmid (ARGFP)-based cloning and long-term plasmid maintenance workflow is a generalizable blueprint for researchers to build and maintain plasmids in strains without using ARGs, potentially reducing the spread of antibiotic resistance.

RESULTS AND DISCUSSION

ARGFP selection and maintenance strategy

We developed a two-part method: first, we constructed plasmids containing essential and auxotrophic gene markers in engineered auxotrophic strains of *E. coli* DH10B using the corresponding auxotrophic genes as selection markers on plasmids (Figure 1A); second, we created working strains missing both the auxotrophic and essential genes, which were provided on our plasmids of interest using modified recombineering plasmids (Figure 1B). After being plated on appropriately selective conditions, the working strain transformants contained only the plasmid of interest and were maintained without any additives to the growth medium.

Auxotrophic selection for plasmid presence in engineered *E. coli*

To test the validity of our approach, we first sought to establish the robustness of auxotrophic-based plasmid selection in *E. coli* strains. We created thymidine (Thy) auxotrophic strains by knocking out the thymidylate synthase gene *thyA* from the *E. coli* DH10B and EcN genomes[52]; similarly, we created N-acetyl-D-glucosamine (NAcG) auxotrophic strains by knocking out the L-glutamine-D-fructose-6-phosphate aminotransferase gene *glmS*.[53] These knockouts are conditionally lethal, i.e., knockout cells are only viable in the presence of exogenous Thy or NAcG or plasmid-borne *thyA* or *glmS*.[18–20,38,46,53–55] We then inserted those genes into a plasmid under the control of a constitutive promoter in low-, medium-, and high-copy plasmid backbones[56,57] containing kanamycin, spectinomycin, and chloramphenicol resistance cassettes, respectively. We measured the efficiency of transforming wild-type (WT) DH10B, DH10B Δ*thyA*, WT EcN, and EcN Δ*thyA* with miniprepped *thyA*-containing plasmids (Figure 2A) and WT DH10B, DH10B Δ*glmS*, WT EcN, and EcN Δ*glmS* with miniprepped *glmS*-containing plasmids (Figure 2B). For all auxotrophic strains tested, the auxotrophic selection efficiency was similar to the efficiency

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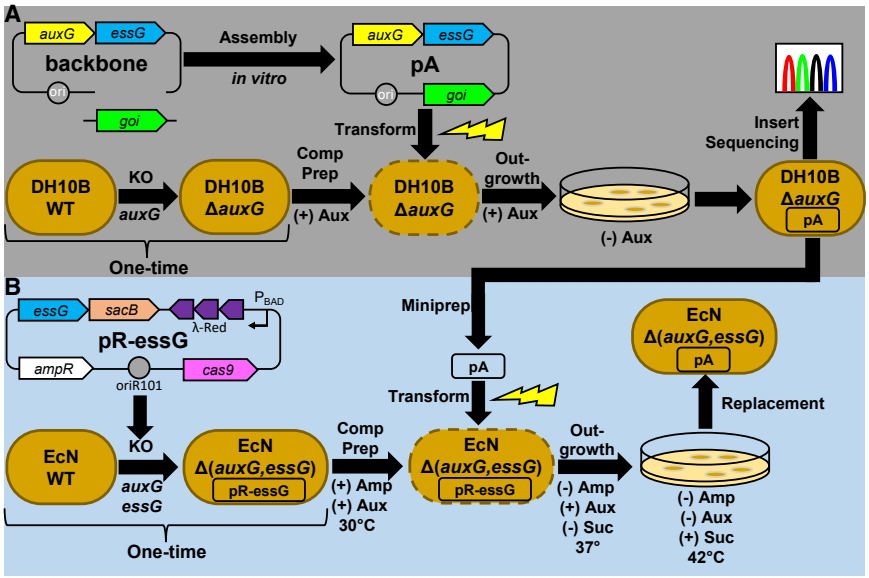

Figure 1. Fully antibiotic-resistance-gene-free plasmid-based cloning and maintenance

(A) Cloning in DH10B auxotrophic strains. To make pA, a gene of interest is assembled into a plasmid backbone containing a bicistronic auxotrophic gene (*auxG*) and essential gene (*essG*) operon and an origin of replication. pA is used to transform a strain of DH10B with the genomic copy of *auxG* knocked out via recombineering, which is grown and made competent in medium containing the auxotrophic compound (Aux) to maintain cell viability. Following outgrowth in medium containing Aux (mimicking excluding the antibiotic during traditional cloning outgrowth), transformants are washed and plated on medium without the Aux, selecting for colonies that contain pA. Colonies are then screened for correct assembly.

(B) Plasmid replacement and maintenance in double-knockout EcN. A special recombineering plasmid, pR-essG, containing an *essG* cassette, a *sacB* cassette, arabinose-inducible λ-Red recombinase genes, a constitutive *cas9* recombination counterselection cassette, an ampicillin (Amp) resistance marker, and a heat-unstable oriR101 origin of replication, is used to knock out both *essG* and *auxG* in EcN while maintaining cell viability. Competent cells, prepared in such a way as to maintain pR-essG, are transformed with pA miniprepped from DH10B auxotrophs, outgrown briefly, washed, and then plated on medium without Amp, without the Aux, and with sucrose (Suc) to select for the replacement of pR-essG with pA. Plates are incubated at 42°C to further promote replacement of pR-essG with pA. Individual colonies on the plate all contain pA and can be subsequently cultured with no medium supplements.

of the antibiotic selection and the antibiotic plus auxotrophic selection, suggesting that all cells contained the plasmid. Statistically significant differences between auxotrophic and antibiotic selection in auxotrophic strains are likely attributable to variations in expression levels of the markers. In short, we validated that auxotrophic plasmid selection is robust for use in a laboratory environment.

Next, we evaluated if our auxotrophic cloning strains may be transformed with *in-vitro*-assembled plasmids. We transformed auxotrophic DH10B strains with Gibson-assembled plasmids and quantified transformation efficiency (Figure 2C). The efficiency was one order of magnitude lower than that of miniprepped plasmids, likely because the assemblies were not methylated or supercoiled like the plasmids used in Figures 2A and 2B. We then screened multiple colonies from the auxotrophic selection condition of each strain-plasmid combination for the presence of plasmid by replating individually resuspended colonies onto plates with and without antibiotics (Figure 2D). As expected, all tested colonies grew in both conditions, indicating that 100% contained the plasmid. These results suggest that auxotrophic genes are practical selection markers in auxotrophic strains of *E. coli* for plasmids assembled *in vitro.*

Replacement of recombineering plasmids with plasmids of interest in auxotroph-essential gene double-knockout strains of EcN

Auxotrophic selection has limitations beyond its use in a controlled environment. Namely, strains can scavenge the auxotrophic compound found in the environment, potentially removing the selection pressure for plasmid maintenance. This may result in a decrease of copy number in the population or a loss of plasmid entirely.[36,37] To address this shortcoming, we used the plasmid-based expression of an essential gene to make our working strain, EcN, completely dependent on the plasmid of interest. We previously demonstrated that such an essential gene marker works to maintain a gRNA expression plasmid for a CRISPR-Cas9 bacterial kill switch in the mouse gut, but that plasmid still contained an antibiotic resistance cassette to facilitate original cloning.[39]

To create a streamlined process and overcome reliance on antibiotics, we knocked out the essential translation initiation factor 1 gene *infA* in plasmid-free EcN Δ*thyA* and the essential NH_3-dependent NAD^+ synthetase gene *nadE* in plasmid-free EcN Δ*glmS* to create EcN Δ(*thyA, infA*) and Δ(*glmS, nadE*), respectively. Both are small (*infA* is 219 bp and *nadE* is 828 bp) and well insulated from the surrounding genomic context, limiting the size of the plasmid marker and making them straightforward to knock out without disrupting surrounding genes, respectively. To maintain cell viability after knocking out the essential genes, we created pR-essG recombineering plasmids pR-infA and pR-nadE that contain constitutive *infA* and *nadE* expression cassettes, respectively (Figure 3A). We also incorporated a ribosome-binding site and *infA* immediately downstream of *thyA* to create "p1" variants of pA and did the same with *nadE* and *glmS* to create "p2" variants of pA (Figure 3B). We maintained the presence of antibiotic resistance cassettes as *goi1* and *goi2* on p1 and p2, respectively (Figure 3B), for downstream plasmid presence verification experiments (Figure 3C).

Curing pR-essG from cells transformed with pA is crucial to our strategy because the ampicillin ARG must not propagate and because maintaining pR-essG would be burdensome for the cell. To make the method as streamlined as possible, we also desired to cure pR-essG while simultaneous transforming strains with pA. Since pR-essG contains the *Bacillus subtilis*

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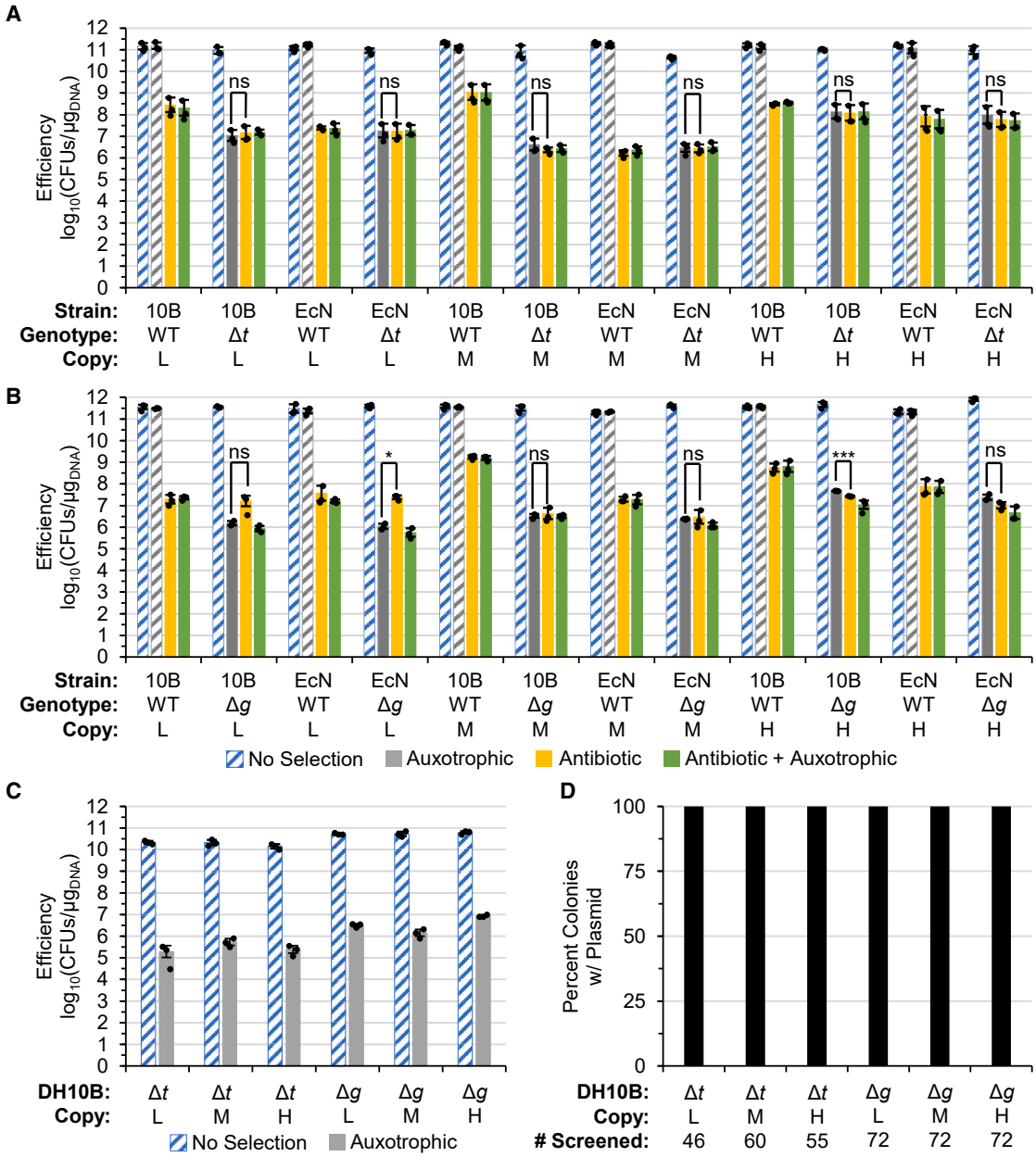

Figure 2. Plasmid-based expression of an auxotrophic gene enables selection of transformants missing the genomic copy of the auxotrophic gene

(A and B) Transformation efficiency of strains using miniprepped plasmids containing (A) *thyA* or (B) *glmS* and low-, medium-, and high-copy origins.

(C) Transformation efficiency of auxotrophic strains with *in vitro* Gibson-assembled low-, medium, and high-copy plasmids containing *thyA* or *glmS* expression cassettes. Dashed bars represent a theoretical maximum transformation efficiency and are not indicative of the number of cells containing plasmid.

(D) Percentage of transformants from (C) containing plasmid, determined by replating colonies on plates with or without antibiotic. "No Selection" medium consisted of M9 + 2% glucose + 0.5% tryptone + 0.01% (w/v) thiamine (M9t) supplemented with 0.01% (w/v) thymidine (Thy) or 0.1% (w/v) N-acetyl-D-glucosamine (NAcG) as appropriate; "Auxotrophic" consisted of M9t only; "Antibiotic" consisted of M9t supplemented with Thy or NAcG and kanamycin (Kan), spectinomycin (Spec), or chloramphenicol (Cm) as appropriate; and "Antibiotic + Auxotrophic" consisted of M9t supplemented with Kan, Spec, or Cm as appropriate. Low-copy plasmids contained a pSC101 origin of replication and a Kan resistance cassette; medium-copy plasmids contained a p15A origin and Spec resistance cassette; and high-copy plasmids contained a colE1 origin and Cm resistance cassette.

In (A)–(C), values are the log mean of biological triplicates (defined as 3 unique transformations), with each replicate in black dots and error bars representing the standard error. In (D), values are a percentage of the number of colonies indicated, with each colony considered a distinct replicate. In (A) and (B), statistical comparisons were performed with a heteroscedastic two-tailed Student's t test (not significant [ns], $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

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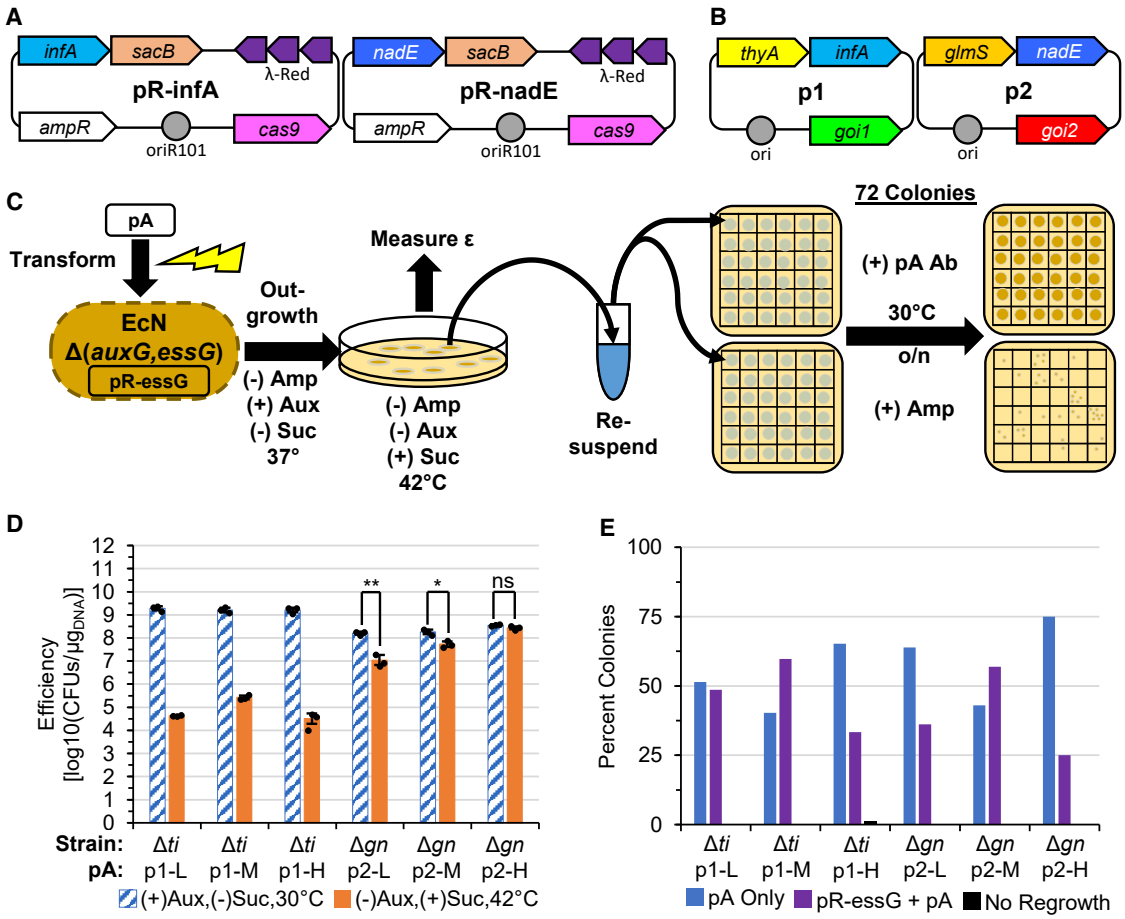

Figure 3. Engineered EcN missing both an auxotrophic and an essential gene is transformable with plasmids expressing the missing auxotrophic and essential genes

(A) Schematics of pR-essG variants pR-infA and pR-nadE.

(B) Schematics of pA variants p1 and p2. Each contains an operon consisting of an auxotrophic gene and an essential gene (*thyA-infA* and *glmS-nadE*, respectively), an origin of replication (low copy [L], pSC101; medium copy [M], p15A; high copy [H], colE1), and a gene of interest (*kanR*, *specR*, and *cmR* for low, medium, and high copy, respectively).

(C) Experimental workflow. Double-knockout EcN strains containing pR-essG are transformed with pA and plated on conditions to promote replacement of pR-essG with pA. Transformation efficiency (ε) is measured, and then 72 individual colonies of each transformant are resuspended in 50 μL PBS. 5 μL each resuspension is replated on plates containing the corresponding pA antibiotic (pA Ab) or Amp to determine which plasmids are present following incubation at 30°C overnight (o/n).

(D) Transformation efficiency of EcN Δ(*thyA, infA*) (Δ*ti*) with p1 variants and EcN Δ(*glmS, nadE*) (Δ*gn*) with p2 variants. Values are the log mean of biological triplicate samples (3 unique transformations), with error bars representing the standard error and dots representing each singular replicate value. Dashed bars represent a theoretical maximum transformation efficiency and are not indicative of the number of cells containing plasmid. Statistical comparisons were performed with a heteroscedastic two-tailed Student's t test (ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

(E) Percentage of transformants containing pA, both pRec-essG and pA, or that did not regrow after being replated. Each colony tested (72 for each strain-plasmid combination) was considered a unique replicate.

levansucrase gene *sacB* and the heat-unstable *oriR101* origin of replication in addition to the ampicillin resistance cassette, we hypothesized that we could replace it with pA in a single transformation step by plating on medium with sucrose and without ampicillin incubated at 42°C (Figure 1B). pA was selected via expression of both the essential gene and the auxotrophic gene since the plates also lacked the auxotrophic compound.

To test this hypothesis, we first measured the transformation efficiency of the pR-infA replacement with p1 in EcN Δ(*thyA, infA*) and the pR-nadE replacement with p2 in EcN Δ(*glmS, nadE*). We plated transformants on medium (1) without sucrose, with the auxotrophic compound, and incubated at 30°C (non-selective control) and (2) with sucrose, without the auxotrophic compound, and incubated at 42°C (replacement selective) (Figure 3C). While the replacement of pR-infA with p1 in EcN Δ(*thyA, infA*) resulted in an expected large (~10^4-fold) difference between the theoretical maximum efficiency and the selective conditions, the pR-nadE-p2 replacement in EcN Δ(*glmS, nadE*) ideally, yet unexpectedly, yielded a number of colonies nearly equal to those of the theoretical maximum efficiency (Figure 3D).

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To understand and quantify which plasmids the transformants contained, we replated individual resuspensions of 72 colonies of each strain-plasmid combination from the replacement-selective conditions on medium with ampicillin or with the antibiotic for which the pA variant conferred resistance (Figure 3C). As expected, all the colonies that regrew contained pA, yet many still contained pR-essG (Figure 3E). However, when we quantified how well the colonies regrew on the new plates, we found that regrowth on the pA antibiotic led to an indistinct lawn covering the area of the plated droplet (Figures S1A–S1C), while regrowth on ampicillin resulted in few translucent colonies or none at all (Figure S1B). This indicated that pR-essG was essentially cured from the population even if a few cells of the original colony still contained it.

Next, we systematically altered the stringency of the replacement selection and demonstrated that the dual auxotrophic-essential gene marker is more effective than a single essential gene at promoting plasmid replacement. We performed the plasmid presence testing experiment after transforming EcN Δ(*thyA, infA*) and a single-knockout EcN Δ*infA* (both initially containing pR-infA) and quantified regrowth of transformants plated in the presence or absence of sucrose and Thy at low and high plate incubation temperatures: 37°C or 42°C, respectively (Figures S2 and S3). We found that the presence of sucrose and a 42°C incubation were sufficient to select for the presence of p1 in both strains. Quantifying regrowth revealed more granular details of the replacement. Of the Δ*infA* colonies originally plated on sucrose-containing medium and grown at 42°C, some grew well when replated on ampicillin, indicating that pR-infA still propagated in these populations and justifying our double-knockout approach for streamlined transformation. Unexpectedly, in Δ(*thyA, infA*) strains, incubating at a high temperature while including sucrose or excluding Thy was sufficient to always select for cells containing p1 while curing pR-infA. However, if sucrose was excluded and Thy was included, many colonies did not regrow at all, indicating false positives on the original transformation plates. To guarantee full replacement, we proceeded with the most stringent selection conditions: 42°C incubation, presence of sucrose, and exclusion of auxotrophic compound. Overall, we established that the recombineering plasmid can be swapped with a plasmid of interest in our double-knockout EcN strains in a single transformation step by plating on appropriately selective conditions. Importantly, our system requires no additional steps following plasmid preparation from the cloning strain or following plasmid transformation of the working strains, which need to be created only once, to remove ARGs.

Finally, we sought to understand how the knockout strains containing pA variants grew in rich (Figures S4I–S4N) and minimal (Figures S5I–S5N) media compared to both plasmid-free (Figure S4B and S5B) and plasmid-containing WT strains (Figures S4C–S4H and S5C–S5H). As expected, the plasmid-free WT strain had the highest calculated growth rate and shortest lag time in rich medium. Two WT replicates containing p1-L and one WT replicate containing p2-L did not grow in rich medium, perhaps due to insufficient ARG expression from the low-copy plasmid. We used a one-way ANOVA with Sidak's method to compare strain-plasmid growth rate and lag time. The following had statistically significant lower growth rates than the WT plasmid-free strain: WT + p2-L ($p < 0.0001$), WT + p2-H ($p = 0.0043$), Δ(*thyA, infA*) + p1-H ($p = 0.036$), and Δ(*glmS, nadE*) + p2-H ($p = 0.0027$). More strains had significantly longer lag times than plasmid-free WT: WT + p1-L ($p < 0.0001$), WT + p1-H ($p = 0.0031$), WT + p2-L ($p < 0.0001$), WT + p2-H ($p < 0.0001$), Δ(*thyA, infA*) + p1-H ($p < 0.0001$), and Δ(*glmS, nadE*) + p2-H ($p < 0.0001$). When comparing knockout strains to WT strains containing the same plasmid, no knockouts had a significantly lower growth rate, and only WT + p1-H had a significantly shorter lag time than Δ(*thyA, infA*) + p1-H ($p < 0.0001$). In minimal medium, plasmid-free WT did not have the highest growth rate nor the shortest lag time; furthermore, two WT + p1-L replicates did not grow, and all strains containing high-copy plasmids grew poorly or not at all. WT strains containing p2-L and p2-M had a higher growth rate than the Δ(*glmS, nadE*) strains containing the same ($p < 0.0001$ and $p = 0.0084$, respectively), and only WT + p2-L had a shorter lag time than Δ(*glmS, nadE*) ($p = 0.0053$). We believe that the data, taken all together, indicate that the double-knockout strains do not inherently have a growth defect compared to WT; rather, individual plasmids may have insufficient marker expression or be overburdensome.[58,59] This issue can be addressed in future works by optimizing the expression levels of markers and genes of interest.

ARG-independent long-term plasmid maintenance

We next sought to show that our double-knockout strains could maintain a plasmid for multiple weeks, mirroring long residence time in a non-laboratory environment, and to demonstrate why our dual auxotrophic gene-essential gene system is preferable to an auxotrophic one in an environment where bacteria can scavenge auxotrophic compounds. To this end, we serially passaged double knockouts and single auxotrophic knockouts containing medium-copy p1 or p2 plasmids with a spectinomycin resistance cassette as the gene of interest for a month while constantly including or excluding the auxotrophic compound. On select days, we measured the fraction of the population that contained plasmid (Figure 4); raw colony-forming unit (CFU) counts from the experiment are shown in Figure S6. As expected, Δ*thyA* cells in the presence of Thy showed significant plasmid loss beginning at day 24 (Figure 4A), while the *ΔthyA* without Thy and Δ(*thyA, infA*) strains never lost the plasmid in either condition (Figure 4B). Unexpectedly, the Δ*glmS* strain in the presence of NAcG did not significantly lose its plasmid (Figure 4C), while the Δ(*glmS, nadE*) strain maintained the plasmid in both conditions as expected (Figure 4D). This may have occurred because the antibiotic resistance cassette was not highly burdensome, the plasmid-borne *glmS* conferred some sort of growth advantage even in the presence of NacG, and/or our antibiotic-resistance-based assay exerted unavoidable selection pressure for cells containing plasmid. Overall, our results indicate that working strains can maintain the plasmid for a month, even when strains can scavenge auxotrophic compounds.

Fully ARGFP-based cloning and maintenance demonstration

Next, we set out to remove all ARGs from our plasmids and to demonstrate a full workflow with our system. We replaced the

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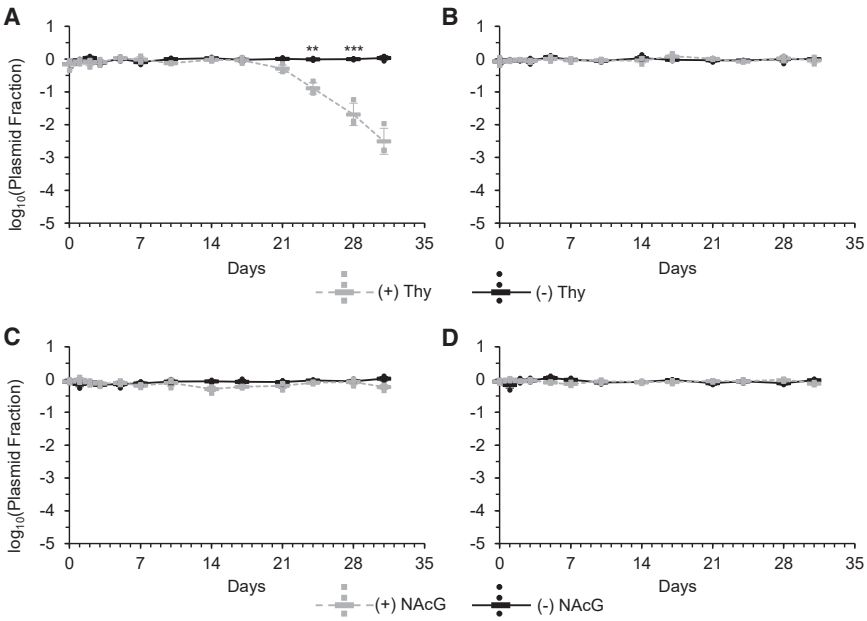

Figure 4. p1 and p2 are maintained long term in the presence of exogenous auxotrophy-recovering compounds in engineered EcN missing both auxotrophic and essential genes

(A and B) Fractions of EcN (A) Δ*thyA* and EcN (B) Δ(*infA, thyA*) still containing p1 after a month of repeated passages with presence or absence of Thy (±Thy).

(C and D) Fractions of EcN (C) Δ*glmS* and EcN (D) Δ(*grpE, glmS*) still containing p2 after a month of repeated passages with presence or absence of NAcG (±NAcG).

Fractions were measured by washing, serially diluting, and then spotting dilutions of stationary phase cultures onto M9t with or without Ab. Values are the log mean of biological triplicates (3 cultures started with 3 different colonies), with error bars representing the standard error and dots representing individual replicates. Statistical comparisons were performed using a two-tailed mixed-model ANOVA with Sidak's multiple comparisons (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

antibiotic resistance cassettes of p1 and p2 with constitutive *gfpmut3* and *mCherry* expression cassettes as *goi1* and *goi2*, respectively (Figure 3B). We transformed DH10B Δ*thyA* with newly assembled p1 variants and DH10B Δ*glmS* with p2 variants and measured the transformation efficiency (Figure 5A). We screened eight transformant colonies of each plasmid variant via colony PCR (Figures S7A and S7E), purposefully picking fluorescent and non-fluorescent colonies as visualized on a transilluminator. Bands that were too short or undetectable corresponded to non-fluorescent colonies, as confirmed upon restreak (Figures S7B–S7D and S7F–S7H). Sanger sequencing of purified colony PCR products of the expected length confirmed correct plasmid assemblies in fluorescent colonies, while non-fluorescent ones had no inserts, likely due to an assembly error rather than mutation during transformation outgrowth or overnight incubation. We grew a single sequence-verified restreaked colony of each DH10B Δ*thyA* + p1 variant and DH10B Δ*glmS* + p2 variant overnight in M9t medium, measured growth (Figures S7I and S7J, respectively) and fluorescence (Figures S7K and S7L, respectively), and subsequently miniprepped the new plasmids. Overall, we validated that auxotrophic DH10B strains are useful cloning strains when no antibiotic marker is present.

Next, we demonstrated that pR-essG in double-knockout EcN strains may be replaced with pA containing no antibiotic resistance markers. We performed pR-infA-p1 replacements in EcN Δ(*thyA, infA*) and pR-nadE-p2 replacements in EcN Δ(*glmS, nadE*) with the newly miniprepped plasmids and measured the transformational efficiency (Figure 5B). Compared to the antibiotic resistance cassette plasmids (Figure 3D), the efficiencies tended to be lower, but this might be attributable to differential expression levels of the fluorophore genes of interest versus the original ARGs. Subsequently, to confirm that the swaps took place, we inoculated three colonies of each transformant into liquid medium with or without ampicillin, grew them overnight, and then measured their growth (Figure 5C) and normalized fluorescence (Figure S8). Only the single replicate controls with pR-essG grew in the presence of ampicillin, while the double-knockout strains were all fluorescent, showing that full replacements occurred and further proving that the recombineering plasmids were cured from the population (Figures 3E, S1, and S3).

We also compared growth rates of double-knockout strains containing ARGFPs (Figures S4O–S4T and S5O–S5T) to plasmid-free WT (Figure S4B and S5B) and double knockouts with ARG-containing plasmids (Figures S4I–S4N and S5I–S5N) in rich (Figure S4) and minimal (Figure S5) media, again using one-way ANOVA with Sidak's method for identifying significant differences. In rich medium, Δ(*thyA, infA*) + p1-L-*gfp* ($p = 0.0031$) and Δ(*thyA, infA*) + p1-H-*gfp* ($p < 0.0001$) had a lower growth rate than the plasmid-free WT strain, and Δ(*thyA, infA*) + p1-H-*gfp* ($p < 0.0001$) and Δ(*glmS, nadE*) + p2-H-*mCherry* ($p < 0.0001$) had a longer lag time. Of the non-ARGFP plasmid-bearing strains, only Δ(*thyA, infA*) + p1-H-*cmR* had a higher growth rate than p1-H-*gfp* ($p < 0.0001$); no significant differences in lag time were observed. In minimal medium, no significant differences in growth rate were found, and only Δ(*glmS, nadE*) + p2-*M-specR* had a shorter lag time than the same strain containing p2-*M-mCherry* (high-copy plasmids were excluded from the analysis due to lack of growth). Overall, these results further reinforce that there are no growth differences between WT and double-knockout strains and merely that high-copy plasmids are overly burdensome and need to be optimized in future work.

Last, we sought to demonstrate that ARGFPs are maintained in double-knockout strains with or without exogenous auxotrophic compound. We performed the same study as in Figure 4 with fluorescent reporters, and we identified cells containing plasmid as those with fluorescence greater than the mean plus

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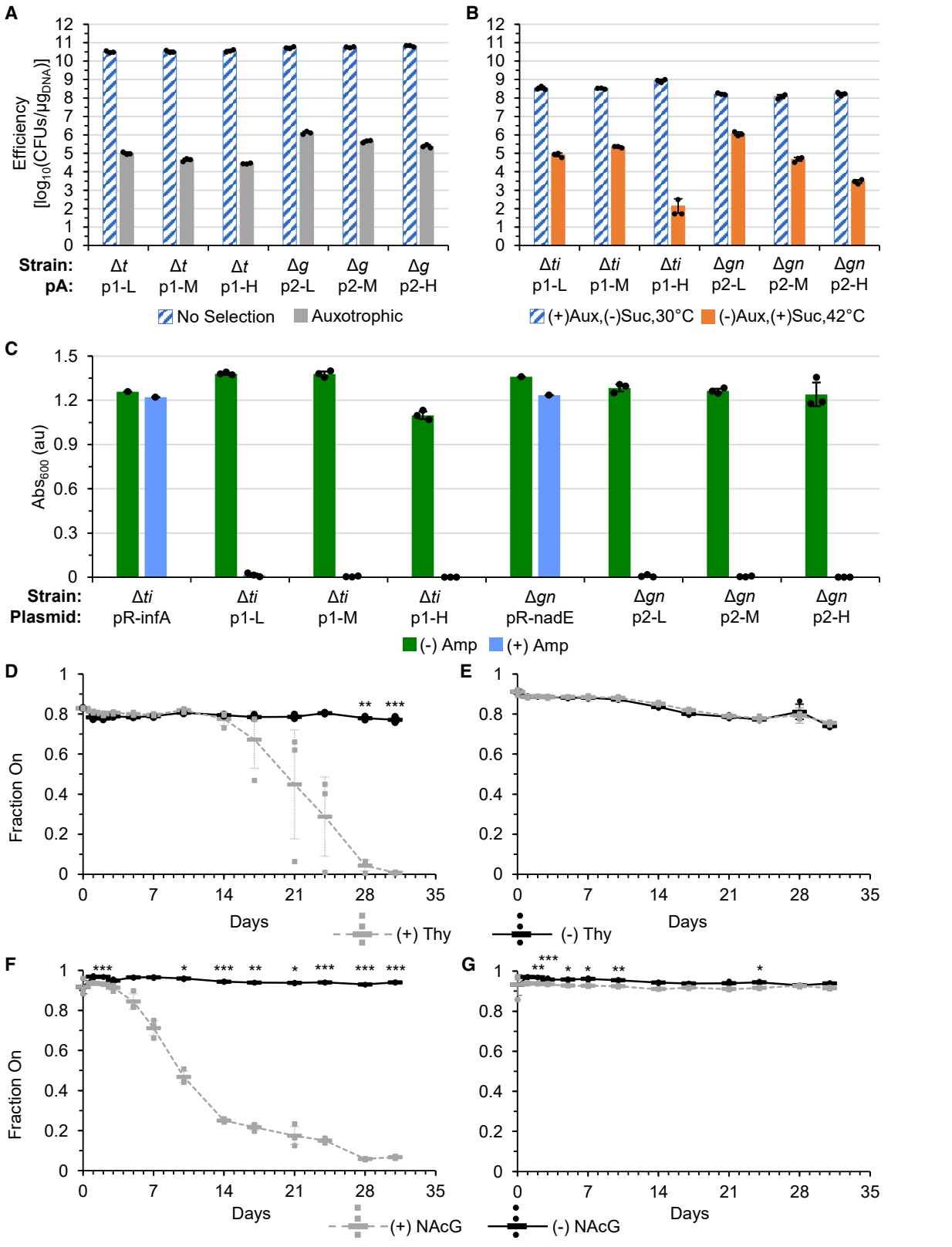

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two standard deviations of a non-fluorescent cell population. On select days of the experiment, we quantified the "Fraction On" of each culture (Figure S9; STAR Methods). Consistent with our hypothesis and previous results, only the auxotrophic knockouts cultured in the presence of the relevant auxotrophic compound had a decrease in the proportion of fluorescent cells over time (Figures 5D and 5F). EcN *ΔthyA* cultures in the presence of Thy had statistically significant fewer fluorescent cells at day 28, and the same was found in EcN Δ*glmS* cultures in the presence of NAcG at day 10. The observed loss in the Δ*glmS* strain occurred rapidly, unlike the ARG-carrying plasmids (Figure 4C), perhaps due to a greater burden associated with fluorescent protein production or the lack of selection pressure associated with measuring fluorescence. The mean population fluorescence of every cultured tended to decrease over time (Figure S10), likely attributable to decreased cellular burden via mutational loss of fluorophore expression; however, only the auxotrophic strains exhibited a near complete loss of population fluorescence (Figures S10A and S10C). While statistically significant differences between Fractions On of the EcN Δ(*glmS, nadE*) culture conditions exist across multiple time points (Figure 5G), we consider these negligible due to (1) small magnitudes of these differences, (2) very low variance associated among each triplicate sample, and (3) different population fluorescence (Figure S10D) between the two conditions caused by the presence or lack of NAcG influencing plasmid copy number. In short, we have demonstrated a fully streamlined ARG-free system in cloning and plasmid maintenance.

Expansion of ARGFPs to *E. coli* MG1655 and demonstration of ARGFP usage in the mouse gut

Finally, we desired to demonstrate the portability of ARGFPs to other strains and to describe their usage in an uncontrolled environment. To this end, we engineered *E. coli* K12 MG1655 strains capable of maintaining ARGFPs (Figures 6A–6C and S11), demonstrated the superiority of ARGFP maintenance to ARG-based plasmid maintenance (Figure S12), and demonstrated ARGFP usage in EcN in the mouse gut (Figures 6D, 6E, and S13).

We created Δ(*thyA, infA*) and Δ(*glmS, nadE*) strains of MG1655, transformed them with all p1 and p2 variants, respectively, and measured transformation efficiency (Figure 6A). Next, we validated that transformants contained the gene of interest. In cases where that gene of interest was an ARG, we performed the same experiment shown in Figure 3C with 36 replicates of each strain-colony combination (Figure 6B) and found that all colonies contained pA and effectively cured pR-essG. For pA variants with fluorophores in place of ARGs, we performed the same experiment shown in Figures 5C and S8 (Figures 6C and S11) and found that all tested colonies contained only pA. Notably, two MG1655 Δ(*thyA, infA*) colonies containing p1-H had very low fluorescence (presumably from a loss-of-expression mutation), and all MG1655 Δ(*glmS, nadE*) colonies containing p2-H had a severe growth defect, requiring 2 days of growth; we believe these results are attributable to overly burdensome expression of fluorophores in high-copy plasmids, consistent with the growth of EcN strains harboring these plasmids (Figures S4G, S4H, S5G, and S5H). By applying ARGFPs to the common laboratory strain *E. coli* MG1655, we demonstrated the portability of ARGFPs to a relevant strain of *E. coli*.

As the culmination of our work, we sought to demonstrate that our system resulted in superior plasmid maintenance compared to a WT strain containing a plasmid with an ARG. We decided to compare the maintenance of a new p1-M variant containing both *gfp* and *specR* (p1-*M-gfp-specR*) in WT and Δ(*thyA, infA*) strains *in vitro* and in the mouse gut. First, we established that p1-*M-gfp-specR* was not maintained in WT *in vitro* unless spectinomycin was present (Figures S12A and S12C); a significant portion of the population measurably lost plasmid by day 7 (measured by counting antibiotic-resistant colonies), with fewer than one in 1,000 cells containing plasmid at 21 days. Δ(*thyA, infA*) strains never exhibited plasmid loss, with or without spectinomycin present. Next, we sought to demonstrate this maintenance ability in strains transiting the mouse gut. We colonized C57BL/6 mice with EcN WT containing p1-*M-gfp-specR,* and with Δ(*thyA, infA*) containing either p1-*M-gfp-specR* or p1-*M-gfp* after antibiotic knockdown and starvation to allow for strain engraftment (Figure 6D). We observed that all strains colonized the mouse gut stably for at least 12 days (Figure S13A). Using qPCR with GFP- and EcN-specific primers (Figures S13B and S13C), we show that over 12 days, Δ(*thyA, infA*), regardless of *specR* presence, displays significant superiority in plasmid maintenance compared to the WT strain (Figure 6E), consistent

Figure 5. Antibiotic-resistance-gene-free plasmids (ARGFPs)

The gene of interest was swapped to *gfpmut3* and *mCherry* in p1 and p2 variants, respectively, from Ab resistance cassettes via Gibson assembly.

(A) Initial transformation efficiencies of DH10B Δ*thyA* (Δ*t*) and Δ*glmS* (Δ*g*) with p1 and p2, respectively.

(B) Replacement transformation efficiencies of EcN Δ(*infA, thyA*) (Δ*ti*) and EcN Δ(*glmS, nadE*) (Δ*gn*) with p1 variants miniprepped from DH10B Δ*thyA* and with p2 variants miniprepped from DH10B Δ*glmS*, respectively.

Values in (A) and (B) are the log-mean efficiency of biological triplicates (3 unique transformations), and error bars represent the standard error associated with log-mean transformation with dots representing individual replicates. Dashed bars represent a theoretical maximum transformation efficiency and are not indicative of the number of cells containing plasmid.

(C) Plasmid swap verification in EcN Δ(*thyA, infA*) and EcN Δ(*glmS, nadE*) determined by inoculating individual colonies into liquid medium with and without Amp. Values are the arithmetic mean of biological triplicates (3 distinct colonies), with error bars representing one standard deviation and dots representing individual replicates.

(D and E) Month-long medium-copy p1 maintenance in EcN (D) Δ*thyA* and (E) Δ(*thyA, infA*) with and without exogenous Thy.

(F and G) Month-long p2 maintenance in EcN (F) Δ*glmS* and (G) Δ(*glmS, nadE*) with and without exogenous NAcG. Fraction On was determined by measuring single-cell fluorescence of cultures, fitting a normal distribution to an empty vector (non-fluorescent) cell population, and counting the number of p1- or p2-containing cells with fluorescence greater than the mean plus two standard deviations of the empty vector. Horizontal bars are the average of biological triplicate cultures (3 unique colonies), with error bars representing a single standard deviation. Circles and squares are the individual replicate values for each. Statistical comparisons in (D)–(G) were performed using a two-tailed mixed-model ANOVA with Sidak's multiple comparisons (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

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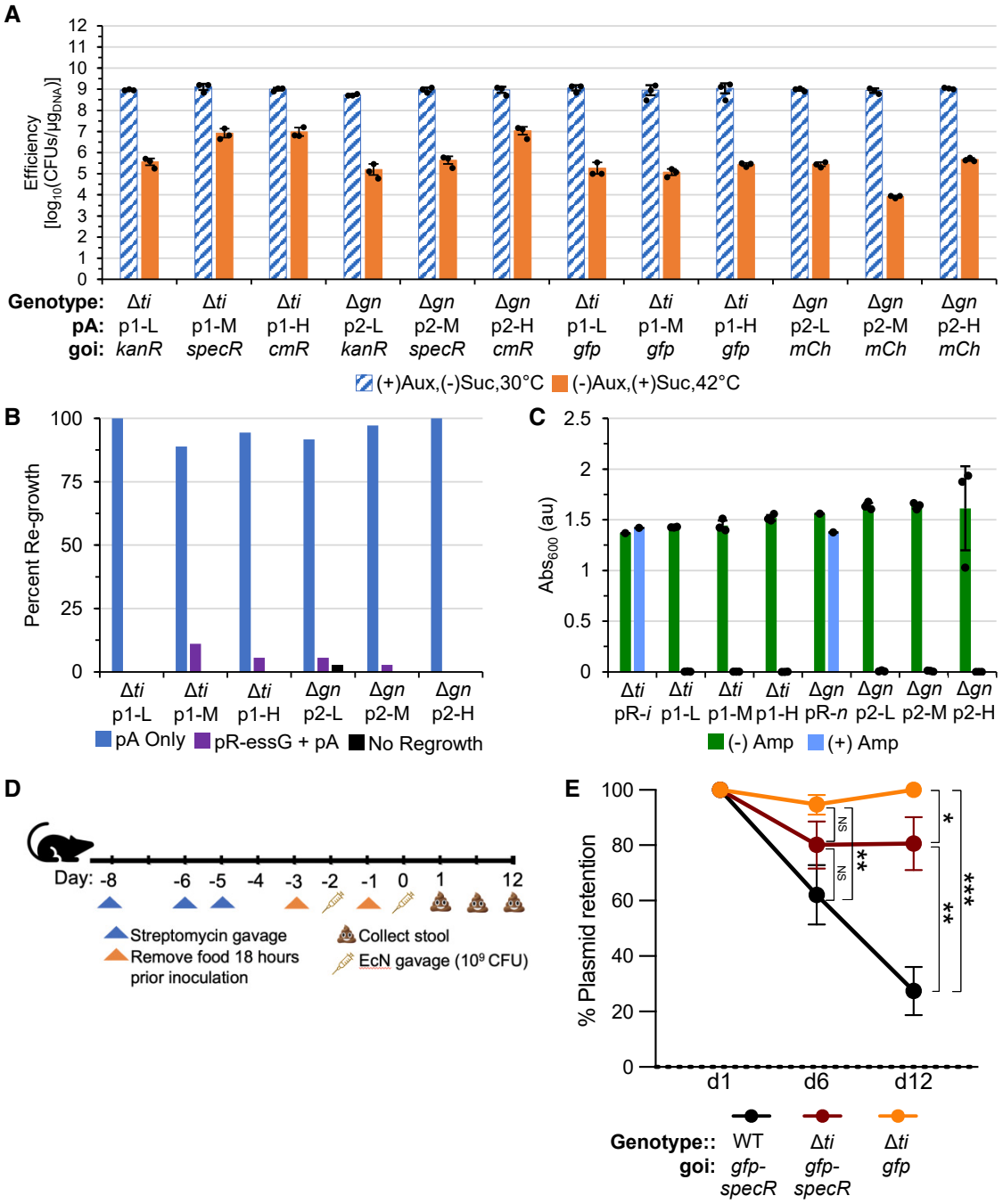

Figure 6. Expanding ARGFPs to *E. coli* MG1655 and demonstrating their use in EcN in the mouse gut

(A) Transformation efficiency of MG1655 Δ(*thyA, infA)* (Δ*ti*) with p1 variants and MG1655 Δ(*glmS, nadE*) (Δ*gn*) with p2 variants. Values are the log mean of biological triplicate samples (3 unique transformations), with error bars representing the standard error and dots representing each replicate value. mCh, mCherry. Dashed bars represent a theoretical maximum transformation efficiency and are not indicative of the number of cells containing plasmid.

(B) Percentage of transformants containing pA (with an ARG), both pR-essG and pA, or that did not regrow after being replated. Each colony tested (36 for each strain-plasmid combination) was considered a unique replicate. Per the metrics defined in Figure S1, pR-essG regrowth always was poor or very poor.

(C) Plasmid replacement verification with ARG-free pA in MG1655 Δ(*thyA, infA*) and MG1655 Δ(*glmS, nadE*) determined by inoculating individual colonies into liquid medium with and without Amp. Values are the arithmetic mean of biological triplicates (3 distinct colonies), with error bars representing one standard deviation and dots representing individual replicates.

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with our *in vitro* data (Figure S12). We speculate that the difference between the two Δ(*thyA, infA*) strains may be attributable to increased metabolic burden associated with expressing two heterologous genes versus only one. In conclusion, our system is superior to ARG-based plasmids when selection pressure is removed.

Conclusion

In this work, we developed a generalizable, streamlined pipeline for fully ARGFP-based cloning and long-term plasmid maintenance. We established that plasmid-born auxotrophic markers can select for auxotrophic strains of *E. coli* DH10B and Nissle 1917 and determined that combining auxotrophic and essential gene markers missing from double-knockout EcN and MG1655 strains may be used to select for plasmid presence in a single transformation step. Next, we showed that those same plasmids can be maintained in our engineered EcN strains and that these strains do not have growth defects. Finally, we demonstrated a completely ARG-free cloning and long-term plasmid maintenance workflow. Compared to previous methods,[60,61] our work systematically quantifies transformation efficiency in auxotrophic selection, never requires removal of ARGs from plasmids during the workflow, necessitates gene knockout in both the cloning and working strains only once, stably maintains plasmids for 1 month even in the presence of the auxotrophic compound *in vitro*, and shows superior plasmid maintenance *in vivo*.

Limitations of the study

Our pipeline is not without limitations. For example, plasmid copy number varies widely among single cells in a bulk population[56]; thus, genomic integration of heterologous genetic constructs has the dual advantages of construct maintenance and less cell-to-cell variability. However, integration still requires re-optimization of construct expression levels, rendering our ARGFP system preferable when bulk population performance is the most relevant metric. Our system does not address how loss-of-function mutations in the gene-of-interest expression cassette can degrade population performance; however, introducing functional redundancy,[39] conditional gene expression,[47,48] and/or co-expressing the genetic payloads with the essential and auxotrophic markers may alleviate this concern. Additionally, antibiotic-resistance-based selection helps prevent contamination of cultures by bacteria that do not contain the plasmid, but we believe that careful aseptic technique addresses this concern, as we have demonstrated in this work. In future work, we plan to address how expression levels of the markers influence selection stringency, transformational efficiency, plasmid copy number, and cellular burden, and we will demonstrate functionality of relevant genetic circuits beyond simple genes in uncontrolled environments.

Our results have positive implications for the field of microbiology, especially as it moves outside of research laboratories. Our knockout strains do not require recreation upon each transformation nor removal of antibiotic resistance markers, and the plasmid selection system in EcN makes it easier to generate new constructs for this strain without the need of initial testing and insert optimization. Our approach is generalizable to other strains of *E. coli*, including those used for large-scale bioproduction. Beyond, our strategy is implementable in non-*E. coli* species commonly used outside of laboratory conditions, although it may require the development of shuttle vectors with *E. coli*-specific auxotrophic markers and origins of replication and new species-specific essential gene markers and origins. It also requires robust recombineering tools that are not reliant upon ARG removal or are able to subsequently remove ARGs from the genome.[62] While the basic gene knockout procedure may require modification, our work provides a blueprint for researchers seeking to develop ARGFP systems in other species.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2023.100669.

(D) Schematic for testing p1-M containing both *gfp* and *specR* or only *gfp in vivo*: 7-week-old C57BL/6 female mice were gavaged with streptomycin, followed by starvation for 18 h and different strains of EcN gavage separated by 2 days. Fecal samples were collected over 12 days since last EcN gavage.
(E) Fraction of EcN strains retaining the GFP-containing plasmid over 12 days *in vivo*, determined by extracting stool DNA and quantifying ratio of $2^{\Delta Ct}$ (GFP-EcN). Each dot represents the mean of 9–10 mice per group, with the bar representing standard error of the mean. Statistical comparison was performed using a Mann-Whitney U non-parametric test (ns, $p > 0.05$; *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$).

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AUTHOR CONTRIBUTIONS

T.S.M. conceived and supervised the entire work; G.D. supervised the animal work; M.B.A., S.R., S.T.V., E.S.R.T., and L.L. performed experiments; M.B.A., S.R., G.D., and T.S.M. analyzed data; and M.B.A., S.R., G.D., and T.S.M. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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REFERENCES

1. Monroe, M.R.. Plasmids 101: What is a plasmid?. https://blog.addgene.org/plasmids-101-what-is-a-plasmid.
2. Cohen, S.N., Chang, A.C.Y., Boyer, H.W., and Helling, R.B. (1973). Construction of Biologically Functional Bacterial Plasmids In Vitro. Proc Natl Acad Sci USA *70*, 3240–3244.
3. Hanczyc, M.M. (2020). Engineering Life: A Review of Synthetic Biology. Artif. Life *26*, 260–273.
4. Slusarczyk, A.L., Lin, A., and Weiss, R. (2012). Foundations for the design and implementation of synthetic genetic circuits. Nat. Rev. Genet. *13*, 406–420.
5. Gardner, T.S., Cantor, C.R., and Collins, J.J. (2000). Construction of a genetic toggle switch in Escherichia coli. Nature *403*, 339–342.
6. Elowitz, M.B., and Leibler, S. (2000). A synthetic oscillatory network of transcriptional regulators. Nature *403*, 335–338.
7. Gräslund, S., Nordlund, P., Weigelt, J., Hallberg, B.M., Bray, J., Gileadi, O., Knapp, S., Oppermann, U., Arrowsmith, C., Hui, R., et al. (2008). Protein production and purification. Nat. Methods *5*, 135–146.
8. Xie, D. (2022). Continuous biomanufacturing with microbes — upstream progresses and challenges. Curr. Opin. Biotechnol. *78*, 102793.
9. Singh, J.S., Abhilash, P.C., Singh, H.B., Singh, R.P., and Singh, D.P. (2011). Genetically engineered bacteria: An emerging tool for environmental remediation and future research perspectives. Gene *480*, 1–9.
10. Moon, T.S. (2022). SynMADE: synthetic microbiota across diverse ecosystems. Trends Biotechnol.
11. Lan, L., Xie, Y., Kong, X., Li, C., and Liu, D. (2021). Investigation of reduction in risk from antibiotic resistance genes in laboratory wastewater by using O3, ultrasound, and autoclaving. Water Environ. Res. *93*, 479–486.
12. Wang, H., Wang, J., Li, S., Li, J., and Jing, C. (2019). Prevalence of antibiotic resistance genes in cell culture liquid waste and the virulence assess for isolated resistant strains. Environ. Sci. Pollut. Res. *26*, 32040–32049.
13. Karkman, A., Do, T.T., Walsh, F., and Virta, M.P.J. (2018). Antibiotic-Resistance Genes in Waste Water. Trends Microbiol. *26*, 220–228.
14. Chen, J., Jin, M., Qiu, Z.-G., Guo, C., Chen, Z.-L., Shen, Z.-Q., Wang, X.-W., and Li, J.-W. (2012). A Survey of Drug Resistance bla Genes Originating from Synthetic Plasmid Vectors in Six Chinese Rivers. Environ. Sci. Technol. *46*, 13448–13454.
15. Bengtsson-Palme, J., Kristiansson, E., and Larsson, D.G.J. (2018). Environmental factors influencing the development and spread of antibiotic resistance. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. *42*, fux053.
16. Cheng, Y.-Y., Zhou, Z., Papadopoulos, J.M., Zuke, J.D., Falbel, T.G., Anantharaman, K., Burton, B.M., and Venturelli, O.S. (2023). Efficient plasmid transfer via natural competence in a microbial co-culture. Mol. Syst. Biol., e11406.
17. Nielsen, K.M., Johnsen, P.J., Bensasson, D., and Daffonchio, D. (2007). Release and persistence of extracellular DNA in the environment. Environ. Biosafety Res. *6*, 37–53.
18. Isabella, V.M., Ha, B.N., Castillo, M.J., Lubkowicz, D.J., Rowe, S.E., Millet, Y.A., Anderson, C.L., Li, N., Fisher, A.B., West, K.A., et al. (2018). Development of a synthetic live bacterial therapeutic for the human metabolic disease phenylketonuria. Nat. Biotechnol. *36*, 857–864.
19. Kurtz, C.B., Millet, Y.A., Puurunen, M.K., Perreault, M., Charbonneau, M.R., Isabella, V.M., Kotula, J.W., Antipov, E., Dagon, Y., Denney, W.S., et al. (2019). An engineered E. coli Nissle improves hyperammonemia and survival in mice and shows dose-dependent exposure in healthy humans. Sci. Transl. Med. *11*, eaau7975.
20. Leventhal, D.S., Sokolovska, A., Li, N., Plescia, C., Kolodziej, S.A., Gallant, C.W., Christmas, R., Gao, J.-R., James, M.J., Abin-Fuentes, A., et al. (2020). Immunotherapy with engineered bacteria by targeting the STING pathway for anti-tumor immunity. Nat. Commun. *11*, 2739.
21. Mimee, M., Tucker, A.C., Voigt, C.A., and Lu, T.K. (2015). Programming a Human Commensal Bacterium, Bacteroides thetaiotaomicron, to Sense and Respond to Stimuli in the Murine Gut Microbiota. cels *1*, 62–71.
22. Kotula, J.W., Kerns, S.J., Shaket, L.A., Siraj, L., Collins, J.J., Way, J.C., and Silver, P.A. (2014). Programmable bacteria detect and record an environmental signal in the mammalian gut. Proc. Natl. Acad. Sci. USA *111*, 4838–4843.
23. Chowdhury, S., Castro, S., Coker, C., Hinchliffe, T.E., Arpaia, N., and Danino, T. (2019). Programmable bacteria induce durable tumor regression and systemic antitumor immunity. Nat. Med. *25*, 1057–1063.
24. Terrinoni, M., Nordqvist, S.L., Källgård, S., Holmgren, J., and Lebens, M. (2018). A Novel Nonantibiotic, lgt-Based Selection System for Stable Maintenance of Expression Vectors in Escherichia coli and Vibrio cholerae. Appl. Environ. Microbiol. *84*, 21433–e2217.
25. Hwang, I.Y., Koh, E., Wong, A., March, J.C., Bentley, W.E., Lee, Y.S., and Chang, M.W. (2017). Engineered probiotic Escherichia coli can eliminate and prevent Pseudomonas aeruginosa gut infection in animal models. Nat. Commun. *8*, 15028.
26. Ho, C.L., Tan, H.Q., Chua, K.J., Kang, A., Lim, K.H., Ling, K.L., Yew, W.S., Lee, Y.S., Thiery, J.P., and Chang, M.W. (2018). Engineered commensal microbes for diet-mediated colorectal-cancer chemoprevention. Nat. Biomed. Eng. *2*, 27.
27. Koh, E., Hwang, I.Y., Lee, H.L., De Sotto, R., Lee, J.W.J., Lee, Y.S., March, J.C., and Chang, M.W. (2022). Engineering probiotics to inhibit Clostridioides difficile infection by dynamic regulation of intestinal metabolism. Nat. Commun. *13*, 3834.
28. Pines, G., Freed, E.F., Winkler, J.D., and Gill, R.T. (2015). Bacterial Recombineering: Genome Engineering via Phage-Based Homologous Recombination. ACS Synth. Biol. *4*, 1176–1185.

29. Wannier, T.M., Ciaccia, P.N., Ellington, A.D., Filsinger, G.T., Isaacs, F.J., Javanmardi, K., Jones, M.A., Kunjapur, A.M., Nyerges, A., Pal, C., et al. (2021). Recombineering and MAGE. Nat. Rev. Methods Primers *1*, 1–24.
30. Kan, A., Gelfat, I., Emani, S., Praveschotinunt, P., and Joshi, N.S. (2021). Plasmid Vectors for in Vivo Selection-Free Use with the Probiotic E. coli Nissle 1917. ACS Synth. Biol. *10*, 94–106.
31. Naseem, S., and Konopka, J.B. (2015). N-acetylglucosamine Regulates Virulence Properties in Microbial Pathogens. PLoS Pathog. *11*, e1004947.
32. Noto Guillen, M., Rosener, B., Sayin, S., and Mitchell, A. (2021). Assembling stable syntrophic Escherichia coli communities by comprehensively identifying beneficiaries of secreted goods. Cell Systems *12*, 1064–1078.e7.
33. Vidal, L., Pinsach, J., Striedner, G., Caminal, G., and Ferrer, P. (2008). Development of an antibiotic-free plasmid selection system based on glycine auxotrophy for recombinant protein overproduction in Escherichia coli. J. Biotechnol. *134*, 127–136.
34. Marie, C., Vandermeulen, G., Quiviger, M., Richard, M., Préat, V., and Scherman, D. (2010). pFARs, Plasmids free of antibiotic resistance markers, display high-level transgene expression in muscle, skin and tumour cells. J. Gene Med. *12*, 323–332.
35. Soubrier, F., Cameron, B., Manse, B., Somarriba, S., Dubertret, C., Jaslin, G., Jung, G., Caer, C.L., Dang, D., Mouvault, J.M., et al. (1999). pCOR: a new design of plasmid vectors for nonviral gene therapy. Gene Ther. *6*, 1482–1488.
36. Kang, C.W., Lim, H.G., Yang, J., Noh, M.H., Seo, S.W., and Jung, G.Y. (2018). Synthetic auxotrophs for stable and tunable maintenance of plasmid copy number. Metab. Eng. *48*, 121–128.
37. Hägg, P., de Pohl, J.W., Abdulkarim, F., and Isaksson, L.A. (2004). A host/plasmid system that is not dependent on antibiotics and antibiotic resistance genes for stable plasmid maintenance in Escherichia coli. J. Biotechnol. *111*, 17–30.
38. Kim, K., Jeong, J.H., Lim, D., Hong, Y., Yun, M., Min, J.-J., Kwak, S.-J., and Choy, H.E. (2013). A Novel Balanced-Lethal Host-Vector System Based on glmS. PLoS One *8*, e60511.
39. Rottinghaus, A.G., Ferreiro, A., Fishbein, S.R.S., Dantas, G., and Moon, T.S. (2022). Genetically stable CRISPR-based kill switches for engineered microbes. Nat. Commun. *13*, 672.
40. Mao, N., Cubillos-Ruiz, A., Cameron, D.E., and Collins, J.J. (2018). Probiotic strains detect and suppress cholera in mice. Sci. Transl. Med. *10*, eaao2586.
41. Mimee, M., Nadeau, P., Hayward, A., Carim, S., Flanagan, S., Jerger, L., Collins, J., McDonnell, S., Swartwout, R., Citorik, R.J., et al. (2018). An ingestible bacterial-electronic system to monitor gastrointestinal health. Science *360*, 915–918.
42. Munck, C., Sheth, R.U., Freedberg, D.E., and Wang, H.H. (2020). Recording mobile DNA in the gut microbiota using an Escherichia coli CRISPR-Cas spacer acquisition platform. Nat. Commun. *11*, 95.
43. Ronda, C., Chen, S.P., Cabral, V., Yaung, S.J., and Wang, H.H. (2019). Metagenomic engineering of the mammalian gut microbiome in situ. Nat. Methods *16*, 167–170.
44. Crook, N., Ferreiro, A., Gasparrini, A.J., Pesesky, M.W., Gibson, M.K., Wang, B., Sun, X., Condiotte, Z., Dobrowolski, S., Peterson, D., et al. (2019). Adaptive Strategies of the Candidate Probiotic E. coli Nissle in the Mammalian Gut. Cell Host Microbe *25*, 499–512.e8.
45. Russell, B.J., Brown, S.D., Siguenza, N., Mai, I., Saran, A.R., Lingaraju, A., Maissy, E.S., Dantas Machado, A.C., Pinto, A.F.M., Sanchez, C., et al. (2022). Intestinal transgene delivery with native E. coli chassis allows persistent physiological changes. Cell *185*, 3263–3277.e15.
46. Wu, G., Sun, Y., Qu, W., Huang, Y., Lu, L., Li, L., and Shao, W. (2011). Application of GFAT as a Novel Selection Marker to Mediate Gene Expression. PLoS One *6*, e17082.
47. Amrofell, M.B., Rottinghaus, A.G., and Moon, T.S. (2020). Engineering microbial diagnostics and therapeutics with smart control. Curr. Opin. Biotechnol. *66*, 11–17.
48. Rottinghaus, A.G., Amrofell, M.B., and Moon, T.S. (2020). Biosensing in Smart Engineered Probiotics. Biotechnol. J. *15*, e1900319.
49. Charbonneau, M.R., Isabella, V.M., Li, N., and Kurtz, C.B. (2020). Developing a new class of engineered live bacterial therapeutics to treat human diseases. Nat. Commun. *11*, 1738.
50. Riglar, D.T., and Silver, P.A. (2018). Engineering bacteria for diagnostic and therapeutic applications. Nat. Rev. Microbiol. *16*, 214–225.
51. McNerney, M.P., Doiron, K.E., Ng, T.L., Chang, T.Z., and Silver, P.A. (2021). Theranostic cells: emerging clinical applications of synthetic biology. Nat. Rev. Genet. *22*, 730–746.
52. Carreras, C.W., and Santi, D.V. (1995). The Catalytic Mechanism and Structure of Thymidylate Synthase. Annu. Rev. Biochem. *64*, 721–762.
53. Wu, H.C., and Wu, T.C. (1971). Isolation and Characterization of a Glucosamine-Requiring Mutant of Escherichia coli K-12 Defective in Glucosamine-6-Phosphate Synthetase. J. Bacteriol. *105*, 455–466.
54. Wright, O., Delmans, M., Stan, G.-B., and Ellis, T. (2015). GeneGuard: A Modular Plasmid System Designed for Biosafety. ACS Synth. Biol. *4*, 307–316.
55. Cohen, S.S., and Barner, H.D. (1954). Studies on unbalanced growth in escherichia coli. Proc Natl Acad Sci USA *40*, 885–893.
56. Shao, B., Rammohan, J., Anderson, D.A., Alperovich, N., Ross, D., and Voigt, C.A. (2021). Single-cell measurement of plasmid copy number and promoter activity. Nat. Commun. *12*, 1475.
57. Shopera, T., He, L., Oyetunde, T., Tang, Y.J., and Moon, T.S. (2017). Decoupling Resource-Coupled Gene Expression in Living Cells. ACS Synth. Biol. *6*, 1596–1604.
58. Ceroni, F., Algar, R., Stan, G.B., and Ellis, T. (2015). Quantifying cellular capacity identifies gene expression designs with reduced burden. Nat. Methods *12*, 415–418.
59. Ceroni, F., Boo, A., Furini, S., Gorochowski, T.E., Borkowski, O., Ladak, Y.N., Awan, A.R., Gilbert, C., Stan, G.-B., and Ellis, T. (2018). Burden-driven feedback control of gene expression. Nat. Methods *15*, 387–393.
60. Oliveira, P.H., and Mairhofer, J. (2013). Marker-free plasmids for biotechnological applications – implications and perspectives. Trends Biotechnol. *31*, 539–547.
61. Vandermeulen, G., Marie, C., Scherman, D., and Préat, V. (2011). New Generation of Plasmid Backbones Devoid of Antibiotic Resistance Marker for Gene Therapy Trials. Mol. Ther. *19*, 1942–1949.
62. Wannier, T.M., Nyerges, A., Kuchwara, H.M., Czikkely, M., Balogh, D., Filsinger, G.T., Borders, N.C., Gregg, C.J., Lajoie, M.J., Rios, X., et al. (2020). Improved bacterial recombineering by parallelized protein discovery. Proc. Natl. Acad. Sci. USA *117*, 13689–13698.
63. Kurtz, C., Denney, W.S., Blankstein, L., Guilmain, S.E., Machinani, S., Kotula, J., Saha, S., Miller, P., and Brennan, A.M. (2018). Translational Development of Microbiome-Based Therapeutics: Kinetics of E. coli Nissle and Engineered Strains in Humans and Nonhuman Primates. Clin. Transl. Sci. *11*, 200–207.
64. Mehrer, C.R., Incha, M.R., Politz, M.C., and Pfleger, B.F. (2018). Anaerobic production of medium-chain fatty alcohols via a β-reduction pathway. Metab. Eng. *48*, 63–71.
65. Guo, J., Wang, T., Guan, C., Liu, B., Luo, C., Xie, Z., Zhang, C., and Xing, X.-H. (2018). Improved sgRNA design in bacteria via genome-wide activity profiling. Nucleic Acids Res. *46*, 7052–7069.
66. Balkay, L. (2022). fca_readfcs. MATLAB Central File Exchange. https://www.mathworks.com/matlabcentral/fileexchange/9608-fca_readfcs.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli DH10B	Invitrogen	EC0113
E. coli DH10B Δ*thyA*	This work	sMBA281
E. coli DH10B Δ*glmS*	This work	sMBA279
E. coli K12 MG1655 (plasmid free)	CGSC	6300
E. coli K12 MG1655 Δ(*thyA, infA*) + pAGR424	This work	sMBA556
E. coli K12 MG1655 Δ(*glmS, nadE*) + pMBA321	This work	sMBA557
E. coli Nissle 1917 (plasmid free) [EcN]	DSMZ	DSM 16700
E. coli Nissle 1917 (plasmid free) Δ*glmS*	This work	sMBA280
E. coli Nissle 1917 Δ(*glmS, nadE*) + pMBA321	This work	sMBA520
E. coli Nissle 1917 Δ*infA* + pAGR424	This work	sMBA471
E. coli Nissle 1917 (plasmid free) Δ*thyA*	This work	sMBA282
E. coli Nissle 1917 Δ(*thyA, infA*) + pAGR424	This work	sMBA472
Chemicals, peptides, and recombinant proteins		
Agar	Sigma	A1296
Ampicillin sodium salt (Amp)	Gold Biotechnology	A-301
Anhydrotetracycline HCl (aTc)	Sigma	37919
L-(+)-arabinose	Sigma	A3256
BsaI-HFv2	New England Biolabs	R3733
$CaCl_2$ $2H_2O$	Sigma	C5080
Chloramphenicol (Cm)	Gold Biotechnology Fisher Scientific	C-105 AC227920250
Deoxynucleotide Set (dNTPs)	G-Biosciences	786–460
1,4-Dithiothreitol (DTT)	Sigma	3483-12-3
DpnI	New England Biolabs	R0176
D-(+)-Glucose	Sigma	G8270
ExoSAP-IT	Thermo Fisher	78201
Glycerol	Fisher Scientific	BP229-1
GoTaq Master Mix	Promega	M7123
Kanamycin monosulfate (Kan)	Gold Biotechnology	K-120
KCl	Sigma	P5405
KH_2PO_4	Sigma	P9791
LB broth, Miller	VWR	J106
MacConkey Agar	MP Biomedicals	091006017
M9 minimal salts, 5X	Sigma	M6030
$MgCl_2$	Sigma	7786-30-3
$MgSO_4$ $7H_2O$	Sigma	63138
N-acetyl-D-glucosamine (NAcG)	Sigma	A3286
Na_2HPO_4	Sigma	S5136
NaCl	Sigma	S7653
NAD^+	Sigma	53-84-9
PEG-8000	Sigma	1546605
Phosphate buffered saline (PBS), 10x concentrate	Sigma	P5493
Phusion high-fidelity DNA polymerase	New England Biolabs	M0530
SapI	New England Biolabs	R0569

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Spectinomycin dihydrochloride pentahydrate (Spec)	Gold Biotechnology Sigma	S-140 S4014
Sucrose	Sigma	S0389
T4 DNA ligase	New England Biolabs	M0202
T5 exonuclease	New England Biolabs	M0663
Taq DNA Ligase	New England Biolabs	M0208
Thiamine hydrochloride	Sigma	T1270
Thymidine (Thy)	Sigma	T1895
Tris base	Sigma	77-86-1
Tryptone (vegetable)	Sigma	16922
Yeast extract	Sigma Gibco	Y1625 212750
Recombinant DNA		
See Tables S1, S2, and S3		
Software and algorithms		
GraphPad Prism v9.4.1	GraphPad Software	N/A
MATLAB R2020b	MathWorks	N/A
Other		
2 cm electroporation cuvettes	Bulldog Bio	12358–346
14 mL round bottom tubes	Falcon	352059
24-well plates with lids	Greiner Bio-One	662160
96-deep well plates	Eppendorf	951032808
96-well black assay microplates	Falcon Greiner Bio-One	353219 655096
96-well clear round bottom assay microplates	Falcon	353910
AeraSeal	Excel Scientific	Z721573
Biorad CFX96 qPCR machine	Biorad	1845097
MagAttract PowerSoil DNA Kit	Qiagen	27100
KiCqStart SYBR Green qPCR Ready Mix	Sigma	KCQS00
PCR reaction strips	Simport	T320-2N
Petri dishes, 100 × 15 mm Style	Corning	351029
Petri dishes, Specialty, 100 × 100mm Square	Fisher	FB0875711A
Powerbead tubes	Qiagen	19301
PureLink Quick Plasmid Miniprep Kit	Invitrogen	K210011
Zymoclean Gel DNA Recovery Kit	ZYMO Research	D4008

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Tae Seok Moon (tsmoon7@gmail.com).

Materials availability

Plasmids and strains generated in this paper are available upon request from the lead contact. This study did not generate new unique reagents.

Data and code availability

(1) The data that support the findings of this study are provided in the main text, supplemental information, or Source Data file (Data S1).
(2) All original code is available in the paper's supplemental information.

(3) Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal husbandry and mouse colonization protocol

All mouse experiments were approved by the Washington University in St. Louis School of Medicine (WUSM) Institutional Animal and Use Committee (Protocol number: 21–0160) and performed in AAALAC-accredited facilities in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Mouse experiments were performed in female, 7-week old C57BL/6 mice (Jackson Labs C57BL/6J #000664). Mice were housed in a specific pathogen free barrier facility maintained by WUSM Division of Comparative Medicine at 30–70% humidity and 68–79°F under a 12:12 h light:dark cycle. Mice were provided with feed and water *ad libitum* except as noted. Mice were co-housed with up to 5 mice per cage.

Mice were gavaged using 18ga x 38mm plastic feeding tubes (FTP-18-38, Instech). To ablate the native microbiome for EcN engraftment, each mouse was gavaged with 20 mg streptomycin sulfate salt (S6501, Sigma Aldrich) in 200 μL H_20[39] on d-8, d-6, and d-5. 58 h later, food was withheld for 18 h, and mice were gavaged with ~10^9 CFU of different EcN strains in 200 μL PBS. 48 h later, another gavage with 10^9 CFU of EcN strains in 200 μL PBS was performed after 18 h of starvation. Fecal samples were collected at d1, d6 and d12 after last gavage of EcN strains.

Measuring EcN colonization in mice

Fecal samples were collected in sterile 2 mL microtubes at time points indicated, resuspended in sterile PBS at a concentration of 20 mg/mL of stool/PBS. They were then plated on MacConkey Agar (MP Biomedicals) at varying dilutions, and CFU was counted a day later. We performed PCR on ~3 randomly selected colonies from each mouse at each time to ensure that all cultured bacteria were EcN by using primers: forward 5′- GGCGCGGCGCTACAC-3′ and reverse 5′-TGCAATTCGAATCATCTTCTCATC-3.

Measuring plasmid maintenance in colonized mice

Fecal samples were collected in sterile 2 mL microtubes for timepoints indicated, and genomic fecal DNA was extracted using the MagAttract PowerSoil DNA Kit (Qiagen, Hilden Germany) with Powerbead tubes (Qiagen) per manufacturer instructions with two rounds of bead beating for 2 min each at 2500 oscillations/min on a Mini-Beadbeater 24 (Biospec Products, Bartlesville, OK, USA) spaced by placement on ice for 2 min. qPCR was performed on the extracted DNA on a BioRad CFX96 qPCR machine (BioRad, Hercules, CA, USA) using the KiCqStart SYBR Green qPCR Ready Mix (Sigma, Burlington, MA, USA), and the following primers synthesized by Integrated DNA Technologies (Coralville, IA, USA): GFP primers (self-designed), forward 5′-ATGGCCCTGTCCTTTTACCAG-3′ and reverse 5′- GCGTAGTTTTCGTCGTTTGC-3'; EcN[63] primers, forward 5′- GGCG CGGCGCTACAC-3′ and reverse 5′-TGCAATTCGAATCATCTTCTCATC-3. All values of cycle thresholds (Ct) above the average Ct or within standard deviation of replicates from Ct of water were interpreted as negative, or in the case of mouse experiments, no longer containing either EcN or GFP. The specificity of primers and ratio of plasmid per EcN (Ratio of GFP Ct over EcN Ct) were assessed by testing against genomic DNA from pure cultures of strains isolated using QIAamp Biostic Bacteremia DNA Kit (Qiagen) and mouse naive fecal DNA extracted as above. Primer efficiency was determined by performing a standard curve. Efficiency for EcN and GFP primers was 102% and 110%, respectively, which allows for accurate comparative *Ct* analysis. The percent plasmid retention for day 6 and day 12 (day n) compared to day 1 was obtained using Equation 1:

$$\%\ Plasmid\ Retention = 100 * \left(\frac{\left(2^{-(Ct_{GFP} - Ct_{EcN})}\right)_{Day\ n}}{\left(2^{-(Ct_{GFP} - Ct_{EcN})}\right)_{Day\ 1}} \right) \quad \text{(Equation 1)}$$

Bacterial models

Information on all *E. coli* strains used and generated in this study can be found in the key resources table.

METHOD DETAILS

Bacterial culturing

Cells were grown in liquid or on solid LB medium or M9t medium (1x M9 salts, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 2% (w/v) glucose, 0.5% tryptone, and 0.01% (w/v) thiamine hydrochloride) supplemented with ampicillin (Amp, 0.1 mg/mL), kanamycin (Kan, 0.02 mg/mL), spectinomycin (Spec, 0.1 mg/mL), chloramphenicol (Cm, 0.034 mg/mL), thymidine (Thy, 0.1 mg/mL), N-acetyl-D-glucosamine (NAcG, 1 mg/mL), and/or sucrose (Suc, 50 mg/mL) as appropriate. Liquid cultures were incubated at 30°C (to maintain recombineering plasmids as necessary) or 37°C and were agitated at 250 rpm. Solid cultures were incubated at 30°C (to maintain pR-essG), 37°C (normal growth), or 42°C (to cure pR-essG) as necessary.

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Cloning

Linear PCR products were produced using Phusion polymerase and custom primers from Integrated DNA Technologies (IDT, Coralville, IA, USA). Templates were *E. coli* genomic DNA or previously constructed plasmids as appropriate. Correct size of linear amplicons was confirmed by gel electrophoresis, and amplicons were gel purified with a Zymoclean Gel DNA Recovery kit (ZYMO Research, Irvine, CA, USA). Circularized plasmids were constructed from linear fragments via Gibson or GoldenGate assembly, and double-stranded recombineering oligonucleotides were assembled via overlap extension PCR to join upstream and downstream homology regions. *E. coli* DH10B WT, unless otherwise noted, was transformed with assembled plasmids and grown overnight on LB plates supplemented with the appropriate antibiotic at 30°C or 37°C. Plasmid assembly was confirmed by Sanger sequencing (Genewiz, Chelmsford, MA, USA) of transformant colony PCR product amplified by GoTaq polymerase (Promega, Madison, WI, USA) purified with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA). Colonies containing sequence-verified plasmids were inoculated into 5.5 mL liquid medium in a 14 mL culture tube, grown overnight, and stocked in 15% (v/v) glycerol, and plasmids were purified from the remaining culture using a PureLink Quick Plasmid Miniprep kit (Invitrogen, Waltham, MA, USA).

Absorbance and fluorimetry measurements

Liquid cultures were diluted 2- or 4-fold in phosphate buffered saline (PBS) to a final volume of 0.2 mL in 96-well black assay microplates. Absorbance at 600 nm was measured with an Infinite 200pro plate reader (Tecan, Männedorf, Switzerland), as were GFPmut3 fluorescence (483 nm/530 nm excitation/emission) and mCherry fluorescence (572 nm/609 nm), both at 80 gain. Absorbance was calculated as the measured absorbance of a given sample minus the absorbance of a blank. Normalized fluorescence was calculated using Equation 2, where F_{sample} is the measured raw fluorescence of a sample, F_{blank} is the measured raw background fluorescence, $Abs_{600,sample}$ is the measured absorbance of a sample, $Abs_{600,blank}$ is the measured background absorbance, F_{empty} is the measured raw fluorescence of an empty vector culture, and $Abs_{600,empty}$ is the measured absorbance of an empty vector culture:

$$Normalized\ Fluorescence = \frac{F_{sample} - F_{blank}}{Abs_{600,sample} - Abs_{600,blank}} - \frac{F_{empty} - F_{blank}}{Abs_{600,empty} - Abs_{600,blank}} \quad \text{(Equation 2)}$$

Competent cell preparation

Cells were prepared for transformation via electroporation by inoculating 2.5 or more mL of LB medium with necessary supplements in a 14 mL round bottom tube with a single colony or small amount of glycerol stock and grown overnight. The next day, 50 mL of fresh LB medium with necessary supplements in a 250 mL baffled shake flask was inoculated with 2.5 mL of overnight culture and grown for 1–1.5 h until early-to-mid-exponential phase. The culture was chilled on ice for 10 min, then pelleted at 4000 g for 8 min in a swing bucket tabletop centrifuge. Supernatant was discarded, the cells were resuspended in 50 mL ice-cold deionized water, then pelleted at 4000 g for 10 min. Supernatant was discarded, the cells were resuspended in 25 mL ice-cold 10% (v/v) glycerol, then pelleted at 4000 g for 12 min. Supernatant was discarded, the cells were resuspended in 10 mL ice-cold 10% (v/v) glycerol, and then pelleted at 4000g for 15 min. Supernatant was discarded, and the cells were re-suspended in 0.5 mL ice-cold 10% (v/v) glycerol. For experiments not involving replacing pR-essG with pA in double-knockout EcN, 50 μL aliquots were made and frozen immediately at −80°C. For replacement experiments, 100 μL aliquots were made and immediately used for transformation to maximize transformation efficiency.

Genomic knockouts

Knockouts were performed by lambda red-mediated CRISPR-Cas9 recombineering as previously described.[39,64] Relevant plasmids are listed in Table S1, relevant gene sequences are listed in Table S2, and relevant oligonucleotides are listed in Table S3. gRNAs were designed using the gRNA designer from Atum (atum.bio) or chosen from the genome-scale library developed by Guo et al.[65] (Table S3A). gRNAs were ordered from IDT (Coralville, IA, USA) as two single-stranded complementary DNA oligos, annealed, and assembled with GoldenGate into a pgRNAcm-derived backbone. ssDNA oligos for knockouts were designed with 30 bp arms homologous to the lagging strand of DNA synthesis flanking the knockout region (Table S3B). Linear dsDNA fragments for knockouts were designed with 250+ bp homologous to the knockout region and assembled with overlap extension PCR (Table S3C). For auxotrophic knockouts in DH10B and EcN, plasmid-free strains were transformed with pMP11, plated on LB containing Amp, and grown overnight at 30°C. The next day, 2 mL of LB + Amp in a 14 mL round bottom tube was inoculated with a single colony or a previously made glycerol stock and grown overnight at 30°C, 250 rpm. The following day, 25 mL of fresh medium containing Amp and 1% (w/v) arabinose (to induce lambda red recombinases) in a 250 mL baffled shake flask was inoculated with 0.5 mL of overnight culture and grown at 30°C, 250 rpm until mid-exponential phase and then harvested for competent cell preparation as described above. 50 μL aliquots of competent cells were frozen or used immediately. Competent cells were electroporated in a 2 cm electroporation cuvette in an electroporator set to 2000 mV in the presence of 100 ng of gRNA plasmid, 100 ng dsDNA fragment, and 100 nmol ssDNA oligo. Transformants were incubated for 3 h in 1 mL SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose) supplemented with Thy or NAcG as necessary to enable newly auxotrophic strain's growth. Transformants were then plated on LB plates supplemented with Amp, Cm, and Thy or NAcG and incubated overnight at 30°C. Following this, individual colonies were screened for successful knockouts by colony PCR with primers complementary to the genome just outside of the double-stranded homology arms (Table S3D). PCR products were screened by

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gel electrophoresis and presumptive positive knockouts were confirmed by Sanger sequencing (Genewiz, Chelmsford, MA, USA). To cure gRNA plasmids from knockouts, 2 mL of LB supplemented with Amp, Thy or NAcG, and 250 ng/mL anhydrotetracycline (to induce expression of a gRNA from pMP11 that targets the origin of the knockout gRNA plasmid) was inoculated with the knockout strain and grown overnight at 30°C, 250 rpm. Cultures were then streaked out on 1) an LB plate supplemented only with Thy or NAcG incubated at 42°C to cure pMP11 and recover a plasmid-free strain and 2) an LB plate supplemented with Amp and Thy or NAcG incubated at 30°C to maintain pMP11. Following overnight incubation, single colonies were grown in liquid culture with or without requisite antibiotics to verify plasmid curing. Successful knockouts were glycerol stocked in 15% (v/v) glycerol at −80°C. For essential gene knockouts, the same procedure was used with the following modifications: the recombineering plasmid had an essential gene and *sacB* expression cassettes inserted (pR-essG), the gRNA complementary to the genome was designed to bind outside of the reading frame of the essential gene to prevent cleavage of pR-essG, and only the gRNA plasmid was cured following knockout confirmation.

Transformation efficiency quantification

Thawed aliquots of competent cells were electroporated in a 2 cm electroporation cuvette in an electroporator set to 2000 mV in the presence of 1 (Figures 2A and 2B), 10 (Figures 2C and 5A), or 100 ng (Figures 3D, 5B, 6A) of DNA. Each strain was transformed with a given plasmid in triplicate. Transformed cells were incubated at 30°C or 37°C and 250 rpm for 1 h in 1 mL SOC medium supplemented with Thy or NAcG as necessary. To remove Thy or NAcG and to prevent their interference with experimental results, transformants were washed with PBS. Washed transformants were then serially 10-fold diluted to a volume of 1 mL seven times in a 96-deep well plate. 10 μL of each serial dilution was spotted on M9t plates supplemented with Amp, Kan, Spec, Cm, Thy, NAcG, and/or Suc as noted. Plates were incubated overnight at 30°C, 37°C, or 42°C as necessary. The following day, colonies were counted on each serial dilution, and the transformation efficiency was determined using Equation 3, where CFUs are the colony forming units counted at a given dilution, Dil is the fold-dilution at which the CFUs were counted, Vf is the volume fraction of a given dilution that was plated, and m_{DNA} is the mass of DNA in micrograms that was used to transform the cells. For triplicate samples, the log-mean efficiency values were found, with standard error calculated by Equation 4, where se is the standard error, σ is the standard deviation of the non-log-transformed data, and μ is the mean of the non-log-transformed data:

$$Efficiency = \frac{CFUs \times Dil \times {}^{1}/_{Vf}}{m_{DNA}} \quad \text{(Equation 3)}$$

$$se = 0.434 \times \frac{\sigma}{\mu} \quad \text{(Equation 4)}$$

Growth curve measurement

To compare growth rates of various strains in rich medium, individual colonies were inoculated in 0.6 mL LB medium with antibiotics as necessary in a 96 deep well plate and grown overnight at 37°C and 250 rpm. Cultures were diluted 100-fold into 0.6 mL fresh medium and grown for 2 h at 37°C and 250 rpm. Then, cultures were diluted 50-fold into 0.2 mL fresh medium in a 96 well measurement plate. The plate was covered with a lid, and growth was measured kinetically in an Infinite 200pro plate reader by orbitally shaking at 37°C with Abs_{600} measurements every 15 min for 8 h. To compare growth rates of various strains in minimal medium, individual colonies were inoculated in 0.6 mL M9 + 2% (w/v) glucose medium with antibiotics as necessary in a 96 deep well plate and grown overnight at 37°C and 250 rpm. Cultures were diluted 200-fold into 0.6 mL fresh medium, and Abs_{600} measurements were taken every hour for 12 h. The measured absorbance values were $\log_{10}$-transformed, and growth rate was calculated by identifying the linear region (where growth was exponential) and using Equation 5, where μ is growth rate (h^{-1}), SLOPE is the Excel function to fit a slope to a linear dataset, and $Abs_{600,n}$:$Abs_{600,n+i}$ and t_n:t_{n+i} are the regions where the slope is linear. Lag time (t_L, h), was calculated using Equation 6, where $Abs_{600,n-1}$ is the last measured value before growth became exponential, and INTERCEPT is the Excel function to fit a y-intercept to a linear dataset.

$$\mu = 2.303 * SLOPE(log_{10}(Abs_{600,n}) : log_{10}(Abs_{600,n+i}), t_n : t_{n+i})_{linear} \quad \text{(Equation 5)}$$

$$t_L = \frac{log_{10}(Abs_{600,n-1}) - INTERCEPT(log_{10}(Abs_{600,n}) : log_{10}(Abs_{600,n+i}), t_n : t_{n+i})_{linear}}{SLOPE(log_{10}(Abs_{600,n}) : log_{10}(Abs_{600,n+i}), t_n : t_{n+i})_{linear}} \quad \text{(Equation 6)}$$

Antibiotic-based plasmid presence verification

To determine which plasmids, if any, the transformants contained, individual colonies were resuspended in 50 μL PBS. Colonies were re-plated by spotting 5 μL of resuspension on gridded agar plates containing the antibiotic selecting for a given plasmid as well as Thy or NAcG as necessary. Plates were incubated at 30°C or 37°C overnight as necessary. The following day, the colonies that grew on

each condition were quantified. To semi-quantitatively describe how many cells in the original colony contained the plasmid, regrowth on a given plate was classified by the following metrics: very poor, 1–2 CFUs (often small and translucent); poor, 3–10 CFUs (usually small and translucent); moderate, 11–50 CFUs (healthy, but distinguishable); good, 51–100 CFUs (healthy, semi-distinguishable); very good, 101+ CFUs (healthy, indistinguishable [lawn]).

Long-term plasmid maintenance with antibiotic-based plasmid presence verification

Auxotrophic and double knockout EcN strains were transformed with medium-copy plasmids containing either *thyA-infA* or *glmS-nadE* cassettes and a spectinomycin resistance cassette. In triplicate for each strain-plasmid combination, single colonies were inoculated into 1 well of a 24-well plate containing 1 mL M9t medium. The plate was covered with an AeraSeal membrane and lid, then incubated overnight at 37°C and 250 rpm. The next day, cultures were diluted 1000x into 1 mL fresh M9t medium in the presence or absence of Thy or NAcG as necessary, and then grown overnight again. This was continued daily for 31 days. At days 0, 1, 2, 3, 5, 7, 10, 14, 17, 21, 24, 28, and 31, growth was measured by Abs_{600}, all cultures were stocked in glycerol (15% v/v) and frozen, and cultures were 10-fold serially diluted seven times in a 96-deep well plate. 10 μL of each serial dilution was plated on an M9t + Spec plate and M9t + Thy or NAcG plate, as necessary, to determine how many CFUs in the culture contained plasmid and how many CFUs were total in the culture, respectively. Equation 7 was used to calculate the fraction of cells that contained the plasmid, where CFUs are colony forming units, Dil is the dilution factor, and Vf is the volume fraction of serial dilution plated. For p1-*M-gfp-specR* long-term maintenance procedures, the same procedure was used with the following modifications: 14 mL round-bottom culture tubes containing 2 mL of medium were used instead of 24-well plates, and samples were only taken at days 0, 3, 7, 10, 14, 17, and 21.

$$Plasmid\ Fraction = \frac{\left[CFUs \times Dil \times {}^{1}/_{Vf}\right]_{M9t+Spec}}{\left[CFUs \times Dil \times {}^{1}/_{Vf}\right]_{M9t\ Only}} \quad \text{(Equation 7)}$$

Long-term plasmid maintenance with flow cytometry-based plasmid presence verification

Medium-copy *infA-thyA* or *glmS-nadE* plasmids containing *gfpmut3* or *mCherry* cassettes, respectively, in place of Spec resistance cassettes, were subjected to the same long-term culturing procedure as described above. For flow cytometry measurements, frozen stocks were thawed and then diluted 200-fold into cold 0.2 mL PBS + 2 mg/mL Kan in a 96-well clear round bottom assay microplate. Samples were chilled at 4°C for over 1 h, then loaded into a Guava EasyCyte HT flow cytometer (Luminex, Austin, TX, USA). 100,000 events, gated by forward and side scatter, were collected per sample. Samples were excited by a 488 nm laser, and GFPmut3 fluorescence emission was collected by a 525 nm detector while mCherry fluorescence emission was collected by a 695 nm detector. Data files were exported and analyzed in MATLAB 2020b (MathWorks, Natick, MA, USA) using the ''fca_readfcs'' function to convert FCS 3.0 raw data files into a MATLAB-readable format (Methods S1).[66] To determine how many cells were fluorescent, the green and red fluorescence distributions of a non-GFPmut3- and non-mCherry-containing population were modeled as a normal distribution using the MATLAB ''histfit'' function. The Fraction On of each population was calculated using Equation 8, where F is the fluorescence of a given cell, μ_{empty} is the mean of the modeled normal distribution of the empty vector, and σ_{empty} is the accompanying standard deviation (Figure S9, Methods S1). Fraction On values were averaged for triplicate samples. Mean fluorescence for each sample was also calculated by subtracting the mean fluorescence of the empty vector control from that of a sample.

$$Fraction\ On = \frac{\sum_{cells}\left[F > \mu_{empty} + 2\sigma_{empty}\right]}{\sum_{cells}} \quad \text{(Equation 8)}$$

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical details of experiments, including significance criteria and sample size, can be found in the figure legends or main text. No sample size calculations were performed during the design of experiments. No samples were excluded. Specific p-values are detailed in the source data file (Data S1).