Genome-scale promoter engineering by coselection maGe

Harris H Wang[1,2,6], Hwangbeom Kim[3,6], Le Cong[1,4,5], Jaehwan Jeong[3], Duhee Bang[3] & George M Church[1,4]

multiplex automated genome engineering (maGe) uses short oligonucleotides to scarlessly modify genomes; however, insertions >10 bases are still inefficient but can be improved substantially by selection of highly modified chromosomes. here we describe 'coselection' maGe (cos-maGe) to optimize biosynthesis of aromatic amino acid derivatives by combinatorially inserting multiple t7 promoters simultaneously into 12 genomic operons. Promoter libraries can be quickly generated to study gain-of-function epistatic interactions in gene networks.

The ability to directly manipulate and add new genetic regulatory elements to the chromosome is highly desirable as it bypasses the need to resort to plasmid-based systems in which copy-number variation can substantially affect the dynamics and robustness of synthetic networks[1]. Previously, we had developed the MAGE method to combinatorially introduce sequence changes to many sites in the *Escherichia coli* genome using libraries of 90-base synthetic oligonucleotides[2,3]. Although the efficiency for making small modifications is high (>30% for <4 base pairs (bp))[2,4], insertion efficiency per MAGE cycle drops substantially for larger inserts (<2% for >20 bp), thus limiting its utility for integration of regulatory elements or larger sequence motifs. However, we had unexpectedly found that a small fraction of isolates contained more than one mutated site in each cycle of MAGE. We hypothesized that isolation of clones containing a mutation at one site may increase the likelihood of finding other mutated sites because a certain cell subpopulation may be more electrocompetent or more available to perform oligo-based allelic replacement. We call the process of population enrichment for highly mutated genomes CoS-MAGE, which we demonstrate here as an enhanced scarless genome engineering approach to efficiently introduce larger regulatory elements such as promoters into targeted regions on the chromosome.

We chose the 20-bp T7 promoter (5′-TAATACGACTCACTATAGGG-3′) for insertion upstream of 12 genes or operons in the *E. coli* genome that are associated with the biosynthesis of aromatic amino acids (**Fig. 1** and **Supplementary Fig. 1**). In the presence of T7 RNA polymerase, transcription from the T7 promoter is strong and orthogonal to that at endogenous promoters. In *E. coli*, the industrially relevant blue dye, indigo, and a compound used in leukemia treatment[5], indirubin, can be produced from the tryptophan-biosynthesis intermediate, indole, by heterologous expression of a *Methylophaga* sp. flavin-containing monooxygenase, encoded by *bfmo*[6]. Using the MAGE-competent EcNR2 strain[2], we first transformed the bfmo-encoding pJ401 plasmid to generate EcHW7. We then removed known feedback regulation and allosteric inhibition associated with tryptophan biosynthesis[7–10], which involved nonsense inactivation of *trpR* and introducing mutations in *aroF* (to encode a P148L mutant protein), *trpE* (to encode a M293T mutant protein) and in *aroG* (to encode a D146N mutant protein). We then inactivated chromosomal *galK*, *malK*, *cat* and *bla* by introducing a revertible premature stop codon in each gene with high efficiency by MAGE using 90-bp oligos to generate the strain EcHW47 (Online Methods). We transformed EcHW7 and EcHW47 with pN249 to produce strains E7N and E47N, respectively.

To perform CoS-MAGE, we spiked the mixed oligo pool that targets all 12 insertion sites at a molar ratio of 50:1 with a small amount of an oligo (called CoS oligo) that reverted the function of an inactivated genomic selectable marker (that is, $galK^-$, $malK^-$, cat^- or bla^-). Restoration to $galK^+$ or $malK^+$ enabled growth on M9 minimal medium supplemented with galactose or maltose, respectively, as the sole carbon source. Restoration to cat^+ or bla^+ conferred antibiotic resistance to chloramphenicol or carbenicillin, respectively.

In the first coselection stage, we performed four MAGE cycles targeting 12 sites and cat^-. At the end of the coselection stage, we isolated 96 colonies plated with and without chloramphenicol and genotyped them by multiplex allele-specific colony PCR[3] to query for T7 promoter insertions at the 12 target sites (Online Methods). Among the isolated clones, target sites near the coselection marker were highly enriched for T7 promoter insertions only when the chloramphenicol coselection was applied (**Fig. 1b**). At these sites, the frequency of T7 promoter integration was as high as 25% with coselection in comparison to 2–3% without it. We observed this increased frequency of integration after only two MAGE cycles (**Fig. 1b**). We hypothesize that oligo-mediated genomic modifications near the coselection marker were enhanced by capturing cells whose replication forks were transiently in the open state and available for allelic replacement (P. Carr and H.H.W., unpublished data).

By selecting against cells that do not generate oligo-mediated allelic replacements, we can substantially enrich the population for individual clones with multiple T7 promoter insertions. This coselection effect can be enhanced through multiple MAGE cycles.

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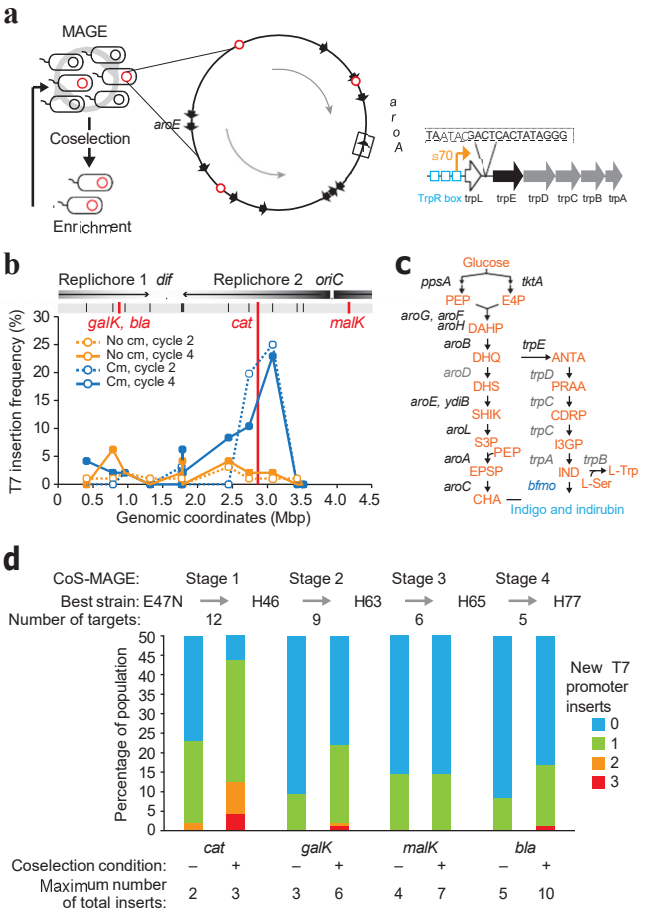


figure 1 | CoS-MAGE strategy for enriching highly modified genomes. (**a**) Cells undergoing cycles of MAGE were enriched by a coselection stage through phenotypic selection of a revertible genomic locus (*malK*, *galK*, *cat* or *bla*) and used for subsequent iterative cycles of MAGE (left). Twelve genomic operons were targeted for insertion of the T7 promoter sequence upstream of the first relevant open reading frame. For example, T7 promoter sequence (bottom right) targeting *trpE* was inserted upstream of the first relevant open reading frame. TrpR box, tryptophan repressor binding sequence; σ70, endogenous promoter. (**b**) T7 promoter insertion frequency near the *cat* locus with and without coselection by chloramphenicol (cm) after two and four MAGE cycles as determined by multiplex allele-specific colony PCR (Online Methods). Genomic coordinates based on the coordinate designations in ancestral strain MG1655 (GenBank U00096.2). (**c**) Schematic of indigo and indirubin biosynthesis. First gene in each relevant operon shown in black; targeted genes that are not first in the operon are shown in gray. (**d**) Percentage of colonies with 0-3 new T7 promoter insertions for the indicated conditions. The most enriched strains at the end of each coselection stage are labeled.

Without coselection, 10–25% of the population contained cells with one T7 promoter after four MAGE cycles. With coselection, more than 40% of cells had at least one T7 promoter insertion and 5% had three insertions (**Fig. 1d**). Using clone H46 that contained the most T7 promoter insertions (3 of 12 sites), we performed a second stage of CoS-MAGE by targeting the remaining nine sites using another coselection marker, *galK*. This stage produced clones with up to three more T7 promoter insertions. Two more stages of CoS-MAGE generated a clone with ten T7 promoter insertions (**Fig. 1d**). In comparison, we could only generate up to five insertions in a single clone in the absence of coselection. Coselection with *malK* during the third stage yielded mutants with only one additional insertion, likely because *malK* is far from any target site (>500 kilobases; **Supplementary Table 1**), providing support for the replication-dependency hypothesis in which coselection affects neighboring chromosomal regions. These results demonstrate that CoS-MAGE can substantially enhance the generation of genomic insertions (**Supplementary Note**). We inserted T7 promoter into the two remaining sites by CoS-MAGE to produce a clone that contains all 12 T7 promoters to orthogonally drive gene expression.

One important feature of CoS-MAGE is the capacity to produce combinatorial libraries of variants. In the process of generating the 12-target strain, we isolated intermediate strains with fewer T7 promoter insertions but in different combinations (**Fig. 2**).

We recovered 80 unique variants (named H1–H80) with 1–12 T7 promoter insertions including all 12 single inserts and two complete variants of 11 double inserts (*aroG* or *trpE* with all others). Using combinatorial T7 promoter expression, we probed the tunable parameters in the biosynthesis network. We indirectly measured the amount of tryptophan produced through expression of *bfmo* from each strain in the library when driven by a plasmid-encoded T7 polymerase (pN249). We readily extracted indigo and indirubin pigments, the major products of the bfmo monooxygenase[6], by dimethyl sulfoxide (DMSO) treatment (Online Methods).

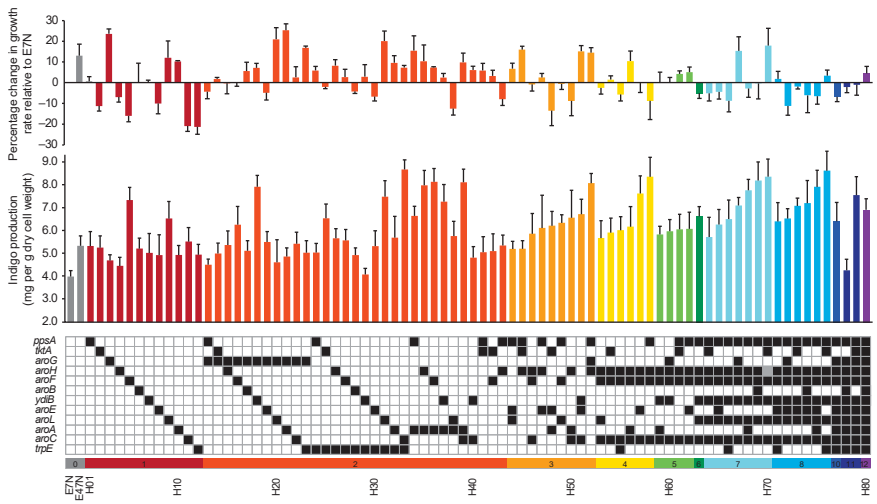
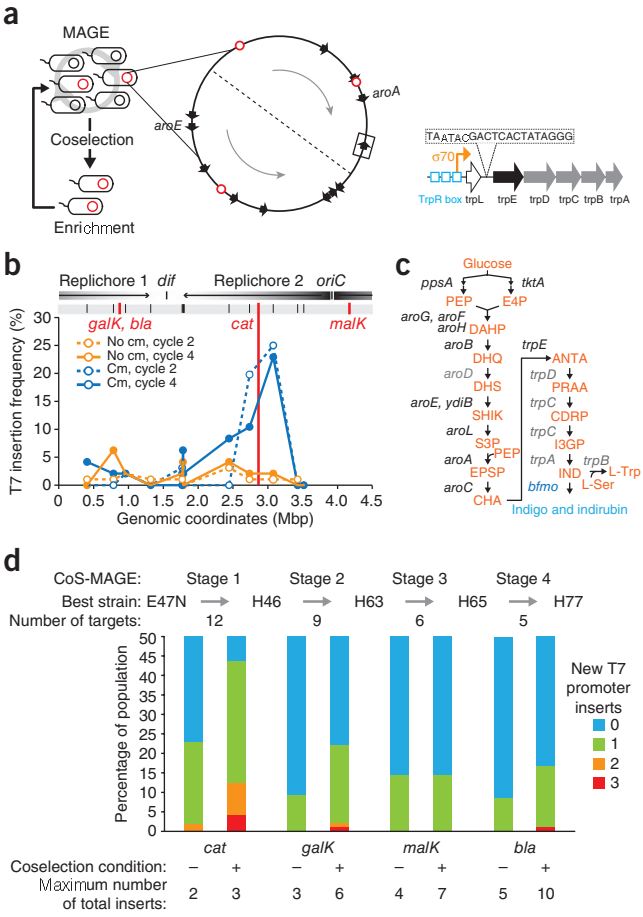


figure 2 | Indigo production from synthetic T7 promoter-regulated operons. Relative change in growth rate for E47N (E7N derivative with inactivated *trpR*, and mutated *aroF*, *trpE* and *aroG*) and H1-H80 (derivatives of E47N with T7 promoter insertions) was normalized to growth rate of E7N (E2N-derivative with *bfmo*-containing pJ401 plasmid) as $(y - x)/y$, where x is the doubling time of each strain and y is doubling time of the E7N wild type (top). Indigo production based on measured indirubin amounts (middle; Online Methods). All error bars, s.e.m. ($n = 3$). Color coding reflects grouping by the number of T7 insertions as indicated on the bottom. Genotypes for each clone are marked by black boxes and ordered by their relative location along the biosynthesis pathway (*ppsA* is proximal and *trpE* is distal). The gray box of strain H70 corresponds to an insert in *aroH* with a mutated T7 sequence (5′TAATACACTCAGTATGGG-3′).



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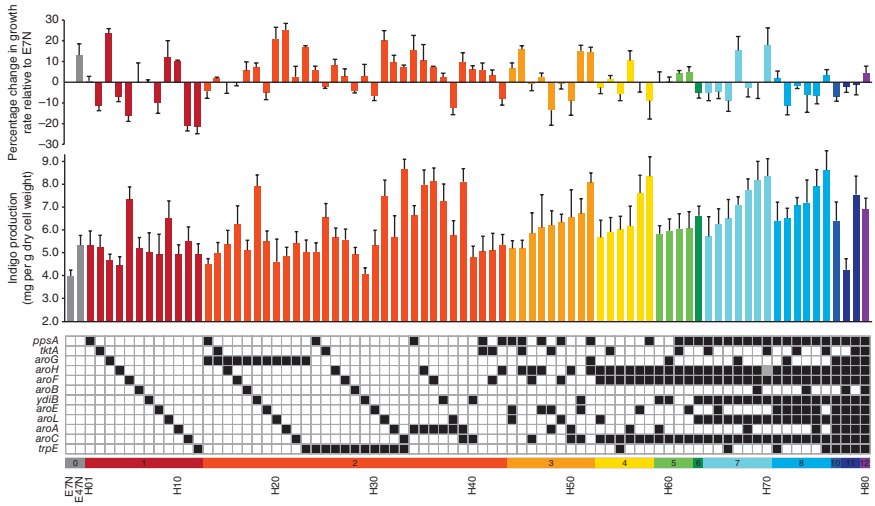

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Analysis of higher-order mutants revealed more unexpected results. Strain H77, with 10 of 12 inserts, showed a 20% improvement in indigo production over the E47N strain ($P = 2.5 \times 10^{-3}$), but the addition of the T7 promoter insert in *aroB* in the H78 strain significantly reduced the yield to 20% below that in the E47N strain ($P = 4.2 \times 10^{-3}$). Addition of the final T7 promoter insert in *tktA* in the 12-promoter H80 strain returned yield to 29% above that in the E47N strain ($P = 2.5 \times 10^{-3}$). Such large and opposite effects of *aroB* insertion and subsequent *tktA* insertion suggest that interactions in this gene network are unexpectedly complex, nonlinear and epistatic. Growth rate measurements of each strain under *bmfo*-induced conditions also revealed changes ranging from −30% to +30% relative to the ancestral strain (**Fig. 2**). However, we extracted no notable correlation between growth rate and indigo amount (**Supplementary Fig. 3**). We hypothesize that such complex epistatic interactions are more prevalent in endogenous networks than previously expected, especially in the context of large-scale modulation of gene expression using synthetic regulatory modules.

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methods

Methods and any associated references are available in the online version of the paper.

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acknowledgments

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author contributions

H.H.W., H.K., D.B. and G.M.C. designed the study. H.H.W., H.K., L.C. and J.J. performed the experiments. H.H.W., H.K. and D.B. analyzed the data and prepared the initial manuscript. All authors edited and revised the final manuscript.

competing financial interests

The authors declare competing financial interests: details are available in the online version of the paper.

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Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.H.W., H.K., D.B. and G.M.C. designed the study. H.H.W., H.K., L.C. and J.J. performed the experiments. H.H.W., H.K. and D.B. analyzed the data and prepared the initial manuscript. All authors edited and revised the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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online methods

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A streamlined CoS-MAGE protocol is described here. Each cycle of MAGE takes ~2 h, and four cycles can be easily done in a single day, ending with plating on the selection medium plates (for the coselection step). Overnight selection on plates will yield colonies in the morning, which can be picked directly into MASC-PCRs to identify the best clone (2 h turnaround). At the same time, each colony can be grown in 96-well format in parallel to the PCR-genotyping step. The best clone (taken from the already 2-h–grown 96-well plate) can then be used for the next four cycles of MAGE in the same day (day 2) and iterated in subsequent days in the same manner. For selection that required a minimal medium growth step (M9-Gal and M9-Mal), an additional day was needed to allow the colonies to form for each coselection step, thus doubling the total time needed. We note that four cycles of MAGE is not needed each day before the coselection enrichment is applied (two or even one cycle may be sufficient). We performed four cycles per day because it was practically as easy as two cycles with low sample numbers, and some minor benefit can be gained from additional rounds of standard MAGE. This coselection method is also a practical way of implementing MAGE in the absence of an automated instrument to perform MAGE, while still achieving a high frequency of site-directed mutagenesis.

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inactivated in EcHW7 by MAGE using trpR_KO_Oligo. MASC-PCR screening of 48 colonies (using primer set trpR_KO_f/fm/r) identified several clones, one of which was designated EcHW44. To remove tryptophan-associated allosteric feedback, single nucleotide mutations were introduced to *aroF trpE*, and *aroG* of EcHW44 using aroF(P148L)_oligo, trpE(M293T)_oligo, aroG(D146N)_oligo by applying four cycles of MAGE along with oligo galK_mut45_oligo. After the four MAGE cycles, cells were plated on Mac-Gal with kanamycin plates and 48 *galK*$^-$ white colonies were picked after overnight growth, pooled and regrown together for another two cycles of MAGE with aroF(P148L)_oligo, trpE(M293T)_oligo, aroG(D146N)_oligo and galK_mut45_oligo (total oligo concentration of 10 mM). After the two MAGE cycles, cells were plated on Mac-Gal with kanamycin plates, and 32 *malK*$^-$ white colonies were picked after overnight growth and genotyped for the *aroF*, *trpE* and *aroG* mutations by MASC-PCR using primer sets aroF(P148L)_f/fm/r, trpE(M293T)_f/fm/r and aroG(D146N)_f/fm/r, which identified clone EcHW45 that had all the mutations. EcHW45 was used to generate the inactivated *cat*$^-$ genotype using cat_mut45_oligo and subsequently made *bla*$^-$ with bla_mut45_oligo to generate EcHW47. Screening of *cat*$^-$ and *bla*$^-$ was easily done by replica plating from LB plates onto LB with chloramphenicol and carbenicillin or more quickly done by picking ~30 colonies into LB and LB chloramphenicol and carbenicillin to identify chloramphenicol- or carbenicillin-sensitive strains. EcHW47 contained *aroF*(P148L), *trpE*(M293T), *aroG*(D146N) and revertible nonsense mutations in the inactivated *galK*$^-$, *malK*$^-$, *cat*$^-$ and *bla*$^-$ loci. Transformation of the IPTG-inducible T7 RNA polymerase gene on pN249 (courtesy of C. Voigt, Massachusetts Institute of Technology) into EcNR2, EcHW7 and EcHW47 and selection on LB with spectinomycin yielded E2N, E7N and E47N, respectively.

T7 promoter insertions by CoS-MAGE. The E47N strain was used to insert T7 promoters into 12 genomic loci 35 bp upstream of *aroL*, *aroG*, *aroA*, *trpE*, *ydiB*, *ppsA*, *aroH*, *aroC*, *aroF*, *tktA*, *aroE* and *aroB*. For the first stage of CoS-MAGE, 12 oligos were pooled together in equal molar ratio for a final concentration of 1 mM per oligo. The cat_restore_oligo (CoS oligo at final concentration of 0.02 mM) was added to this pool. The E47N strain was cycled four times by MAGE using the oligo pool over the course of 8 h (a typical workday). In general, the number of *cat*$^+$ cells in the population was ~1 in 2,000, so dilutions were made accordingly when plating. The cell population was diluted and spread on LB with chloramphenicol (for coselection for *cat*) or on LB (without coselection) overnight. We isolated and genotyped 96 colonies from each plate by MASC-PCR for presence of the T7 promoter sequences at each of the 12 target sites. Of the coselected colonies assayed, clone H46 had the most T7 promoter inserts (*aroH*, *aroC* and *aroF*), so it was used for the second stage of CoS-MAGE. In the second stage, 9 oligos corresponding to the remaining targets were pooled together in equal molar ratio as well as the galK_restore_oligo (CoS oligo at final per oligo concentration of 1 mM). We increased the CoS oligo to target oligo ratio because at the original (1:50 ratio) we expected to find 1 red *galK*$^+$ colony in ~2,000 colonies on MAC-Gal, which would be infeasible for clonal isolation. Boosting the CoS oligo concentration can increase the number of *galK*$^+$ colonies to levels screenable by plating. Alternatively, plating on M9-Gal for *galK*$^+$ colonies was also feasible and would not need higher *galK* CoS oligo concentration, but at a longer delay owing to slower colony growth on minimal medium (~2.5× of wild type). The 9 target plus *galK* CoS oligo pool was used in the second coselection stage through four cycles of MAGE on H46 strain, and the cell population was plated on Mac-Gal. Isolation and MASC-PCR genotyping of 96 red *galK*$^+$ colonies grown overnight resulted many additional T7 inserts. The best clone (H63) had six inserts in total (*aroF*, *aroC*, *aroH*, *ppsA*, *ydiB* and *aroL*). The third coselection stage was done on the remaining six targets similarly as described above using malK_restore_oligo as the CoS oligo and plating on Mac-Mal to isolate red *malK*$^+$ colonies. The best clone (H65) containing seven inserts (*aroE*, *aroF*, *aroC*, *aroH*, *ppsA*, *ydiB* and *aroL*) was isolated. The fourth coselection stage was done on the remaining five targets using bla_restore_oligo. Selection on LB with carbenicillin and isolation of 96 colonies resulted in the best clone, H77, that contained ten inserts (without *tktA* and *aroB*). We reiterated CoS-MAGE on the remaining targets to generate the H80 clone, which contained all 12 desired T7 promoter insertions as well as other clones containing unique insertions through MASC-PCR every two MAGE cycles. A summary of genomic distance of each locus to the relevant coselectable marker is shown in **Supplementary Table 1**. In total, 80 genotypically unique strains (H1-H80) were isolated containing 1–12 T7 promoter insertions. We transformed the T7 polymerase–encoding pN249 plasmid into each strain and selected on LB with spectinomycin for the presence of the plasmid.

Sequencing verification. We sequenced all 12 target sites for the expected T7 promoter sequence in each of the 80 strains (H1-H80). The PCR primers amplified ~150–180 bp around each T7 insertion locus by a standard protocol. All sequenced sites were verified to contain the correct T7 sequence (TAATACGACTCACTATAGGG) except site *aroH* of strain H70, in which the T7 sequence was TAATACACTCAGTATGGG.

Pigment extraction and quantification. To quantify the amount of upregulation in the modified biosynthesis pathway, we measured indigo and indirubin production in strains H1–H80 and ances- tral strains E2N, E7N and E47N following previously described methods[3]. Each strain was grown in 10 ml of LB with kanamycin, spectinomycin and IPTG for 24 h at 30 °C before extraction. Strain E2N was grown in LB with spectinomycin and IPTG only. For pigment extraction, 1 ml of each strain was concentrated by centrifugation in a deep-well 96-well plate. Excess medium was discarded, and each cell pellet was resuspended in 1 ml of DMSO. The samples were sonicated for 10 s and centrifuged again to concentrate the cell debris, which appeared white. The blue supernatant was removed and stored at room temperature in the dark to allow accurate measurement of indigo and indirubin levels. After 3 d, 200 ml of each supernatant was transferred to a 96-well plate, and absorbance at 550 nm was measured on a Molecular Devices spectrophotometer to quantify relative indirubin amounts. Indigo at 620 nm was also measured. However, the absorbance values decayed over time (**Supplementary Fig. 2**), likely owing to indigo instability. Thus, to determine absolute indigo production levels, we took the relative indirubin values against the amounts in strain H33 (best producer) and normalized them against the measured indigo production level (at Abs_{620}) of an immediately extracted H33 strain sample. Indigo amounts were determined by mapping the Abs_{620} to a calibration curve generated using dilutions

inactivated in EcHW7 by MAGE using trpR_KO_Oligo. MASC-PCR screening of 48 colonies (using primer set trpR_KO_f/fm/r) identified several clones, one of which was designated EcHW44. To remove tryptophan-associated allosteric feedback, single nucleotide mutations were introduced to *aroF trpE*, and *aroG* of EcHW44 using aroF(P148L)_oligo, trpE(M293T)_oligo, aroG(D146N)_oligo by applying four cycles of MAGE along with oligo galK_mut45_oligo. After the four MAGE cycles, cells were plated on Mac-Gal with kanamycin plates and 48 *galK*$^-$ white colonies were picked after overnight growth, pooled and regrown together for another two cycles of MAGE with aroF(P148L)_oligo, trpE(M293T)_oligo, aroG(D146N)_oligo and galK_mut45_oligo (total oligo concentration of 10 μM). After the two MAGE cycles, cells were plated on Mac-Gal with kanamycin plates, and 32 *malK*$^-$ white colonies were picked after overnight growth and genotyped for the *aroF*, *trpE* and *aroG* mutations by MASC-PCR using primer sets aroF(P148L)_f/fm/r, trpE(M293T)_f/fm/r and aroG(D146N)_f/fm/r, which identified clone EcHW45 that had all the mutations. EcHW45 was used to generate the inactivated *cat*$^-$ genotype using cat_mut45_oligo and subsequently made *bla*$^-$ with bla_mut45_oligo to generate EcHW47. Screening of *cat*$^-$ and *bla*$^-$ was easily done by replica plating from LB plates onto LB with chloramphenicol and carbenicillin or more quickly done by picking ~30 colonies into LB and LB chloramphenicol and carbenicillin to identify chloramphenicol- or carbenicillin-sensitive strains. EcHW47 contained *aroF*(P148L), *trpE*(M293T), *aroG*(D146N) and revertible nonsense mutations in the inactivated *galK*$^-$, *malK*$^-$, *cat*$^-$ and *bla*$^-$ loci. Transformation of the IPTG-inducible T7 RNA polymerase gene on pN249 (courtesy of C. Voigt, Massachusetts Institute of Technology) into EcNR2, EcHW7 and EcHW47 and selection on LB with spectinomycin yielded E2N, E7N and E47N, respectively.

T7 promoter insertions by CoS-MAGE. The E47N strain was used to insert T7 promoters into 12 genomic loci 35 bp upstream of *aroL*, *aroG*, *aroA*, *trpE*, *ydiB*, *ppsA*, *aroH*, *aroC*, *aroF*, *tktA*, *aroE* and *aroB*. For the first stage of CoS-MAGE, 12 oligos were pooled together in equal molar ratio for a final concentration of 1 μM per oligo. The cat_restore_oligo (CoS oligo at final concentration of 0.02 μM) was added to this pool. The E47N strain was cycled four times by MAGE using the oligo pool over the course of 8 h (a typical workday). In general, the number of *cat*$^+$ cells in the population was ~1 in 2,000, so dilutions were made accordingly when plating. The cell population was diluted and spread on LB with chloramphenicol (for coselection for *cat*) or on LB (without coselection) overnight. We isolated and genotyped 96 colonies from each plate by MASC-PCR for presence of the T7 promoter sequences at each of the 12 target sites. Of the coselected colonies assayed, clone H46 had the most T7 promoter inserts (*aroH*, *aroC* and *aroF*), so it was used for the second stage of CoS-MAGE. In the second stage, 9 oligos corresponding to the remaining targets were pooled together in equal molar ratio as well as the galK_restore_oligo (CoS oligo at final per oligo concentration of 1 μM). We increased the CoS oligo to target oligo ratio because at the original (1:50 ratio) we expected to find 1 red *galK*$^+$ colony in ~2,000 colonies on MAC-Gal, which would be infeasible for clonal isolation. Boosting the CoS oligo concentration can increase the number of *galK*$^+$ colonies to levels screenable by plating. Alternatively, plating on M9-Gal for *galK*$^+$ colonies was also feasible and would not need higher *galK* CoS oligo concentration, but at a longer delay owing to slower colony growth on minimal medium (~2.5× of wild type). The 9 target plus *galK* CoS oligo pool was used in the second coselection stage through four cycles of MAGE on H46 strain, and the cell population was plated on Mac-Gal. Isolation and MASC-PCR genotyping of 96 red *galK*$^+$ colonies grown overnight resulted many additional T7 inserts. The best clone (H63) had six inserts in total (*aroF*, *aroC*, *aroH*, *ppsA*, *ydiB* and *aroL*). The third coselection stage was done on the remaining six targets similarly as described above using malK_restore_oligo as the CoS oligo and plating on Mac-Mal to isolate red *malK*$^+$ colonies. The best clone (H65) containing seven inserts (*aroE, aroF*, *aroC*, *aroH*, *ppsA*, *ydiB* and *aroL*) was isolated. The fourth coselection stage was done on the remaining five targets using bla_restore_oligo. Selection on LB with carbenicillin and isolation of 96 colonies resulted in the best clone, H77, that contained ten inserts (without *tktA* and *aroB*). We reiterated CoS-MAGE on the remaining targets to generate the H80 clone, which contained all 12 desired T7 promoter insertions as well as other clones containing unique insertions through MASC-PCR every two MAGE cycles. A summary of genomic distance of each locus to the relevant coselectable marker is shown in **Supplementary Table 1**. In total, 80 genotypically unique strains (H1-H80) were isolated containing 1–12 T7 promoter insertions. We transformed the T7 polymerase–encoding pN249 plasmid into each strain and selected on LB with spectinomycin for the presence of the plasmid.

Sequencing verification. We sequenced all 12 target sites for the expected T7 promoter sequence in each of the 80 strains (H1-H80). The PCR primers amplified ~150–180 bp around each T7 insertion locus by a standard protocol. All sequenced sites were verified to contain the correct T7 sequence (TAATACGACTCACTATAGGG) except site *aroH* of strain H70, in which the T7 sequence was TAATACACTCAGTATGGG.

Pigment extraction and quantification. To quantify the amount of upregulation in the modified biosynthesis pathway, we measured indigo and indirubin production in strains H1–H80 and ancestral strains E2N, E7N and E47N following previously described methods[3]. Each strain was grown in 10 ml of LB with kanamycin, spectinomycin and IPTG for 24 h at 30 °C before extraction. Strain E2N was grown in LB with spectinomycin and IPTG only. For pigment extraction, 1 ml of each strain was concentrated by centrifugation in a deep-well 96-well plate. Excess medium was discarded, and each cell pellet was resuspended in 1 ml of DMSO. The samples were sonicated for 10 s and centrifuged again to concentrate the cell debris, which appeared white. The blue supernatant was removed and stored at room temperature in the dark to allow accurate measurement of indigo and indirubin levels. After 3 d, 200 μl of each supernatant was transferred to a 96-well plate, and absorbance at 550 nm was measured on a Molecular Devices spectrophotometer to quantify relative indirubin amounts. Indigo at 620 nm was also measured. However, the absorbance values decayed over time (**Supplementary Fig. 2**), likely owing to indigo instability. Thus, to determine absolute indigo production levels, we took the relative indirubin values against the amounts in strain H33 (best producer) and normalized them against the measured indigo production level (at Abs_{620}) of an immediately extracted H33 strain sample. Indigo amounts were determined by mapping the Abs_{620} to a calibration curve generated using dilutions

of commercially obtained indigo powder. Five milliliters of cells were dried and weighed to derive the dry cell weight. Pigment extractions were repeated four separate times on different days for each strain to ensure statistical confidence.

Statistical analysis. P values described in the paper were determined by performing the Student's t test between the two sample groups to derive the t score, which is then used to compute the P value.

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