Transcriptional potential determines the adaptability of *Escherichia coli* strains with different fitness backgrounds

Running title: Transcriptional potential determines the adaptability of *Escherichia coli*

Kitae Kim\(^a\), Soon-Kyeong Kwon\(^{a,b}\), Pil Kim\(^c\), Jihyun F. Kim\(^{a,d}\)

\(^a\)Department of Systems Biology, Division of Life Sciences, and Institute for Life Science and Biotechnology, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

\(^b\)Division of Applied Life Science (BK21), Gyeongsang National University, 501 Jinju-daero, Jinju-si, Gyeongsangnam-do 52828, Republic of Korea

\(^c\)Department of Biotechnology, The Catholic University of Korea, 43 Jibong-ro, Bucheon-si, Gyeonggi-do 14662, Republic of Korea

\(^d\)Microbiome Initiative, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

Address correspondence to Jihyun F. Kim, jfk1@yonsei.ac.kr, or Soon-Kyeong Kwon, skkwon@gnu.ac.kr.
SUPPLEMENTAL MATERIAL

Supplemental material contains five supplementary tables and seven supplementary figures.

Table S1. Strains and plasmids used in this study.

Table S2. Extended view of the single nucleotide polymorphisms (SNPs), deletions, insertions, and other polymorphisms (DIPs) identified in the evolving populations.

Table S3. DEGs commonly up- or down-regulated in WT or O3 populations.

Table S4. Oligonucleotides used in this study.

Table S5. Summary statistics of DNA and RNA sequencing.

Figure S1. Phenotypic difference between O3 lacZ dadX and O3 lacZ dadX araA.

Figure S2. Experimental design of experimental evolution.

Figure S3. Growth curves of ancestral and evolved populations.

Figure S4. Effect of dominant mutations on fitness.

Figure S5. Position-dependent differentially expressed gene (DEG) cluster analysis.

Figure S6. DNA sequencing read quality after read trimming.

Figure S7. RNA sequencing read quality after read trimming.
### SUPPLEMENTAL TABLES

**Table S1.** Strains and plasmids used in this study.

| Strains, plasmids | Relevant characteristics | Source          |
|-------------------|--------------------------|-----------------|
| **Strains**       |                          |                 |
| WT                | *Escherichia coli* K-12 MG1655 wild type | Laboratory stock |
| O3 *lacZ dadX*    | *E. coli* K-12 MG1655 ΔlacZ::oriC-mioC ΔdadX/cvrA::oriC-mioC | (1)             |
| O3 *lacZ dadX araA* | O3 *lacZ dadX*, REL606 *araA*, ΔyeaJ-yeaV | This study      |
| REL606            | Ara'                     | Laboratory stock |
| 68WT-1            | Evolved WT lineage 1 at day 68 | This study      |
| 68WT-2            | Evolved WT lineage 2 at day 68 | This study      |
| 68WT-3            | Evolved WT lineage 3 at day 68 | This study      |
| 68O3-1            | Evolved O3 lineage 1 at day 68 | This study      |
| 68O3-2            | Evolved O3 lineage 2 at day 68 | This study      |
| 68O3-3            | Evolved O3 lineage 3 at day 68 | This study      |
| WT *mrdB*         | WT *mrdB* (R69H)          | This study      |
| Gene Combination         | Mutated Genes | References    |
|-------------------------|---------------|---------------|
| WT *rpoB*               | *rpoB* (A1055V) | This study    |
| WT *mrdB* *rpoB*        | *rpoB* (A1055V) | This study    |
| WT *rpoB* *pyrE*-rph    | *rpoB* (A1055V), *pyrE*-rph (Δ82) | This study    |
| WT *cysE*               | *cysE* (S224A) | This study    |
| WT *rpoC*               | *rpoC* (R1075C) | This study    |
| WT *cysE* *rpoC*        | *cysE* (S224A), *rpoC* (R1075C) | This study    |
| WT *rlmH*               | *rlmH* (S121*) | This study    |
| WT *ydhZ/pykF*          | *ydhZ/pykF* (intergenic (-284/-273) Δ1) | This study    |
| WT *pykF*               | *pykF* (R385L) | This study    |
| WT *gtrB* *rpoB*        | *gtrB* (S267L), *rpoB* (I524M) | This study    |
| WT *pyfK* *rpoB*        | *pyfK* (R385L), *rpoB* (I524M) | This study    |
| WT *yeiH*               | *yeiH* (I137F) | This study    |
| WT *yeiH* *ptsP*        | *yeiH* (I137F), *ptsP* (Q340*) | This study    |
| WT *ptsP* *nusA*        | *ptsP* (Q340*), *nusA* (coding (1457/1488 nt)+G) | This study    |
| WT *ptsP*               | *ptsP* (L111*) | This study    |
| WT *mreB*               | *mreB* (A82S) | This study    |
| Strain | Description | Reference |
|--------|-------------|-----------|
| WT pyrE-rph | WT pyrE-rph (Δ82) | This study |
| WT nagA | WT nagA (coding (422/1149 nt) Δ1) | This study |
| WT cyaA | WT cyaA (R160L) | This study |
| WT cyaA mreB | WT cyaA (R160L), mreB (A82S) | This study |
| O3 mrdB | O3 lacZ dadX mrdB (R69H) | This study |
| O3 pyrE-rph | O3 lacZ dadX pyrE-rph (Δ82) | This study |
| O3 cysE | O3 lacZ dadX cysE (S224A) | This study |
| O3 cysE rpoC | O3 lacZ dadX cysE (S224A), rpoC (R1075C) | This study |
| O3 ydhZ/pykF | O3 lacZ dadX ydhZ/pykF (intergenic (-284/-273)Δ1) | This study |
| O3 rlmH ydhZ/pykF | O3 lacZ dadX rlmH (S121*) ydhZ/pykF (intergenic (-284/-273)Δ1) | This study |
| O3 gtrB | O3 lacZ dadX gtrB (S267L) | This study |
| O3 pykF | O3 lacZ dadX pykF (R385L) | This study |
| O3 rpoB | O3 lacZ dadX rpoB (I524M) | This study |
| O3 gtrB rpoB | O3 lacZ dadX gtrB (S267L), rpoB (I524M) | This study |
| O3 pykF gtrB rpoB | O3 lacZ dadX pykF (R385L), gtrB (S267L), rpoB (I524M) | This study |
| O3 ptsP | O3 lacZ dadX ptsP (Q340*) | This study |
| O3 nagA                  | O3 lacZ dadX nagA (coding (422/1149 nt) Δ1) | This study |
|-------------------------|--------------------------------------------|------------|
| O3 yeiH                 | O3 lacZ dadX yeiH (I137F)                  | This study |
| O3 cyaA                 | O3 lacZ dadX cyaA (R160L)                  | This study |

**Plasmids**

| Plasmid     | Description                                                                 | Reference |
|-------------|-----------------------------------------------------------------------------|-----------|
| pKD46       | Used for λ-red recombination, bfa, γ, β, exo (Red recombinase), temperature-conditional origin of replication | (2)       |
| pKD3        | Template plasmid for chloramphenicol acetyltransferase (CAT)                | (2)       |
| pDMS197     | Template plasmid for sacB (sucrose counter selection marker)                | (3)       |
| pTOP TA V2  | TOP cloning plasmid (Enzynomics)                                            |           |
| pTOP TA V2 CAT-sacB | Template plasmid for CAT-sacB selection-counter selection marker       | This study |
| pMA7-SacB   | Helper plasmid in transient mutator multiplex automated genome engineering (TM-MAGE) cycling | (4)       |
Table S2. Extended view of the single nucleotide polymorphisms (SNPs), deletions, insertions, and other polymorphisms (DIPs) identified in the evolving populations.

| Lineage / position | Gene          | Mutation | Allele frequency | Annotation                  | Description                                                                 |
|--------------------|---------------|----------|------------------|----------------------------|----------------------------------------------------------------------------|
| WT-1               | ispH →        | G→C      | 10.9%            | S135T (AGT→ACT)            | 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, 4Fe-4S protein          |
|                    | cusA → / → pheP | T→C      | 10.2%            | intergenic (+16/-86)       | copper/silver efflux system, membrane component/phenylalanine transporter   |
|                    | mrdB ←        | C→T      | 100%             | R69H (CGC→CAC)             | cell wall shape-determining protein                                        |
|                    | fabF →        | T→G      | 35.9%            | Y268D (TAT→GAT)            | 3-oxoacyl-[acyl-carrier-protein] synthase II                                |
|                    | yobF ← / ← yebO | IS1 (+) +9 bp | 13.9%         | intergenic (-45/+617)       | DUF2527 family heat-induced protein/putative inner membrane protein         |
|                    | rrsG ← / ← clpB | A→T      | 17.0%            | intergenic (-300/+143)     | 16S ribosomal RNA of rrnG operon/protein disaggregation chaperone            |
|                    | barA →        | IS1 (+) +9 bp | 12.8%         | coding (906-914/2757 nt)   | hybrid sensory histidine kinase, in two-component regulatory system with UvrY|
|                    | ptsP ←        | A→C      | 17.3%            | F490C (TTC→TGC)            | PEP-protein phosphotransferase enzyme I; GAF domain containing protein     |
|                    | pyrE-rph      | Δ82 bp   | 100%             |                            | pyrE-rph                                                                  |
| Gene | Mutation | Function | Description |
|------|----------|----------|-------------|
| 4,184,408 | rpoB → C→T | 100% | A1055V (GCG→GTG) |
|        |          |          | RNA polymerase, beta subunit |
| WT-2  |          |          |             |
| 696,419  | ubiF → / ← glnX | +TTT | 13.0% | intergenic (+143/+11) |
|         |          |          | 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol oxygenase/tRNA-Gln |
| 702,330  | nagA ← Δ1 bp | 20.7% | coding (422/1149 nt) |
|         |          |          | N-acetylglucosamine-6-phosphate deacetylase |
| 973,367  | elyC ← Δ1 bp | 25.7% | coding (35/780 nt) |
|         |          |          | envelope biogenesis factor; DUF218 superfamily protein |
| 1,019,695 | ompA ← C→T | 29.2% | G120D (GGC→GAC) |
|         |          |          | outer membrane protein A (3a;II*:G;d) |
| 1,293,196 | hns ← / → tdk | IS5 (+) +4 bp | 40% | intergenic (-274/-328) |
|         |          |          | global DNA-binding transcriptional dual regulator H-NS/thymidine kinase/deoxyuridine kinase |
| 2,311,785 | ompC ← +CAACAT | 19.0% | coding (965/1104 nt) |
|         |          |          | outer membrane porin protein C |
| 2,912,408 | relA ← A→C | 14.7% | V415G (GTG→GGC) |
|         |          |          | (p)ppGpp synthetase I/GTP pyrophosphokinase |
| 2,913,235 | relA ← T→G | 11.0% | L139F (TTA→TTG) |
|         |          |          | (p)ppGpp synthetase I/GTP pyrophosphokinase |
| 3,696,149 | yhjR ← C→T | 27.9% | A13T (GCG→ACG) |
|         |          |          | DUF2629 family protein |
| 3,781,893 | cysE ← A→C | 100% | S224A (TCC→GCC) |
|         |          |          | serine acetyltransferase |
| 4,188,572 | rpoC → C→T | 100% | R1075C (CGT→TGT) |
|         |          |          | RNA polymerase, beta prime subunit |
| 4,627,797 | nadR ← IS1 (+) +9 bp | 14.2% | coding (483-491/1233 nt) |
|         |          |          | nicotinamide mononucleotide adenyltransferase, ribosynicotinamide kinase, transcriptional repressor |
| WT-3   |          |          |             |
Microbiology Spectrum

Kim et al.

571,172  
*ybcL* →  
IS2 (+) +5 bp  
87.9%  
coding (280-284/552 nt)  
inactive polymorphonuclear leukocyte migration suppressor; DLP12 prophage; UPF0098 family secreted protein

668,354  
*rlmH* ←  
G→T  
100%  
S121* (TCG→TAG)  
23S rRNA m(3)Psi1915 pseudouridine methyltransferase, SAM-dependent

1,755,425  
*ydhZ* ← / →  
*pykF* Δ1 bp  
100%  
intergenic (-284/-273)  
uncharacterized protein/pyruvate kinase I

1,915,319  
*proQ* ←  
+T  
23.8%  
coding (216/699 nt)  
RNA chaperone, putative ProP translation regulator

O3-1

1,756,482  
*pykF* →  
G→T  
100%  
R385L (CGC→CTC)  
pyruvate kinase I

2,459,131  
*gtrB* →  
C→T  
100%  
S267L (TCA→TTA)  
CPS-53 (KpLE1) prophage; bactoprenol glucosyl transferase

3,812,557  
*sprT* →  
T→G  
91.0%  
Y14D (TAC→GAC)  
bi-functional (p)ppGpp synthetase II/guanosine-3’,5’-bis pyrophosphate 3’-pyrophosphohydrolase

4,172,934  
*rpoB* →  
T→G  
100%  
I524M (ATT→ATG)  
RNA polymerase, beta subunit

O3-2

701,363  
*nagA* ←  
Δ1 bp  
51.6%  
coding (422/1149 nt)  
N-acetylglucosamine-6-phosphate deacetylase

2,240,243  
*yeiH* →  
A→T  
100%  
I137F (ATC→ITC)  
UPF0324 family inner membrane protein

2,957,535  
*ptsP* ←  
G→A  
100%  
Q340* (CAG→TAG)  
Pep-protein phosphotransferase enzyme I; GAF domain containing protein

3,306,188  
*nusA* ←  
+G  
100%  
coding (1457/1488 nt)  
transcription termination/antitermination L factor
| Chromosome Location | Gene Pair | Direction | Length (bp) | Percentage | Description |
|---------------------|-----------|-----------|-------------|------------|-------------|
| 3,805,919           | pyrE ← / ← rph | Δ1 bp | 100% | intergenic (-33/+33) | orotate phosphoribosyltransferase/ribonuclease PH (defective); enzyme; Degradation of RNA; RNase PH |
| O3-3                |           |          |             |            |             |
| 1,465,043           | ydbA → | IS1 (+) +9 bp | 66.7% | pseudogene (21-29/2513 nt) | pseudogene, autotransporter homolog; interrupted by IS2 and IS30 |
| 1,499,782           | ydcK ← | Δ1 bp | 68.9% | coding (299/981 nt) | uncharacterized protein |
| 1,897,746           | yof ← / ← yebO | IS2 (+) +5 bp | 24.9% | intergenic (-37/+629) | DUF2527 family heat-induced protein/putative inner membrane protein |
| 2,958,221           | ptsP ← | A→C | 100% | L111* (TTA→TGA) | PEP-protein phosphotransferase enzyme I; GAF domain containing protein |
| 3,307,050           | nusA ← | G→A | 32.3% | R199C (CGT→TGT) | transcription termination/antitermination L factor |
| 3,390,962           | mreB ← | C→A | 100% | A82S (GCC→TCC) | cell wall structural complex MreBCD, actin-like component MreB |
| 3,805,977           | pyrE-rph | Δ82 bp | 100% |  | pyrE-rph |
| 3,981,749           | cyaA → | G→T | 64.4% | R160L (CGC→CTC) | adenylate cyclase |
| 4,171,929           | rpoB → | T→A | 25.8% | D189E (GAT→GAA) | RNA polymerase, beta subunit |
| 4,533,728           | fimA ← | Δ1 :: IS186 (−) +6 bp :: Δ1 | 36.8% | coding (494-499/549 nt) | major type 1 subunit fimbrin (pilin) |
Table S3. DEGs that are commonly up- or down-regulated in WT or O3 populations.

| Lineage/gene | Function                                      | Average Log2FC | Average q-value |
|--------------|-----------------------------------------------|----------------|----------------|
| WT           |                                               |                |                |
| ydaM         | diguanylate cyclase DgcM                      | -1.68896       | 0.000818       |
| amn          | AMP nucleosidase                              | -0.80977       | 0.008372       |
| rnhB         | RNase HII                                     | 0.709147       | 0.007159       |
| yqfA         | transmembrane homeostasis protein A           | -1.50936       | 0.008587       |
| ibaG         | acid stress protein IbaG                     | 0.635081       | 0.016929       |
| srkA         | stress response kinase A                     | 0.741528       | 1.02E-05       |
| mltA         | membrane-bound lytic murein transglycosylase A| 0.961929       | 5.35E-05       |
| ybfA         | DUF2517 domain-containing protein YbfA        | -1.02759       | 0.013195       |
| ybgA         | DUF1722 domain-containing protein YbgA        | -1.93694       | 0.018844       |
| dnaG         | DNA primase                                   | 0.815044       | 0.000232       |
| csgG         | curli secretion channel                       | -1.53791       | 0.002594       |
| proP         | osmolyte:H+ symporter ProP                   | -1.25239       | 0.015265       |
| Gene | Description | Fold Change | P-value |
|------|-------------|-------------|---------|
| yqgC | protein YqgC | 1.006299 | 0.022184 |
| yaaU | putative transporter YaaU | -1.26135 | 0.026508 |
| yhjD | putative transporter YhjD | -1.08171 | 0.022303 |
| dtpD | dipeptide:H+ symporter DtpD | 1.187037 | 0.016882 |
| dnaE | DNA polymerase III subunit α | 0.73969 | 0.007525 |
| cspD | DNA replication inhibitor CspD | -0.94156 | 0.005748 |
| pbpC | peptidoglycan glycosyltransferase PbpC | 0.728647 | 0.020088 |
| gadC | L-glutamate:4-aminobutyrate antiporter | -1.45671 | 0.019172 |
| rlmN | 23S rRNA m2A2503 methyltransferase/tRNA m2A37 methyltransferase | 0.752209 | 0.004434 |
| ppiA | peptidyl-prolyl cis-trans isomerase A | 0.715015 | 0.023499 |
| glcB | malate synthase G | -0.76428 | 0.020917 |
| wcaC | colanic acid biosynthesis galactosyltransferase WcaC | -1.55763 | 0.010894 |
| dkgA | methylglyoxal reductase DkgA | -1.57133 | 0.015858 |
| dps | stationary phase nucleoid component that sequesters iron and protects DNA from damage | -2.4165 | 0.01543 |
| gadB | gadB | -1.96865 | 0.001756 |
| accA | acetyl-CoA carboxyltransferase subunit α | 0.688628 | 0.011405 |
| Gene | Description                               | Value       | P-value  |
|------|-------------------------------------------|-------------|----------|
| wzc  | protein-tyrosine kinase Wzc               | -1.39008    | 0.02307  |
| O3   |                                           |             |          |
| racR | Rac prophage; DNA-binding transcriptional repressor RacR | -0.79431    | 0.001077 |
| yffB | putative reductase YffB                   | 0.807003    | 0.003224 |
**Table S4.** Oligonucleotides used in this study.

| Oligonucleotides     | Sequence (5’ to 3’)                                                                 |
|----------------------|-------------------------------------------------------------------------------------|
| **Marker integration** |                                                                                     |
| cat_F                | TGTAGGCTGGAGCTGCTTC                                                                |
| cat_BamH1_R          | CGGGATCCCATATGAATATCCTCCTAGTTCC                                                   |
| sacB_F               | CTG ACA TGG GAA TTC TGA TCC                                                        |
| sacB_BamH1_R         | GAA TAC GGT TAG CCA TTT GCC GGA TCC CG                                             |
| araA_cat-sacB_F      | ATGACGATTTTTGATAATTATGAAGTGTGTTGTTCATTGGCAGCCAGCAGAGCACGCTTTAGAGCCTC               |
| araA_cat-sacB_R      | TTAGCGACGAACCCCGATAATACACTTCGTCCAGCGACGCGCTCTTTAACATATGAATATCCCTCCTAGTTCC         |
| REL606_araA_F        | ATGACGATTTTTGATAATTATGAAG                                                          |
| REL606_araA_R        | TTAGCGACGAACCCCGTAAT                                                              |
| araA_conf_F          | GCGCTTTGCTTATTCCGGCCCTAC                                                          |
| araA_conf_R          | GAGCGCCGAACAACACTATCTTCC                                                          |
| cat_outward          | CGGTCTGGTTATAGGTACATTTCC                                                          |
| sacB_outward         | CGCAACGCTGGATAGTTAGGCC                                                            |
| Contamination check                  |                 |
|-------------------------------------|----------------|
| MG1655_F                            | TTCGCAACACGATGATGAATCG |
| W3110_F                             | GCATAGCTCCACCATCTCTG |
| yjaA_R                              | AGGCAGCGTCTCCTGACAC |
| O3_lacZ_conf_F                      | GGTGTGATGTAGCTGACCACATCGG |
| O3_lacZ_conf_R                      | GAAATCCCAGATCTCCTATGGG |
| 27F                                 | AGAGTTTGATCTGACACG |
| 1492R                               | TACGGYTACCCTGTACCTG |

**TM-MAGE**

|                                |                                  |
|--------------------------------|----------------------------------|
| WT1_mrbD                        | GCGCAAAATGCCACTCCACaTGTTTAGAGCTGCTGCCCTATATCTCTATATCGTATTATTGTGGGTGCGGTAGATGCTTTCCGTT |
| WT1O31O33_rph                    | CTCATACGTCGGGCTCCCTTTAAAAATCAGTTTCCGACGCGCCCTTCTGCGG TTCCTGCCTCCTTGCACCTCAATAGATGCGGCCGTCCTCCTGGTCATCACACT |
| WT1_rpoB                        | TGATCTTAGAATACCCCTTGGTACCCAGACCGCAGCTGCGATCCATCTTGGTTACCGGAGCTGCTTTAACCaCCAGATATACCTTAA |
| WT2_cysE                        | GGTGCAGCCACCGGGGCACCTGGGCGGCAATCTTTCCGCGCCGGGCCAACTTCAATTGGGCGGATTTTTCCGCGCCCGCGCGCAATCATAACCATAC |
| WT2_rpoC                        | GCATATCGGTACCTGGGAGAACGCTCGGTTACCCCTGAGCATCAACGGTAAAACTTGGTTACCCGGGAGCTGCTTGAGATGCTGCAGCTG |
| WT3_rlmH                        | CGACGTGGTCGTCTACTCTGGTGTTGCGGCGCTGGAAGGGTTGTCGCCCTGCTGTAAGCGCGCGCGCTGAGCAGCTGGGTaGCTGTCCTGCCGCTG |
| WT3_rlmH2                       | AGGGTTGTCGCGCTGCTTAAAGCGCGCGGCTGAGCAGGATGGTTGTCGCGTACCTCCTCCCTCCCATCGCTGGTTCGGCGGCTG |

15
| Gene     | Sequence                                                |
|----------|---------------------------------------------------------|
| WT3_ydh | ACCTTTTCCCTGGAACGTTAATCTTTTGATAAATATTTATGTCTAACAAGTTGTATATTTTTTTTGAAACGCTGTTTTTTCTTTTTTGG       |
| O31_pykF  | GGCGGTAATCTGCTGCAGCAGTACGAATACTTCCGCCGGATGCGACACCACCTCCTGTCGACTGAGACCAACCGAAAAACGGGCTCCTCAGTTGG |
| O31_grtrB | ATGGGGCGTGGAGGATTATTTAGATACACTACATATTGGAAATGCTGTTAGGGGATATCCTTCACTACTTCTTTGTTGTTGTTAGCAGTTT |
| O31_spoT  | TCGTTAATCAACAAAGCGGGTCGCCCTTTGTATCTGTTTTGAAAGCTGAATCTCAACCTTGACACATTTTTTGGCGAGATTGCTTTTGGT |
| O31_wecE  | ACAAAGTGCGAATTACCCTACGCTCAGTTTGGACGGCGACGATGTTGAAATGCTGTTAGGGGATATCCTTCACTACTTCTTTGTTGTTAGCAGTTT |
| O31_rpoB  | GAACCTCGAAGCTTGCACGTTTCCAGGGGTCAAGACCGCGCTGCTGACTTCCCTGACACGGCACTGACGTTTGGTTGTCGATATTTCAGACAGCGGGTTG |
| O31_nagA  | AGTGGTCCGTTGGAATCTGGAATTAATGAAGACACCAATAATATATCTGTTTGTGCTAAGGAGCGGGCTGCTGATTTCCTGTGTTGAA       |
| O32_yeiH  | TTCTGTCTGGCTTGCTTCTGCGCCAGAAAGTGTTTTGGCTGGAATAAGCACAACACCTCGATGTTGCTGCGGCTGTTGCTTTCTGTAAGCAGTTG |
| O32_ptsP  | ACTTTTATACGCTAAGCTGGTTTTTTTTAATACTAAGCGGAAATGGAGGTTGCTCTACCTCTGAGCGGAGCACTGACGTTTGGCTTTTGGTTCAGTTG |
| O32_nusA  | AATCGTACATCTGTCGCTTCTTGCTCTGCTCTGACTAGTTTATGCTCGGCTCAGCCAGACAGCAGAAAAATATACGAGGAGCCATATACGAGT |
| O32_pyrErph | GCGGAAATTCCAATAATGGGCGTATATGTTTTGCTGCTCTGCTCTACACTTTACTTTCTCTACAAGCAAAAAAGCGAGACTCAATCGT |
| O33_ydcK  | GCTAGGCTACTTATATGACCGCGTTGCTTGTTTTCACTATATCGATGCGGTCACTTCTCCAGACCGACGTTTGGGTATTAGGAAAGGCTGTTG |
| O33_mreB  | CCACCAGTCACCAAGCGCAACTGGGCAAGCTGTTAATATGTGTTACGGCCCTAAACACCGCGGAAACGTCTTTTCTTCTACGGAGGGGATATT |
| O33_cyaA  | CTTTCATTATGCGGAAGAGTTTTCTCCAGGAGAAAGGCTGTACTTCCACCGAGGCGGAGCACGGCAGGCAATATATT |
| MASC-PCR  | Kim et al.                                              |
WT1_mrdB_WT_F  GGGGCCAGCCTTCATAACGC
WT1_mrdB_M_F  GGGGCCAGCCTTCATAACGT
WT1_mrdB_R  TGGCATGATGGAGCGTAAAATCGGCC
WT1O31O33_rph_WT_F  CCAGCGCCGCTTTCTGCG
WT1O31O33_rph_M_F  CCAGCGCCGCTTTCTGCC
WT1O31O33_rph_R  CCGCAGTTTTCTGTCGAATTGTGAACG
WT1_rpoB_WT_F  GCCGTGCTGAAAGATTGTTAAGGTATATCTGCG
WT1_rpoB_M_F  GCCGTGCTGAAAGATTGTTAAGGTATATCTGGT
WT1_rpoB_R  GACCGATGTTTCATACGAGACGGTACGC
WT2_cysE_WT_F  ACCGTTGCAAGCACCACCGA
WT2_cysE_M_F  CCGGTTCAGCACACCACGC
WT2_cysE_R  CAGCAAAAATTGTCGCGGTATCATGC
WT2_rpoC_WT_F  ACGTACCAGCGTGTTAAGATCTGC
WT2_rpoC_M_F  AGAACGTACCAGCGTGTTAAGATCTGT
WT2_rpoC_R  GAAGGAAACGATACGCTGATTTCAGCCA
WT3_rlmH_WT_F  GAGGGTAAGCGCCGACAGCG
WT3_rlmH_M_F  GGAGGTAAGCAGCGACAGCT
WT3_rlmH_R    AAACTCTGGAGTTAATGCGTGAAGCTGCA
WT3_ydhZ/pykF_WT_F GTCTAACAAGTGTATATTTTTGAACGAGCGCTGTTTTT
WT3_ydhZ/pykF_M_F TCTAACAAGTGTATATTTTTGAACGAGCGCTGTTTTG
WT3_ydhZ/pykF_R AGTTTGTCTCATCAGTTGCGCAGATTC
O31_pykF_WT_F  GCTACTCAGGGCGTAATCTGCTCG
O31_pykF_M_F  TGCTACTCAGGGCGTAATCTGCTCT
O31_pykF_R    CAACTGATGAGGCCGTTTTTCTTTTGGTGG
O31_spoT_WT_F  GTATCTGTTTGAAGCCTGAATCAACTGATTCAACCT
O31_spoT_M_F  CTGTTTGAAGCCTGAATCAACTGATTCAACCG
O31_spoT_R    TATCCTGTTAGGTCGCGGAGTATCTTCA
O31_wecE_WT_F  CCAAGAGAGCGAGCGCCTGCT
O31_wecE_M_F  CAAAGAGAGCGAGCGCCTGCC
O31_wecE_R    TACTGGGCAACGTATTTGTTACACCGGTAAG
O31_rpoB_WT_F  GACCAGAAACACCAGCTGCTGAGATT
O31_rpoB_M_F  ACCAGAAACACCAGCTGCTGAGATG
O31_rpoB_R     GGTCGCGGCTGAACAAGCTGG
O31_gtrB_WT_F  TGAATTCCACCTAAACAGTATTG
O31_gtrB_M_F   CTGAATTCCACCTAAACAGTATTA
O31_gtrB_R     AACTGATGGACGCCTGAAGC
O31_gtrB_R     AACTGATGGACGCCTGAAGC
O32_nagA_WT_F  CTTACGCACAAATTCGGATTATGGGTGCC
O32_nagA_M_F   GCTTACGCACAAATTCGGATTATGGGTGCT
O32_nagA_R     GCCCTGGCTCCTGCTCAGGG
O32_yeiH_WT_F  GTCTGGATAAGCACACCAGCTGGTGA
O32_yeiH_M_F   GTCTGGATAAGCACACCAGCTGGTTGT
O32_yeiH_R     CAGTGCCATCGGAGGTTTTCGC
O32_ptsP_WT_F  AGCATGACTTTTATACGCTACCGCTTTTAACTG
O32_ptsP_M_F   GAGCATGACTTTTATACGCTACCGCTTTTAACTA
O32_ptsP_R     TCTGGAACGCAGACTGACC
O32_nusA_WT_F  CGTCACCGAACCAGCAAATATTACGGGC
O32_nusA_M_F   CGTCACCGAACCAGCAAATATTACGGGG
O32_nusA_R     AGTGCCGAAATCGGCGAAGAAGTGA
| Gene          | WT Forward | M Forward | R Reverse |
|--------------|------------|-----------|-----------|
| O32_pyrE/rph | GGCGACTGATGAGTCGCC | GCGACTGATGAGTCGCC | CATCTACCAGCATTACCGTC |
| O33_ydcK     | CTGTCACCTATATATGCACCCTGGCTGATTTCACTATT | GCTGTCACCTATATATGCACCCTGGCTGATTTCACTATA | GATCGATCGCGAAACGTTGTTAGCAC |
| O33_ptsP     | CTGGCGACGTTAATAATTGGTGACGCTTA | TGGCGACGTTAATAATTGGTGACGCTTC | CTGTTCGGTCTACCTGGCCGATCATG |
| O33_mreB     | GAGCATTTTTTCAGTCAGCAAGAAGTCGGC | GGAGCATTTTTTCAGTCAGCAAGAAGTCGGA | CCCAGCTTTTCAGATTATCCCTTATGATTTGAAAAATTCG |
| O33_cyaA     | GGAAGTCAGCTTCTTCCTGATTGAGAAACCG | GGAAATCGCTTTCTCTCAGGATTGATTTGAAAAACCG | TGGAAATCGCTTTCTCTCAGGATTGATTTGAAAAACCT |
|              |            |           | GCCAGCAGACGTGGGTTCCG |
Table S5. Summary statistics of DNA and RNA sequencing.

| DNA sample | Number of reads after trimming | Total nucleotides | Mapping coverage in replication rate analysis |
|------------|-------------------------------|-------------------|-----------------------------------------------|
| WT         | 27,408,896                    | 2,751,469,002     | 591.5                                         |
| O3         | 18,485,284                    | 1,849,121,811     | 398.7                                         |
| 68WT-1     | 42,885,790                    | 4,304,381,946     | 927.2                                         |
| 68WT-2     | 31,031,580                    | 3,113,542,915     | 670.7                                         |
| 68WT-3     | 35,851,672                    | 3,596,492,772     | 774.7                                         |
| 68O3-1     | 30,502,760                    | 3,060,069,118     | 660.7                                         |
| 68O3-2     | 30,277,846                    | 3,037,049,923     | 655.7                                         |
| 68O3-3     | 32,031,690                    | 3,214,386,677     | 694                                           |

| RNA sample | Number of reads after trimming | Total nucleotides | Mapping coverage in RNA-Seq |
|------------|-------------------------------|-------------------|-----------------------------|
| WT replicate 1 | 26,809,176                  | 2,410,164,990     | 404.4                       |
| WT replicate 2 | 31,968,566                  | 3,135,369,779     | 503.1                       |
| O3 replicate 1 | 27,879,452                  | 2,758,656,031     | 458.7                       |
| O3 replicate 2 | 31,298,852                  | 3,009,089,965     | 486.5                       |
|                | Microbiology Spectrum | Kim et al. |
|----------------|-----------------------|------------|
| 68WT-1 replicate 1 | 26,723,722           | 2,635,340,009 | 446 |
| 68WT-1 replicate 2 | 33,294,134           | 3,254,862,968 | 529.5 |
| 68WT-2 replicate 1 | 27,074,456           | 2,667,130,885 | 445.6 |
| 68WT-2 replicate 2 | 38,831,588           | 3,845,954,857 | 580.7 |
| 68WT-3 replicate 1 | 30,401,556           | 2,964,122,954 | 485.9 |
| 68WT-3 replicate 2 | 27,790,244           | 2,746,941,128 | 453.3 |
| 68O3-1 replicate 1 | 30,092,928           | 2,986,114,490 | 490.1 |
| 68O3-1 replicate 2 | 29,176,030           | 2,863,813,890 | 497.6 |
| 68O3-2 replicate 1 | 29,930,186           | 2,913,359,163 | 481.6 |
| 68O3-2 replicate 2 | 30,194,776           | 2,975,981,498 | 489.6 |
| 68O3-3 replicate 1 | 27,111,316           | 2,654,066,683 | 472.5 |
| 68O3-3 replicate 2 | 33,374,454           | 3,297,369,466 | 519.3 |
Figure S1. Phenotypic difference between O3 lacZ dadX and O3 lacZ dadX araA (A) Growth curves of O3 lacZ dadX and O3 lacZ dadX araA strains. Optical densities were measured every 30 min in 200 μl M9 minimal medium supplemented with 0.4% glucose using a 96-well microplate. (B) Competitive fitness of O3 lacZ dadX araA against O3 lacZ dadX. O3 lacZ dadX, a derivative of E. coli MG1655 with two additional replication origins. O3 lacZ dadX araA, a derivative of E. coli MG1655 with two additional replication origins and arabinose utilization marker.
Figure S2. Experimental design of experimental evolution. During the experimental evolution, cells were transferred into a flask with a fresh M9 medium containing 0.4% glucose; each ancestor underwent evolution in three independent flasks (WT-1, WT-2, WT-3; O3-1, O3-2, O3-3). WT, wild-type *E. coli* MG1655; O3, a derivative of *E. coli* MG1655 with two additional replication origins and arabinose utilization marker.
Figure S3. Growth curves of ancestral and evolved populations. Optical densities were measured every 30 min in 200 μl M9 minimal medium supplemented with 0.4% glucose using a 96-well microplate. WT, wild-type *E. coli* MG1655; O3, a derivative of *E. coli* MG1655 with two additional replication origins and arabinose utilization marker.
Figure S4. Effect of dominant mutations on fitness. Growth rates were measured in M9 minimal medium supplemented with 0.4% glucose. Relative fitness was calculated by dividing the growth rates of mutant strains by ancestral strain. Grey line, the relative growth rate of each recipient strain. WT, wild-type *E. coli* MG1655; O3, a derivative of *E. coli* MG1655 with two additional replication origins.
**Figure S5.** Position-dependent differentially expressed gene (DEG) cluster analysis. DEG cluster analysis was conducted on the final descendant populations (68WT-1 – 68O3-3). DEGs were calculated using the respective ancestral strains as a control. WT, wild-type *E. coli* MG1655; O3, a derivative of *E. coli* MG1655 with two additional replication origins and arabinose utilization marker.
Figure S6. DNA sequencing read quality after read trimming. (A) Length of sequencing reads. (B) GC-contents of sequencing reads. (C) PHRED score of sequencing reads.
Figure A: Histograms showing the distribution of read lengths for different replicates. The x-axis represents read length, and the y-axis represents the percentage of reads.

Figure B: Histograms illustrating the distribution of GC percent for different replicates. The x-axis represents GC percent, and the y-axis represents the percentage of reads.

Figure C: Histograms depicting the distribution of PHRED scores for different replicates. The x-axis represents PHRED score, and the y-axis represents the percentage of reads.
Figure S7. RNA sequencing read quality after read trimming. (A) Length of sequencing reads. (B) GC-contents of sequencing reads. (C) PHRED score of sequencing reads.
SUPPLEMENTAL REFERENCES

1. Yang HJ, Kim K, Kwon SK, Kim JF. 2022. *Escherichia coli* cell factories with altered chromosomal replication scenarios exhibit accelerated growth and rapid biomass production. Microb Cell Fact 21:125.

2. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-5.

3. Edwards RA, Keller LH, Schifferli DM. 1998. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene 207:149-57.

4. Lennen RM, Nilsson Wallin AI, Pedersen M, Bonde M, Luo H, Herrgard MJ, Sommer MO. 2016. Transient overexpression of DNA adenine methylase enables efficient and mobile genome engineering with reduced off-target effects. Nucleic Acids Res 44:e36.