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Evolutionary Biology

Experimental evolution for the recovery of growth loss due to genome reduction

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Abstract

As the genome encodes the information crucial for cell growth, a sizeable genomic deficiency often causes a significant decrease in growth fitness. Whether and how the decreased growth fitness caused by genome reduction could be compensated by evolution was investigated here. Experimental evolution with an *Escherichia coli* strain carrying a reduced genome was conducted in multiple lineages for approximately 1,000 generations. The growth rate, which was largely declined due to genome reduction, was considerably recovered, associated with the improved carrying capacity. Genome mutations accumulated during evolution were significantly varied across the evolutionary lineages and were randomly localized on the reduced genome. Transcriptome reorganization showed a common evolutionary direction and conserved the chromosomal periodicity, regardless of highly diversified gene categories, regulons, and pathways enriched in the differentially expressed genes. Genome mutations and transcriptome reorganization caused by evolution, which were found to be dissimilar to those caused by genome reduction, must have followed divergent mechanisms in individual evolutionary lineages. Gene network reconstruction successfully identified three gene modules functionally differentiated, which were responsible for the evolutionary changes of the reduced genome in growth fitness, genome mutation, and gene expression, respectively. The diversity in evolutionary approaches improved the growth fitness associated with the homeostatic transcriptome architecture as if the evolutionary compensation for genome reduction was like all roads leading to Rome.

eLife assessment

This is an **important** study of the recovery of genome-reduced bacterial cells in laboratory evolution experiments, to understand how they regain their fitness. Through the analysis of gene expression and a series of tests, the authors present **convincing** evidence indicating distinct molecular changes in the evolved bacterial strains, although the precise mechanisms remain uncharacterized. These findings imply that diverse mechanisms are employed to offset the effects of a reduced genome, offering intriguing insights into genome evolution.

Introduction

The genome encodes the information for cell growth, and its size is likely the evolutionary consequence [1–3]. To determine the essential genomic sequence of modern cells, removing redundant DNA sequences from the wild-type genome in bacteria, so-called genome reduction, has been challenged to a large extent [4–7]. These efforts have been made to discover the minimal genetic requirement for a free-living organism growing under the defined conditions [8–10]. It resulted in the finding of the coordination of genome with cell growth, i.e., genome reduction significantly decreased the growth rate of *Escherichia coli* (*E. coli*) cells independent of culture media or growth forms [11–13]. Slow growth or fitness decline was commonly observed in the genetically reduced [13,14] and chemically synthesized genomes [10,15].

The growth decrease could be recovered by experimental evolution. Growth fitness generally represents the adaptiveness of the living organism to the defined environment [16]. Although the reduced genomes somehow showed differentiated evolvability compared to the wild-type genomes [17–19], their evolutionary adaptation to the environmental changes has been successfully achieved [20–22]. Experimental evolution under the defined culture condition successfully increased the decreased growth rates of the reduced genomes [17,20,21] and fastened the slow-growing synthetic genome [23]. The evolutionary rescue of the growth rate must be associated with the changes in genomic sequence and gene expression benefited for the growth fitness, as what happened in nature adaptive evolution [24–26]. Our previous study showed that the decreased growth rate caused by the absence of a sizeable genomic sequence could be complemented by introducing the mutations elsewhere in the genome [20,21]. Additionally, the changes in growth rate caused by either genome reduction or experimental evolution were dependent on the genome size but not the specific gene function [20].

A genome-wide understanding of the evolutionary compensated fitness increase of the reduced genome is required. The experimental evolution compensated for the genome reduction-mediated growth changes were considered stringently related to transcriptome reorganization. Previous studies have identified conserved features in transcriptome reorganization, despite significant disruption to gene expression patterns resulting from either genome reduction or experimental evolution [27–29]. The findings indicated that experimental evolution might reinstate growth rates that have been disrupted by genome reduction to maintain homeostasis in growing cells. Although the reduced growth rate caused by genome reduction could be recovered by experimental evolution, it remains unclear whether such an evolutionary improvement in growth fitness was a general feature of the reduced genome and how the genome-wide changes occurred to match the growth fitness increase. In the present study, we performed the experimental evolution with a reduced genome in multiple lineages and analyzed the evolutionary changes of the genome and transcriptome.

Results

Fitness recovery of the reduced genome by experimental evolution

Experimental evolution of the reduced genome was conducted to regain the growth fitness, which was decreased due to genome reduction. The *E. coli* strain carrying a reduced genome, derived from the wild-type genome W3110, showed a significant decline in its growth rate in the minimal medium compared to the wild-type strain [13]. To improve the genome reduction-mediated decreased growth rate, the serial transfer of the genome-reduced strain was performed with multiple dilution rates to keep the bacterial growth within the exponential phase (Fig. S1), as

described [17,20]. Nine evolutionary lineages were conducted independently (Fig. S2, Table S1). A gradual increase in growth rate was observed along with the generation passage, which was combined with a rapid increase in the early evolutionary phase and a slow increase in the later phase (**Fig. 1A**). As the growth increases were calculated according to the initial and final records, the exponential growth rates of the ancestor and evolved populations were obtained according to the growth curves for a precise evaluation of the evolutionary changes in growth. The results demonstrated that most evolved populations (Evos) showed improved growth rates, in which eight out of nine Evos were highly significant (**Fig. 1B**, upper). However, the magnitudes of growth improvement were considerably varied, and the evolutionary dynamics of the nine lineages were somehow divergent (Fig. S2). It indicated the diversity in the evolutionary approaches for improved fitness. Eight of nine Evos achieved faster growth than the genome-reduced ancestor (Anc), whereas all Evos decreased in their saturated population densities (**Fig. 1B**, bottom). In comparison to the wild-type strain carrying the full-length genome (WT), the primarily decreased growth rate caused by genome reduction was significantly improved by experimental evolution (**Fig. 1C**, upper), associated with the considerable reduction in saturated density (**Fig. 1C**, bottom). It demonstrated that the experimental evolution could compensate for the genome reduction with a trade-off in population size (carrying capacity), consistent with the previous findings [20,21].

Intriguingly, a positive correlation was observed between the growth fitness and the carrying capacity of the Evos (**Fig. 1D**). It was somehow consistent with the positive correlations between the colony growth rate and the colony size of a genome-reduced strain [11] and between the growth rates and the saturated population size of an assortment of genome reduced strains [13]. Nevertheless, the negative correlation between growth rate and carrying capacity, known as the *r/K* selection[30,31] was often observed as the trade-off relationship between *r* and *K* in the evolution and ecology studies [32] [33,34]. As the *r/K* trade-off was proposed to balance the cellular metabolism that resulted from the cost of enzymes involved [34], the deleted genes might play a role in maintaining the metabolism balance for the *r/K* correlation. On the other hand, the experimental evolution (i.e., serial transfer) was strictly performed within the exponential growth phase; thus, the evolutionary selection was supposed to be driven by the growth rate without selective pressure to maintain the carrying capacity. The declined carrying capacity might have been its neutral “drift” but not a trade-off to the growth rate. Independent and parallel experimental evolution of the reduced genomes selecting either *r* or *K* is required to clarify the actual mechanisms.

Significant variation and random localization of genome mutations

Genome resequencing (Table S2) identified a total of 65 mutations fixed in the nine Evos (**Table 1**). The number of mutations largely varied among the nine Evos, from 2 to 13, and no common mutation was detected in all nine Evos (Table S3). A 1,199-bp deletion of *insH* was frequently found in the Evos (Table S3, highlighted), which well agreed with its function as a transposable sequence. 51 out of 65 mutations occurred in the genes, and 45 out of 51 were SNPs. As 36 out of 45 SNPs were nonsynonymous, which was highly significant compared to random mutations ($p < 0.01$), the mutated genes might benefit fitness increase. In addition, the abundance of mutations was unlikely to be related to the magnitude of fitness increase. There was no significant correlation between the number of mutations and the growth rate in a statistical view ($p > 0.1$). Even from an individual close-up viewpoint, the abundance of mutations poorly explained the fitness increase. For instance, A2 accumulating only two mutations presented a highly increased growth rate compared to F2 of eight mutations (**Table 1**), which poorly improved the growth (**Fig. 1B**). B2, D2, and E2 all succeeded in increasing fitness to an equivalent degree (**Fig. 1B**), whereas they fixed 13, 7, and 3 mutations, respectively (**Table 1**). The mutated genes were varied in 14 gene categories, somehow more frequently in the gene categories of Transporter, Enzyme, and Unknown function (Fig. S3). They seemed highly related to essentiality[7] (*https://*

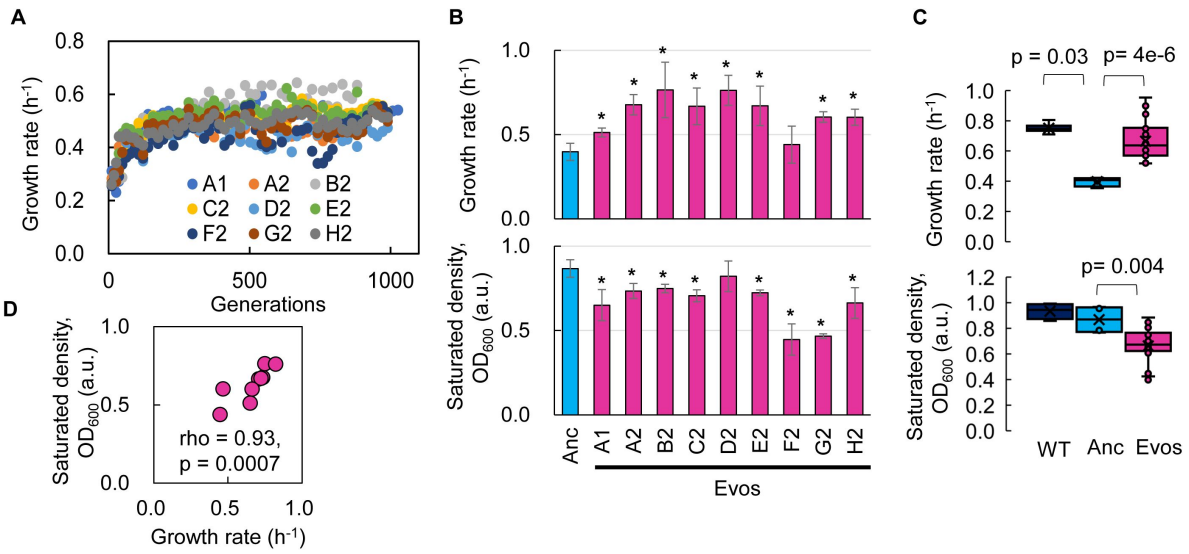

Figure 1

Fitness increase of the reduced genome mediated by experimental evolution.

A. Temporal changes in growth rate. Color variation indicates the nine evolutionary lineages. **B.** Growth rate and maximal population size of the reduced genome. Blue and pink indicate the common ancestor and the nine evolved populations, respectively. Standard errors are shown according to the biological replications (N=4~6). **C.** Boxplots of growth rate and maximum. Cross and open circles indicate the mean and individual values, respectively. Statistic significance evaluated by Mann-Whitney U tests is indicated. **D.** Correlation between growth rate and maximum. Spearman's rank correlation coefficient and p-value are indicated.

shigen.nig.ac.jp/ecoli/pec/genes.jsp), as 11 out of 49 mutated genes were essential (Table S3). Although the essentiality of genes might differ between the wild-type and reduced genomes, the experimentally determined 302 essential genes in the wild-type *E. coli* strain were used for the analysis, of which 286 were annotated in the reduced genome. The ratio of essential genes in the mutated genes was significantly higher than in the total genes (286 out of 3290 genes, Chi-square test p=0.008). As the essential genes were determined according to the growth[35] and were known to be more conserved than nonessential ones [36,37], the high frequency of the mutations fixed in the essential genes was highly intriguing and reasonable. The large variety of genome mutations fixed in the independent lineages might result from a highly rugged fitness landscape [38]. Nevertheless, it was unclear whether and how these mutations were explicitly responsible for recovering the growth rate of the reduced genome.

Additionally, there were no overlapping genomic positions for the 65 mutations in the nine Evos (**Fig. 2A**). Random simulation was performed to verify whether there was any bias or hotspot in the genomic location for mutation accumulation due to the genome reduction. A total of 65 mutations were randomly generated on the reduced genome (**Fig. 2B**), and the genomic distances from the mutations to the nearest genome reduction-mediated scars were calculated. Welch's t-test was performed to evaluate whether the genomic distances calculated from random mutations significantly differed from those from the mutations accumulated in the Evos. As the mean of p values (1,000 times of random simulations) was insignificant (**Fig. 2C**, $\mu_p > 0.05$), the mutations fixed on the reduced genome were either closer or farther to the genomic scars, indicating there was no locational bias for mutation accumulation caused by genome reduction.

Common evolutionary direction and homeostasis in transcriptome reorganization

Since no specificity was detected in the genome mutations, whether these mutations disturbed the genome-wide gene expression pattern was investigated. Hierarchical clustering and principal component analysis (PCA) showed that the evolved transcriptomes were directed to similar patterns but divergent to the WT transcriptome (Fig. S4). The evolutionary direction of the transcriptomes of the reduced genome was not approaching the wild-type transcriptome. As the *E. coli* chromosome was structured, whether the genome reduction caused the changes in its architecture, which led to the differentiated transcriptome reorganization in the Evos, was investigated. The chromosomal periodicity of gene expression was analyzed to determine the structural feature of genome-wide pattern, as previously described [28,39]. The analytical results showed that the transcriptomes of all Evos presented a common six-period with statistical significance, equivalent to those of the wild-type and ancestral reduced genomes (**Fig. 3A**, Table S4). It demonstrated that the chromosomal architecture of gene expression patterns remained highly conserved, regardless of the considerably varied mutations. The homeostatic periodicity was consistent with our previous findings that the chromosomal periodicity of the transcriptome was independent of genomic or environmental variation [27,28].

In addition, the genomic locations of the mutations seemed irrelevant to chromosomal periodicity (**Fig. 3A**, red lines). No mutagenesis hotspot was observed even if these mutations were accumulated on a single genome (**Fig. 2A**). Alternatively, the expression levels of the mutated genes were somehow higher than those of the remaining genes (**Fig. 3B**). As the regulatory mechanisms or the gene functions were supposed to be disturbed by the mutations, the expression levels of individual genes might have been either up- or down-regulated. Nevertheless, the overall expression levels of all mutated genes tended to be increased. One of the reasons was assumed to be the mutation essentiality, which remained to be experimentally verified. The ratio of the essential genes in the mutated genes was ~ 22% (Table S3), much higher than the ratio (~9%) of essential to all genes (286 out of 3,290) in the reduced genome. As the essential genes showed higher expression levels than the nonessential ones [40], the high essentiality of the mutated

Evos	All	Intergenic	Genic		Genic SNP	
			Indel	SNP	N	S
A1	3	0	0	2	1	1
A2	2	1	0	1	1	0
B2	13	0	1	11	9	2
C2	11	1	0	9	8	1
D2	7	0	1	5	4	1
E2	3	1	0	1	1	0
F2	8	1	0	6	5	1
G2	12	2	2	6	4	2
H2	6	0	2	4	3	1
Sum	65	6	6	45	36	9

Table 1

Overview of fixed genome mutations.

The number of mutations in the nine Evos are shown separately and summed. SNP, N and S indicate single nucleotide substitution, nonsynonymous and synonymous SNP, respectively.

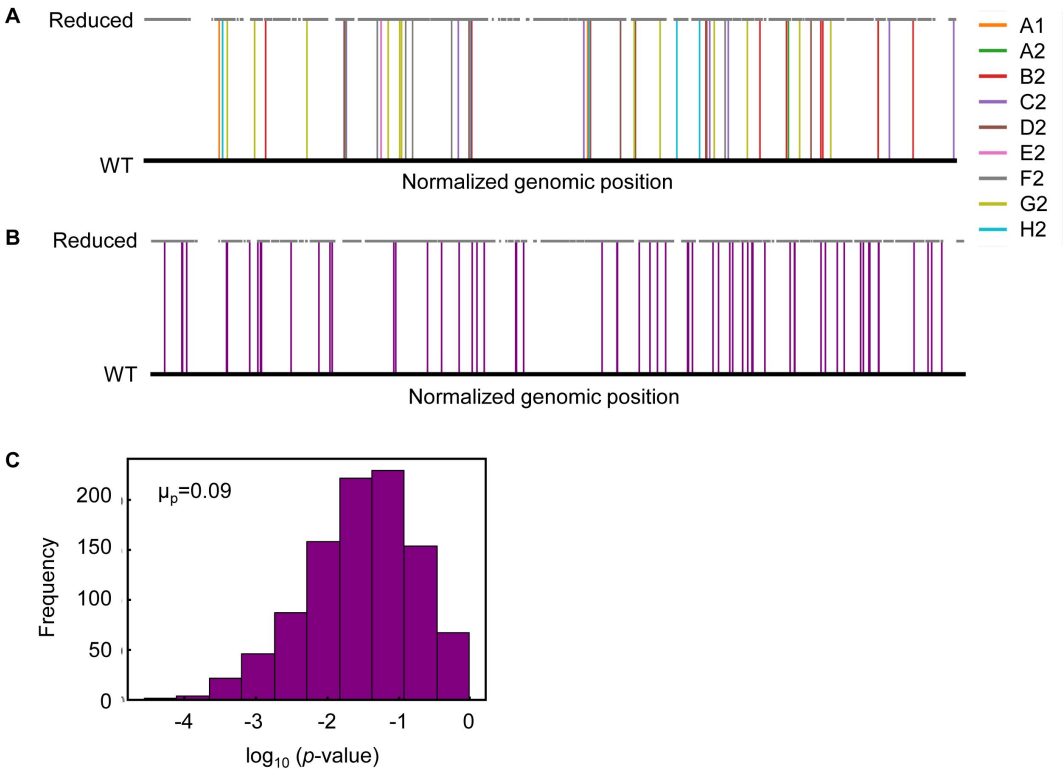

Figure 2

Genomic localization of mutations.

A. Normalized genomic positions of all mutations. The vertical lines highlight the total 65 mutations fixed in the nine Evos. Color variation indicates the nine Evos. WT and Reduced represent the wild-type and reduced genomes used in the present study. **B.** Normalized genomic positions of random mutations. The simulation of 65 mutations randomly fixed in the reduced genome was performed 1,000 times. As an example of the simulation, the genomic positions of 65 random mutations are shown. The vertical lines in purple indicate the mutations. **C.** Statistic significance of the genome locational bias of mutations. The distance from the mutated location to the nearest genomic scar caused by genome reduction was calculated. The mutations accumulated in the nine Evos and the 1,000-time random simulation were all subjected to the calculation. The significance of genome locational bias of the mutations in Evos was evaluated by Welch's t-test. The histogram of 1,000 tests for 1,000 simulated results is shown. The mean of *p*-values (μ_p) is indicated, which is within the 95% confidence interval ($0.07< \mu_p < 0.09$).

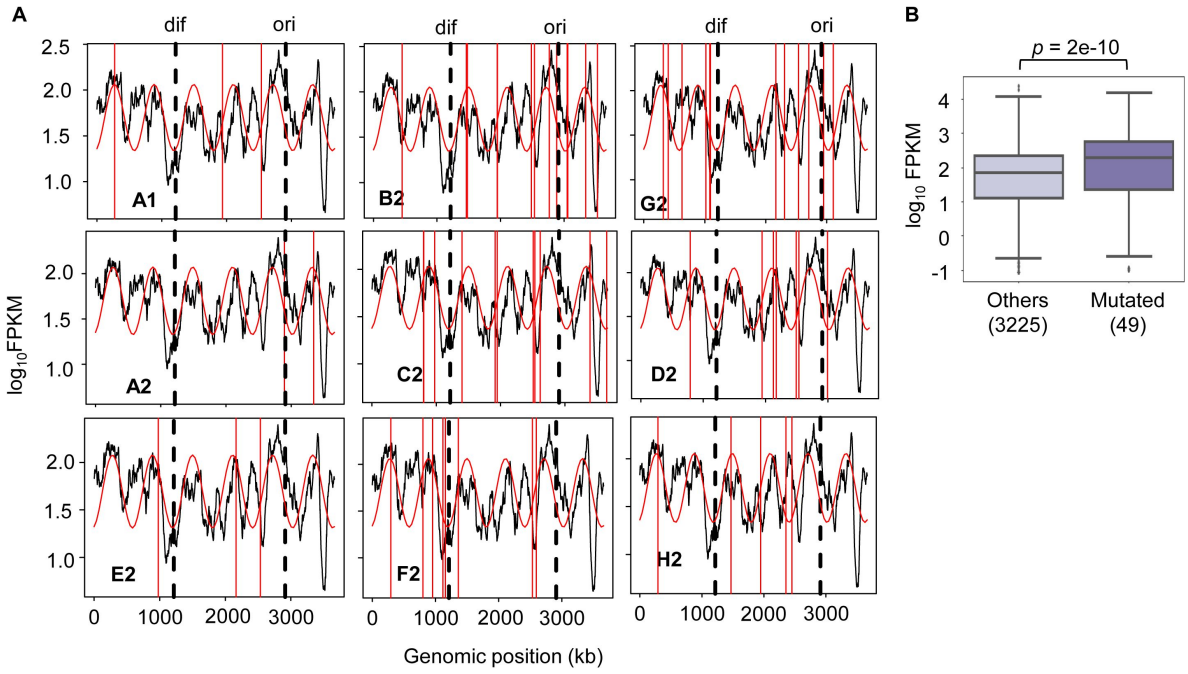

Figure 3

Chromosomal periodicity of transcriptome and mutated gene expression.

A. Chromosomal periodicity of transcriptomes. The transcriptomes of the nine Evos are shown. Black lines, red curves, and red vertical lines indicate the gene expression levels, fitted periods, and locations of mutations, respectively. *Ori* and *dif* are indicated with the vertical broken lines. **B.** Boxplot of gene expression levels. Gene expression levels of the 49 mutated genes in the nine Evos and the remaining 3,225 genes are shown. Statistic significance evaluated by Welch's t-test is indicated.

genes might result in a higher mean expression level. On the other hand, the high frequency of the mutations fixed in the essential genes was unexpected because the essential genes were generally more conserved than nonessential ones [36]. The essentiality of the mutations might have participated in maintaining the homeostatic transcriptome architecture of the reduced genome.

Diversified functions and pathways of the differentially expressed genes

As the evolved transcriptomes were differentiated from those of the WT and reduced genomes (Fig. S4), the differentially expressed genes (DEGs) were further identified to discover the gene functions or biological processes contributing to the fitness changes. Instead of an in-depth survey on the directional changes of the DEGs, the abundance and functional enrichment of DEGs were investigated to achieve an overview of how significant the genome-wide fluctuation in gene expression, which ignored the details of individual genes. The abundance of DEGs among the Evos varied from 333 to 1,130 genes and of few overlaps (**Fig. 4A**). Most DEGs were unique to each evolutionary lineage, and the common DEGs across all Evos were only 108 genes (Table S5). The number of DEGs partially overlapped among the Evos declined along with the increased lineages of Evos (**Fig. 4B**). Enrichment analysis showed that only the histidine-related pathways were significantly enriched in the common DEGs (**Fig. 4C**). Functional enrichment of the DEGs in individual Evos showed that the amino acid metabolism considerably participated and that the enriched pathways were poorly overlapped (Fig. S5). These findings strongly suggested no universal rule for evolutionary changes of the reduced genome.

In comparison, 1,226 DEGs were induced by genome reduction. The common DEGs of genome reduction and evolution varied from 168 to 540, fewer than half of the DEGs responsible for genome reduction in all Evos (**Fig. 5A**). The conclusion remained consistent even if the DEGs were determined with an alternative method, RankProd (Fig. S6). Functional enrichment of the DEGs observed the specific transcriptional regulation and metabolic pathways participating in the transcriptome reorganization in response to genome reduction and evolution. Only σ38 was enriched in the genome reduction-mediated DEGs, which was the only regulon that partially overlapped with the Evos (**Fig. 5B**). No regulon in common was enriched, besides a few partially overlapped regulons, i.e., GadW, GadX, and RcsB (**Fig. 5B**). In addition, both the number of enriched pathways and their overlaps were significantly differentiated among the nine Evos or between Evos and reduced genome (**Fig. 5C**). No common pathways were commonly enriched between the genome reduction-mediated DEGs and Evos, no matter annotated the metabolic pathways with GO or KEGG (Fig. S7). The flagellar assembly, the only enriched pathway in genome reduction-mediated DEGs, was absent in all Evos (**Fig. 5D**). Alternatively, the amino acids-related metabolisms were frequently detected in the Evos, e.g., histidine metabolism, biosynthesis of amino acids, etc. (**Fig. 5D**, Fig. S7). The variable pathways in the Evos indicated that evolution compensated for the genome reduction in various ways, which differed from how the genome reduction was caused.

Gene modules responsible for the evolutionary changes of the reduced genome

Genome mutation analysis and transcriptome analysis failed to identify the common gene categories or pathways that correlated to the evolution of the reduced genome; thus, the gene modules correlated to evolution were newly evaluated. The weighted gene co-expression network analysis (WGCNA) [41] was performed toward the evolved transcriptomes as tested previously [27]. Reconstruction of the 3,290 genes in the reduced genome led to 21 gene modules, comprising 8 to 320 genes per module (Fig. S8). Hierarchical clustering of these modules showed that roughly three major classes could be primarily divided (**Fig. 6A**). Functional correlation analysis showed that three of 21 modules (M2, M10, and M16) were highly significantly correlated to the growth

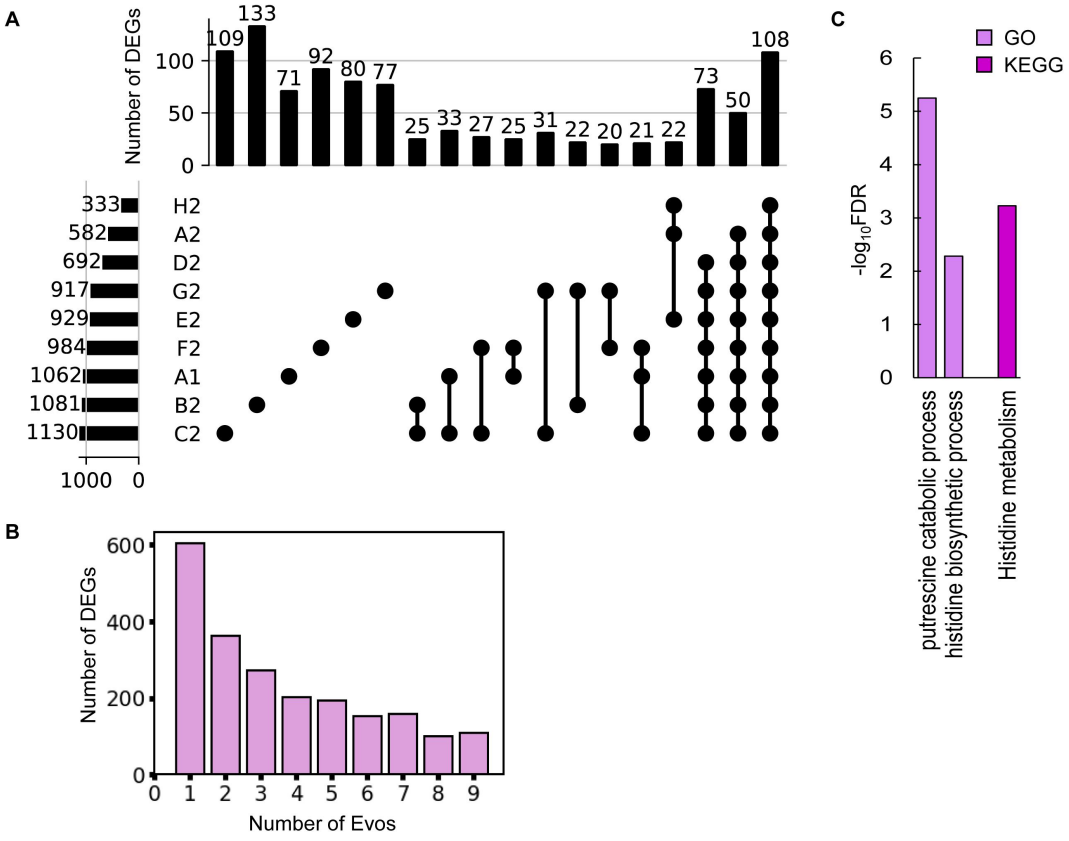

Figure 4

Differentially expressed genes (DEGs) and their enriched functions.

A. Commonality of DEGs in the nine Evos. Closed circles represent the combinations of the Evos. Vertical and horizontal bars indicate the number of the overlapped DEGs in the combinations and the number of all DEGs in each Evos, respectively. The combinations with more than 20 DEGs in common are shown. **B.** The number of DEGs overlapped among the Evos. The numbers of DEGs overlapped across 2 to 9 Evos are shown. The number of Evos detected in the single Evos is indicated as 1. **C.** Enriched function in common. The KEGG and GO terms enriched in the common DEGs across the nine Evos are shown. The statistical significance (FDR) of the enriched pathways and biological processes is shown on a logarithmic scale represented by color gradation.

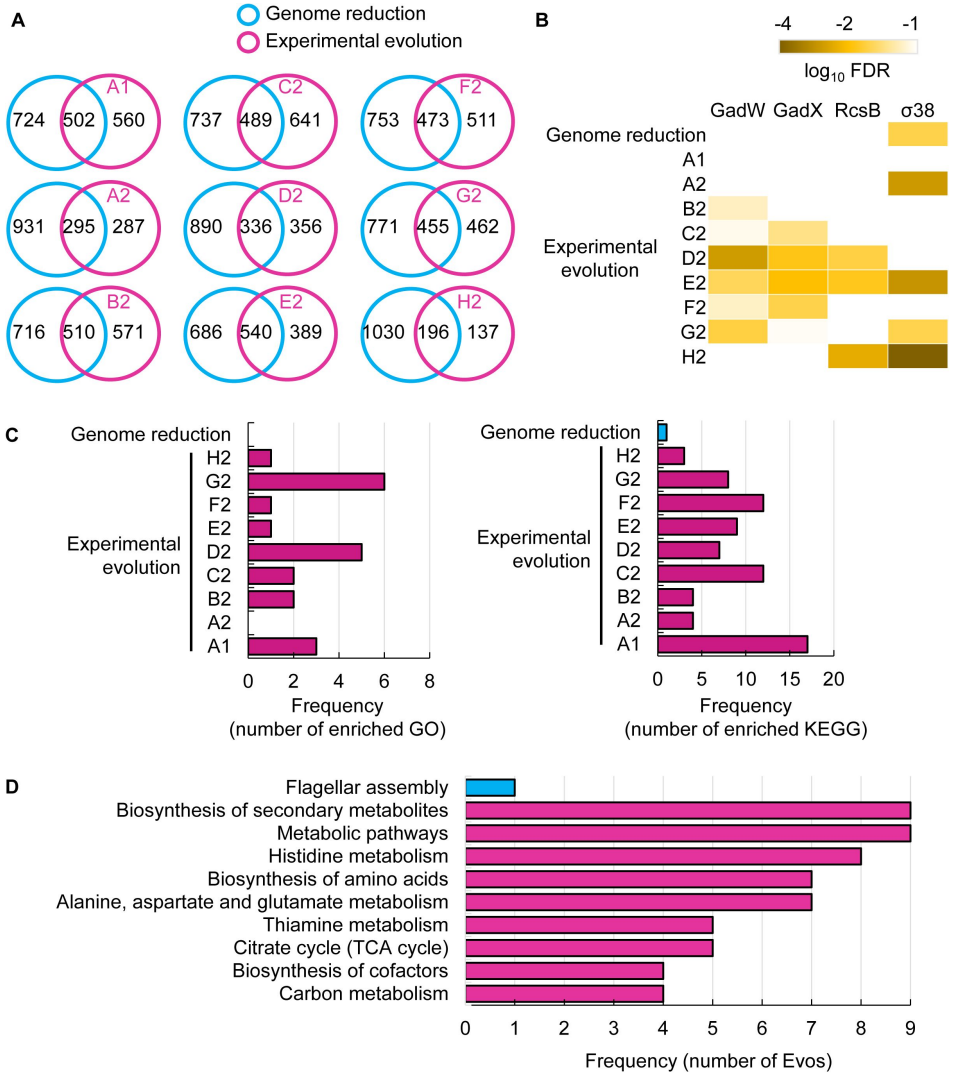

Figure 5

Transcriptome comparison between genome reduction and evolution.

A. Venn diagrams of DEGs induced by genome reduction and evolution. The numbers of individual and overlapped DEGs are indicated. **B.** Heatmap of enriched regulons. Statistically significant regulons are shown with the FDR values on a logarithmic scale. **C.** Number of enriched functions in common. Left and right panels indicate the numbers of enriched GO terms and KEGG pathways caused by genome reduction and evolution, respectively. **D.** Enriched functions in common. The overlapped GO terms enriched in the nine Evos and genome reduction are shown. Blue and pink represent genome reduction and evolution, respectively.

fitness, the number of DEGs, and mutation frequency, respectively (**Fig. 6A**). These modules M2, M10 and M16 might be considered as the hotspots for the genes responsible for growth fitness, transcriptional reorganization, and mutation accumulation of the reduced genome in evolution, respectively. It indicated that the three modules were highly essential and functionally differentiated for growth control, transcriptional change, and mutagenesis.

Enrichment analyses further identified the gene categories, regulons, and metabolisms that significantly appeared in the three modules (**Fig. 6B**). Two gene categories of nonessential function were enriched in M10, and the module correlated to the number of DEGs; in contrast, no gene category was enriched in M2 and M16 (**Fig. 6B**, left). All three modules successfully enriched the regulons without overlaps (**Fig. 6B**, middle). It indicates that the main regulatory mechanisms participating in the three modules for growth control, transcriptional change, and mutagenesis were divergent. GO enrichment resulted in various biological processes in the three modules, roughly related to transport, transposition, and translation in M2, M10, and M16, respectively (**Fig. 6B**, right). Compared to the enriched functions of the genes that disappeared due to genome reduction (**Fig. 6B**, bottom), the gene categories of phage and unknown function and the biological processes related to DNA transposition and integration were commonly identified in M10. It strongly indicated that M10 was responsible for genome reduction. The newly constructed gene networks successfully identified three modules correlated to mutation, DEGs, and growth, revealing the functional differentiation responsible for evolution to maintain the homeostatic transcriptome architecture for a growing cell.

Discussion

The evolutionary compensation for genome reduction was directed toward an identical goal of increased fitness but differentiated in the manner of genomic changes. Firstly, various genetic functions seemed to trigger the increased growth rates of the Evos. A few mutations could compensate for the sizeable genomic deficiency. In particular, mutations in the essential genes, such as RNA polymerases (*rpoA*, *rpoB*, *rpoD*) identified in three Evos (Table S3), were supposed to participate in the global regulation for improved growth. Nevertheless, the considerable variation in the fixed mutations without overlaps among the nine Evos (**Table 1**) implied no common mutagenetic strategy for the evolutionary improvement of growth fitness. It was supported by the fact that no genomic locational bias for mutations fixed in the evolution (**Fig. 2**). Secondly, the transcriptomes presented conserved chromosomal architectures (**Fig. 3**) and universal directional changes (Fig. S4), regardless of the significant and differentiated changes in gene expression in response to evolution (**Fig. 4**, Fig. S5). Although the periodicity of chromosomal architecture was well known [42,43] and coordinated with the bacterial growth rate[28,44], employing its homeostasis as the evolutionary consequence provided a conceptual and unique understanding of growing cells.

It's unclear whether the differentiation in evolutionary paths was particularly significant for the reduced genome used in the present study. Evolution studies often focus on finding the common mutations accumulated in multiple evolutionary lineages to obtain the reasonable mechanism responsible for the adaptation to the defined condition. Common mutations[22,45] or identical genetic functions[46] were reported in the experimental evolution with different reduced genomes, commonly known as parallel evolution (**Fig. 7**, i). In addition, as not all mutations contribute to the evolved fitness [22,46], another strategy for varied phenotypes was known as divergent evolution (**Fig. 7**, ii). The present study accentuated the variety of mutations fixed during evolution. Considering the high essentiality of the mutated genes (Table S3), most or all mutations were assumed to benefit the fitness increase, partially demonstrated previously [20]. Nevertheless, the evolved transcriptomes presented a homeostatic architecture, revealing the divergent to convergent evolutionary strategy (**Fig. 7**, iii). Multiple evolutionary paths for the reduced genome to improve growth fitness were likely all roads leading to Rome.

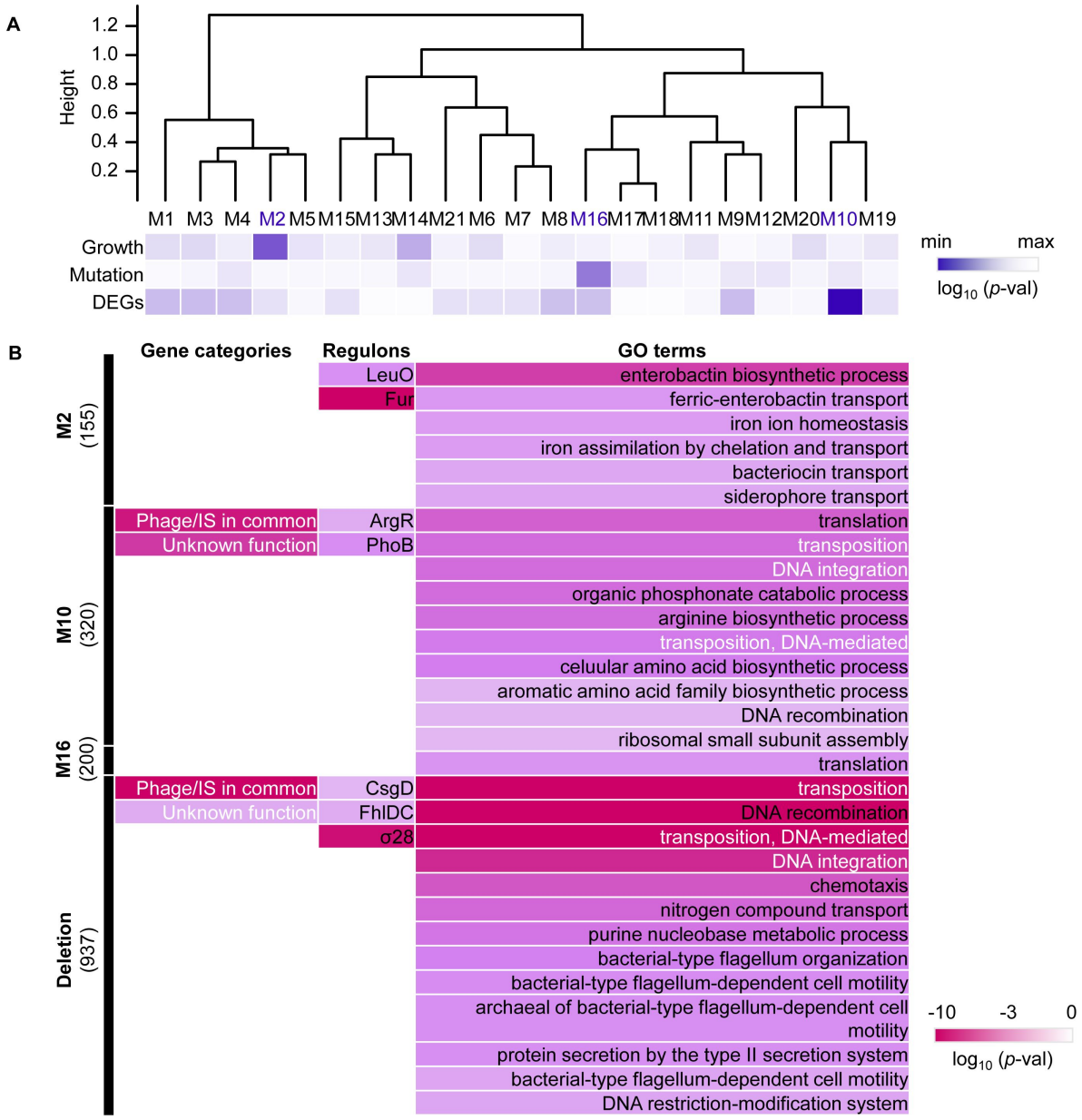

Figure 6

Reconstructed gene modules.

A. Cluster dendrogram of the gene modules reconstructed by WGCNA. A total of 21 gene modules (M1~M21) were reconstructed. The significance of the correlation coefficients of the gene modules to growth, mutation, and expression is represented in purple gradation. From light to dark indicates the logarithmic p-values from high to low. **B.** Enriched functions of gene modules and deletion. Enriched gene categories, regulons, and GO terms are shown from left to right. The numbers of the genes assigned in the three gene modules and the genomic deletion for genome reduction are indicated in the brackets. Color gradation indicates the normalized *p* values on a logarithmic scale.

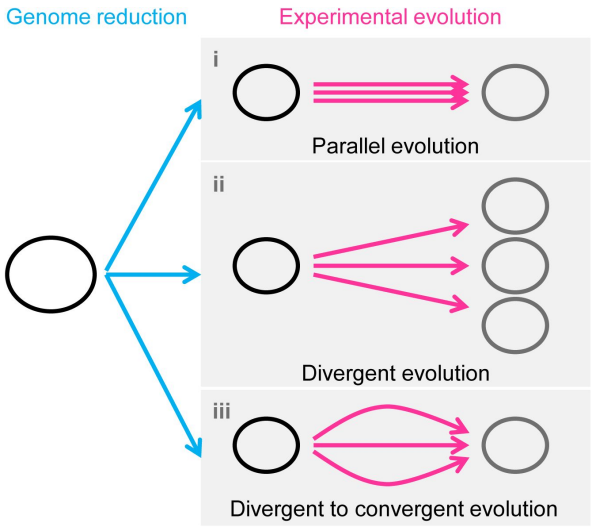

Figure 7

Schematic drawing of evolutionary approaches for the reduced genome.

Three evolutionary strategies are proposed. Pink and blue arrowed lines indicate experimental evolution and genome reduction, respectively. The size of the open cycles represents the genome size. Black and grey indicate the ancestor and evolved genomes, respectively.

In addition, the transcriptome reorganization for fitness increase triggered by evolution differed from that for fitness decrease caused by genome reduction. General analyses failed to detect the regulatory network or genetic function mediated by genome reduction and evolution in common. Instead, the newly constructed gene modules successfully enriched the gene categories of mobile elements and unknown functions (**Fig. 6B**, left) as the evolutionary compensation for genome reduction. The represented mobile elements, flagella, were known to be responsive to environmental stresses such as hypoosmotic pressure or pH [47,48]. Genome reduction and evolution seemed equivalent to the stress response in *E. coli*. These findings were reasonable as enterobactin protected *E. coli* from oxidative stress [49,50], and enterobactin biosynthesis was upregulated for biofilm formation in genome-reduced *E. coli* [51]. The compensation of evolution to genome reduction not only verified the known function and mechanism from a global regulatory viewpoint but also revealed a novel understanding of the molecular mechanisms and gene functions.

The discriminated functions of gene modules might play a crucial role in response to genomic and evolutionary changes. WGCNA was conducted to discover the potential correlation of gene expression to growth fitness. It succeeded in finding the genes participating in the evolutionary changes of the reduced genome to regain growth fitness. Three enriched gene modules were assumed separately responsible for replication, transcription, and population dynamics (**Fig. 6B**). The growth-correlated gene module significantly enriched the iron-related biological functions (M2). Although the translation was commonly reported to be correlated to the growth rate [52,53], it was enriched in the gene module coordinated to transcriptional changes (M10). Such functional differentiation of the gene modules might connect with the differentiated medium components responsible for varied bacterial growth phases, which was observed using the high-throughput growth assay in hundreds of medium combinations combined with machine learning [54,55]. We assumed that the various chemicals disturbed different metabolic fluxes in which different gene modules might have participated. The biological meaningfulness of the gene modules suggested an alternative genetic classification besides the commonly used clustering criteria, such as Gene Orthology [56] and Regulon [57]. In summary, the present study provided a representative example showing multiple evolutionary paths (i.e., gene mutation and expression) directed the reduced genome to the improved fitness with the homeostatic transcriptome.

Materials and Methods

E. coli strains and culture medium

The *E. coli* K-12 W3110 wild-type and its genome-reduced strains were initially distributed by the National BioResource Project of the National Institute of Genetics. The reduced genome has been constructed by multiple deletions of large genomic fragments [58], which led to an approximately 21% smaller size than its parent wild-type genome W3110. The minimal medium M63 was used as described in detail elsewhere [13,59]. In brief, the medium contains 62 mM dipotassium hydrogen phosphate, 39 mM potassium dihydrogen phosphate, 15 mM ammonium sulfate, 15 μM thiamine hydrochloride, 1.8 μM Iron (II) sulfate, 0.2 mM magnesium sulfate, and 22 mM glucose.

Experimental evolution

The genome-reduced *E. coli* strain was evolved in 2 mL of the M63 medium by serial transfer, as previously described [17,20]. Nine evolutionary lineages were all initiated from the identical culture stock prepared in advance. The 24-well microplates specific for microbe culture (IWAKI) were used, and every four wells of four tenfold serial dilutions, e.g., 10^3~10^6, were used for each lineage. Multiple dilutions changing in order promised at least one of the wells within the exponential growth phase after the overnight culture. The microplates were incubated in a microplate bioshaker (Deep Well Maximizer, Taitec) at 37°C, with rotation at 500 rpm. The serial transfer was performed at ~24-h intervals. Only one of the four wells (dilutions) showing growth

in the early exponential phase (OD_{600} = 0.01-0.1) was selected and diluted into four wells of a new microplate using four dilution ratios. Serial transfer was repeated until all evolutionary lineages reached approximately 1,000 generations, which required approximately two months per lineage. A total of nine lineages were conducted, and the daily records of the nine lineages were summarized in Table S1. The evolutionary generations (*G*) and the growth rates (μ) were calculated according to the following equations (Eq. 1 and Eq. 2).

$$G = \log_2\left(\frac{N_t}{N_0}\right) \qquad \text{(Eq. 1)}$$

$$\mu = \frac{\ln\left(\frac{N_t}{N_0}\right)}{\Delta t} \qquad \text{(Eq. 2)}$$

Growth assay

Both the ancestor and the evolved *E. coli* populations of the reduced genome were subjected to the growth assay, as previously described [13,59]. In brief, every 200 μl of culture was dispensed to each well in the 96-well microplate (Coaster). The microplate was incubated at 37°C in a plate reader (EPOCH2, BioTek), shaking at 567 cpm (cycles per minute) for 48 h. The temporal changes in OD_{600} were measured at 30-min intervals. The growth fitness (*r*) was calculated using the following equation between any two consecutive points (Eq. 3).

$$r_i = \frac{\ln\left(\frac{C_{i+1}}{C_i}\right)}{t_{i+1} - t_i} \qquad \text{(Eq. 3)}$$

Here, t_i and t_{i+1} are the culture times at the two consecutive measurement points, and C_i and C_{i+1} are the OD_{600} at time points t_i and t_{i+1}. The growth rate was determined as the average of three consecutive r_i, showing the largest mean and minor variance to avoid the unreliable calculation caused by the occasionally occurring values. The details of the experimental and analytical processes can be found at *https://doi.org/10.3791/56197*. The mean of the biological triplicates was defined as the growth fitness and used in the analyses.

Genome resequencing and mutation analysis

The *E. coli* cells were collected at the stationary growth phase (i.e., OD_{600} > 1.0) and subjected to genome resequencing, as described previously [20]. In brief, the bacterial culture was stopped by adding rifampicin at 300 μg/mL. The cell pellet was collected for genomic DNA purification using a Wizard Genomic DNA Purification Kit (Promega) under the manufacturer's instructions. The sequencing library was prepared using the Nextera XT DNA Sample Prep Kit (Illumina), and the paired-end sequencing (300 bp × 2) was performed using the Illumina HiSeq platform. The raw datasets of DNA-seq were deposited in the DDBJ Sequence Read Archive under the accession number DRA013661. The sequencing reads were aligned to the reference genome *E. coli* W3110 (AP009048.1, GenBank), and the mutation analysis was performed with the Breseq pipeline [60,61]. The DNA sequencing and mapping statistics are summarized in Table S2. The mutations fixed in the coding regions due to evolution are shown in Table S3.

Calculation and simulation of the genomic positions of mutations

The distances from the genomic positions of the genome mutations fixed in the nine Evos to the nearest genomic scars caused by the genome reduction were calculated as previously described [13,39]. The mutation simulation was performed with Python in the following steps. A total of 65 mutations were randomly generated on the reduced genome, and the distances from the mutated genomic locations to the nearest genomic scars caused by genome reduction were calculated. Subsequently, Welch's t-test was performed to evaluate whether the distances calculated from the random mutations were significantly longer or shorter than those calculated from the mutations that occurred in Evos. The random simulation, distance calculation, and statistic test were

performed 1,000 times, which resulted in 1,000 p values. Finally, the mean of p values (μ_p) was calculated, and a 95% reliable region was applied. It was used to evaluate whether the 65 mutations in the Evos were significantly close to the genomic scars, i.e., the locational bias.

RNA sequencing

The *E. coli* cells were collected at the exponential growth phase (i.e., $5\times10^7 \sim 2\times10^8$ cells/mL) and subjected to RNA sequencing, as described previously [27,44]. In brief, the bacterial growth was stopped by mixing with the iced 10% phenol ethanol solution. The cell pellet was collected to purify the total RNAs using the RNeasy Mini Kit (QIAGEN) and RNase-Free DNase Set (QIAGEN) according to product instructions. The paired-end sequencing (150 bp × 2) was performed using the Novaseq6000 next-generation sequencer (Illumina). The rRNAs were removed from the total RNAs using the Ribo-Zero Plus rRNA Depletion Kit (Illumina), and the mRNA libraries were prepared using the Ultra Directional RNA Library Prep Kit for Illumina (NEBNext). Biological replicates were performed for all conditions (N=2∼4). The raw datasets were deposited in the DDBJ Sequence Read Archive under the accession number DRA013662.

Data processing and normalization

The FASTQ files were mapped to the reference genome W3110 (accession number AP009048.1, GenBank) using the mapping software Bowtie2 [62], as described previously [27,44]. The obtained read counts were converted to FPKM values according to the gene length and total read count values. Global normalization of the FPKM values was performed to reach an identical mean value in the logarithmic scale in all datasets. The resulting normalized FPKM values were statistically equivalent to TPM. The gene expression level was determined as the logarithmic value of FPKM, and the mean values of biological replicates were used for the following analyses (Table S6).

Computational analysis

The normalized datasets were subjected to the computational analyses performed with the R statistical analysis software. A total of 3290 genes were used for hierarchical clustering and principal component analysis (PCA), which were performed with the R functions of “hclust” and “prcomp”, respectively, as described previously[27]. The corresponding parameters of “method” and “scale” were set as “average” and “F”, respectively. The R package of DESeq2[63] was used to determine the differentially expressed genes (DEGs), based on the false discovery rate (FDR < 0.05) [64]. The read counts were used as the input data for DESeq2, in which the data normalization was performed at each run for pair comparison.

Enrichment analysis

Functional enrichments were performed according to the features of gene category[65], transcriptional regulation[57], gene ontology[56], and metabolic pathways[66,67]. Twenty-one gene categories and 46 regulons, which comprised more than 15 genes and 15 regulatees, were subjected to the enrichment, respectively. The statistical significance was evaluated by the binomial test with Bonferroni correction. The enrichment analysis of gene ontology (GO terms) [56,68] and metabolic pathway (Kyoto encyclopedia of genes and genomes, KEGG) [66,67] was performed using DAVID, a web-based tool for visualizing the characteristics of gene clusters with expression variation [69,70]. The statistical significance was according to FDR.

Chromosomal periodicity analysis

Fourier transform was used to evaluate the chromosomal periodicity of the transcriptome, as previously described [28,44]. The genome was divided into compartments of 1 kb each, and the mean expression level of the genes within the corresponding sections was calculated. Gene expression levels were smoothed with a moving average of 100 kb and subjected to the periodicity

analysis using the function “periodogram” in R. The max peak (periodic wavelength) of the periodogram was fitted to the gene expression data using the function “nls” in R by the least squares method according to the following equation (Eq. 4).

$$\mathrm{Exp}\,(x) = a * \sin\left(x + \frac{b}{T}\right) + c \quad \text{(Eq. 4)}$$

Here, a, b, T, and c represent the periodic amplitude, the periodic phase, the periodic wavelength indicated by the max peak, and the mean expression level of the whole transcriptome as a constant, respectively. Note that altering the moving average did not change the max peak. The statistical significance of the periodicity was assessed with Fisher’s g test (Table S4), as described previously [28,44]. The genomic position of *ori* was determined according to the previous reports [44,71]. In addition, the function “abline” in R was used to point out the genomic positions of the mutations.

Gene network analysis

The weighted gene co-expression network analysis (WGCNA) of the nine Evos was performed with the R package of WGCNA [41], as described previously [27]. A Step-by-Step method was used to determine the parameters for constructing the gene networks. The soft threshold was set at 12, where the R^2 of Scale Free Topology Model Fit was approximately 0.9, as recommended by the developer’s instruction. The resultant gene networks were clustered with the “hclust” function (method=average) and reconstructed by merging similar modules using the “mergeCloseModules” function with a height cut of 0.25 in the “dynamic tree cut” method. Finally, a total of 21 modules were determined for 3,290 genes. The correlation coefficients and p-values between the expression of the gene modules and the other global features (growth rates, DEGs, and mutations) were evaluated using the functions “cor” and “corPvalueStudent” in R, respectively. FDR correction was applied to the p-values to account for multiplicity. Functional enrichment of the gene modules was performed, and the statistical significance was evaluated by the binomial test with Bonferroni correction as described above.

Acknowledgements

We thank NBRP for providing the *E. coli* strains carrying the wild-type and reduced genomes (W3110 and KHK collection). This work was supported by the JSPS KAKENHI Grant-in-Aid for Scientific Research (B) (grant number 19H03215) and partially by Grant-in-Aid for Challenging Exploratory Research (grant number 21K19815).

Competing interests

The authors declare that there are no competing interests.

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Editors

Reviewer #1 (Public Review):

In this study, the authors explored how the reduced growth fitness, resulting from genome reduction, can be compensated through evolution. They conducted an evolution experiment with a strain of Escherichia coli that carried a reduced genome, over approximately 1,000 generations. The authors carried out sequencing, and found no clear genetic signatures of evolution across replicate populations. They carry out transcriptomics and a series of analyses that lead them to conclude that there are divergent mechanisms at play in individual evolutionary lineages. The authors used gene network reconstruction to identify three gene modules functionally differentiated, correlating with changes in growth fitness, genome mutation, and gene expression, respectively, due to evolutionary changes in the reduced genome.

I think that this study addresses an interesting question. Many microbial evolution experiments evolve by loss of function mutations, but presumably a cell that has already lost so much of its genome needs to find other mechanisms to adapt. Experiments like this have the potential to study "constructive" rather than "destructive" evolution.

Comments on revised version:

I think the authors have carefully gone through the manuscript and addressed all of my concerns.

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Reviewer #2 (Public Review):

This manuscript describes an adaptive laboratory evolution (ALE) study with a previously constructed genome-reduced E. coli. The growth performance of the end-point lineages evolved in M63 medium was comparable to the full-length wild-type level at lower cell densities.

Subsequent mutation profiling and RNA-Seq analysis revealed many changes on the genome and transcriptomes of the evolved lineages. The authors did a great deal on analyzing the patterns of evolutionary changes between independent lineages, such as the chromosomal periodicity of transcriptomes, pathway enrichment analysis, weight gene co-expression analysis, and so on. They observed a striking diversity in the molecular characteristics amongst the evolved lineages, which, as they suggest, reflect divergent evolutionary strategies adopted by the genome-reduced organism.

As for the overall quality of the manuscript, I am rather torn. The manuscript leans towards elaborating observed findings, rather than explaining their biological significance. For this reason, readers are left with more questions than answers. For example, fitness assay on reconstituted (single and combinatorial) mutants was not performed, nor any supporting

evidence on the proposed contributions of each mutants provided. This leaves the nature of mutations - be them beneficial, neutral or deleterious, the presence of epistatic interactions, and the magnitude of fitness contribution, largely elusive. Also, it is difficult to tell whether the RNA-Seq analysis in this study managed to draw biologically meaningful conclusions, or instill insight into the nature of genome-reduced bacteria. The analysis primarily highlighted the differences in transcriptome profiles among each lineage based on metrics such as 'DEG counts' and the 'GO enrichment'. However, I could not see any specific implications regarding the biology of the evolved minimal genome drawn. In their concluding remark, 'Multiple evolutionary paths for the reduced genome to improve growth fitness were likely all roads leading to Rome,' the authors observed the first half of the sentence, but the distinctive characteristics of 'all roads' or 'evolutionary paths', which I think should have been the key aspect in this investigation, remains elusive.

Comments on revised version:

I appreciate the author's responses. They responded to most of the comments, but I still think that there is room for improvement. Please refer to the following comments. Quoted below are the author's responses.

"We agree that our study leaned towards elaborating observed findings rather than explaining the detailed biological mechanisms."
- Comment: I doubt if there are scientific merits in merely elaborating observed findings. The conclusion of this study suggests that evolutionary paths in reduced genomes are highly diverse. But if you think about the nature of adaptive evolution, which relies upon the spontaneous mutation event followed by selection, certain degree of divergence is always expected. The problem with current experimental setting is that there are no ways to quantitively assess whether the degree of evolutionary divergence increases as the function of genome reduction, as the authors claimed. In addition, this notion is in direct contradiction to the prediction that genome reduction constraints evolution by reducing the number of solution space. It is more logical to think and predict that genome reduction would, in turn, lead to the loss of evolutionary divergence. We are also interested to know whether solution space to the optimization problem altered in response to the genome reduction. In this regard, a control ALE experiment on non-reduced wild-type seems to be a mandatory experimental control. I highly suggest that authors present a control experiment, as it was done for "JCVI syn3.0B vs non-minimal M. mycoides" (doi: 10.1038/s41586 023 06288 x) and "E. coli eMS57 vs MG1655" (doi: 10.1038/s41467 019 08888 6).
"We focused on the genome wide biological features rather than the specific biological functions."
- Comment: The 'biological features' delivered in current manuscript does not give insight as to which genomic changes translated into strain fitness improvement. Rather than explaining the genotype-phenotype relationships and/or the mechanistic basis of fitness improvement, authors merely elaborated on the observed phenotypes. I question the scientific merits of such 'findings'.
"Although the reduced growth rate caused by genome reduction could be recovered by experimental evolution, it remains unclear whether such an evolutionary improvement in growth fitness was a general feature of the reduced genome and how the genome wide changes occurred to match the growth fitness increase."
- Comment: This response is very confusing to understand. "it remains unclear whether such an evolutionary improvement in growth fitness was a general feature of the reduced genome" - what aspects remain unclear?? What assumption led the authors to believe that reduced genome's fitness cannot be evolutionarily improved?
- Comment: "and how the genome wide changes occurred to match the growth fitness increase" - this is exactly the aspect that authors should deliver, instead of just elaborating the observed findings. Why don't authors select one or two fastest-growing (or the fittest)

lineages and specifically analyze underlying adaptive changes (i.e. genotype-phenotype relationships)?

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Reviewer #3 (Public Review):

Summary:

Studying evolutionary trajectories provides important insight in genetic architecture of adaptation and provide potential contribution to evaluating the predictability (or unpredictability) in biological processes involving adaptation. While many papers in the field address adaptation to environmental challenges, the number of studies on how genomic contexts, such as large-scale variation, can impact evolutionary outcomes adaptation is relatively low. This research experimentally evolved a genome-reduced strain for ~1000 generations with 9 replicates and dissected their evolutionary changes. Using the fitness assay of OD measurement, the authors claimed there is a general trend of increasing growth rate and decreasing carrying capacity, despite a positive correlation among all replicates. The authors also performed genomic and transcriptomic research at the end of experimental evolution, claiming the dissimilarity in the evolution at the molecular level.

Strengths:

The experimental evolution approach with a high number of replicates provides a good way to reveal the generality/diversity of the evolutionary routes.

The assay of fitness, genome, and transcriptome all together allows a more thorough understanding of the evolutionary scenarios and genetic mechanisms.

Comments on revised version:

#5 in the last round of comments: When the authors mentioned no overlapping in single mutation level, I thought the authors would directly use this statement to support their next sentence about no bias of these mutations. As the author's responded, I was suspecting no overlapping for 65 mutation across the entire genome is likely to be not statistically significant. In the revised version, the authors emphasized and specified their simulation and argument in the following sentences, so I do not have questions on this point anymore.

#14 in the last round of comments: As what authors responded, "short-term responses" meant transcriptional or physiological changes within a few hours after environmental or genetic fluctuation. "long-term responses" involve new compensatory mutations and selection. The point was that, the authors found that "the transcriptome reorganization for fitness increase triggered by evolution differed from that for fitness decrease caused by genome reduction." That is short vs long-term responses to genetic perturbation. Some other experimental evolution did short vs long-term responses to environmental perturbation and usually also found that the short-term responses are reverted in the long-term responses (e.g., https://academic.oup.com/mbe/article/33/1/25/2579742). I hope this explanation makes more sense. And I think the authors can make their own decisions on whether they would like to add this discussion or not.

https://doi.org/10.7554/eLife.93520.2.sa0

Author response:

The following is the authors' response to the original reviews.

Thank you for the careful reading and the positive evaluation of our manuscript. As you mentioned, the present study tried to address the question of how the lost genomic functions could be compensated by evolutionary adaptation, indicating the potential mechanism of "constructive" rather than "destructive" evolution. Thank you for the instructive comments that helped us to improve the manuscript. We sincerely hope the revised manuscript and the following point-to-point response meet your concerns.

- *Line 80 "Growth Fitness" is this growth rate?*

Yes. The sentence was revised as follows.

(L87-88) “The results demonstrated that most evolved populations (Evos) showed improved growth rates, in which eight out of nine Evos were highly significant (Fig. 1B, upper).”

- *Line 94 a more nuanced understanding of r/K selection theory, allows for trade-ups between R and K, as well as trade-offs. This may explain why you did not see a trade-off between growth and carrying capacity in this study. See this paper https://doi.org/10.1038/s41396-023-01543-5. Overall, your evos lineages evolved higher growth rates and lower carrying capacity (Figures 1B, C, E). If selection was driving the evolution of higher growth rates, it may have been that there was no selective pressure to maintain high carrying capacity. This means that the evolutionary change you observed in carrying capacity may have been neutral "drift" of the carrying capacity trait, during selection for growth rate, not because of a trade-off between R and K. This is especially likely since carrying capacity declined during evolution. Unless the authors have convincing evidence for a tradeoff, I suggest they remove this claim.*

- *Line 96 the authors introduce a previous result where they use colony size to measure growth rate, this finding needs to be properly introduced and explained so that we can understand the context of the conclusion.*

- *Line 97 This sentence "the collapse of the trade-off law likely resulted from genome reduction." I am not sure how the authors can draw this conclusion, what is the evidence supporting that the genome size reduction causes the breakdown of the tradeoff between R and K (if there was a tradeoff)?*

Thank you for the reference information and the thoughtful comments. The recommended paper was newly cited, and the description of the trade-off collapse was deleted. Accordingly, the corresponding paragraph was rewritten as follows.

(L100-115) “Intriguingly, a positive correlation was observed between the growth fitness and the carrying capacity of the Evos (Fig. 1D). It was somehow consistent with the positive correlations between the colony growth rate and the colony size of a genome-reduced strain 11 and between the growth rates and the saturated population size of an assortment of genome reduced strains 13. Nevertheless, the negative correlation between growth rate and carrying capacity, known as the r/K selection30,31 was often observed as the trade-off relationship between r and K in the evolution and ecology studies 32 33,34. As the r/K trade-off was proposed to balance the cellular metabolism that resulted from the cost of enzymes involved 34, the deleted genes might play a role in maintaining the metabolism balance for the r/K correlation. On the other hand, the experimental evolution (i.e., serial transfer) was strictly performed within the exponential growth phase; thus, the evolutionary selection was

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