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MEGA (Multiple Essential Genes Assembling) Deletion and Replacement Method for Genome Reduction in *Escherichia coli*

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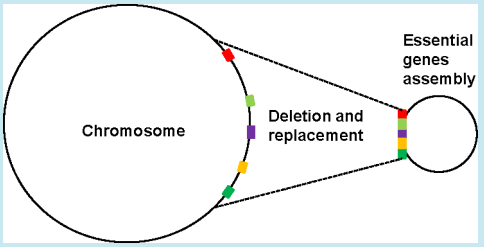
Supporting Information

ABSTRACT: Top-down reduction of the bacterial genome to construct desired chassis cells is important for synthetic biology. However, the current progress in the field of genome reduction is greatly hindered by indispensable life-essential genes that are interspersed throughout the chromosomal loci. Here, we described a new method designated as "MEGA (Multiple Essential Genes Assembling) deletion and replacement" that functions by assembling multiple essential genes in an *E. coli*–*S. cerevisiae* shuttle vector, removing targeted chromosomal regions containing essential and nonessential genes using a one-round deletion, and then integrating the cloned essential genes into the *in situ* chromosomal loci via I-SceI endonuclease cleavage. As a proof of concept, we separately generated three large deletions (80–205 kbp) in the *E. coli* MDS42 chromosome. We believe that the MEGA deletion and replacement method has potential to become widely used in large-scale genome reductions in other sequenced organisms in addition to *E. coli*.



KEYWORDS: *genome engineering, essential gene, gene assembly, genome reduction*

Bacterial genome reduction decreases genome complexity and enhances the predictability and operability of the genetic regulations, thus facilitating the rational design and construction of desired chassis cells.[1,2] For example, *Escherichia coli* MDS42, which contains a 663.3-kbp deletion in its mobile elements, prophages, and virulence genes, shows beneficial properties in comparison to its parent strain (MG1655, 4.6 Mbp), such as enhanced genetic stability and higher electroporation efficiency.[3] Additionally, the removal of nonessential metabolism burdens to increase goal product yield is important for industrial applications. The genome-reduced strain MGF-01 (containing a 1.0-Mbp deletion from the 4.6-Mbp genome of strain W3110) is able to produce higher levels of L-threonine compared to the parent strain.[4] The MGF-01-derived strain DGF-298, which contains a further-reduced genome of 2.98 Mbp, still maintains robust growth and would therefore be a suitable host for research and industrial applications.[5]

Several methods for generating precise genomic deletions have been developed, such as the Lambda Red system using linear DNA recombination,[6−8] suicide plasmid-mediated homologous recombination,[9] and the site-specific recombination system.[10] However, the progress in genome reduction is relatively slow because each deletion can only remove one chromosomal segment between two essential genes. Essential genes are required for functional life under a given set of growth conditions; therefore, they are not dispensable.[11] For example, a genome-wide single-gene deletion study in *E. coli* K-12 identified 303 essential genes,[12] approximately 7% of the total 4453 open reading frames (ORFs). Deletions or mutations of essential genes can only be accomplished when complemented with another copy of the intact essential genes, supplied via either free plasmids[13,14] or chromosomally integrated plasmids.[15,16] Systematic genomic deletions in *E. coli* MG1655 have indicated that all of the regions of the chromosome, with the exception of the origin of replication, can be deleted in the presence of the corresponding essential genes complemented *in trans*.[17] However, as far as we know, no more than one essential gene has been complemented per time per deletion.

In this study, we presented "MEGA (Multiple Essential Genes Assembling) deletion and replacement", a new method for large genomic reduction via the cloning and assembly of multiple essential genes in an *E. coli*-yeast shuttle vector, followed by targeted deletion of the corresponding chromosomal region. The cloned essential genes can also be integrated into the chromosome using the endonuclease I-SceI to cause double-stranded DNA breakage to promote homologous recombination. We chose three large chromosomal regions (80–205 kbp) containing 2–8 essential genes, which would normally require multiple rounds of deletions if using the previous methods. Using MEGA deletion and replacement, we rapidly removed each of the large chromosomal regions via a one-round deletion in *E. coli* MDS42.

RESULTS

Principle and Design Specifications of the MEGA Deletion and Replacement Method. The MEGA deletion

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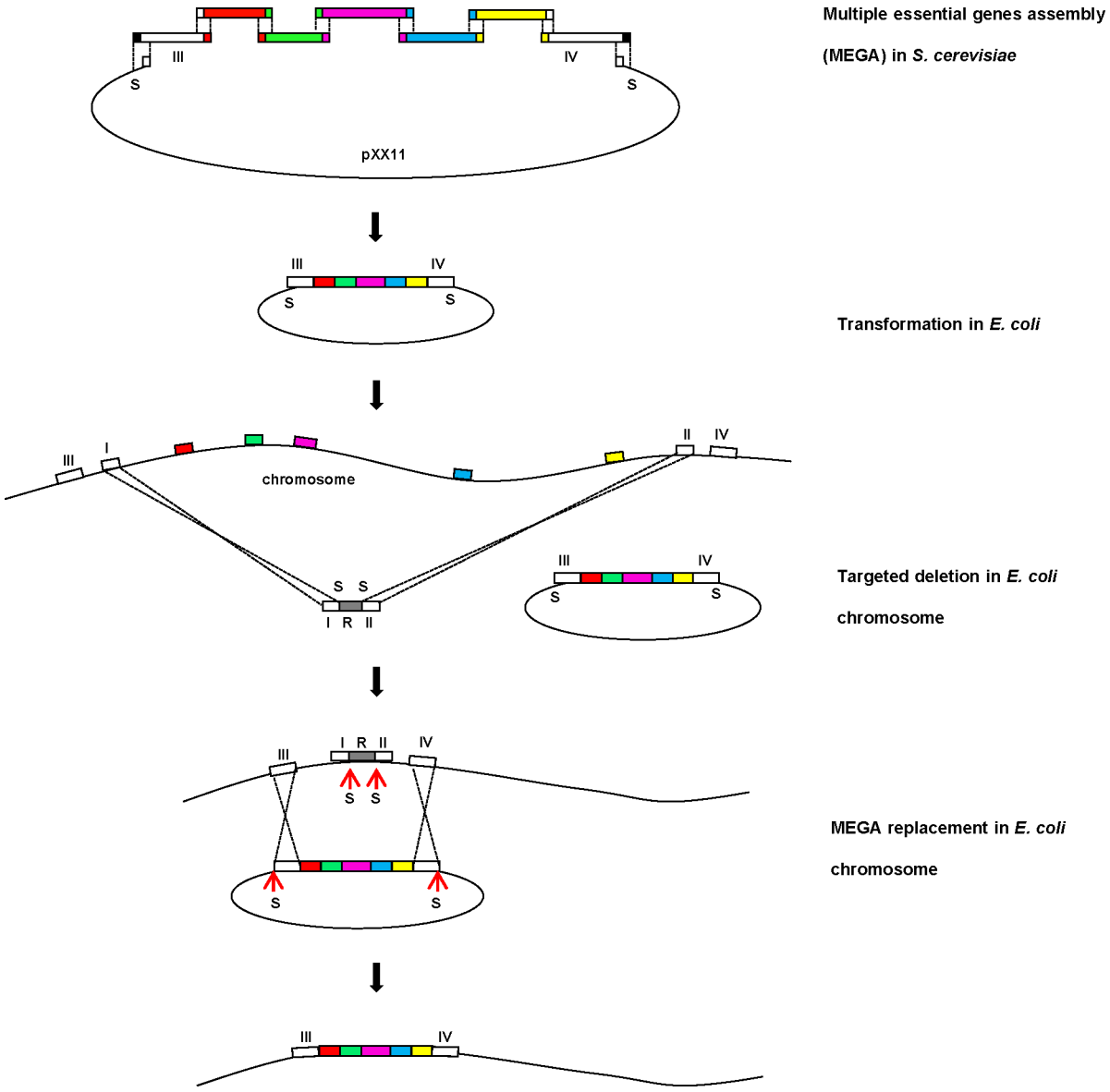

Figure 1. Schematic diagram of the MEGA deletion and replacement method. The essential genes (colored bars), the left and right chromosomal homologous arms (III, IV) for integration, and the pXX11 vector were PCR-amplified. Up to 100-bp overlaps to the adjacent DNA fragments, indicated by colored boxes, were introduced at the 3′ ends of the primers. The DNA fragments were mixed and introduced via transformation into *S. cerevisiae* for *in vivo* assembly. The assembled plasmid was introduced via electroporation into *E. coli*, and the targeted chromosomal region was deleted in the presence of the essential genes complemented *in trans*. The induction of I-SceI caused four double-stranded DNA breaks (indicated by red arrows) and initiated homologous recombination at regions III and IV between the chromosome and the complementing plasmid, resulting in the integration of the cloned essential genes into the host chromosome. I, II: homologous arms of the targeted region. R: apramycin resistance gene. S: I-SceI recognition site.

and replacement method was developed in this study to provide a solution to the indispensability of essential genes, which has held back the progress in genome reduction. As shown in Figure 1, the MEGA deletion step requires polymerase chain reaction (PCR) amplifications of multiple essential genes *in vitro*, followed by assembly using highly efficient yeast homologous recombination *in vivo*. In the presence of multiple *in trans*-complemented essential genes in the *E. coli*, the targeted chromosomal region was then completely deleted via a one-round deletion. The MEGA replacement step integrated the complementing essential genes into the corresponding chromosomal loci *in situ*, avoiding the potential *trans*-acting effects of the essential genes.

To minimize the risk of missing life-essential genes and to increase the probability of success in the large genomic deletions, the essential genes in the *E. coli* genome were identified based on a combination of the results of bioinformatic predictions[18,19] and from single-gene deletion experiments.[12] Moreover, experimental data provided from the EcoCyc[20] and PEC (Profiling of *E. coli* Chromosome)[21] databases were also integrated.

Precautions were taken to ensure the functionality of the cloned essential genes. Experimental data regarding the confirmed coding sequences, promoters, and terminators of the essential genes were collected from the EcoCyc database. If no experimental or predicted data were available, then the regions 200−300-bp upstream and 50−100-bp downstream of

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Table 1. Large Chromosomal Deletions Generated by MEGA Deletion and Replacement in *E. coli* Strain MDS42

deletion region	location in the MDS42 genome (bp)	complemented plasmid (essential genes listed)	complementation length (kbp)	deletion length (kbp)	actual genome reduction (kbp)	positive deletion rate (%)
D	249 731−334 906	pWT438 (*yagG*[a], *hemB*)	5	85	80	100
E	1 656 605−1 877 912	pWT439 (*asnT*, *yefM*, *dcd*, *metG*, *folE*, *rplY*, *yejM*, *proL*)	16	221	205	100
H	3 688 435−3 799 416	pWT462 (*dnaB*, *ssb*, *alsK*, *pheU*)	4	111	107	45[b]

[a]*yagG* was deleted in MDS42; however, it was suggested as an essential gene in a previous study.[12] Therefore, it was added as an essential gene for the complementation. [b]Shorter homologous arms (50−60 bp) for targeting region H were used, which could partially explain the lower positive rate following H deletion. In comparison, the homologous arms for targeting regions D and E were approximately 300 bp in length. For the exact positions of the homologous arms, please refer to Supporting Information Table S2.

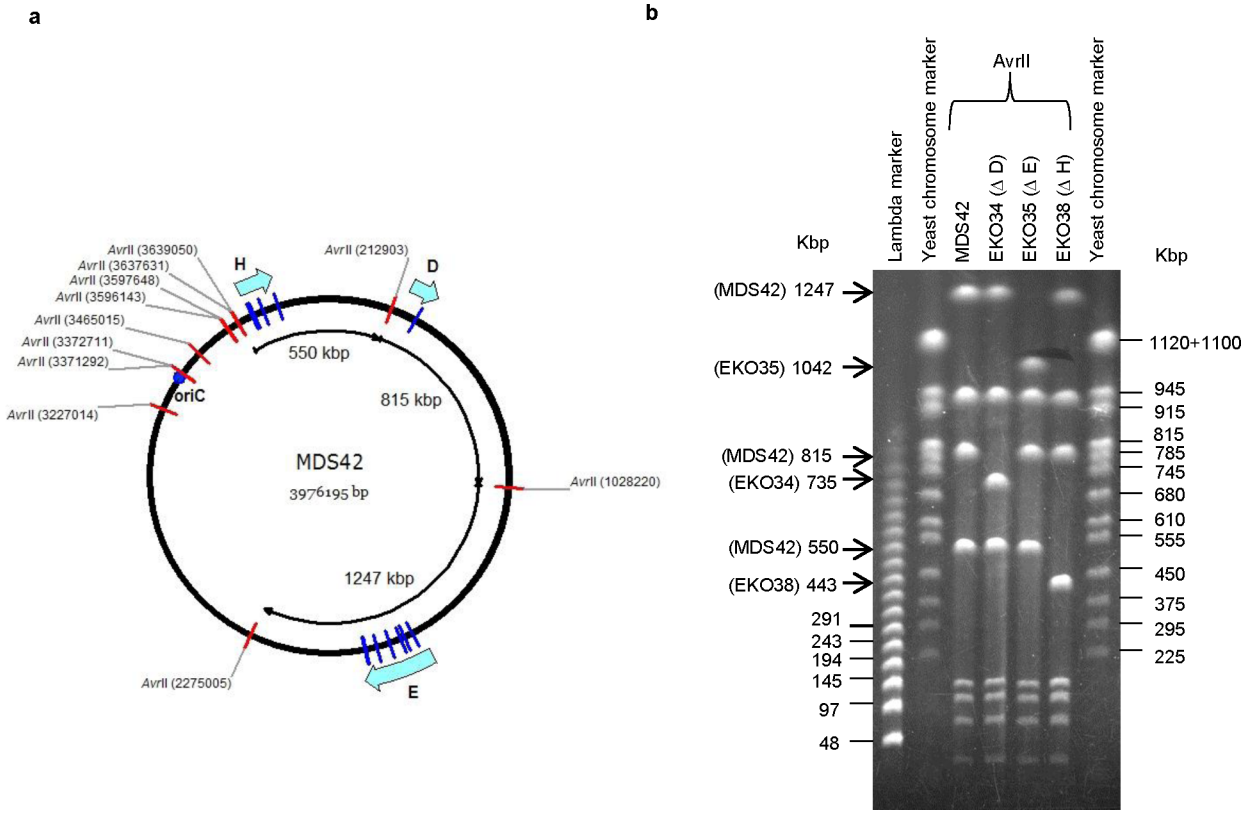

Figure 2. Large chromosomal deletions generated using MEGA deletion and replacement. (A) The genomic context of the three deletion regions in *E. coli* strain MDS42. The related positions of the D, E, and H regions are shown with cyan arrowheads, while the essential genes within these regions are marked with blue lines. The AvrII cutting sites on the chromosome are marked with red lines. Region D lies between the AvrII cutting sites at 212 903 and 1 028 220 bp (815 kbp), region E is located within the AvrII cutting sites at 1 028 220 and 2 275 005 bp (1247 kbp), and region H is between the AvrII cutting sites at 3 639 050 and 212 903 bp (550 kbp). (B) The pulsed-field gel electrophoresis (PFGE) confirmation of the large deletions. The genomes of MDS42 (parent strain) and the deletion mutant strains with the essential gene integrated into the chromosome (EKO34 [ΔD], EKO35 [ΔE], and EKO38 [ΔH]) were subjected to AvrII digestion. The digestion of MDS42 resulted in 1247, 952, 815, 550, 144, 131, 92, and 40 kbp bands. In comparison to MDS42, the deletion of the D region resulted in a band shift from 815 to 735 kbp in strain EKO34, while the removal of the E region caused a band shift from 1247 to 1042 kbp in strain EKO35. Strain EKO38 had a band shift from 550 to 443 kbp due to the loss of the H region. The digestion patterns were consistent with the theoretical calculation. The gel was run at 6 V/cm and 14 °C for 40 h with an initial pulse time of 60 s and a final pulse time of 120 s.

the coding sequences of the essential genes were chosen as the potential promoter and terminator regions. The upstream genes that cotranscribed with the essential genes were also PCR-amplified. Furthermore, the transcription direction and the relative order of the cloned essential genes were maintained in the same orientation as those on the native chromosome.

Our attempts to directly clone more than three DNA fragments containing essential genes into a plasmid in the *E. coli* were unsuccessful. The highly efficient yeast homologous recombination system enables the assembly of multiple DNA fragments to construct large DNA molecules, such as biochemical pathways,[22] and even the entire genome.[23,24] Therefore, in this study, we adapted the yeast homologous recombination system to assemble multiple *E. coli* essential genes. To reduce any discrepant assembly in the yeast[22] and to minimize the unpredicted effect of increased copies of the essential genes in the *E. coli*, the assembly vector pXX11 was constructed. This vector contained the Bacterial Artificial Chromosome (BAC) and the yeast replication element (CEN6 ARS4) that propagated as a single copy in both the *E. coli* and *S. cerevisiae* cells (Supporting Information Figure S1).

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The assembly vector pXX11, the essential genes, and the homologous arms (1.5−2.5 kbp) of the targeted integration regions (marked as III and IV in Figure 1) were PCR-amplified to contain 60−100-bp overlaps to the adjacent fragments, which were introduced at the 3′ ends of the primers (Figure 1). The lithium acetate transformation procedure in yeast[25] was used in this study because it was simple and fast and had satisfying transformation efficiency. The assembled plasmid containing the essential genes was introduced via electroporation into the *E. coli* MDS42 cells harboring pREDIA,[26] which contains the arabinose-inducible Lambda Red system (*exo*, *beta*, and *gam*) and the rhamnose-inducible endonuclease I-SceI. A linear DNA cassette containing two homologous arms and a positive selective marker (apramycin resistance gene) was then introduced via electrotransformation into the arabinose-induced cells, resulting in homologous recombination and targeted deletion.

The MEGA replacement step allowed chromosomal integration of the cloned essential genes to avoid any potential *trans*-acting effects of the essential genes. During the assembly and deletion steps, two I-SceI sites (indicated by arrowheads and the letter S in Figure 1) were added at the termini of the assembled homologous arms, while another two I-SceI sites were added at the termini of the disruption cassette by using pXX22 (Supporting Information Figure S1) as a template for the PCR amplification. Induction of the I-SceI endonuclease caused DNA breaks at the four I-SceI sites, and the Lambda Red system mediated the homologous recombination between the chromosome and the plasmid, resulting in the integration of the cloned essential genes into the host chromosome.

MEGA Deletion and Replacement Is Efficient for *E. coli* Genome Reduction. To test the efficiency and the universality of the MEGA deletion and replacement method, we chose three large regions (Table 1) in the *E. coli* MDS42 chromosome. Each region contained 2−8 essential genes (Figure 2A) that were unable to be deleted in only one round using the traditional deletion methods. For example, the E region (221 kbp, mapping to 1 656 840−1 878 436 bp in the MDS42 chromosome), contains 8 essential genes (*asnT*, *yefM*, *dcd*, *metG*, *folE*, *rplY*, *yejM*, and *proL*) interspersed among the 320 nonessential genes. The corresponding region in the parent strain (MG1655) was incompletely deleted by five separate deletions in a previous study.[27] In this study, the above essential genes within the E region were PCR-amplified and assembled in pXX11 to construct the complementing plasmid pWT439 (Table 1). In the presence of pWT439, the complete 221-kbp segment of the E region was successfully deleted from the MDS42 chromosome via a one-round deletion. By including the complementing essential genes (16 kbp) that were integrated into the chromosome *in situ*, the actual genome reduction in region E was 205 kbp. Regions D and H were similarly deleted via MEGA deletion and replacement. The positive rate of deletion was 45−100% (Table 1), depending on the targeting area and the length of the homologous arms used in the disruption cassettes. The integration of the essential genes was efficient, with a positive rate of 80−90% after 5 rounds of induction of I-SceI and the Lambda Red system. The deletions were confirmed by sequencing (Supporting Information Table S3) and using pulsed-field gel electrophoresis (PFGE).[28] Figure 2B shows the PFGE of the AvrII-digested genomic DNA from the parent strain (MDS42) and the genome reduction strains with the essential genes integrated into the chromosome. The AvrII digestion of the MDS42 genome released 8 fragments (1247, 952, 815, 550, 144, 131, 92, and 40 kbp). The deletion of regions D, E, and H caused band shifts from 815 to 735 kbp (80-kbp reduction), 1247 to 1042 kbp (205 kbp) and 550 to 443 kbp (107 kbp), respectively.

The growth of the deletion and replacement strains was measured, and the corresponding generation time and the final optical density were used for a one-way analysis of variance (ANOVA) analysis (Supporting Information Table S4). Interestingly, a nearly identical growth pattern was detected in the wild type MDS42, both with and without the presence of an additional copy of the multiple essential genes *in trans* (Figure 3A−C). The deletion of the largest region (E) had a mild effect on the growth of the *E. coli* cells. Moreover, the deletion mutants with the essential genes either complemented *in trans* (EKO32) or integrated into the chromosome (EKO35) exhibited similar growth patterns. Similar results were obtained for the deletions and replacements of regions D and H in the MDS42 chromosome (Figure 3B and C). Therefore, we believe that this method is practical for large genomic reductions, and it is possible to use only the MEGA deletion step or the deletion step combined with the replacement step, depending on the research purposes.

DISCUSSION

The genome deletion methods reported in previous studies can only delete one nonessential region between two essential genes at a chromosomal locus. In this study, we developed the MEGA deletion and replacement method to provide a solution to the obstruction of life-essential genes in *E. coli* genomic deletions. We demonstrated the efficiency of this method by separately generating three large genomic deletions and replacements (80−205 kbp) in *E. coli*. Because this method eliminates the vector, it could be useful in sequential deletions to construct *E. coli* strains with further-reduced genomes. Moreover, this method can be easily adapted for genome-wide testing and modifications other than genome deletion. For example, it could be useful for testing the cumulative effects of growth-important genes in addition to the life-essential genes in a large region by assembling the known essential genes and serial deletions. We tested a larger deletion than the D region and found that the further deletion of its adjacent region (42 kbp, mapping to 207 817−250 773 bp in the MDS42 genome) resulted in severe growth defects. The identification of the exact genes that affect growth still requires further research. We tried larger deletion fragments (>300 kb and 500 kb), however, to no success. We do not yet understand what limits deletions sizes beyond this achieved in this manuscript (∼200 kbp). Nevertheless, this method has good portability, we expect that it could be useful in large-scale genome engineering in other organisms, in addition to *E. coli*. Since this method relies on knowing the essential genes locations on the chromosome, it would be suitable for sequenced bacteria.

In contrast to the traditional point of view that the *E. coli* chromosome has a well-conserved structure that limits its genome plasticity,[29] our results suggested that the locations of the life-essential genes on the *E. coli* chromosome vary, based on the fact that the genome could tolerate large deletions in the presence of multiple essential genes acting simultaneously *in trans*. Because the presence of essential genes is a fundamental criterion that distinguishes plasmids from chromosomes,[30] the disparity of the chromosome and plasmid would be very small, as was shown in this study. We suggest that it is possible to

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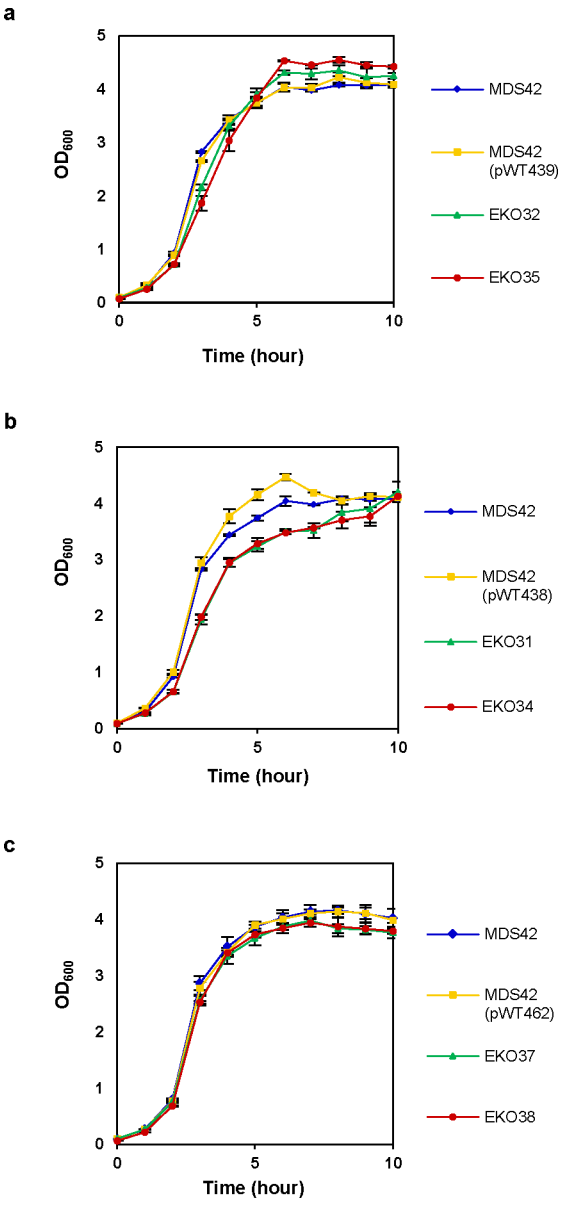

Figure 3. Growth curves of the genome reduction mutants. Parts A, B, and C show the growth of the mutants with deletions in regions E (EKO32 and EKO35), D (EKO31 and EKO34), and H (EKO37 and EKO38). Mutants EKO32, EKO31, and EKO37 had essential genes complemented *in trans*, while EKO35, EKO34, and EKO38 had chromosomally integrated essential genes. In each figure, the growth of the parent strain (MDS42) was used as a control. An additional control (MDS42) contained the complementing plasmids before deletion: pWT439, pWT438, and pWT462 carried the essential genes within regions E, D, and H, respectively. Overnight cultures of each strain were diluted in fresh MOPS-rich medium to an optical density of approximately 0.1 at 600 nm, and growth was measured hourly for 10 h. At least two independent experiments were performed, and triplicate biological replicates were measured each time. Representative results are shown.

assemble more essential genes to reconstruct a synthetic chromosome and that the original chromosome could be continuously deleted and even completely displaced by the synthetic chromosome if the latter contains all of the genes essential for sustaining life.

METHODS

Bacterial Strains, Media, and Reagents. *E. coli* strain MDS42 (Scarab Genome, WI, U.S.A.) was used in all of the genomic deletion experiments, and *E. coli* strain DH10B was used as a cloning host. The assembly host, *Saccharomyces cerevisiae* strain BY4742,[31] was kindly provided by Dr. Sheng Yang. The *E. coli* strains were cultivated in Luria−Bertani (LB) medium for the routine genetic modifications. For the *E. coli* growth experiments, the bacterial cells were cultivated in MOPS EZ rich defined (MOPS-rich) medium (Teknova, CA USA). Antibiotics were added to the bacterial cultures at the following concentrations: ampicillin, 100 μg/mL; chloramphenicol, 17 μg/mL, and apramycin, 50 μg/mL. The yeast cells were cultured in YPAD medium (1% [w/v] Bacto yeast extract, 2% [w/v] Bacto peptone, 2% [w/v] glucose, and 80 mg/L adenine hemisulfate) or synthetic complete drop-out medium lacking uracil (SC-U).[25]

The restriction endonucleases and DNA size markers were purchased from Thermo Fermentas or New England BioLabs (NEB, MA, U.S.A.). All of the chemicals were obtained from Sigma-Aldrich or Sangon Biotech unless otherwise specified. Phanta Super Fidelity DNA Polymerase (Vazyme, Jiangsu, China) and KOD-Plus-Neo DNA Polymerase (Toyobo, Osaka, Japan) were used for the PCR amplification of the essential genes and the homologous arms. KDO-FX (Toyobo, Osaka, Japan) DNA Polymerase was used in the colony PCR confirmation. The primers were synthesized by Genescript (Jiangsu, China) or JIE LI Biology (Shanghai, China). The Wizard SV Gel and PCR Clean-up System from Promega (WI, U.S.A.) was used for the DNA purification. The E.N.Z.A. Plasmid Mini Kit I and the BAC/PAC DNA Isolation Kit for plasmid preparation were purchased from Omega Bio-Tek (GA, U.S.A.).

Construction of the Assembly Vector (pXX11) and the Template Plasmid (pXX22). The 2-kbp fragment containing the yeast origin of replication, a centromere, and a uracil selection marker was PCR-amplified from the *E. coli*-yeast shuttle vector pSH47.[32] The PCR product was gel-purified and inserted in the EcoRV-digested pBluescript II SK(+) (Stratagene, CA, U.S.A.) vector to construct pXX10. The correct insert was confirmed by sequencing and was released via *Hpa*I/*Bam*HI digestion. The released fragment was inserted in the *Hpa*I/*Bam*HI-digested pBeloBAC11 (NEB) to construct the assembly vector (pXX11).

The template plasmid (pXX22) was constructed to facilitate the amplification of the disruption cassettes used in the MEGA replacement step. The intermediate plasmid (pXX21) was constructed using a blunt-end ligation of the purified PCR product generated using pHY773[33] as a template and using the primers pXX21-F/R to introduce one I-SceI site. The primers used in the vector construction are listed in Supporting Information Table S1. Another I-SceI site was introduced in a similar fashion using the primers pXX22-F/R and the pXX21 template for the PCR amplification. Blunt-end ligation of the PCR product generated the plasmid pXX22. The existence of two I-SceI sites was confirmed via sequencing.

Assembly of the Multiple Essential Genes. The pXX11 vector, the essential genes, and the homologous arms for the targeted integration were PCR-amplified. The regions amplified for the essential genes and homologous arms are listed in Supporting Information Table S2. The PCR products were subjected to agarose gel electrophoresis and purification. The

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purified vector fragments were subjected to DpnI digestion to remove the template DNA.

For the assembly, 100 ng of the vector DNA and equal molar volumes of each of the DNA fragments (1:3 molar ratio to the vector) were mixed and introduced into *S. cerevisiae* BY4742 using the lithium acetate transformation procedure.[25] The transformants were verified via colony PCR. Yeast cells harboring the correct assembly were cultivated in 3 mL of SC-U medium at 30 °C for 24−48 h to reach the early stationary phase. The cells were collected and resuspended in 500 μL of cell wall-disrupting buffer (1 M sorbitol, 0.1 M EDTA (pH 7.5), 1.5% snailase, 100 mM DTT) and were incubated at 37 °C for 2 h. The pellets were collected by centrifugation at 2000*g* for 10 min. A BAC/PAC DNA isolation kit was used for the plasmid isolation.

The isolated plasmids were introduced via electrotransformation into *E. coli* DH10B cells. The plasmids isolated from the *E. coli* were subjected to enzymatic digestion to ensure that each assembly DNA fragment appeared only once.

Preparation of the Disruption Cassettes and the Targeted Genomic Deletion. The disruption cassettes containing the selection marker (apramycin resistance gene) were PCR-amplified using pXX22 as the template. The long (approximately 300 bp) homologous arms of the targeted regions were amplified from the MDS42 genome, while the short (50−60 bp) homologous arms were introduced at the 3′ ends of the primers. The exact positions of the homologous arms are listed in Supporting Information Table S2. Approximately 300 ng of DNA was transformed into arabinose-induced *E. coli* MDS42 harboring both the helper plasmid (pREDIA,[26] kindly provided by Dr. Sheng Yang) and the complementing plasmid with the essential genes, according to previously described procedures.[6,8] The correct deletions were verified via colony PCR and sequencing.

Integration of the Cloned Essential Genes into the Chromosome. Single colonies of the deletion mutants were cultivated in fresh liquid LB medium supplied with 10 mM L-rhamnose and L-arabinose at 30 °C to induce the I-SceI Lambda Red system. The overnight cultures were diluted 100-fold into 5 mL of fresh liquid LB medium containing 10 mM L-rhamnose and L-arabinose and were incubated at 30 °C for 12 h. After 4−5 rounds of serial cultivation, the cells were streaked out on LB plates. The single colonies were subjected to apramycin and chloramphenicol antibiotic sensitivity tests and PCR confirmation to ensure the integration of the essential genes and curation of the vector.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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