Supplementary Materials for

Programmed chromosome fission and fusion enable precise large-scale genome rearrangement and assembly

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Other Supplementary Material for this manuscript includes the following:
(available at science.sciencemag.org/content/365/6456/922/suppl/DC1)

Tables S1, S2, and S6 as separate Excel files
Data Files S1 to S7 as a separate .zip file

Data file S1 – *E. coli* MDS42 genome
GenBank file of a wildtype *E. coli* MDS42 genome, which served as a starting genome for fission experiments.

Data file S2 – Fission BAC
Annotated GenBank file of a "fission BAC", used for performing fission experiments. The GenBank accession number for this construct is MN226640.

Data file S3 – pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer plasmid
Annotated GenBank file of a pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer plasmid, which encodes the spacers required for performing Cas9 cuts during fission and fusion experiments. The GenBank accession number for this construct is MN226641.

Data file S4 – pKW5_MB1$_{Kan}$_Tracr^{K}_Spacer plasmid
Annotated GenBank file of a pKW5_MB1$_{Kan}$_Tracr^{K}_Spacer plasmid, which encodes the spacers required for performing Cas9 cuts for fission of a 1.55 Mb section (**Fig. 2**). The GenBank accession number for this construct is MN226642.

Data file S5 – Genome with watermarked region 1
GenBank file of the genome with a watermarked region 1 (Section C) spanning 1,454,024 to 1,979,777. The watermark scheme consists of the systematic substitution of TCG, TCA and TAG codons - in annotated genes - by their synonymous AGC, AGT and TAA, respectively. The GenBank accession number for this sequence is CP042184.

Data file S6 – Genome with watermarked region 2
GenBank file of the genome with a watermarked region 2 (Section A) spanning 436,924-939,332. The watermark scheme consists of the systematic substitution of TCG, TCA and TAG codons - in annotated genes - by their synonymous AGC, AGT and TAA, respectively. The GenBank accession number for this sequence is CP042183.

Data file S7 – Genome containing watermarked regions 1 and 2
Sequence of the chimeric genome containing watermarked regions 1 and 2, resulting from the combination of genome fission, chromosomal transplantation and chromosomal fusion. The GenBank accession number for this sequence is CP042182.

Table S1 – Table of spacers for fission and fusion
Table containing the spacer sequences for Cas9 cleavage used for performing all the genome fission and fusion experiments described here.

Table S2 – Homology regions for fission experiments
Table containing the genomic coordinates of genome splitting in the different fission experiments and the sequences of the homology regions used in fission.

Table S6 – Table of primers used for lambda-red recombinations
Table of primers used for lambda-red recombination experiments involving replacement of double selection markers and integration of fusion sequences.

Materials and Methods

Strains and plasmids used in this study

The positive-negative selection cassettes used in fission and fusion are -1/+1 (*pheS*$^{T251A\_A294G}$*-Kan*^{R}), -2/+2 (*sacB-Cm*^{R}) and -1/+3 (*pheS*$^{T251A\_A294G}$*-Hyg*^{R}). -2/+2 and -1/+3 are described previously (*7, 23*). In -1/+1, *pheS*$^{T251A\_A294G}$ is dominant lethal in the presence of 4-chloro-phenylalanine, and *Kan*^{R} confers resistance to kanamycin. Both proteins are expressed polycistronically under control of the EM7 promoter. The -1/+3 cassette was synthesised *de novo*. The -1/+3 cassette is also referred to as *pheS*-Hyg*^{R}. All cloning procedures described in this section were performed in *E. coli* DH10b, which carries an *rpsL*K43R mutation that confers resistance to streptomycin.

Fission BACs harboured the Linker 1 sequence (containing the *luxABCDE* operon), used to recircularize the genomic dsDNA breaks and complete Chromosome 1, as well as *sacB-Cm*^{R} and the Linker 2 sequence (containing the BAC partitioning elements) used to recircularize and complete the newly formed Chromosome 2. Fission BACs were constructed by Gibson assembly of two PCR products and two gBlocks: a PCR amplified *luxABCDE* tether construct, a PCR amplified *sacB-Cm*^{R} with BAC partitioning genes (*repE, sopA-C*), and two gBlocks (IDT) containing *rpsL* constructs with flanking protospacers (see **Data file S2** for an annotated Genbank file of a fission BAC; see **Table S4** for homology sequences).

Fusion and conjugation experiments (see below) are performed with a BAC backbone in which the *sacB-Cm*^{R} cassette is replaced by an *oriT-pheS*-Kan*^{R} cassette. To generate this variant, plasmid pSC101_oriT-pheS*-Kan was first constructed by 4-piece Gibson assembly, where the PCR products were a pSC101 backbone, an *oriT* sequence, an EM7-pheS*

sequence, and a kanamycin resistance sequence. This plasmid served as a template for amplification of the *oriT-pheS*-Kan*^{R} cassette for further recombination (see below).

The plasmid pKW20_CDFtet_pAraRedCas9_tracrRNA used throughout this study harbours the genes for Cas9 and the lambda-red recombination components alpha/beta/gamma under the control of an arabinose-inducible promoter, as well as a tracrRNA under its native promoter, as previously described (*7*).

The fission spacers are encoded in the plasmid pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer (**Data file S3**), which contains a pMB1 origin of replication, an ampicillin resistance marker, the spacer array under the control of its endogenous promoter as previously described (*7*), and a tracrRNA upstream of the spacer array (*5*).

For each fission procedure, the pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer plasmid was derivatized to contain a spacer/direct repeat array containing six spacers: four correspond to the target protospacer sequences for cutting the fission BAC, and two correspond to the target protospacer sequences for cutting the genome. The individual spacer arrays were assembled from overlapping oligos through multiple rounds of overlap extension PCR – the products were inserted by Gibson assembly between restriction sites AccI and EcoRI in the backbone of the pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer plasmid. All spacer array plasmids were verified by Sanger sequencing to be free of mutations; see **Table S5** for the sequences of spacers used in this study. From this plasmid a derivative was constructed that swaps the ampicillin resistance gene for the kanamycin resistance gene; this plasmid was generated by Gibson assembly and named pKW5_MB1$_{Kan}$_Tracr^{K}_Spacer (**Data file S4**). All fission experiments in this study were performed with the pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer plasmid, with the

exception that the fission of a 1.54 Mb section (Section ABC) was performed with the pKW5_MB1$_{Kan}$_Tracr^{K}_ Spacer plasmid.

The fusion spacers are encoded in an analogous plasmid as to that used for the fission spacers (pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer), except fusion uses only four spacers: two target Chromosome 1 (with the *oriC* origin of replication) and two target Chromosome 2 (with the BAC origin of replication). Fusion spacer plasmids were constructed in the same manner as described above.

Fission and fusion experiments were performed in the *E. coli* strain MDS42 (*1*), which has been utilized for recent genome engineering projects (*5*). The MDS42$^{rpsL\text{K43R}}$ strain (*7*) was used throughout our experiments with K43R mutation introduced into the *rpsL* gene to confer resistance to streptomycin in the absence of an additional wild-type copy of *rpsL* and sensitivity to streptomycin in the presence of any additional copy of wildtype *rpsL*.

The partially recoded MDS42$^{rpsL\text{k43R}}$ strain utilized for demonstration of Chromosome fusion, inversion and translocation contains a watermarked region 1 of approximately 0.5 Mb between 1,454,024 and 1,979,777 (**Data file S5**), with a defined serine codon and stop codon recoding scheme (TCG to AGC, TCA to AGT, and TAG to TAA). Similarly, the recipient strain of the chimeric genome generation experiment contains a watermarked region 2 with the same recoding scheme between coordinates 436,924-939,332 (**Data file S6**). Both of these strains were generated by GENESIS as described in detail previously (*5*). The resulting strain for watermarked region 1 contained an *rpsL-Kan^{R}* cassette at position 1,979,778-1,979,783, and the resulting strain for watermarked region 2 contained a *sacB-Cm^{R}* cassette at

position 1,050,809-1,050,814 – these were removed from the genome by lambda-red mediated recombination prior to genome rearrangement experiments.

Fission procedure

To perform fission, the strains of interest were electroporated sequentially, first with pKW20_CDFtet_pAraRedCas9_tracrRNA, and then with the corresponding fission BAC. Cells harbouring both of these plasmids were made competent with 0.5% L-arabinose induction for 1 hour, as described previously (*7*), to express Cas9 and the lambda-recombination machinery. 100 µL of induced electrocompetent cells were electroporated with ~8 µg of the corresponding pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer or pKW5_MB1$_{Kan}$_Tracr^{K}_Spacer plasmid, encoding spacer RNAs for the 6 necessary Cas9 cleavages. Cells were then recovered in 4 mL of SOB shaking at 37 °C for 1 hour. 80 µL of 25% L-arabinose were then added for a final concentration of 0.5%, and incubated for another hour. Cells were subsequently transferred to 100 mL of LB + 5 ng/µL tetracycline + 18 ng/µL chloramphenicol + 100 ng/µL ampicillin (for fissions performed with pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer) *or* 50 ng/µL kanamycin (for fissions performed with pKW5_MB1$_{Kan}$_Tracr^{K}_Spacer), and incubated shaking at 37 °C for 4 hours.

Cells were then pelleted by centrifugation, resuspended in Milli-Q filtered water and spread in serial dilutions in LB agar plates supplemented with 5 ng/µL tetracycline + 100 ng/µL streptomycin + 18 ng/µL chloramphenicol + 100 ng/µL ampicillin (for fissions performed with pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer) *or* 50 ng/µL kanamycin (for fissions performed with pKW5_MB1$_{Kan}$_Tracr^{K}_Spacer). From the resulting colonies, clones were identified that had some luminescent signal, but where this signal was lower than that of a pre-fission control. Candidate clones were assessed phenotypically by resuspending colonies in Milli-Q water

and stamping them on the selection plates indicated in the main text. From overnight cultures of candidate clones, genomic DNA extractions were performed with the QIAgen Blood and Tissue kit, as per manufacturer's instructions. From the genomic DNA template, PCR reactions were performed with primers targeting the hypothetical new junctions, i) flanking either side of the *luxABCDE* operon in the genome, and ii) flanking either side of the BAC backbone.

Construction of watermarked Chr. 2 bearing oriT-pheS*-Kan^R

The partially recoded MDS42$^{rpsL\text{K43R}}$ strain (**Data file S5**) contains the watermarked region 1 (1,454,024 and 1,979,777). Fission was used to partition this watermarked region into Chr. 2. The *sacB-Cm^R* in the Linker sequence 2 of Chr. 2 was subsequently replaced by an *oriT-pheS*-Kan^R* using DOSER (*7*). Post-fission cells harbouring pKW20_CDFtet_pAraRedCas9_tracrRNA were made electrocompetent with of 0.5% L-arabinose induction for 1 hour. 100 µL of electrocompetent cells were electroporated with ~8 µg of *oriT-pheS*-Kan^R* PCR product, amplified from pSC101_oriT-pheS*-Kan with primers containing homology to the *SacB-Cm^R* cassette in Chr. 2 (see **Table S6** for primer sequences). Cells were recovered for 4 hours (SOB, 200 rpm, 37 °C), pelleted by centrifugation, resuspended in 1 mL of Milli-Q water and plated on LB agar plates containing 7.5% sucrose and 50 ng/µL kanamycin. Clones that had undergone the replacement were identified by PCR flanking the *oriT-pheS*-KanR* integration site in Chr. 2, followed by Sanger sequencing. The phenotype of the correct clones was validated by stamping cell suspensions on 20 ng/µL chloramphenicol, 7.5% sucrose, 2.5 mM 4-chloro-phenylalanine, 50 ng/µL or a combination of these.

Insertion of pH with homologies for fusion

To recombineer *pheS*-Hyg*^{R} selection cassettes into the positions targeted for fusion in the genome, the selection cassette was first PCR amplified with primers containing homology regions HR1 and HR2 (**Table S2** for homology sequences, **Table S6** for primer sequences). Briefly, post-fission MDS42*rpsL*K43R cells cured of the corresponding pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer fission plasmid, and harbouring pKW20_CDFtet_pAraRedCas9_tracrRNA were made electrocompetent with 1 hour of 0.5% L-arabinose induction as described previously (*7*), to express Cas9 and lambda-red machinery. 3 µg of column-purified HR1-*pheS*-Hyg*^{R}-HR2 PCR product was electroporated into 100 µL of these competent cells. Transformed cells were recovered in 4 mL of super optimal broth (SOB) medium for 1 hour at 37 °C, diluted to 100 mL of LB with tetracycline (5 µg/mL), and incubated for 4 hours at 37 °C with shaking at 200 r.p.m. The culture was then spun down and re-suspended in 1 mL of Milli-Q water and spread in serial dilutions on selection plates of LB agar supplemented with 200 µg/mL hygromycin B.

Fusion procedure

After introduction of a *pheS*-Hyg*^{R} at the desired locus for fusion, cells were made electrocompetent with L-arabinose induction, as described above, to drive expression of Cas9 and the lambda-red machinery from pKW20_CDFtet_pAraRedCas9_tracrRNA. 100uL of electrocompetent cells were electroporated with ~8 µg of the corresponding pKW3_MB1$_{Amp}$_Tracr^{K}_Spacer fusion plasmid, encoding spacer RNAs for 4 Cas9 cleavages that initiate fusion. Cells were recovered for 2 hours in 4 mL of SOB, with addition of 0.5% L-arabinose after 1 hour (as above). They were then transferred to 100 mL LB + 5 ng/µL

tetracycline + 100 ng/μL ampicillin and recovered for 4 hours. Cells were then pelleted by centrifugation, resuspended in Milli-Q water and spread on LB agar plates containing 5 ng/μL tetracycline + 100 ng/μL ampicillin + 2.5 mM 4-chloro-phenylalanine. Resulting colonies were resuspended in Milli-Q water and stamped on selection plates (as above). Genomic DNA was prepared with the QIAgen Blood and Tissue kit as above, and PCR reactions were performed with primers flanking either side of the two new junctions generated by fusion.

Chromosomal transplant

The donor cell is post-fission MDS42^{rpsLk43R} with a watermarked Chromosome 2 containing *oriT-pheS*-Kan*^{R}. The recipient cell is post-fission MDS42^{rpsLk43R} with a non-watermarked Chromosome 2 containing *SacB-Cm*^{R} and a watermarked region in Chromosome 1 (see above). First, the *luxABCDE* operon in Chromosome 1 of the recipient cell was replaced with a *pheS*-Hyg*^{R} cassette, as above. Then, the donor and recipient strains were respectively grown in LB + 50 ng/μL kanamycin or 18 ng/μL chloramphenicol, in the absence of tetracycline or ampicillin to isolate clones cured of both pKW3_fission_C and pKW20_CDFtet_pAraRedCas9_tracrRNA. Post-fission donor cells were subsequently electroporated with an immobilised F' plasmid - pJF146 – which carries apramycin resistance (*5*). This plasmid harbours the components required for conjugation but contains a truncated *oriT* sequence, which renders it incapable of self-transfer.

For conjugative transplant, the donor strain was grown overnight in 25 ng/μL apramycin, and the recipient in 200 ng/μL hygromycin B. 5 mL of cell suspension of OD_{600} = 2.4 were washed three times in LB + 2% glucose, and resuspended in 200 μL LB + 2% glucose. 100 μL of donor cell suspension and 100 μL of recipient suspension were gently mixed by

pipetting, and the mixture spotted in 10-20 µL droplets on 37 °C pre-warmed TYE agar. The spots were left to dry and then incubated for 2 hours at 37 °C. Cells were washed off the plate with LB, diluted in 200 mL LB + 50 ng/µL kanamycin and 200 ng/µL hygromycin B, and incubated while shaking at 37 °C for 3 hours. The entire culture was pelleted by centrifugation, resuspended in Milli-Q water and plated in serial dilutions in LB agar supplemented with 50 ng/µL kanamycin + 200 ng/µL hygromycin B + 7.5% sucrose. Colonies were resuspended in Milli-Q and stamped on relevant selection plates.

Next-generation sequencing

For Illumina sequencing, *E. coli* genomic DNA was purified from overnight cultures using the DNEasy Blood and Tissue Kit (QIAgen) as per manufacturer's instructions. Paired-end Illumina sequencing libraries were prepared using the Nextera XT Kit as per manufacturer's instructions. Sequencing data was obtained in the Illumina MiSeq, running 2 x 300 or 2 x 75 cycles with MiSeq Reagent Kit v3.

For Oxford Nanopore sequencing, *E. coli* overnight cultures were purified with the PureGene Yeast/Bacteria Kit (QIAgen) as per manufacturer's instructions, taking care to avoid vigorous shaking so as to minimise DNA shearing. Sequencing libraries were prepared with the Rapid Barcoding Kit (SQK-RBK004) with the following modifications. Purified DNA samples were diluted 1:10 in water and quantified using a Qubit Fluorometer with a dsDNA High Sensitivity assay. For a given sequencing run, equal masses of different undiluted DNA samples (at least 600 ng) were subject to fragmentation with 3.5 µL of barcoded fragmentation mix, adding water to a total volume of 20 µL, and incubated for 1min 30s at 30 °C followed by 1 minute at 80 °C. AmpureXP bead clean up was omitted and the barcoded libraries were pooled directly in equimass ratios, mixed with an equal volume of AMPure XP

beads, washed twice with 500 µL of 70% EtOH and eluted in 11 µL of 10 mM Tris-HCl (pH 8.0) + 50 mM NaCl. 1 µL of pooled library was quantified in a Qubit Fluorometer with a dsDNA High Sensitivity assay, and 1 µL of RAP was added to the remaining 10 µL. The library loading mix was prepared with 11 µL of library, 34 µL of SQB, 25.5 µL of loading beads and 4.5 µL of nuclease-free water. The typical total input into the flowcell was ~1 µg of DNA. Sequencing was performed on a MinION Mk1B, using the MinKNOW software with standard 48 hour protocols.

Sequencing data analysis

Illumina reads were trimmed with Trimmomatic to remove adapter sequences (*24*). Oxford Nanopore (ONT) reads were basecalled using Guppy basecaller v2.3.1, with a mean quality cut-off filter of 6. Reads that passed the quality filter were subject to adapter trimming and demultiplexing with Porechop v0.2.4 (available at https://github.com/rrwick/Porechop), discarding the reads that contain internal adapters. Demultiplexed reads derived from the same physical DNA sample but obtained in different sequencing runs were in some instances pooled together to increase coverage of ONT reads.

Hybrid *de novo* assemblies combining Illumina and ONT data were performed using Unicycler (*22*) with bridge application set to 'normal', except for Fission G which was performed in 'bold'. Assemblies were visualised using Bandage (*25*). The assembly graphs shown in this work are a direct output of Unicycler and Bandage and were not subject to manual refinement.

Growth rate measurement and analysis

Clones resulting from fission and fusion experiments were streaked in LB agar + 100 ng/µL streptomycin plates. 5 colonies from each were picked and grown overnight in LB + 100

ng/µL streptomycin. Overnight cultures were diluted 1:100 in LB + 100 ng/µL streptomycin in a 96 well plate. OD_{600} measurements were taken every 5 minutes in a Tecan Infinite Microplate reader.

To determine doubling times, the growth curves were log2-transformed. At a linear phase of the curve during exponential growth, the first derivative was determined (d(log2(x))/dt) and ten consecutive time-points with the maximal log2-derivatives were used to calculate the doubling time for each replicate. A total of 5 independently grown biological replicates were measured for each strain. The mean doubling time and standard deviation from the mean were calculated for all n=5 replicates, except when indicated in the text.

Pulse-field gel electrophoresis analysis of fission strains

Clones resulting from fission experiments were grown overnight at 37 °C in 3mL of LB medium supplemented with appropriate antibiotics; 50 µg/mL of kanamycin for cells with Chr. 2 harbouring a *pheS*$^{T251A\_A294G}$ *-Kan*^{R} selection cassette, 20 µg/mL chloramphenicol for *sacB-Cm*^{R}, and 100 µg/mL streptomycin for the pre-fission MDS42 control. Genomic DNA agarose plugs were prepared from each strain using the BioRad CHEF Genomic DNA Kit, as per manufacturer's instructions. Plugs were stored at 4 °C until used.

Plugs were cut in half (~50 µL per half plug), and subject to a restriction digest with AvrII. For this, half-plugs were rinsed in 0.1X BioRad CHEF Wash Buffer and equilibrated twice in 1X New England Biolabs Cutsmart buffer for 20 minutes at room temperature. The buffer was aspirated and each half-plug was then treated with 25U of AvrII in 100 µL of 1X NEB Cutsmart buffer, and incubated for 2 hours at 37 °C. Following restriction digestion the buffer was aspirated, 150 µL of BioRad CHEF Proteinase K solution was added to each half-

plug, and plugs were incubated for another 30 minutes at 37 °C. Plugs were then rinsed with 800 µL of 0.5x TBE, and loaded into a 1% agarose pulse-field gel. Electrophoresis was conducted in a BioRad CHEF-DR-III pulse-field electrophoresis system, in 0.5x TBE, for 22 hours at 14 °C, with 20-120s switch times, 120° angle and 6 V/cm.

Analysis of genetic stability

Cells were grown at 37 °C in 3 mL volumes of LB medium supplemented with the appropriate antibiotic (e.g. 20 µg/mL of chloramphenicol for strains with Chr. 2 harbouring a *sacB-Cm*^{R} selection cassette or 50 µg/mL of kanamycin for strains with Chr. 2 harbouring a *pheS*$^{T251A\_A294G}$ *-Kan*^{R} selection cassette) while shaking at 200 rpm. The cultures were passaged by diluting 1/1500x once every twelve hours for a total of 5 days, which yielded a total estimated number of generations of 105 (*26*). To assess the genetic stability of post-passaged cells, the resulting cultures were used directly for the preparation of genomic DNA agarose plugs for pulse-field gel electrophoresis, as described above.

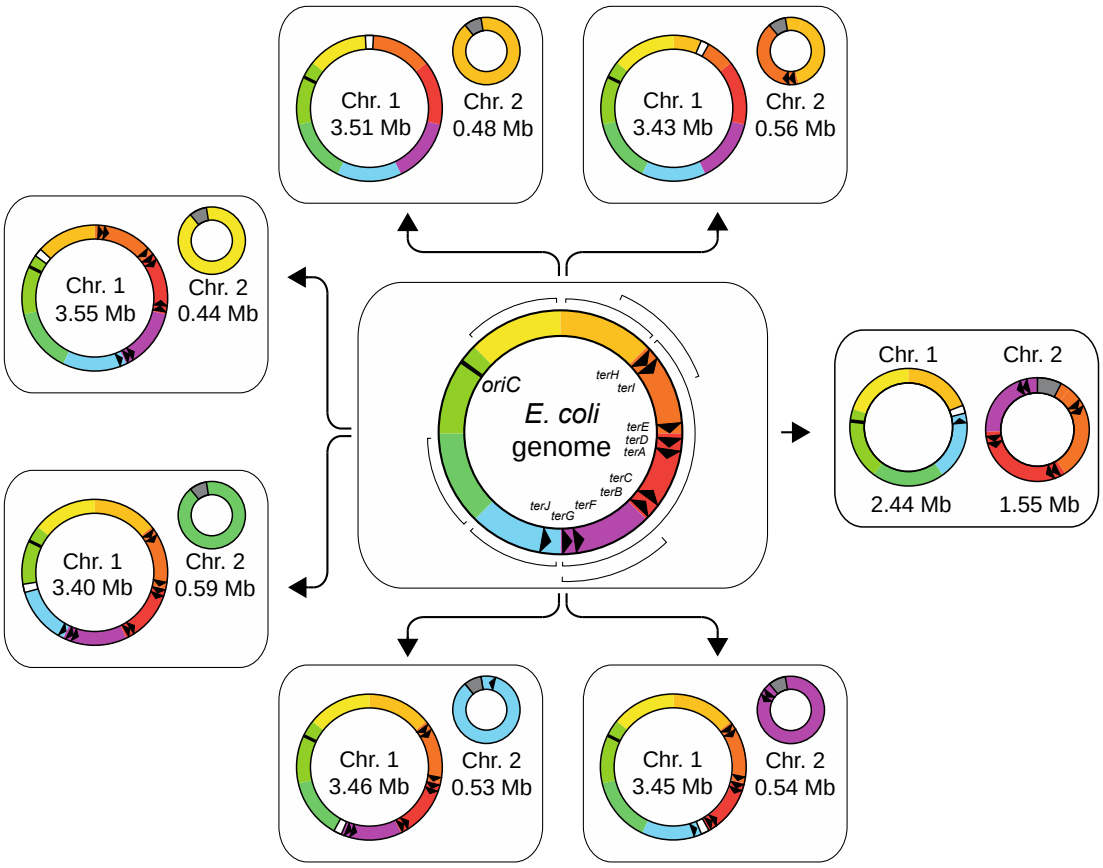
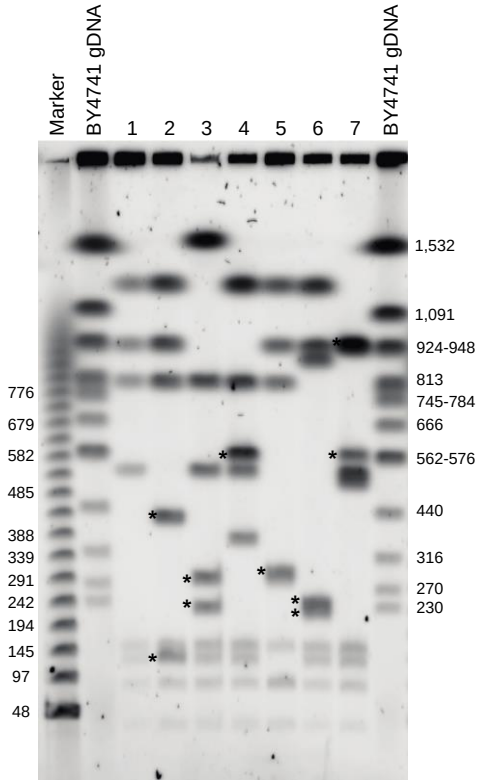


Fig. S1. Details of *E. coli* genomes before and after fission. Different regions of the *E. coli* genome are represented as different colours, with each colour corresponding to approximately 500 kb. Section A (436,924 – 939,016; Dark orange), Section B (938,832 – 1,454,152; Red), Section C (1,453,970 – 1,979,288; Purple), Section D (1,979,116 – 2,502,461; Blue), Section E (2,502,331 – 3,082,013; Dark green), Section F (3,081,850 – 3,508,874; Light green), Section G (3,508,705 – 3,945,69; Yellow), Section H (3,945,525 – 437,084; Light orange). The diagram shows a schematic of successul fissions. All fissions, except the 3.43 Mb, 0.56 Mb fission split the genome at the indicated section boundaries. In Chromosome 1 (Chr.1). Linker sequence 1 is represented as a white bar and *oriC* is represented as a black bar. In Chromosome 2 (Chr.2), Linker sequence 2 is shown in grey. The termination sites, *terH, terI, terE, terD, terA, terC, terB, terF, terG,* and *terJ,* are all represented as black arrows. The location of termination sites before and after fission experiments is shown.



Section in Chr. 2 following fission

Sample # :	MDS42 wild-type 1	Fission 0.56 Mb 2	D 3	E 4	G 5	H 6	ABC 7
	1,246,785	1,246,785	1,681,672	1,246,785	1,246,785	1,246,785	956,536
	952,029	952,009	815,317	815,317	952,009	952,009	952,009
	815,317	814,274	550,099	587,360	815,317	903,828	593,467
	550,099	430,043	298,054	550,099	312,112	245,792	550,099
	144,278	144,278	232,941	378,508	293,369	229,655	525,958
	131,128	134,946	144,278	144,278	144,278	144,278	144,278
	92,304	131,128	131,128	131,128	92,304	131,128	131,128
	39,983	92,304	92,304	92,304	89,605	92,304	92,304
	1,505	39,983	39,983	39,983	39,983	39,983	39,983
	1,419	1,505	1,505	1,505	1,505	1,505	1,505
	1,419	1,419	1,419	1,419	1,419	1,419	1,419
		1,419	1,419	1,419	1,419	1,419	1,419

Fig S2. Pulse-field gel electrophoresis of post-fission strains. Genomic DNA from each of the indicated samples was digested with AvrII and subjected to PFGE. Each sample gave the expected band pattern. Predicted band sizes, in bp, are shown in the table. Diagnostic bands originating from cutting of Chr. 2 are highlighted in the table, and marked with an asterisk in the gel. 'Marker' lanes contain New England Biolabs' Lambda PFG Ladder. The size of the bands in the ladders is indicated in kbp.

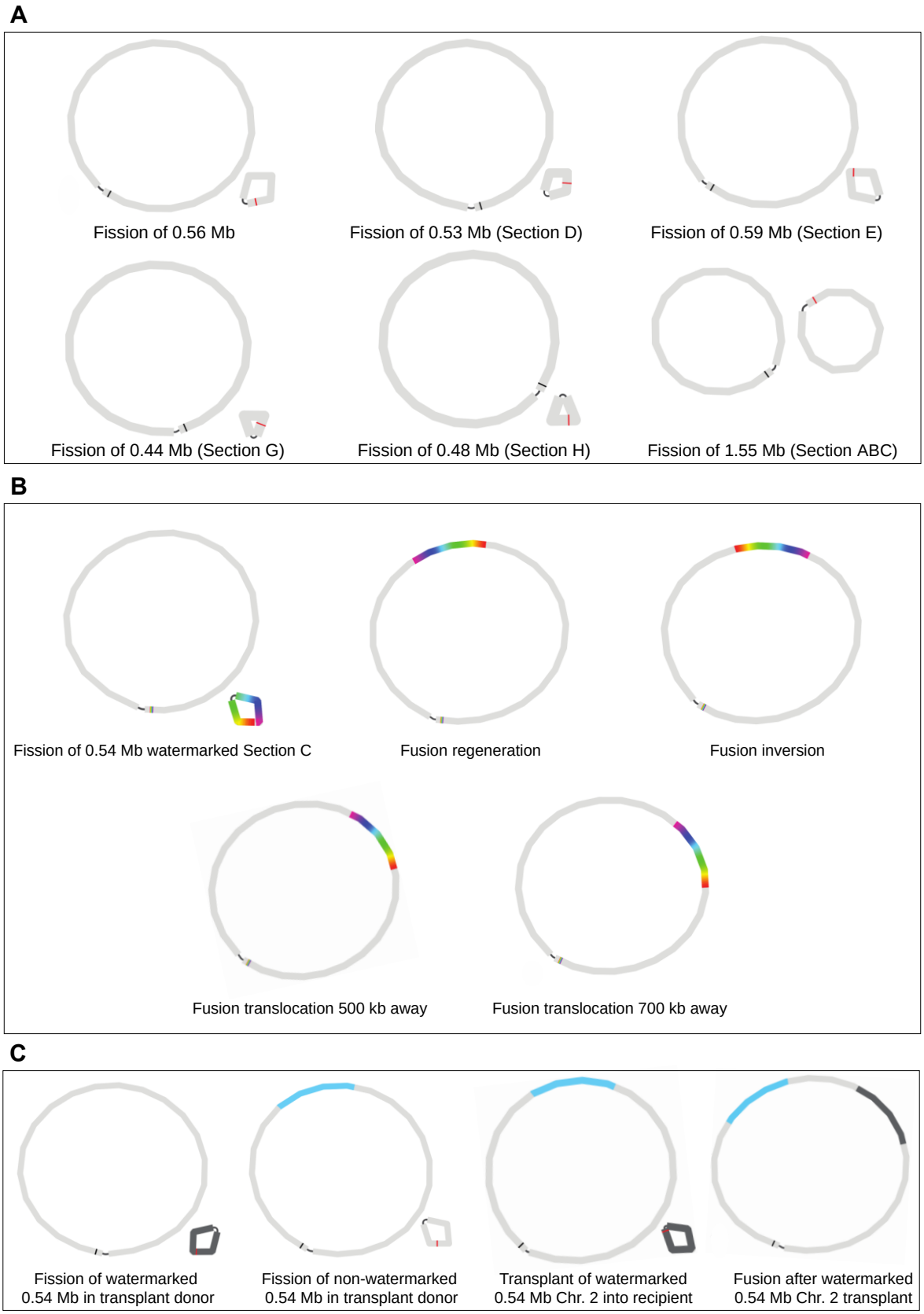


Fig. S3. *De novo* assembly graphs of fission and fusion experiments. (A) Assembly graphs for the *de novo* assembly of six *E. coli* genomes post-fission, each composed of two pairs of

distinct chromosomes (**Fig. 2A**). The regions corresponding to *oriC* and the BAC replication components are shown as black and red lines, respectively. **(B)** *De novo* assembly graph of *E. coli* genomes after fission of watermarked Section C (**Fig. 2, Fig. S1**) and fusion to generate different arrangements (**Fig. 3**). The watermarked region is represented as the long rainbow segment, with directionality 5' (magenta) to 3' (red). The region 10 kbp upstream and downstream of *oriC* is represented as the small rainbow segment for reference, with directionality 5' (magenta) to 3' (red). Fusion with inversion places the watermarked region in an inverted orientation, while regeneration generates the original arrangement. Chromosomal fusion with translocation places the watermarked region 500 or 700 kb away from its original location, preserving its original directionality. **(C)** *De novo* assembly graphs of the donor and recipient strains of chromosomal transplant post-fission, post-transplant of Chromosome 2 and post-fusion into a single chromosome (**Fig. 4**). The watermarked Section C from the donor (**Fig. 2, Fig. S1)** is shown in dark grey. The watermarked Section A in the recipient (**Fig. 2, Fig. S1**) is shown in blue. Fission in the donor results in the transfer of watermarked Section C into Chromosome 2. Fission in the recipient results in the transfer of a non-watermarked Section C into Chromosome 2. After transplant of Chromosome 2 from the donor to the recipient, the recipient cell contains a watermarked Chromosome 2. Fusion generates a cell with a single chromosome where both Section A and Section C are recoded. All *de novo* assemblies were performed with Unicycler (*22*) and visualised with Bandage (*25*). Regions of interest were highlighted and coloured with the Bandage integrated BLAST (*27*) search. Contigs corresponding to the pKW20_CDFtet_pAraRedCas9_tracrRNA and pKW3/5_MB1_Tracr^{K}_Spacer plasmids used in fission and fusion experiments are not shown.

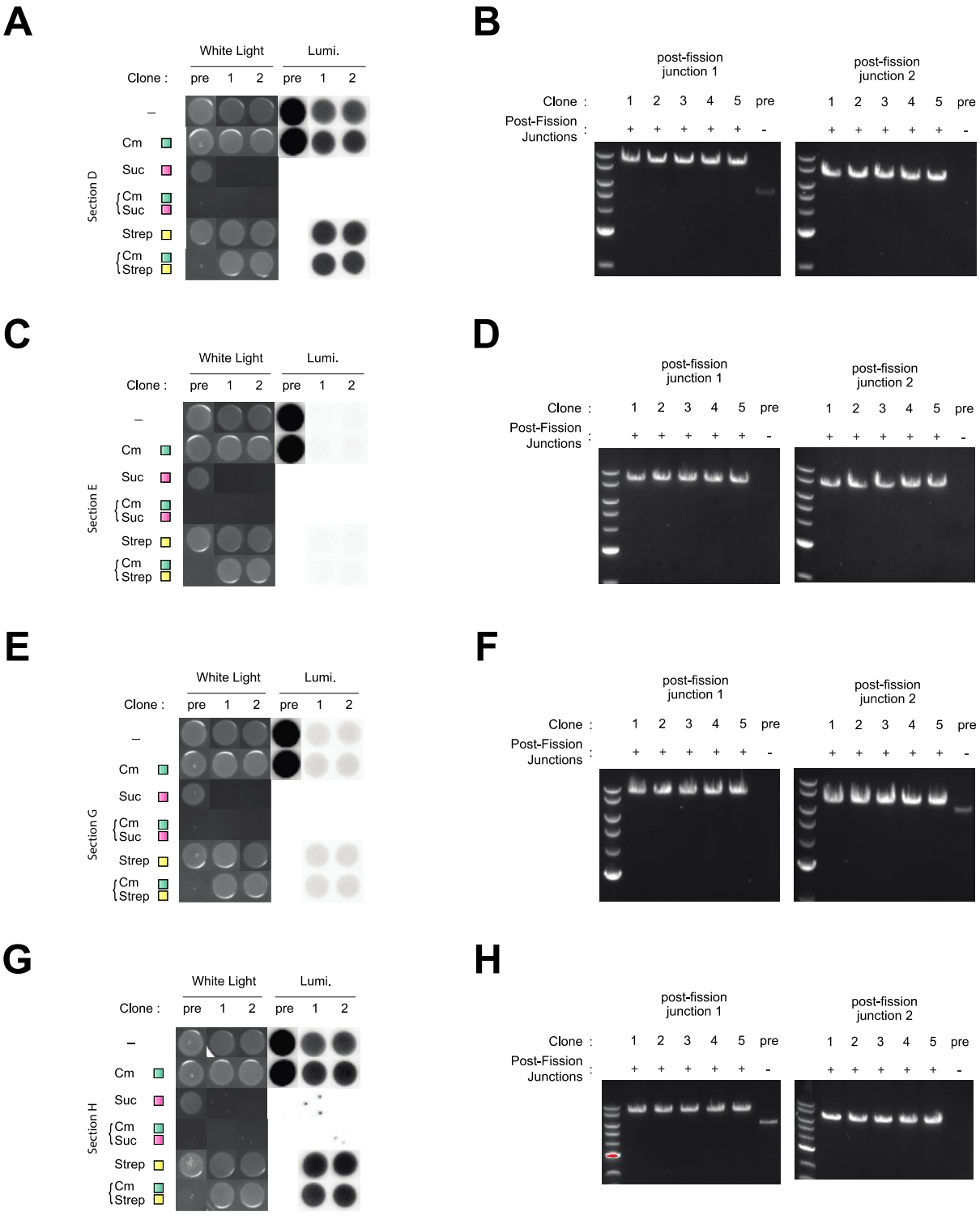


Fig. S4. Selective growth and junction PCR data for Fission experiments performed throughout the *E. coli* genome. (A) Selective growth and luminescence for the fission of a 0.53 Mb Chr. 2 derived from Section D (see **Fig. S1** for a detailed schematic; different ~0.5 Mb regions of the *E. coli* genome are represented as different colours). Post-fission clones (1

and 2) are not able to lose Chr. 2 upon addition of sucrose. The new location of Linker sequence 1 (containing *luxABCDE*) in Chr. 1, as opposed to the fission BAC, results in a drop in luminescence intensity (Lumi.). Cells were assessed for growth in plain LB agar (-), 20 µg/mL chloramphenicol (Cm), 7.5% sucrose (Suc), 100 µg/mL streptomycin (Strep) or a combination of these. "Pre" stands for pre-fission control. **(B)** Junction specific PCR for the fission of a 0.53 Mb Chr. 2 derived from Section D (**Fig. S1**), assessed by PCR across junction 1 (Linker sequence 1 in Chr. 1) and junction 2 (Linker sequence 2 in Chr. 2). Post-fission clones (1-5) exhibit products of the expected size, whereas the pre-fission control (pre) does not. **(C)** Selective growth and luminescence for the fission of a 0.59 Mb Chr. 2 derived from Section E (**Fig. S1**). Post-fission clones (1 and 2) are not able to lose Chr. 2 upon addition of sucrose. The new location of Linker sequence 1 (containing *luxABCDE*) in Chr. 1, as opposed to the fission BAC, results in a drop in luminescence intensity (Lumi.). Cells were assessed for growth in plain LB agar (-), 20 µg/mL chloramphenicol (Cm), 7.5% sucrose (Suc), 100 µg/mL streptomycin (Strep) or a combination of these. "Pre" stands for pre-fission control. **(D)** Junction specific PCR for the fission of a 0.59 Mb Chr. 2 derived from Section E (**Fig. S1**), assessed by PCR across junction 1 (Linker sequence 1 in Chr. 1) and junction 2 (Linker sequence 2 in Chr. 2). Post-fission clones (1-5) exhibit products of the expected size, whereas the pre-fission control (pre) does not. **(E)** Selective growth and luminescence for the fission of a 0.44 Mb Chr. 2 derived from Section G (**Fig. S1**). Post-fission clones (1 and 2) are not able to lose Chr. 2 upon addition of sucrose. The new location of Linker sequence 1 (containing *luxABCDE*) in Chr. 1, as opposed to the fission BAC, results in a drop in luminescence intensity (Lumi.). Cells were assessed for growth in plain LB agar (-), 20 µg/mL chloramphenicol (Cm), 7.5% sucrose (Suc), 100 µg/mL streptomycin (Strep) or a combination of these. "Pre" stands for pre-fission control. **(F)** Junction specific PCR for the fission of a 0.44 Mb Chr. 2 excised from Section G (**Fig. S1**), assessed by PCR

across junction 1 (Linker sequence 1 in Chr. 1) and junction 2 (Linker sequence 2 in Chr. 2). Post-fission clones (1-5) exhibit products of the expected size, whereas the pre-fission control (pre) does not. **(G)** Selective growth and luminescence for the fission of a 0.48 Mb Chr. 2 derived from Section H (**Fig. S1**). Post-fission clones (1 and 2) are not able to lose Chr. 2 upon addition of sucrose. The new location of Linker sequence 1 (containing *luxABCDE*) in Chr. 1, as opposed to the fission BAC, results in a drop in luminescence intensity (Lumi.). Cells were assessed for growth in plain LB agar (-), 20 µg/mL chloramphenicol (Cm), 7.5% sucrose (Suc), 100 µg/mL streptomycin (Strep) or a combination of these. "Pre" stands for pre-fission control. **(H)** Junction specific PCR for the fission of a 0.48 Mb Chr. 2 derived from Section H (Fig. S1), assessed by PCR across junction 1 (Linker sequence 1 in Chr. 1) and junction 2 (Linker sequence 2 in Chr. 2). Post-fission clones (1-5) exhibit products of the expected size, whereas the pre-fission control (pre) does not.

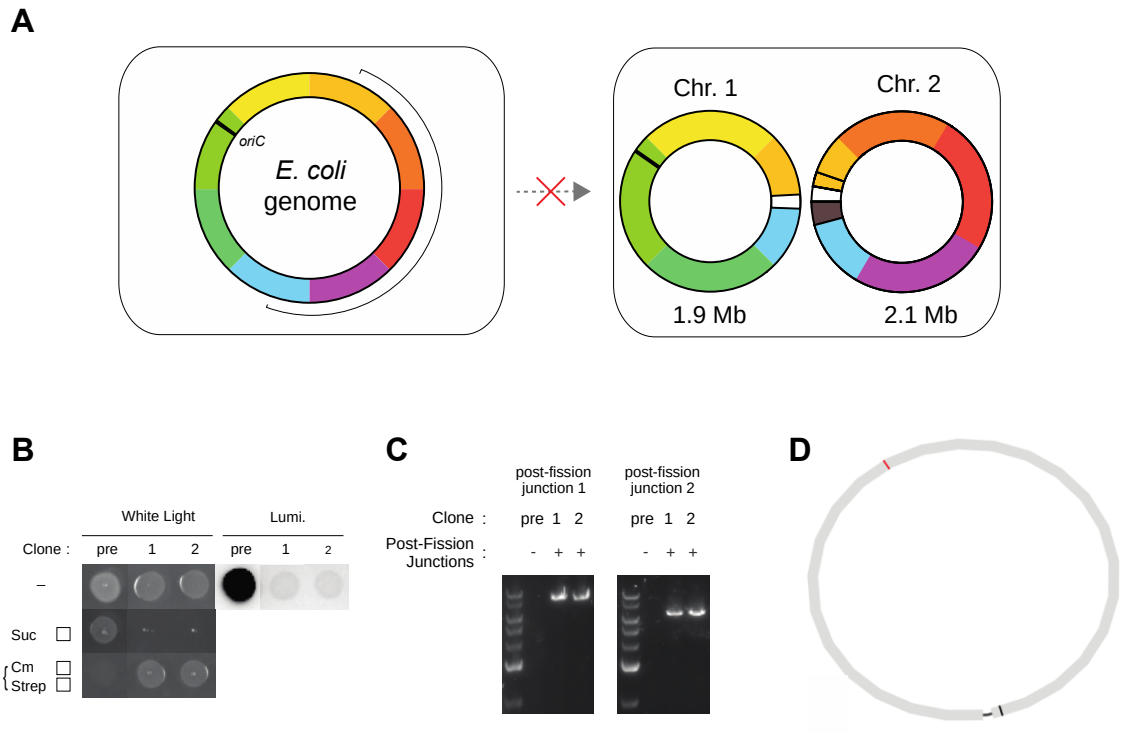


Fig. S5. Characterisation of a failed fission. (A) Diagram of an attempted 2 Mb fission. The colour scheme is as in Fig. 1. **(B)** Cells have the correct selective growth phenotypes. (**C**) PCR across the new junctions forms the correct products. (**D**) Whole genome sequencing and *de novo* assembly of the resulting genomes shows a single contig. In two independent instances, the *de novo* assemblies show that while Linker 1 (*luxABCDE*) and Linker 2 (BAC replication components) are flanked by their respective expected DNA sequences, further recombinations between ribosomal RNA genes mediate the maintenance of a single-chromosome architecture. The accession numbers for the DNA sequences of the assemblies and the sequencing data are listed in **Table S4**. Our data do not allow us to determine whether this fission failed because of the specific chromosomes it would generate or because of their size.

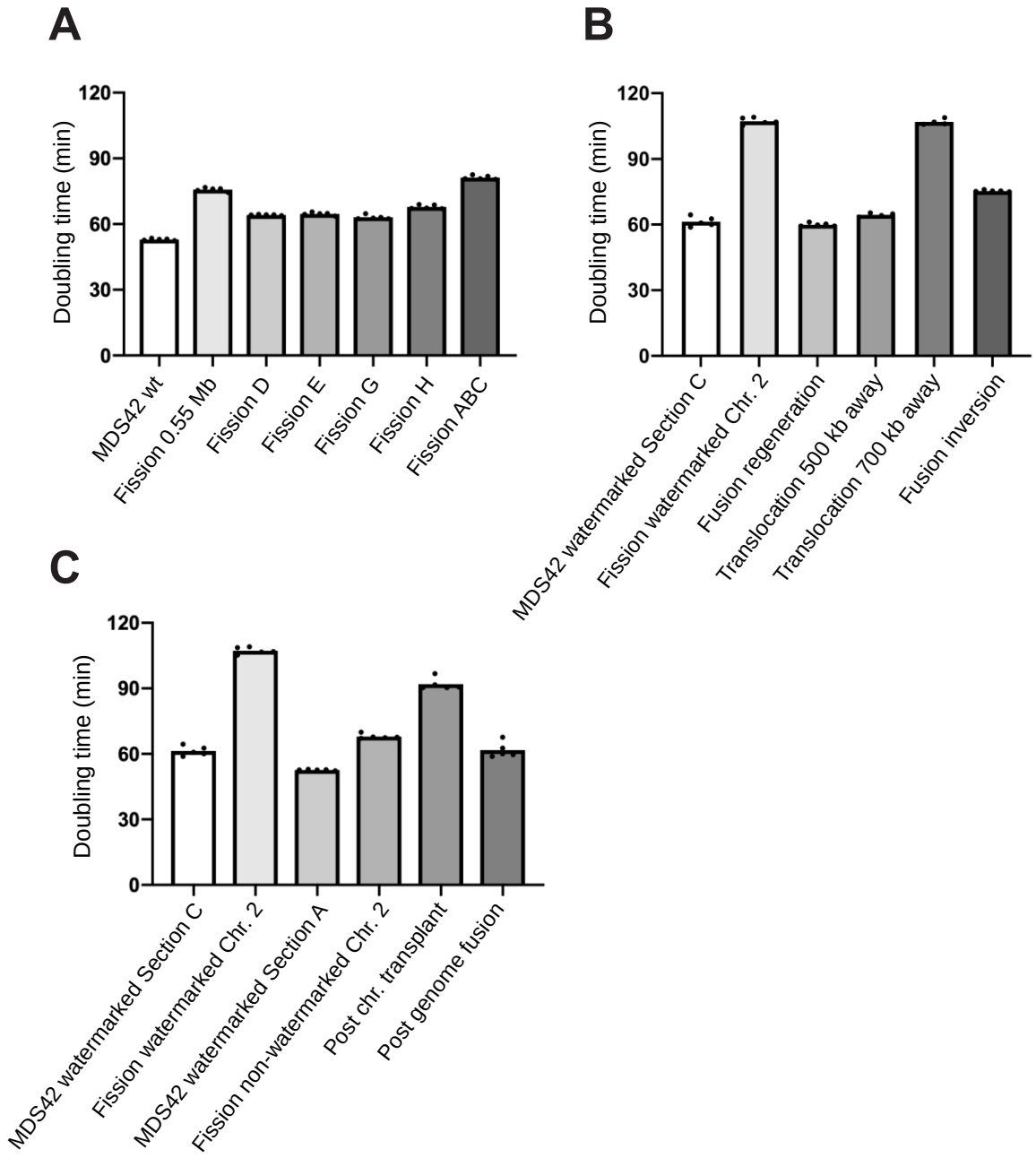
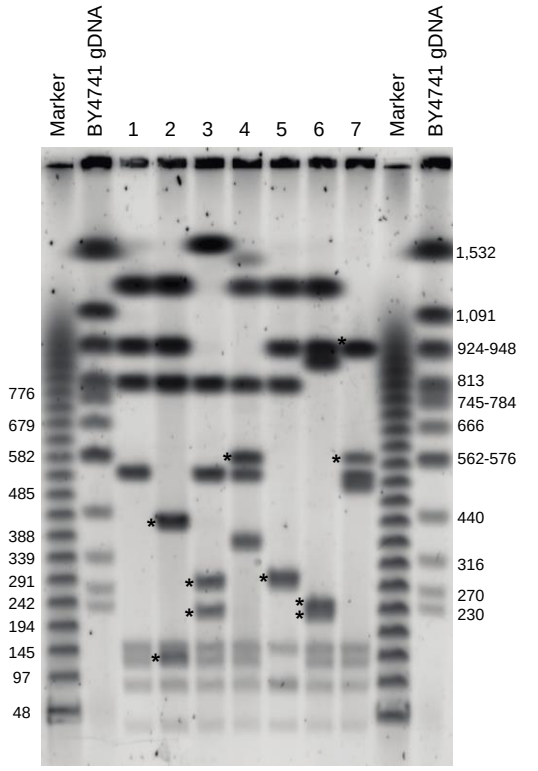


Fig. S6. Doubling times for post-fission and post-fusion clones described in this study. **(A)** Doubling times of all strains resulting from fission experiments described in **Fig. 1, Fig. 2** and **Fig. S1**. Doubling times were measured in LB supplemented with 100 ng/µL streptomycin. Listed are the doubling times in minutes of the different strains: pre-fission parental wildtype MDS42 ("MDS42 wt"), 52.97 ± 0.39; post-fission of 0.56 Mb

Chromosome 2, 75.76 ± 0.8; post-fission of section D (“Fission D”), 64.18 ± 0.21; post-fission of section E, 64.67 ± 0.61; post-fission of section G, 63.1 ± 0.85; post-fission of section H (“Fission H”), 67.83 ± 0.83; post-fission of 1.55 Mb segment ABC (“Fission ABC”), 81.28 ± 0.81. Doubling times represent the average of five independently grown biological replicates of each strain. The individual data points are represented by dots. **(B)** Doubling times of all strains resulting from fission and fusion experiments (described in **Fig. 2, Fig. 3 and Fig. S1**) measured in LB supplemented with 100 ng/μL streptomycin. Listed are the doubling times in minutes of the different strains: pre-fission MDS42 with a watermarked Section C (“MDS42 watermarked Section C”), 61.37 ± 1.97; post-fission of the watermarked Section C (“Fission watermarked Chr. 2”), 107.30 ± 1.39; post-fusion of Chr. 1 and Chr.2 to regenerate the original genomic arrangement (“Fusion regeneration”), 59.94 ± 0.67; translocation of watermarked Section C 500 kb away form its original locus (“Translocation 500 kb away”), 64.45 ± 0.70; post-fusion translocation of watermarked Section C 700 kb away form its original locus (“Translocation 700 kb away”), 106.93 ± 1.19; post-fusion inversion of watermark region 1 (“Fusion inversion”), 75.4 ± 0.36. Doubling times of “Translocation 500 kb away” and “Translocation 700 kb away” represent the average of four independently grown biological replicates of each strain. All other doubling times represent the mean of five biological replicates. The individual data points are represented by dots. **(C)** Doubling times of all strains resulting from fission and fusion experiments described in **Fig. 4,** measured in LB + 100 ng/μL streptomycin. “MDS42 watermarked Section C” and “Fission watermarked Chr. 2” are as described in (**B)**. Listed are the doubling times in minutes of the different strains: pre-fission MDS42 with watermarked Section A (“MDS42 watermarked Section A”), 52.66 ± 0.25; post-fission of non-watermarked Section C in MDS42 with watermarked Section A (“Fission non-watermarked Chr. 2”), 67.98 ± 1.01; post-transplant MDS42 watermarked Section A containing a

watermarked Chr. 2 (“Post chr. transplant”, from **Fig. 4**), 91.95 ± 2.43; post-fusion chimeric genome containing a single chromosome with watermarked Sections C and A (“Post genome fusion”, from **Fig 4**), 61.77 ± 3.20. Doubling times represent the mean of five independently grown biological replicates of each strain ± standard deviation from the mean. The individual data points are represented by dots.



Section in Chr. 2 following fission

	MDS42 wild-type	Fission 0.56 Mb	D	E	G	H	ABC
Sample # :	1	2	3	4	5	6	7
	1,246,785	1,246,785	1,681,672	1,246,785	1,246,785	1,246,785	956,536
	952,029	952,009	815,317	815,317	952,009	952,009	952,009
	815,317	814,274	550,099	587,360	815,317	903,828	593,467
	550,099	430,043	298,054	550,099	312,112	245,792	550,099
	144,278	144,278	232,941	378,508	293,369	229,655	525,958
	131,128	134,946	144,278	144,278	144,278	144,278	144,278
	92,304	131,128	131,128	131,128	92,304	131,128	131,128
	39,983	92,304	92,304	92,304	89,605	92,304	92,304
	1,505	39,983	39,983	39,983	39,983	39,983	39,983
	1,419	1,505	1,505	1,505	1,505	1,505	1,505
	1,419	1,419	1,419	1,419	1,419	1,419	1,419
		1,419	1,419	1,419	1,419	1,419	1,419

Fig S7. Analysis of the stability of fissions by pulse-field gel electrophoresis. Genomic DNA from each of the indicated samples was digested with AvrII and subjected to PFGE. The size of predicted bands, in bp, for each sample are shown in the table. Diagnostic bands originating from cutting of Chr. 2 are highlighted in the table, and marked with an asterisk in the gel. We observed all the expected bands following AvrII digestion of fission products after growing continuously for 105 generations. We observed one minor additional high molecular weight band for the Fission E digest; while this may be the result of a partial digest or contamination of this culture we cannot rule out a minor portion of the Fission E cells contain a rearrangement after prolonged growth. We observed no additional bands for any of the other fissions tested, suggesting they are genetically stable. 'Marker' lanes contain New England Biolabs' Lambda PFG Ladder. The sizes of the bands in the ladders are indicated in kbp.

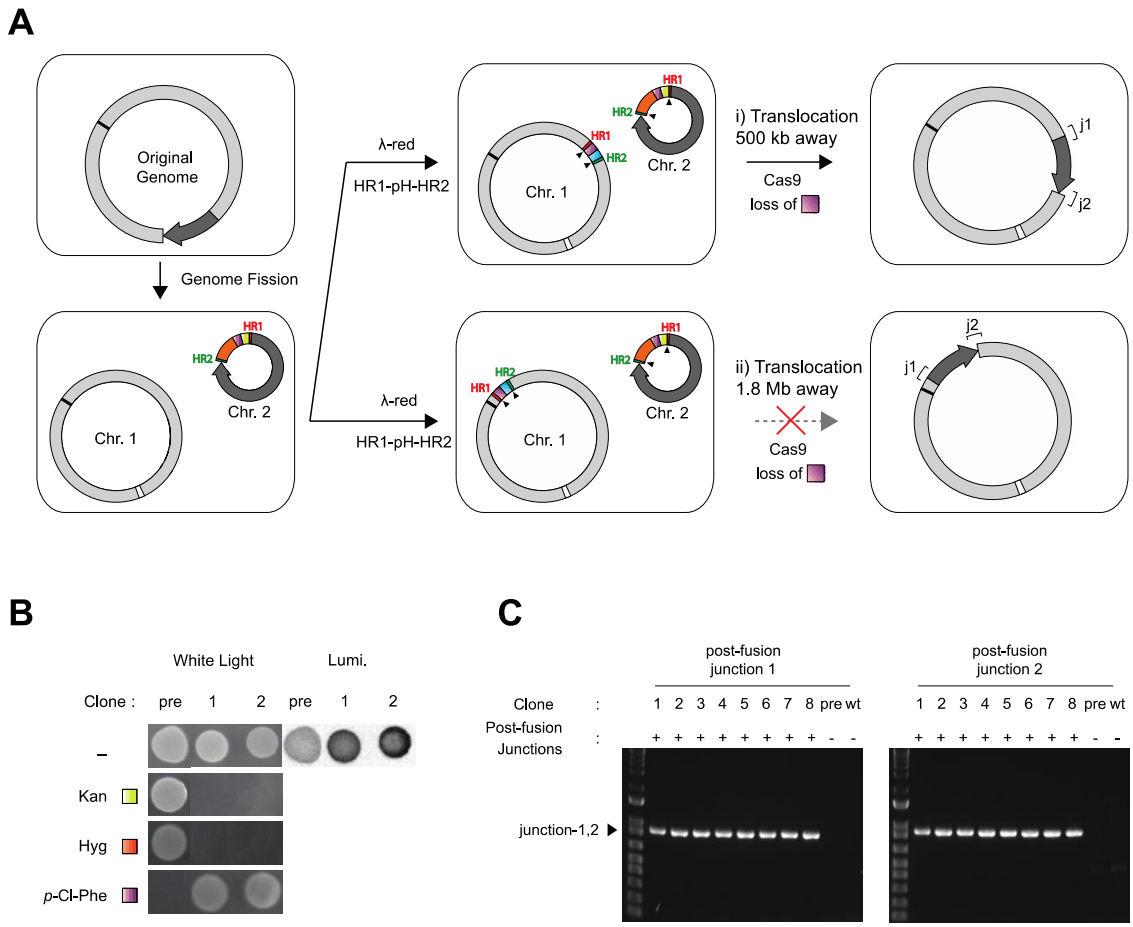


Fig. S8. Programmed translocation of a 0.54 Mb watermarked region to sites 500 kb and 1.8 Mb away from the original genomic locus. (A) A ~0.54 Mb watermarked DNA segment (dark grey) is separated from the rest of the *E. coli* genome (light grey) by fission, yielding Chr. 1 (~3.45 Mb) and watermarked Chr. 2 (0.54 Mb). Chr. 2 is supported by the BAC replication and segregation components (orange), and coupled to a *pheS**-*Kan*^{R} double selection cassette (purple and yellow, respectively). Chr. 2 contains the homology regions HR1 (red) and HR2 (green), which will drive chromosomal fusion. To achieve fusion, a fusion sequence containing a *pheS**-*Hyg*^{R} double selection cassette flanked by HR1 and HR2 is introduced in Chr. 1 by lamba-red recombination. This is followed by expression of Cas9 and lambda-recombination machinery, provision of suitable protospacers for cleavage (black arrows) and selection for fusion products via loss of *pheS** on 4-chloro-phenylalanine. The location and orientation of the fusion sequence in Chr. 1 defines the architecture of the

resulting genome. The figure shows **i)** fusion of Chr. 2 and Chr.1 at a genomic location ~500 kb away from its original location, by integration of the fusion sequence at the target locus, and **ii)** translocation of the 0.54 Mb segment 1.8 Mb away from its original location. Each of these processes produces two junctions, termed j1 and j2. **(B)** Selective growth and luminescence of the fusion-mediated translocation of the 0.54 Mb watermarked region ~500 kb away from its original genomic locus. Post-fusion clones (1 and 2) are resistant to 4-chloro-phenylalanine and sensitive to kanamycin and hygromycin. The pre-fusion (pre) and post-fusion clones are luminescent by virtue Linker sequence 1 (containing *luxABCDE*) in Chr. 1. "Pre" indicates the post-fission, pre-fusion control. **(C)** Junction specific PCR of the fusion-mediated translocation 500 kb away from its original genomic locus with primers flanking either side of the two new junctions j1 (junction 1) and j2 (junction 2). All post-fusion clones (1-8) and the pre-fission control (wt) yield bands of the expected size, whereas the pre-fusion control (pre) does not.

Experiment	Fraction of 200mL culture plated	Total colonies on plate	Colonies too luminescent	Colonies correct luminescence	Colonies no luminescence	Selective growth correct	PCR across junctions correct	De novo assembly correct
Fission 0.56 Mb	0.001	86	1	64	21	16 out of 16	15 out of 16	1 out 1
Fission 2.1 Mb	0.1	192	158	27	7		4 out of 8	0 out of 2
Fission 0.53 Mb (section D)	0.01	67	11	7	49	5 out of 7	5 out of 5	1 out of 1
Fission 0.59 Mb (section E)	0.1	164	54	109	1	9 out of 9	5 out of 5	1 out of 1
Fission 0.44 Mb (section G)	0.1	365	125	13	227	6 out of 8	5 out of 5	1 out of 1
Fission 0.48 Mb (section H)	0.01	114	36	60	18	9 out of 9	5 out of 5	1 out of 1
Fission 1.55 Mb (section ABC)	0.01	81	2	76	3	32 out of 32	16 out of 16	1 out of 1
Fission 0.54 Mb (watermarked Section C)	0.01	47	13	25	9	27 out of 28	10 out of 10	1 out of 1
Fission 0.54 Mb (non-watermarked Section C)	1	35	10	22	3	18 out of 20	10 out of 10	1 out of 1
Fusion regeneration (Section C)	0.1	Thousands	NA	NA	NA	8 out of 8	8 out of 8	1 out of 1
Translocation 700 kb away	0.1	Thousands	NA	NA	NA	8 out of 8	8 out of 8	1 out of 1
Translocation 500 kb away	0.1	Thousands	NA	NA	NA	8 out of 8	8 out of 8	1 out of 1
Translocation 1.8 Mb away	0.1	50	NA	NA	NA	8 out of 8	8 out of 8	0 out of 1
Inversion	0.1	Thousands	NA	NA	NA	8 out of 8	8 out of 8	1 out of 1

Table S3. Efficiencies of fission and fusion experiments

The efficiencies of individual fission or fusion experiments are provided. From the post-fission colonies that exhibited decreased luminescence compared to the pre-fission strain (Colonies correct luminescence), the efficiencies of selective growth and PCR across junctions were determined. For the translocation 1.8 Mb away from the original site, the *de novo* assembly of the clone investigated was incorrect. We do not expect all translocations to be viable, and it seems likely that this translocation failed because it generated an unfavourable genome arrangement.

Experiment	Genbank #	SRA #
Fission 0.56 Mb	VMRF00000000	SRR9671404, SRR9661817
Fission 2.1 Mb clone 1	CP041993	SRR9671398, SRR9661793
Fission 2.1 Mb clone 2	CP041992	SRR9671397, SRR9661791
Fission 0.53 Mb (section D)	VMRE00000000	SRR9671403, SRR9661819
Fission 0.59 Mb (section E)	VMRD00000000	SRR9671406, SRR9661813
Fission 0.44 Mb (section G)	VMRC00000000	SRR9671405, SRR9661815
Fission 0.48 Mb (section H)	VMRB00000000	SRR9671400, SRR9661811
Fission 1.55 Mb (section ABC)	VMRA00000000	SRR9671399, SRR9661799
Fission 0.54 Mb (watermarked Section C)	VMQZ00000000	SRR9671402, SRR9661797
Fission 0.54 Mb (non-watermarked Section C)	VMQY00000000	SRR9671401, SRR9661795
Fusion regeneration (section C)	CP041991	SRR9671412, SRR9661808
Translocation 700 kb away	VMQX00000000	SRR9671410, SRR9661804
Translocation 500 kb away	CP041989	SRR9671409, SRR9661802
Translocation 1.8 Mb away	N/A	SRR9661776
Inversion	CP041990	SRR9671411, SRR9661806
Post-transplant of watermarked Section C into strain with watermarked Section A	VMQW00000000	SRR9671408, SRR9661810
Post-fusion to combine watermarked Sections A and C	CP041988	SRR9671407, SRR9661789

Table S4. Deposition of genome assemblies and raw sequencing data.

Genbank files containing annotated sequences of the strains resulting from fission and fusions experiments were deposited, with the Genbank identifier number (Genbank #) of each indicated strain provided. Raw sequencing data from both Illumina and Oxford Nanopore Technologies NGS platforms was deposited on the sequence read archive (SRA) (https://wwww.ncbi.nlm.nih.gov/sra), with the SRA identifier number (SRA #) provided for each indicated strain.

Experiment	Chr. 2 Relative copy number
Fission 0.56 Mb	1.21x
Fission 0.53 Mb (section D)	1.82x
Fission 0.59 Mb (section E)	1.55x
Fission 0.44 Mb (section G)	1.97x
Fission 0.48 Mb (section H)	1.99x
Fission 1.55 Mb (section ABC)	1.01x
Fission 0.54 Mb (watermarked Section C)	1.47x
Fission 0.54 Mb (non-watermarked Section C)	1.09x

Table S5. Copy number of Chromosome 2 from different fission experiments

The copy number of Chromosome 2 (Chr. 2) generated by fission, relative to Chromosome 1, is provided for the indicated post-fission strain. The copy number was calculated from the *de novo* assembly data by the program Unicycler (*22*).

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