### Supplemental material

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### I. Supplemental Materials and Methods

Table 1. *Escherichia coli* strains

| Strain       | Relevant genotype                                       | Reference |
|--------------|---------------------------------------------------------|-----------|
| C600         | *surE44 hsdR thi-1 thr-1 leuB6*                         | (70)      |
|              | *lacY1 tonA21*                                          |           |
| C600polA1    | As C600 but *fadA:: Tn10*                               | this work |
|              | *polA1*; constructed by P1 transduction from H221       |           |
|              | *zfi3134::Tn10kan*                                      |           |
| CAG18562     |                                                         | (71)      |
| DH5α         | *supE44 ΔlacU169 [Δ 80]*                                 | (72)      |
|              | *lacZΔM15* *hsdR17 recA1*                               |           |
|              | *gyrA96 thi-1 relA1*                                     |           |
| H221         | *fadA::Tn10 polA1*                                       | (73)      |
| MM294        | *supE44 hsdR endA1 pro thi*                             | (74)      |
| MG1655       | wild type                                               | (75)      |
| MGO          | as MG1655 but containing this work                       |           |
|              | pCAHlamO plasmid integrated in the lambda attachment site|           |

Table 2. Plasmids
| Plasmid      | Characteristics and/or construction                                                                 | Reference |
|-------------|------------------------------------------------------------------------------------------------------|-----------|
| pKB2        | Wild-type λ plasmid bearing a *kan* gene                                                              | (76)      |
| pKB2po-     | As pKB2 but bearing a point mutation in the -10 region of the *pO* promoter                           | (58)      |
| pLamber     | A hybrid plasmid bearing *oriλ* and *oriColE1*-like, and *kan* and *bla* genes                       | (73)      |
| pLamberA    | As pLamber but lacking the *kan* gene; constructed by removing of the 916 bp *HindIII*-Neol fragment | This work |
| pLamberB    | As pLamber but lacking the *bla* gene; constructed by removing of the 803 bp *AsuII*-AseI fragment   | This work |
| pdelλO      | A derivative of pLamberB bearing a deletion of a part of the *O* gene, coding for C-terminal domain of *λO*. | This work |
| pdelλOpo-   | As pdelλO but bearing a point mutation in the -10 region of the *pO* promoter                        | This work |
| pdelλOins6,  | A series of plasmids derived from pdelλO, in which insertions of 6, 10, 50, 100 or 500 bp were introduced between *pO* and *oriλ* | This work |
| pdelλOins10, |                                                                                                    |           |
| pdelλOins50, |                                                                                                    |           |
| pdelλOins100, |                                                                                                     |           |
| pdelλOins500 |                                                                                                    |           |
| pIK12       | pBluescript II SK vector bearing *O*<sup>+</sup> and *PtsI*<sup>-</sup>*A66* alleles                 | (77)      |
| pEW1        | As pIK12 but bearing wild-type alleles of *O* and *P*; constructed by replacement of the *EcoRI*-SsrII fragment from pIK12 with an | (44)      |
| Plasmid Name          | Description                                                                 | Reference |
|----------------------|-----------------------------------------------------------------------------|-----------|
| pGP1-2               | A part of the T7 RNA polymerase expression system                            | (78)      |
| pUC19oriλ            | pUC19 bearing oriλ                                                          | this work |
| pUC19it34            | pUC19 containing λ iterons 3 and 4                                          | this work |
| pUC19it4             | pUC19 containing λ iteron 4                                                 | this work |
| pBRoriλ              | pBR322 bearing oriλ                                                         | this work |
| pBRit34              | pBR322 containing λ iterons 3 and 4                                         | this work |
| pBRit4               | pBR322 containing λ iteron 4                                                | this work |
| pCAH56               | contains attP of the λ phage, ptac promoter and cat gene                     | (79)      |
| pCAHlamO             | pCAH56 derivative bearing the O gene under the control of ptac promoter     | this work |
| pINTts               | a helper plasmid overexpressing λ phage integrase, used for integration of pCAH56 plasmid derivatives with the host chromosome | (79)      |
| pKBlin               | as pKB2 but devoid of Cro function                                           | (45)      |
| pKBlinpO−            | as pKBlin but pO−                                                           | This work |
| pTCλ5                | p\text{tet}, tetR, λ replication region under control of p\text{tet}        | This work |
| pTCλ5pO−             | as pTCλ5, but bearing pO− mutation                                          | This work |
| pTCλ1                | p\text{tet}, tetR, λ replication region under control of p\text{tet}        | (49)      |
| pUC19                |                                                                             | (80)      |
| pBR322               |                                                                             | (81)      |
| pTAC3734             | lacZ, ampR                                                                  | (82)      |
### Table 3. Oligonucleotides

| No. | Primer sequence |
|-----|----------------|
| 1.  | 5’ CGACCTGACAAACACAGACTGGAT |
| 2.  | 5’ CACTTGCTGCCGCTCTGAATTGC |
| 3.  | 5’ GATGGTTACGCGAGCTCAGACTATCAAATATG |
| 4.  | 5’ CATATTTGATAGTCTGAGCTCGGCGTAACCATC |
| 5.  | 5’ GAGGGTTATTCGTCTAGAGCGCGGCGAGATCTG |
| 6.  | 5’ CAGATCTGCAGCCGCGCTCTAGACGAATAAGCCTC. |
| 7.  | 5’ AGAGGTAACTTTGCCCCTCGAGGAGCGTAAATGTGGCA |
| 8.  | 5’ TGCCACATTACGCTCTCGAGGGCAAAAGTTACCTCT |
| 9.  | 5’ CTTGCTCGAGTATCGTGCTATCTG |
| 10. | 5’ GGTCTAGATTCAGCCACTGCAT TTCC |
| 11. | 5’ GATTTCTCGAGTTCTCAATGCTG |
| 12. | 5’ CCTGTGCACAGATTAGGTCT |
| 13. | 5’- CACCGATTCTCAACTTAGCGAGATTAC |
| 14. | 5’-CTGGCTGATGGGTGCGATAGTCTTC |
| 15. | 5’-CGGGATCCTCATAGACATCCCTCAAAATGGGGGA TTGCTATCCCTCAAAACAGGGGACGGGATCCGC |
| 16. | 5’-CGGGATCCTCATAGATCCCTCAAAACAGGGGAGGATCCGC |
| 17. | 5’-AGGAGTCCATATGACAAATACAGCAGAAAAATACTCAACTTCGGCAAGGG |
| 18. | 5’-GCGGGATCCTCATAGATCCCTCAGGGGTAATCCAG |
| 19. | 5’-GGGAGGAAATATCTGGATCCGTGCTGTTGAC |
| 20. | 5’-CCGAGCGTTCTGAACAAAGCTTATGGAGTTCTG |
| 21. | 5’-CTGGTCAGAAGCTTCGCCAGAATTCTCTGACG |
Strains and plasmids construction

*E. coli* strains are listed in Table S1 and plasmids are described in Table S2. Bacteriophages λpapa (from our collection) and λpO- (30) were used in density shift experiments. Phage P1vir (from our collection) was used for transduction.

MGO strain was constructed by integration of the plasmid pCAHlamO (Table S2) into the lambda attachment site using lambda site-specific recombination (79).

Plasmids pLamberA and pLamberB were constructed by deletion of fragments, containing *kan* or *bla* gene, respectively, from pLamber plasmid (73), as described in the Table 2. Both those plasmids contain λ phage region encompassing *pr* promoter and genes *cro, cII, O* and *P*, as well as ColE1-type origin of replication.

Plasmid pdelλO was constructed by PCR mutagenesis. Primers 1 and 2 (Table S3) were used in the reaction leading to deletion of the 291 bp fragment from pLamberB, coding for the C-terminal domain of the λO protein.

Plasmid pdelλOpo- was constructed by ligation of the 5.8 kb HindIII- EcoRI fragment of pdelλO and the 1.584 bp HindIII-EcoRI fragment of pKB2po-.

A series of pdelλO-derived plasmids bearing insertions of 6, 10, 50, 100 or 500 bp, called pdelλOins6, pdelλOins10, pdelλOins50, pdelλOins100 and pdelλOins500, respectively, were constructed as follows. 6 bp and 10 bp insertions were introduced into pdelλO plasmid by PCR using following pairs of primers: 3 and 4 or 5 and 6 (Table S3). Plasmids bearing 50 bp and 100 bp insertions were constructed in two steps. First, a unique XhoI site was introduced into a pdelλO plasmid by PCR mutagenesis, using primers 7 and 8 (Table S3). Then, hybridizations of two pairs of the 50 bp-long and 100 bp-long oligonucleotides, whose sequences corresponded to fragments of the spectinomycin-resistance gene, were performed to obtain dsDNAs. Following phosphorylation of the 5’-ends by T4 polynucleotide kinase (1 unit, Promega), the double stranded oligonucleotides were ligated with pdelλO, previously
linearized with XhoI. The 500 bp fragment for construction of pdelλOins500 was PCR-amplified using a template containing spectinomycin-resistance gene and primers 9 and 10 (Table S3). Following digestion with XhoI and XbaI, the 500 bp fragment was ligated with a XhoI-linearized pdelλOins10 plasmid. The series of plasmids based on pdelλO does not express a functional replication initiator O protein.

Plasmids pUC19 (80) bearing various numbers of iterons were constructed by cloning the below listed DNA fragments into BamHI site of pUC19: pUC19oriλ contains a PCR product obtained with primers 13 and 14 (Table S3), pUC19it34 contains a fragment obtained by hybridization of the oligo 15 (Table S3) and its complementary counterpart and subsequent BamHI digest, pUC19it4 bears a fragment obtained by hybridization and BamHI digest of the oligo 16 (Table S3) and its complementary counterpart. Derivatives of pBR322 with λ iteron sequences (pBRoriλ, pBRit34, pBRit4) were constructed analogously.

Plasmid pCAHlamO was constructed by cloning a PCR fragment containing the O gene sequence, obtained using primers 17 and 18 (Table S3), into BamHI-NdeI sites of plasmid pCAH56 (79).

Plasmids containing pR-pO::lacZ (pTac800) and pR-pO-oriλ::lacZ (pTac1400) fusions were constructed by inserting a PCR fragment, amplified using primers 19 and 20 (pTac800wt and pO-) or 19 and 21 (pTac1400 wt and pO-), into BamHI-HindIII sites of pTac3734 (82) (Table S2).

pTCλ5 and pTCλ5pO- plasmid was cloned by inserting 1727 bp fragment (obtained by PCR, using primers 11 and 12 (Table S3) and plasmid pKB2 or pKB2pO-, respectively, as a template), into XhoI-ApaL1 sites of plasmid pTCλ1 (49).

β-galactosidase assay
Overnight cultures were diluted in the LB medium with or without indicated concentrations of the inducer and grown until OD578=0.2. After reaching desired density, cultures were chilled on ice. 100µl of culture were added to 1.5ml of the Z buffer and 100 µl of chloroform and vortexed. Subsequently, solution was incubated for 10 minutes. Following incubation, 400 µl of the ONPG solution was added and the reaction was carried out for 5 minutes. The reaction was stopped by addition of 1ml of 1M sodium carbonate. The absorbance of the solution was measured at 420nm wavelength. β-galactosidase activity was calculated as described (82).

Preparation of Fraction II

*E. coli* C600 strain was grown in LB medium at 37˚ to OD600 = 1.0. The cells were collected by centrifugation, washed once in buffer A (25 mM Hepes- KOH pH 7.6, 5 mM EDTA) and then resuspended in buffer A (2 ml buffer A per 1 ml of cell paste). The cell paste was transferred to a polypropylene tube, quickly frozen in liquid nitrogen and stored at –70˚. After thawing at 0-4˚, the liquid was adjusted to 150 mM KCl and 2mM DTT, and lysozyme was added to 0.5 mg/ml. Following incubation at 0˚ for 30 min, the cells were frozen again in liquid nitrogen, thawed at 0-4˚ and centrifuged in Beckman 50.2 Ti rotor at 30,000 rpm for 30 min at 4˚. The supernatant was collected and ammonium sulfate (0.28 g per 1 ml of the cleared lysate) was slowly added at 0˚ with stirring over a 60 min period. The precipitate was collected by centrifugation at 23,000 rpm for 20 min at 4˚, pellet was resuspended in 100 µl of buffer B (25 mM Hpes – KOH pH 8.0, 0.1 mM EDTA, 2 mM DTT) and dialyzed against 200 ml of buffer B at 0˚ for 30 min. The fraction was frozen in aliquots in liquid nitrogen and stored at –70˚.

In vitro DNA replication

The standard reaction mixture (final volume equal to 25 µl) consisted of: Hepes- KOH pH 7.6, 40 mM; magnesium acetate, 11 mM; bovine serum albumin, 50 μg/ml; creatine phosphate, 40 mM;
creatine kinase, 20 μg/ml; ATP, 2 mM; GTP, CTP, UTP each 500 μM; dATP, dGTP, dCTP, and dTTP, each 100 μM, with [3H]dTTP (150 cpm/pmol); PEG 6000, 8.6 μg/ml; supercoiled DNA, 250 ng; λO protein, 300 ng; λP protein, 80 ng. The mixtures were assembled at 0°C. The reactions were initiated by addition of 1.5 μl of Fraction II, and incubation was carried at 32°C for 2 h. Total nucleotide incorporation was measured by determining radioactivity in a scintillation counter after trichloroacetic acid precipitation.

Density shift experiments

Amount of bacteriophage particles added to bacterial cultures was monitored by titration of the λwt and λpO- phage lysates on the strain used in this experiment. Bacteria were grown in a "light" minimal medium (84) overnight at 37°C, and - after dilution (1:50 v/v) with fresh medium - the cultivation was prolonged to OD500 equal to 0.2. The bacteria were collected by centrifugation, washed with TM buffer (10 mM Tris-HCl pH = 7.2, 10 mM MgSO4) and resuspended in 0.1 volume of this buffer. After 60 min incubation at 37°C, a phage lysate was added to m.o.i. = 10 and incubation was continued for 15 min. The suspension was sedimented, resuspended in the same volume of prewarmed (to 37°C) "heavy" minimal medium (containing [15N]H4Cl and [13C]glucose instead of NH4Cl and glucose, respectively), and incubation was carried on at 37°C. Samples of the infected culture were withdrawn at indicated times, total DNA was isolated and ultracentrifuged in the CsCl density gradient as described previously (Wrzyn et al., 1995). Fractions were collected from the bottom of the tube, DNA was transferred to a nitrocellulose membrane and detected by hybridization with a fluorescein-labeled probe consisting of a replication region of phage λ genome. The probe was obtained by random priming reaction using Gene Images Random Prime Labeling Kit (Amersham Biosciences) and a linearized λ plasmid (pKB2). Signals were detected using Gene Image CDP-Star Detection Kit-to anti-fluorescien-AP conjugate (Amersham).
Hybridization signals were quantified densitometrically, and percent of □ DNA at each position (full-light, heavy-light, and full-heavy) was calculated.

II. Supplemental results

A
**Fig. S1.** A map of the pKB2pO- plasmid. Restriction sites used in the experiments are marked and DNA fragments used as probes are shown as external circles (A), theoretical schemes and results of two-dimensional agarose gel electrophoresis of pKB2pO- (B).
**Fig. S2.** A map of the pKBlin pO- (the same as for pKBlin) plasmid. Restriction sites used in the experiments are marked and DNA fragments used as probes are shown as external circles (A), theoretical schemes and results of two-dimensional agarose gel electrophoresis of pKBlin pO- in comparison to pKBlin (B).
Fig. S3. Maintenance of pKB2 (wild-type λ plasmid; closed circles) and its pO- derivative (pKB2 po-; open circles) in *E. coli* MG1655 strain. Plasmid-bearing bacteria were grown in LB medium at 37°C without an antibiotic (overnight cultures were supplemented with 25 μg/ml kanamycin as the *kan* gene is present on both plasmids). During growth, the culture was successively diluted in a fresh LB medium and maintained in early-middle log phase. Samples were withdrawn at indicated times and percent of kanamycin-resistant cells was calculated by titrating bacteria on LB plates with and without kanamycin.
**Fig. S4.** Effect of pO promoter activity on the efficiency of transcription starting from pR in the presence of oriλ sequence and the λO protein. pR promoter activity was assessed in the presence and absence of pO function using a strain overexpressing the λO protein (MGO), bearing pR-pO-oriλ-lacZ (pTac1400wt and pTac1400pO-) fusions. Activity of the promoter was presented relatively to the value obtained in the absence of IPTG, which induced overproduction of λO. β-galactosidase activity is presented in Miller units.

**Fig. S5.** *In vitro* replication of pdelλO plasmid (marked as wt) and its derivatives, bearing either the pO- mutation (pdelλOpo-, marked as po-) or insertion of 6 (pdelλOins6, marked as 6
bp), 10 (pdelλOins10, marked as 10 bp), 50 (pdelλOins50, marked as 50 bp), 100 (pdelλOins100, marked as 100 bp) and 500 (pdelλOins500, marked as 500 bp) base pairs. The reaction was performed using Fraction II, as described in Materials and Methods. Synthesis of each hybrid λ-ColE1 replicon was estimated in the presence or absence of λO and λP proteins in the replication mixture. Thus, level of λ plasmid DNA replication was calculated as a difference between levels of DNA synthesis in these two experimental systems. The value of 100 % corresponds to 73 pmoles of synthesized DNA. Standard error was depicted by error bars.

**Table S4.** Efficiency of transformation of *E. coli* MG1655 strains with wild-type (pKB2) and pO⁻ (pKB2pO⁻) version of λ plasmid. MG1655 strain bore pUC19 (pUC19oriλ, pUC19it34, pUC19it4) or pBR322 (pBRoriλ, pBRit34, pBRit4) derivatives with four, two or one iteron sequence of the λ origin. The results represent the mean value of three independent experiments.

| MG1655/plasmid | Efficiency of transformation (transformants per 1 μg of DNA) | Ratio pKB2pO⁻/pKB2 (%) |
|----------------|-------------------------------------------------------------|------------------------|
| -              | 5.4 (±0.01) x 10⁴                                          | 66                     |
| pUC19          | 4.3 (±0.13) x 10⁴                                          | 61                     |
| pUC19 1 iteron  | 1.7 (±0.11) x 10⁴                                          | 46                     |
| pUC19 2 iteron  | 7.8 (± 2.0) x 10⁴                                          | 33                     |
| pUC19 4 iteron  | 1.8 (±0.14) x 10³                                          | 17                     |
| pBR322         | 1.6 (±0.02) x 10⁵                                          | 62                     |
| pBR322 literons| 8.8 (±0.09) x 10⁴                                          | 69                     |
| pBR322 2 iteron | 8.3(±0.77) x 10⁴                                          | 58                     |
| pBR322 4 iteron | 9.7(±0.08) x 10⁴                                          | 55                     |

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