Supporting Information

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SI Results and Discussion

Oligomerization of DnaA on the Minimum Region of *datA*. To analyze whether DnaA oligomers are formed in the region between DnaA boxes 2–3, we used *datA* del5, which carries a single GATC sequence between those sites (Fig. S2), and DpnII, a restriction enzyme that specifically cleaves DNA at GATC sequences. DpnII-dependent digestion was protected in a dose-dependent manner when ATP-DnaA was included in the reaction (Fig. S4), suggesting that DnaA oligomers bind throughout this region. Protection was also observed when ADP-DnaA was used, although the level of protection was lower than that of ATP-DnaA (Fig. S4), suggesting that ADP-DnaA oligomers are not as stable as ATP-DnaA oligomers.

Coordination Between Initiation of Replication from *oriC* and Binding of Integration Host Factor to *datA*. To confirm the cell cycle-coordinated binding of integration host factor (IHF) to *datA*, we performed independent ChIP experiments using rifampicin to repress the second round of initiation (Fig. S5). In these experiments, synchronization of the KYA018 [*dnaC2* (Ts)] replication cycle was performed as in Fig. 4 *B* and *C*, except that rifampicin was added 5 min after the temperature shift to 30 °C to inhibit the second round of initiation (Fig. S5). *oriC* was also duplicated within 5 min after the temperature reduction to 30 °C, and the *oriC* level was further maintained for 15 min (Fig. S5*A*). This finding indicates that initiation occurred only once within the initial 5 min at 30 °C. *datA* was duplicated 15–20 min after initiation. IHF dissociated from *datA* before initiation and rebound to *datA* 10–20 min after initiation (Fig. S5*B*). This result is consistent with the data in Fig. 4*C* and the cell cycle-coordinated regulation of IHF binding to *datA* (Fig. S6).

Furthermore, these results explain the mechanism underlying the observation that deletion of *datA* or *ihf* causes rifampicin-resistant (Rif^R) initiations at *oriC* (1, 2). As described above, IHF binds to *datA* even in the presence of rifampicin, which suggests that DDAH (*datA*-dependent DnaA-ATP hydrolysis) is activated. If *datA* or *ihf* is inactivated under such conditions, the ATP-DnaA level should increase due to the defect in DDAH, resulting in Rif^R initiations. *oriC* can use HU instead of IHF for replication initiation (3). Overproduction of WT DnaA also causes extra Rif^R initiation events (4).

SI Materials and Methods

Protein, DNA, and *Escherichia coli* Strains. WT and mutant DnaA proteins were overexpressed and purified from *E. coli* cells, as previously described (5, 6). IHF protein was overexpressed and purified from MC1061 cells, as previously described (7, 8). The purity of each protein was >90% as judged by SDS/PAGE and Coomassie Brilliant blue staining. ATP- and ADP-DnaA were prepared by incubating DnaA and 3 μM ATP or ADP on ice for 15 min, as previously described (5).

A *datA* WT fragment (991 bp) was amplified by PCR using Kohara clone #652 (9) and primers datA-1 and datA-2 (see Table S1 for each sequence), phosphorylated with T4 kinase (Takara), and inserted into the BamHI and HindIII sites of pACYC177 to yield pKX40. The *datA* fragments used for deletion analysis in the in vitro DDAH system were amplified using pKX40 as a template and the following primers (Table S1): datA-1 and datA-2 for *datA* WT and *datA* ΔIBS (IHF binding site), datA-1 and datA-4 for del1, datA-1,and datA-3 for del2, datA-2 and datA-6 for del3, datA-4 and datA-6 for del4, datA-3 and datA-6 for del5 and del5ΔIBS, datA-6 and datA-10 for del6, datA-6 and datA-9 for del7, datA-3 and datA-7 for del8, and datA-2 and datA-5 for del9 (see Table S1 for each sequence). pKX40-derivatives, including *datA* sequence deletion or substitutions, were constructed by inside-out PCR using the following primers (Table S1): delIBS-U and delIBS-L for pKX62 (ΔIBS), Ksh-43 and Ksh-44 for pKX48 (subDnaAbox1), Ksh-37 and Ksh-38 for pKX41 (subDnaAbox2), Ksh-39 and Ksh-40 for pKX42 (subIBS), Ksh-41 and Ksh-42 for pKX43 (subDnaAbox3), Ksh-45 and Ksh-46 for pKX47 (subDnaAbox4), Ksh-47 and Ksh-48 for pKX49 (subDnaAbox5), Uni-del-ins and delC for pKX51 (delC), Uni-del-ins and delD for pKX52 (delD), Uni-del-ins and insB for pKX53 (insB), and Uni-del-ins and insBplus for pKX54 ($insB^+$). The resulting plasmids were used as templates for PCR using the primers datA-1 and datA-2, and the resulting mutant fragments were used in the in vitro DDAH system. The *oriC* fragment FK-9 is the same as F. kaz-9, which was previously described (10). The fragment was amplified by PCR using genomic DNA as a template and primers Kaz-17 and Kaz-18 (Table S1). Oligonucleotides for EMSA, which are listed in Table S1 along with their sequences, were annealed at room temperature overnight (DB1-U and DB1-L for DnaAbox1, subDB1-U and subDB1-L for subDnaAbox1, DB2-U and DB2-L for DnaAbox2, subDB2-U and subDB2-L for subDnaAbox2, DB3-U and DB3-L for DnaAbox3, subDB3-U and subDB3-L for subDnaAbox3, DB4-U and DB4-L for DnaAbox4, subDB4-U and subDB4-L for subDnaAbox4, DB5-U and DB5-L for DB5, subDB5-U and subDB5-L for subDnaAbox5, IBS-U and IBS-L for IBS, and subIBS-U and subIBS-L for subIBS).

All *E. coli* strains used in this study are listed in Table S3. Δ*ihfA*::*frt-kan*, Δ*ihfB*::*frt-kan*, and Δ*hupA*::*frt-kan* derived from the Keio collection were introduced into MK86 cells using P1 transduction, yielding KX30, KX31, and KX32, respectively. Δ*datA*::*kan,* derived from RSD448 cells, was introduced into MK86 cells using P1 transduction, yielding KX93. The *cat* gene was introduced near *dnaC* (i.e., between *yjjN* and *mdoB*) in PC2 using RED system (11), and the resultant strain was used as a donor of P1 transduction to MG1655, yielding KYA018. M9 medium was supplemented with 0.2% glucose, 0.2% casamino acid, and 5 μg/mL thiamine.

DpnII Inhibition by ATP-DnaA. The *datA* del5 DNA fragment (0.05 pmol) was incubated at 30 °C for 5 min with DpnII (New England Biolabs; 0.1 U) and various amounts of ATP- or ADP-DnaA in buffer H (15 μL) in the absence of ATP. After the reaction was terminated in 0.5% (wt/vol) SDS, DNA fragments were purified and analyzed by 6% (wt/vol) PAGE and ethidium bromide staining.

EMSA. For the experiments shown in Fig. 3*D*, various amounts of ATP- or ADP-DnaA were incubated at 15 °C for 5 min with the *datA* del5DNA fragment (0.15 pmol) in buffer H (10 μL), including 150 mM potassium glutamate, 2 mM ATP or ADP, 150 ng λDNA (as a competitor), and either 0 or 6 pmol of IHF. DNA and DNA-protein complexes were analyzed by 2%(wt/vol) agarose gel electrophoresis at 80 V for 70 min in Tris•Borate buffer, followed by staining with GelStar (Takara).

For the experiments shown in Fig. S1, ATP-DnaA or IHF were incubated with various amounts of DNA at 30 °C for 5 min in buffer H (10 μL) containing either 0 or 100 ng of poly (dI•dC). DNA and DNA-protein complexes were analyzed by 12% or 8% PAGE at 100 V for 60–80 min in Tris•Borate buffer, followed by staining with Gel-Star (Takara).

ChIP with Synchronized Cells. ChIP was performed according to a previously described method (12), with minor modifications. KYA018 (*dnaC2*) cells were grown in supplemented M9 medium (15 mL) at 30 °C, a permissive temperature, until they reached OD A_{660} (absorbance at 660 nm) of 0.03. They were then shifted to 38 °C, a restrictive temperature, and further incubated for 90 min. Cells were then incubated at 30 °C for 5 min, followed by a further incubation at 38 °C for 5–55 min or at 30 °C for 5–15 min in the presence of 300 μg/mL rifampicin. Samples were withdrawn at each of the indicated time-points and cross-linked with 3% formaldehyde (37% solution; Wako) at room temperature for 5 min. The reactions were quenched by incubation in 125 mM glycine for an additional 5 min. The cells were then collected by centrifugation, washed twice with 1 mL of ice-cold TBS [50 mM Tris•HCl (pH 7.5) and 500 mM NaCl], resuspended in 125 μL of lysis buffer [50 mM Tris•HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, complete Mini EDTA-free protease inhibitor mixture (Roche), and 1 mg/mL lysozyme], incubated for 30 min at 37 °C, and lysed by the standard freeze-and-thaw method. Lysates were diluted in 500 μL (final volume) of IP buffer [50 mM Tris•HCl (pH 8.0), 250 mM NaCl, 1 mM EDTA, and 1% (vol/vol) Triton X-100] and sonicated six times for 20 s each. The resulting size of the chromosomal DNA was about 1 kb. Cell debris was then removed by centrifugation at 14 krpm (16,000 × *g*) for 15 min at 4 °C, and the resulting supernatant (400 μL) was removed and termed fraction I. A portion (350 μL) of fraction I was mixed with 5 μL of polyclonal rabbit anti-IHF antiserum and 60 μL of Protein A Sepharose 4 Fast Flow (GE Healthcare) (50% slurry in IP buffer), followed by incubation at 4 °C for 30 min with a gentle rotation. Sepharose beads and bound materials were washed sequentially in 500 μL of IP buffer, 750 μL of buffer WI [50 mM Tris•HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, and 1% (vol/vol) Triton X-100], and 750 μL of buffer WII (buffer WI including 250 mM LiCl instead of 500 mM NaCl), and ultimately resuspended in 1 mL of TE [10 mM Tris•HCl (pH 8.0) and 1 mM EDTA]. After removing TE, the beads and bound materials were resuspended in 100 μL of Elution buffer [50 mM Tris•HCl (pH 8.0), 10 mM EDTA, and 1% SDS] and incubated at 65 °C for 12 h to allow de–cross-linking. DNA in the samples before (Input) and after (ChIP) immunoprecipitation were purified using a Wizard SV Gel and PCR Clean-Up System (Promega). The levels of *oriC*, *datA*, and *ylcC* were quantified by real-time quantitative PCR using SYBR Premix Ex Taq II (Perfect Real Time) (Takara) and the following primers: ORI_1 and KWoriCRev for *oriC*, RTDATAL and RTDATAR for *datA*, and RTYLCC-L and RTYLCC-R for *ylcC* (see Table S1 for each sequence). The amounts of immunoprecipitated DNA were represented as ChIP values. As a quantitative standard, DNA in fraction I (5 μL) was similarly de–cross-linked, purified, and quantified by real-time quantitative PCR; the amount of DNA was represented as the Input value. The *ylcC* region contains no specific IHF binding site and was used as a background control (13). The ratio of ChIP value to Input value (ChIP/Input) for *ylcC* was subtracted from the ChIP/Input value for *oriC* and *datA* to calculate the value for specific IHF binding.

In addition, the amount of *ter* DNA product in fraction I was quantified by real-time quantitative PCR using the SUEterRev1 and TER_2 primers (Table S1), and was used to calculate the *oriC*/*ter* and *datA*/*ter* ratios.

1. Von Freiesleben U, Rasmussen KV, Atlung T, Hansen FG (2000) Rifampicin-resistant initiation of chromosome replication from *oriC* in *ihf* mutants. *Mol Microbiol* 37(5): 1087–1093.
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3. Hwang DS, Kornberg A (1992) Opening of the replication origin of *Escherichia coli* by DnaA protein with protein HU or IHF. *J Biol Chem* 267(32):23083–23086.
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12. Cho BK, Knight EM, Barrett CL, Palsson BO (2008) Genome-wide analysis of Fis binding in *Escherichia coli* indicates a causative role for A-/AT-tracts. *Genome Res* 18(6):900–910.
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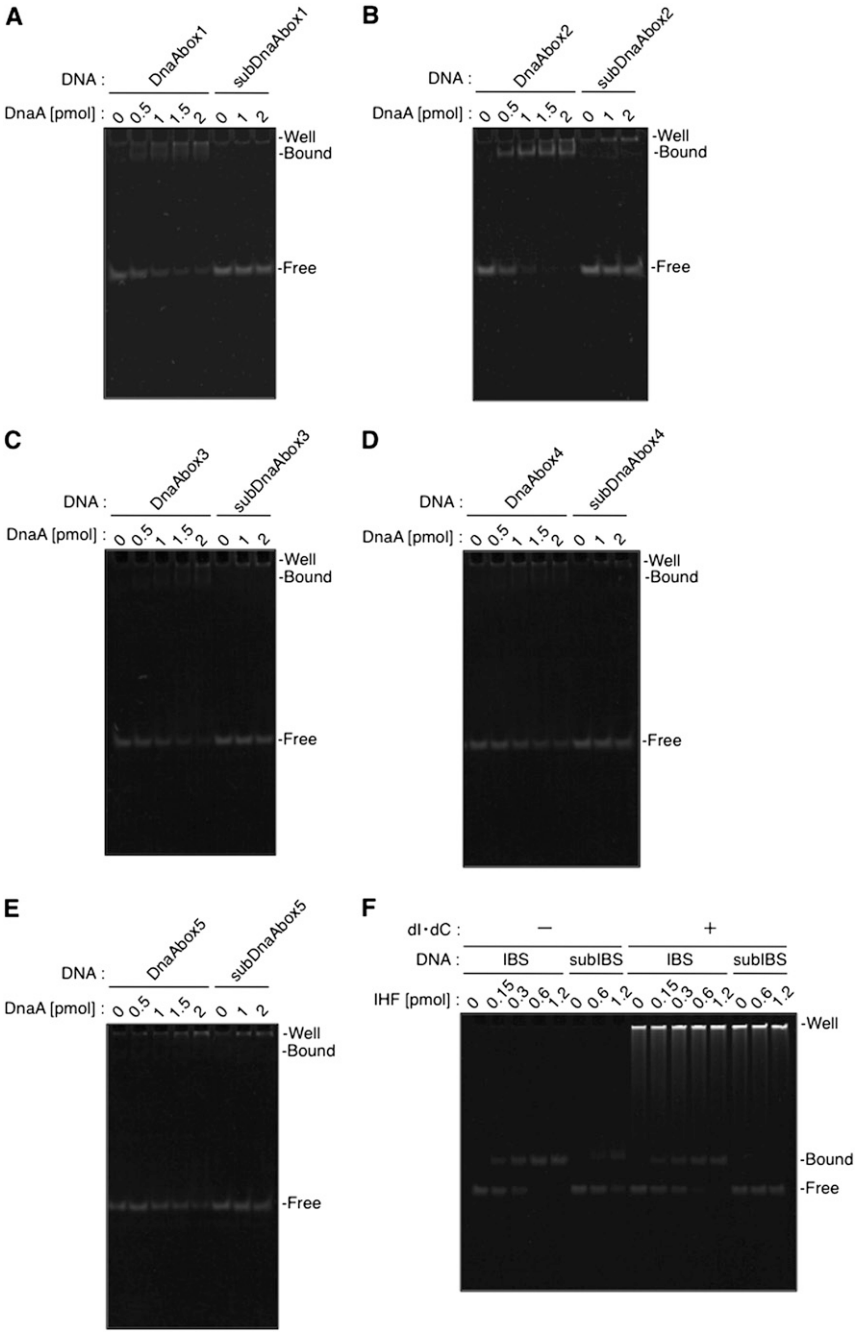

Fig. S1. EMSA of DnaA and IHF binding to *datA* mutant fragments. (*A–E*) The indicated amounts of ATP-DnaA were incubated with 0.5 pmol of each DNA at 30 °C for 5 min, followed by 12% PAGE. Well, gel well; Bound, protein-bound DNA; Free, protein-free DNA. DNA fragments used were DnaAbox1 and sub DnaAbox1 (*A*), DnaAbox2 and subDnaAbox2 (*B*), DnaAbox3 and subDnaAbox3 (*C*), DnaAbox4 and subDnaAbox4 (*D*), and DnaAbox5 and subDnaAbox5 (*E*). (*F*) The indicated amounts of IHF were incubated with 0.3 pmol of IBS or subIBS DNA at 30 °C for 5 min in the presence or absence of 100 ng poly (dI•dC), followed by 8% PAGE.

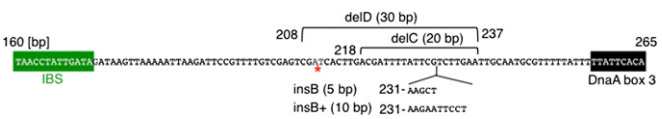

Fig. S2. Sequence between IBS and DnaA box 3. Positions and sequences of *delC*, *delD*, *insB*, and *insB*$^{+}$ mutations in addition to the GATC site (red star) are shown (1). There is only a single GATC site between DnaA boxes 2 and 3.

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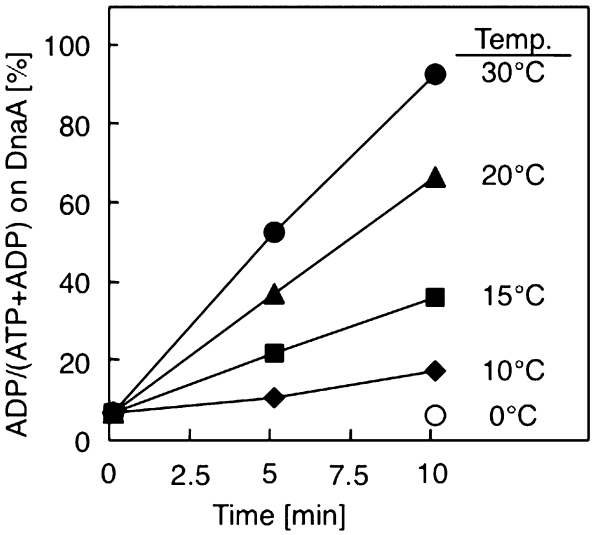

Fig. S3. Analysis of DDAH activity at low temperatures. [α-^{32}P]ATP-DnaA (1 pmol) was incubated with *datA* WT (0.05 pmol) and IHF (0.2 pmol) at 0 °C, 10 °C, 15 °C, or 30 °C for either 5 or 10 min, and then analyzed by thin-layer chromatography. The proportion of ADP-DnaA to the sum of ATP-DnaA and ADP-DnaA molecules is indicated as percentages (%).

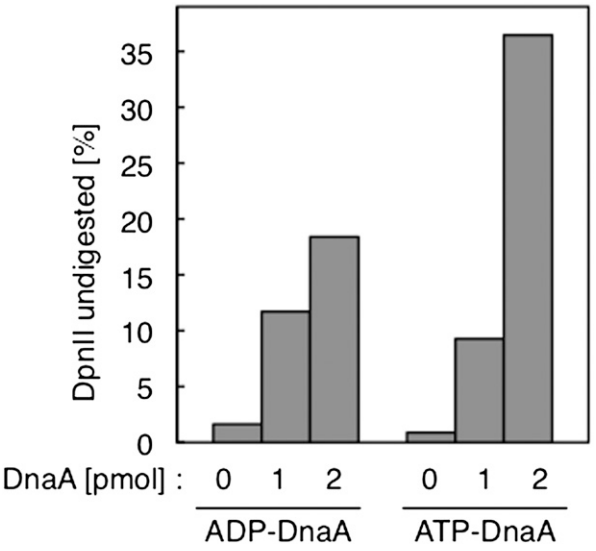

Fig. S4. Analysis of DpnII-digestion of *datA* in the presence of IHF and DnaA. The indicated amounts of ATP- or ADP-DnaA were incubated with *datA* del5 (0.05 pmol) and IHF (0.2 pmol) at 30 °C for 5 min in the presence of DpnII. The proportion of undigested DNA to total DNA is indicated as percentages (%).

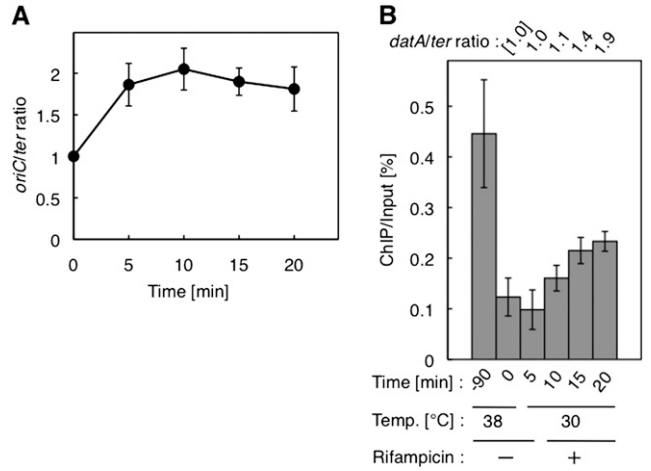

Fig. S5. Replication cycle-coordinated binding of IHF to *datA* in the presence of rifampicin. KYA018 (*dnaC2*) cells growing at 30 °C in supplemented M9 medium were transferred to 38 °C and incubated for 90 min. Next, the cells were transferred to 30 °C (time 0) and incubated for 5 min, followed by addition of 300 μg/mL rifampicin. The cells were then incubated at 30 °C for 5–15 min. At the indicated times, samples were withdrawn. ChIP assay for IHF binding was performed as described in Fig. 4C. *oriC/ter* ratio (*A*) and relative ChIP/Input value for *datA* and *datA/ter* ratio (*B*) are shown. Error bars represent the SD from at least two independent experiments.

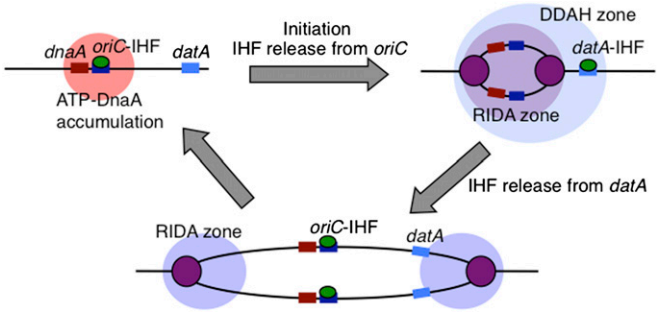

Fig. S6. Model for the coordination of DDAH and RIDA (regulatory inactivation of DnaA) based on replication cycle-coordinated binding of IHF to *datA*. Around the time of initiation, IHF is released from *datA,* and indirectly assists in the reproduction of ATP-DnaA by DARS. After initiation, the clamps are loaded on DNA, activating RIDA, and IHF binds to *datA*, activating DDAH for assisting in RIDA. These events support a rapid decrease in the ATP-DnaA level and repression of untimely initiations. RIDA zones and DDAH zones indicate spaces where DnaA-ATP hydrolysis is promoted by RIDA and DDAH, respectively. After awhile, IHF dissociates from *datA* and RIDA continues DnaA-ATP hydrolysis during replication.

Table S1. List of oligonucleotides

Primers	Sequences
datA-1	CCCGCTCCAAATTCTTCTCTCA
datA-2	TTCTCGAGCGCCCGTTAGCTG
datA-3	CAGCGTCTGGTTCGGGTG
datA-4	CTTATCTGCCCGTGGAATCG
datA-5	ATCAGGCGTCACGCGG
datA-6	GTTGTCTCTGACAAACTCTTGTAAACAG
datA-7	GCCTCAGGCTGTAATCTTAATTTC
datA-9	AAATAAAAACGCATTGCAATTC
datA-10	AGCTTGTGAATAAAAATAAAAACGC
delIBS-U	GATAAGTTAAAAATTAAGATTCCGTTTTGTC
delIBS-L	AAAAAATACTATTCACCGTGCGAAG
Ksh-43	TGTGTGCGACGCGATGCGTTATTGCTGG
Ksh-44	TGTTTTTATTGAGAGAAGAATTTGGAGC
Ksh-37	TGTGTGCCTCAGGCTGTAATCTTAATTTC
Ksh-38	TGTTCTCTGTTTACAAGAGTTTGTCAGAG
Ksh-39	ACGATAAGTTAAAAATTAAGATTCCG
Ksh-40	ACTTTAGGTTAAAAAAATACTATTCACCGTG
Ksh-41	TGTGTAGCTGTGGATGAATCAGGC
Ksh-42	TGTTAAATAAAAACGCATTGCAATTC
Ksh-45	TGTTATCAGGCGTCACGCGG
Ksh-46	TGTGTGCTTGTGAATAAAAATAAAAACGC
Ksh-47	TGTTGTGAGTGCTTACCTGTCCAGC
Ksh-48	TGTGTATCGCGAGGCCGGTTC
Uni-del-ins	TTGCAATGCGTTTTTATTTTTATTCAC
delC	CAAGTGATCGACTCGACAAAACG
delD	ACTCGACAAAACGGAATCTTAATTTTTAAC
insB	TTCAAGAAGCTTCGAATAAAATCGTCAAGTGATCGAC
insBplus	TTCAAGAAGGAATTCTTCGAATAAAATCGTCAAGTGATCGAC
Kaz-17	GGAAGATCATCAGGTTCGGTTGG
Kaz-18	CTGGCTGAAAAGCTGGAAGAGG
ORI_1	CTGTGAATGATCGGTGATC
KWoriCRev	GTGGATAACTCTGTCAGGAAGCTTG
RTDATAL	CCGTGCGAAGTTTCTTTGA
RTDATAR	GCGATGCGTTATTGCTGG
RTYLCC-L	GGCGTGGTAAAGGGTATCG
RTYLCC-R	TCTGCGGGGTGATGGTAAAG
TER_2	TATCTTCCTGCTCAACGGTC
SUEterRev1	GAACTACGCGGGAAATACC
DB1-U	ATAAAATATCCACAGCGAC
DB1-L	GTCGCTGTGGATATTTTAT
subDB1-U	ATAAAAACATGTGTGCGAC
subDB1-L	GTCGCACACATGTTTTTAT
DB2-U	CAGAGTTATCCACAGCCTC
DB2-L	GAGGCTGTGGATAACTCTG
subDB2-U	CAGAGAACATGTGTGCCTC
subDB2-L	GAGGCACACATGTTCTCTG
DB3-U	TATTTTTATTCACAAGCTG
DB3-L	CAGCTTGTGAATAAAAATA
subDB3-U	TATTTAACATGTGTAGCTG
subDB3-L	CAGCTACACATGTTAAATA
DB4-U	CAAGCTGTGGATGAATCAG
DB4-L	CTGATTCATCCACAGCTTG
subDB4-U	CAAGCACACATGTTATCAG
subDB4-L	CTGATAACATGTGTGCTTG
DB5-U	GCGATTGAGGATAAGTGAG
DB5-L	CTCACTTATCCTCAATCGC
subDB5-U	GCGATACACATGTTGTGAG
subDB5-L	CTCACAACATGTGTATCGC
IBS-U	TTTTTTAACCTATTGATAGATAAGTTAAAAATTAAGA
IBS-L	TCTTAATTTTTAACTTATCTATCAATAGGTTAAAAAA
subIBS-U	TTTTTTAACCTAAAGTACGATAAGTTAAAAATTAAGA
subIBS-L	TCTTAATTTTTAACTTATCGTACTTTAGGTTAAAAAA

Table S2. Sequences used for substitution in *datA*

Mutants	Sequences of wild-type	Sequences of mutants
subDnaAbox1	ATATCCACA	A**ATATGTGT**
subDnaAbox2	TTATCCACA	**AACATGTGT**
subIBS	TAACCTATTGATA	TAACCTA**AAGTAC**
subDnaAbox3	TTATTCACA	**AACATGTGT**
subDnaAbox4	TGTGGATGA	**ACACATGTT**
subDnaAbox5	TGAGGATAA	**ACACATGTT**

Substituted sequences are indicated in bold.

Table S3. List of *E. coli* strains

Strains	Relevant genotypes	Source
DH5α	*F- endA1 hsdR17(rk-mk+) supE44 thi1 recA1 gyrA(Nalr) relA1 Δ(lacZYA-argF) U169(φ80lacZΔM15)*	Laboratory stock
MG1655	Wild-type	Laboratory stock
KW262-5	MG1655 *rnhA*::Tn*3 oriC* del-1071::Tn*10*	(1)
MK86	KW262-5 Δ*hda*::*cat*	(1)
KX30	MK86 Δ*ihfA*::*frt-kan*	Present work
KX31	MK86 Δ*ihfB*::*frt-kan*	Present work
KX32	MK86 Δ*hupA*::*frt-kan*	Present work
KX93	MK86 Δ*datA*::*kan*	Present work
KYA018	MG1655 *dnaC2 zjj-18*::*cat*	Present work
RSD448	W3110 Δ*datA*::*kan*	(2), Dr. Tohru Ogawa
PC2	DG75 *dnaC2* (Ts)*	(3), Dr. Tohru Ogawa

*Other genetic markers are *leuB6 thyA47 deoC3 rpsL153*.

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