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DNA Replication Control Is Linked to Genomic Positioning of Control Regions in *Escherichia coli*

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Abstract

Chromosome replication in *Escherichia coli* is in part controlled by three non-coding genomic sequences, *DARS1*, *DARS2*, and *datA* that modulate the activity of the initiator protein DnaA. The relative distance from oriC to the non-coding regions are conserved among *E. coli* species, despite large variations in genome size. Here we use a combination of i) site directed translocation of each region to new positions on the bacterial chromosome and ii) random transposon mediated translocation followed by culture evolution, to show genetic evidence for the importance of position. Here we provide evidence that the genomic locations of these regulatory sequences are important for cell cycle control and bacterial fitness. In addition, our work shows that the functionally redundant *DARS1* and *DARS2* regions play different roles in replication control. *DARS1* is mainly involved in maintaining the origin concentration, while *DARS2* is also involved in maintaining single cell synchrony.

Author Summary

Replication of the *E. coli* chromosome is the central event in the cell cycle, with the control of replication enforced at the level of initiation. DnaA is the key protein responsible for initiation at the origin of replication (oriC), and is active in this when bound to ATP and inactive when bound to ADP. The activity of DnaA is in part controlled by three non-coding DNA regions; *datA*, *DARS1*, and *DARS2*. Here we show that the chromosomal position of *datA*, *DARS1*, and especially *DARS2* relative to *oriC*, is important for cell cycle control and bacterial fitness. Based on this and previous work, we propose that the functionally redundant *DARS1* and *DARS2* regions play different roles in replication control. *DARS1* is mainly involved in maintaining the origin concentration, while *DARS2* is also involved in maintaining single cell synchrony. Both regions are needed for proper replication control, and this also provides an explanation for the conservation observed in all sequenced *E. coli* strains. Further, the present literature indicates that these observations can be applied to other related bacteria.
Introduction

The circular chromosome of *Escherichia coli* is replicated bidirectionally from a single origin, oriC. The DnaA protein is responsible for replication initiation [1]. DnaA belongs to the AAA+ (ATPases Associated with diverse Activities) proteins and can bind both ATP and ADP with similar high affinities [1]. DnaA is active in replication when bound to ATP (DnaA\(^{ATP}\)) [2] and facilitates the unwinding of oriC [3–5]. Integration Host Factor (IHF) [6] and DiaA [7, 8] stimulates initiation from oriC, while initiation is opposed by the binding of Fis to oriC [9–11]. After open complex formation, DnaA loads the DnaB helicase onto the single-stranded DNA, which promotes further duplex opening and assembly of the replisome [5]. After initiation DnaA\(^{ATP}\) is inactivated, i.e. converted to DnaA\(^{ADP}\), by RIDA (Regulatory Inactivation of DnaA) [12–14] and DDAH (datA-dependent DnaA\(^{ATP}\) hydrolysis) [15] to prevent re-initiation. RIDA is more efficient in lowering the DnaA\(^{ATP}\)/DnaA\(^{ADP}\) ratio than DDAH [15]. At later cell cycle stages DnaA\(^{ADP}\) is reactivated at the two DnaA-Reactivating Sequences (DARS1 and DARS2) to allow for the next round of initiation [16, 17]. DARS1 is not regulated by any known pathway [16], while DARS2 activity is modulated by both an IHF- and Fis-dependent pathway [17].

In *E. coli*, there is a selective pressure to maintain chromosome symmetry; i.e. two nearly equal length replication arms [18]. The *datA*, DARS1, and DARS2 regions have the same relative distance to oriC in all *E. coli* strains sequenced [19], but none of the loci alone or in combination are essential for cell viability. Loss of either region is however associated with a fitness cost [19].

The *E. coli* chromosome consists of four insulated macrodomains (MD) and two less constrained regions called nonstructured (NS) regions [20, 21]. DNA recombination occurs preferentially within MDs, while DNA interactions between the different MDs are highly restricted. The two NS regions can however interact with both its flanking MDs [21]. The Ori MD is flanked by the two NS (NS\(^{Right}\) and NS\(^{Left}\)) whereas the Ter MD is flanked by the Left and Right MDs [21]. Both *oriC* and *datA* are located within the Ori MD, DARS2 within the NS\(^{Left}\), DARS1 within the Right MD, and terC in the Ter MD [19]. Chromosomal rearrangements resulting in a mixture of different macrodomains have deleterious effects on cell growth whereas rearrangements within them are better tolerated [22].

Chromosome organization is reported to affect gene expression, mainly at the transcriptional level. A recent study found that a reporter gene cassette, comprised of the lac promoter driving expression of *gfp*, varied ~300-fold depending on its position on the chromosome, in a manner fairly unrelated to the replication-associated gene dosage [23]. Gene expression even varies between insertion sites within the same MD, and both MD- and NS-regions contain high and low activity regions [23], due to intrinsic properties of the region. However, for fast growing bacteria the replication-associated gene dosage determines the organization of the chromosome; i.e. genes involved in translation and transcription (but not other highly expressed genes) are located close to *oriC* [24]. The selective pressure that keeps these genes in the relative proximity to *oriC* could be due to the challenges that *E. coli* faces a very high growth rates, which are hardly observable at lower growth rates [24]. The replication-associated gene dosage could also be important for the activity of non-coding regions such as *datA*, DARS1, and DARS2.

Here we show that the genomic location of *datA*, DARS1, and DARS2 are important for cell cycle control and for bacterial fitness. This provides a direct link between DNA replication control and genomic positioning.
Results

Chromosomal position of dataA, DARS1, and DARS2

The conserved relative distances from oriC to the DARS1, DARS2 and dataA regions in E. coli [25], suggest that their chromosomal positions are important for correctly controlled replication initiation. In order to construct strains carrying dataA, DARS1, and DARS2 at different loci, eight Tn10 insertions from the Singer library were chosen [26, 27] (Fig 1A). Strains had their respective chromosomal dataA, DARS1, or DARS2 loci deleted, and the tetC gene of Tn10 replaced with the dataA, DARS1, or DARS2 loci respectively, resulting in strains carrying a single copy of the respective region at a new chromosomal location. Mutant strains were evaluated by growth- and flow cytometry studies (Materials and Methods).

Wild-type cells exhibited the expected synchronous initiation pattern with the majority of cells containing 2, 4 or 8 replication origins (Fig 1B). Cells deficient in dataA have an increase in the DnaAATP/DnaAADP ratio [15, 28], resulting in an increased origins/mass, and a high degree of initiation asynchrony (Fig 1I) [25]. Rifampicin-resistant initiations, that could be suppressed by increasing the drug concentration, have previously been reported for cells deficient in dataA [29]. However, a fourfold increased rifampicin concentration did not affect any of the parameters measured here. Relocating dataA to any of the positions close to oriC (CAG18499 or CAG18496), dataA (CAG18488), or DARS2 (CAG12173) resulted in cell cycle parameters similar to wild-type (Table 1). Relocation to the positions near DARS1 (CAG18493) or especially terC (CAG12151 and CAG18461) resulted in an increase in origin concentration but no asynchrony (Table 1; Fig 1K). This suggests that although dataA is functional when located close to the termini the cells initiate replication at a decreased initiation mass relative to wild-type. This correlates with previous observations [28]. To test if the reduced dataA function in the terminus region was related to gene dosage we integrated a 2nd dataA region close to terC (ΔdataA CAG12151::dataA CAG18461::dataA; Table 1). A cell with two dataA regions close to terC has an origin concentration slightly below wild-type, but initiates initiation in synchrony. The chromosomal wild-type position of dataA has a gene dosage of 2, while the Tn10 positions CAG12151 and CAG18461 have gene dosages of 1.1 and 1.0, respectively (Materials and Methods). This suggests that the copy number is important for correct dataA function; a single dataA relocated to terC has a reduced function, which can be complemented by a second dataA in terC that brings the overall dataA copy number up to wild-type level.

Cells deficient in DARS1 (Fig 1C) or DARS2 (Fig 1F) have a reduced DnaAATP/DnaAADP ratio compared to wild-type [16, 17]; resulting in a reduced origin concentration compared to wild-type and our data are in agreement with this. Asynchrony of initiation was only observed in ΔDARS2, but not in ΔDARS1 [16].

The chromosomal position of DARS1 did not affect cellular doubling time or single cell initiation synchrony (Table 1). When DARS1 was relocated from its normal position to one of the two positions bordering oriC, the cells contained an elevated number of origins and an increased origin concentration (Fig 1D). We propose that DARS1 located close to oriC results in a phenotype similar to dataA relocated to the termini (see above), where cells initiate replication at a decreased initiation mass relative to wild-type.

Relocation of DARS2 influenced cell cycle parameters to a larger extent than DARS1. A wild-type phenotype was obtained for the five locations, CAG18499, CAG18496, CAG18488, CAG12135, and CAG18493 (Table 1). However when DARS2 were moved to positions close to terC, cells initiated asynchronously and had a decreased origin concentration relative to wild-type, although less than that of a DARS2 deletion (Fig 1H); indicating a still somewhat functional DARS2 in the terminus. In addition we tested the relocation of DARS2 to yafJ, a putative glutamine amidotransferase, located with the same relative distance to oriC as the wild-type
DARS2 position, but on the other replication arm. Here DARS2 had a similar doubling time, origin concentration, and synchrony as the wildtype (Table 1).

In order to assess whether the replication defect of cells carrying DARS2 near terC resulted solely from a gene dosage effect we cloned DARS2 into the F-based plasmid pALO277 [30]. The F plasmid has a copy number of 1–2 plasmids per genome equivalent [31], which is equal to or higher than the copy number of DARS2 at its chromosomal location (Materials and Methods). Replication of the F plasmid is limited by the availability of RepE [32], but DnaA is
also required [33]. To ensure that the presence of DARS2 on pALO277 did not alter the copy number we determined the number of the F plasmid relative to the termini by qPCR analysis for the wild-type cell transformed with either pALO277 or pALO277::DARS2 (S1 Fig). The data shows that the presence of DARS2 on the F plasmid does not alter the copy number. Wild-type cells with pALO277::DARS2 showed an increased origin/mass, indicating a functional DARS2 locus on pALO277::DARS2 (S1 Fig). ΔDARS2 cells were only partly complemented by pALO277::DARS2 as their origin concentration remained below wild-type level and initiation synchrony was never restored. These observations suggest that the contribution of DARS2 to cell cycle control is not solely through copy number. As plasmid F replicates randomly in the cell cycle [34] the coordination of DARS2 replication relative to oriC and other

| Strains                  | RD to oriC | DT | Origin/mass | Asynchrony index |
|--------------------------|------------|----|-------------|-----------------|
| Wild-type                |            |    | 40 min      | 1               | 0.05            |
| ΔdatA                    | 0.10       | 41 min | 1.25 (0.05) | 0.40            |
| ΔDARS1                   | 0.33       | 40 min | 0.82 (0.01) | 0.06            |
| ΔDARS2                   | 0.21       | 41 min | 0.82 (0.01) | 0.30            |
| ΔdatA CAG18499::datA::cat| 0.01      | 36 min | 0.98 (0.01) | 0.06            |
| ΔdatA CAG18496::datA::cat| 0.02      | 37 min | 0.97 (0.02) | 0.06            |
| ΔdatA CAG18488::datA::cat| 0.09      | 40 min | 0.97 (0.04) | 0.06            |
| ΔdatA CAG12135::datA::cat| 0.21      | ND   | ND          | ND              |
| ΔdatA CAG12173::datA::cat| 0.22      | 39 min | 1.01        | 0.06            |
| ΔdatA CAG18493::datA::cat| 0.33      | 38 min | 1.06 (0.01) | 0.05            |
| ΔdatA CAG18461::datA::cat| 0.48      | 40 min | 1.12 (0.02) | 0.06            |
| ΔdatA CAG12151::datA::cat| 0.46      | 38 min | 1.09 (0.01) | 0.06            |
| ΔdatA CAG12151::datA::cat CAG18461::datA | 0.46 | 38 min | 0.95 (0.04) | 0.08 |
| ΔDARS1 CAG18499::DARS1::cat | 0.01 | 41 min | 1.10 (0.04) | 0.09 |
| ΔDARS1 CAG18496::DARS1::cat | 0.02 | 38 min | 1.09 (0.03) | 0.08 |
| ΔDARS1 CAG18488::DARS1::cat | 0.09 | 40 min | 1.03 (0.01) | 0.06 |
| ΔDARS1 CAG12135::DARS1::cat | 0.21 | 40 min | 1.01 (0.04) | 0.09 |
| ΔDARS1 CAG12173::DARS1::cat | 0.22 | 40 min | 0.99 | 0.09 |
| ΔDARS1 CAG18493::DARS1::cat | 0.33 | 43 min | 0.96 (0.02) | 0.08 |
| ΔDARS1 CAG18461::DARS1::cat | 0.48 | 40 min | 0.97 (0.02) | 0.06 |
| ΔDARS1 CAG12151::DARS1::cat | 0.46 | 42 min | 0.97 (0.00) | 0.08 |
| ΔDARS2 CAG18499::DARS2::cat | 0.01 | 41 min | 0.99 (0.04) | 0.10 |
| ΔDARS2 CAG18496::DARS2::cat | 0.02 | 40 min | 1.02 (0.04) | 0.10 |
| ΔDARS2 CAG18488::DARS2::cat | 0.09 | 40 min | 0.99 (0.01) | 0.10 |
| ΔDARS2 CAG12135::DARS2::cat | 0.21 | 42 min | 1.01 (0.02) | 0.08 |
| ΔDARS2 yafJ::DARS2::cat | 0.21 | 40 min | 1.00 | 0.11 |
| ΔDARS2 CAG12173::DARS2::cat | 0.22 | ND | ND | ND |
| ΔDARS2 CAG18493::DARS2::cat | 0.33 | 42 min | 0.98 (0.01) | 0.10 |
| ΔDARS2 CAG18461::DARS2::cat | 0.48 | 42 min | 0.95 (0.05) | 0.27 |
| ΔDARS2 CAG12151::DARS2::cat | 0.46 | 42 min | 0.96 (0.04) | 0.41 |

a Relative distance from oriC to insertion (see Materials and Methods).
b Doubling time in minimal medium supplemented with glucose and casamino acids grown at 37°C.
c Determined as average light scatter from flow cytometric analysis. Numbers are normalized to 1 for wild-type. Standard error is indicated in the brackets.
d Asynchrony index; calculated as described in Materials and Methods.

Table 1. Effect of different chromosomal positions for datA, DARS1, DARS2 in MG1655.

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cis-acting control regions is no longer present and this may explain why DARS2 deficiency cannot be complemented by a plasmid borne DARS2 copy.

Importance of having both DARS1 and DARS2

DARS1 and DARS2 both rejuvenate inactive DnaA<sub>ADP</sub> to active DnaA<sub>ATP</sub> [16, 17]. We proceeded to investigate if loss of either locus could be complemented by an extra copy of the other. The effect of having two functional DARS2 loci were investigated in a ΔDARS1 cell, i.e., retained the original wild-type DARS2 locus, with the addition of a DARS2 inserted into one of seven Tn10 positions.

Loss of DARS1 did not result in initiation asynchrony, and an extra copy of DARS2 only changed this when inserted close to oriC in CAG18499 (although only marginally with and asynchrony index of 0.17) but not in any of the remaining tested positions (Table 2). Why a 2<sup>nd</sup> copy of DARS2 in CAG18499 but not CAG18496 gives asynchrony is unknown. DARS2 reintroduced close to the termini, close to the wildtype DARS2 position and even close to datA, which is located near oriC, failed to fully complement loss of DARS1 with respect to origin concentration (Table 2; S2 Fig). An additional copy of DARS2 only fully complemented DARS1 deficiency, i.e. with respect to origin concentration and synchrony, when introduced at a position close to oriC (CAG18496) or precisely at the wild-type DARS1 position (replacing the chromosomal DARS1 copy with a 2<sup>nd</sup> chromosomal DARS2 copy). This suggests that a relatively high gene dosage of DARS2 is required to complement DARS1 deficiency and that the context of the DARS1 region might be favorable for rejuvenation of DnaAADP to DnaAATP, hence the 2<sup>nd</sup> DARS2 copy here can fully complement to wild-type even though the chromosomal position does not provide a high copy number.

The ability of DARS1 to complement DARS2 deficiency was addressed by the same approach. The decrease in origin concentration observed for DARS2 deficient cells could be fully complemented by an additional DARS1 copy, irrespective of chromosomal position (Table 2; S2 Fig), although slightly elevated when DARS1 were reintroduced close to oriC. However, the additional DARS1 locus failed in all cases to complement the asynchrony phenotype of DARS2 deficient cells. These data suggest that DARS2 is a poor replacement for DARS1 and vice versa. They also indicate that DARS1 and DARS2 serves different functions that are both required for an efficient control of the cell cycle.

Optimal chromosomal position of DARS1 and DARS2 during fast growth

The optimal chromosomal positions of DARS1 and DARS2 were determined by in-culture evolution using a novel transposon mediated approach. Here, the chromosomal DARS1 and DARS2 loci were cloned into the mini Tn10 based transposon NKBOR (on pNKBOR) [35], resulting in NKBOR::DARS1 (pJFM3) and NKBOR::DARS2 (pJFM1), respectively (Materials and Methods). pNKBOR is a R6K-based suicide vector [35]. Hence when pJFM1 or pJFM3 were transformed into the Pir deficient strain MG1655, random insertions of either NKBOR::DARS1 or NKBOR::DARS2 were obtained; these are for simplicity referred to as DARS1 or DARS2 insertions, respectively. Five different experiential set-ups were performed; NKBOR into wild-type, DARS1 into ΔDARS1 and ΔDARS1 ΔDARS2 cells, and DARS2 into ΔDARS2 and ΔDARS1 ΔDARS2 cells, where an estimated 70,000 different transposon insertions were obtained for each, corresponding to an insert pr. 65 base pairs. We feel this is more than adequate to evaluate the importance of the genomic position for the non-coding regions.

The 70,000 different insertions were pooled for each set-up (t = 0) and continuously propagated in LB for a total of 700 generations (t = 700). The hypothesis is that the optimal position of DARS1/DARS2 would result in the fittest clone, which over time would out-compete the
rest. A Southern Blot, probed for NKBOR, was performed to confirm that the output of the direct competition experiment would become more clonal over time (Fig 2). Representative insertion sites from the Input, selected time points and end (t = 700) transposon pools were mapped by full genome sequencing (Materials and Methods). In addition, single clones were isolated after 700 generations from each set-up, for further investigation, and their precise transposon locations were determined by easy gene walking ([36]; Materials and Methods).

The mini-transposon NKBOR (Fig 2A), DARS1 (Fig 2B and 2C), and DARS2 (Fig 2B and 2C) were as expected inserted randomly throughout genome at t = 0. After 700 generations of growth, all NKBOR insertions into wild-type cells were mapped to three locations in the fimE gene (termed NKBOR Clone fimE #1, #2, and #3, Fig 3A, S1 Table). This indicates that under the used experimental settings the disruption of fimE resulted in a fitness advantage. Our Southern Blot showed two distinct bands for NKBOR insertion. However, we do not know the origin of the second band.

The fimE gene encodes FimE that along with FimB are the two recombinases, which mediate inversion of the DNA element containing the promoter for fimA (Type I fimbriae) [37]. We investigated whether NKBOR insertion into the fimE gene altered the level of fimbriae transcription between wild-type and NKBOR fimE mutants using qPCR. The level of fimA transcript in NKBOR and DARS1 insertions into fimE was increased 7- to 31-fold relative to wild-type (S3A Fig), and remnants of pellicle formation were observed (S3B Fig). This indicates that the fitness advantage linked to loss of fimE was due to an increased expression of fimbriae although other yet to be discovered fimE-regulated pathways could be involved.

### Table 2. Effect of having only two functional DARS1 or DARS2 loci.

| Strains                          | RD to oriC | DT | Origin/mass | Asynchrony index |
|----------------------------------|------------|----|-------------|-----------------|
| Wild-type                        | 0.33       | 40 | 1           | 0.05            |
| δDARS1                           | 0.21       | 40 | 0.82 (0.01) | 0.06            |
| δDARS2                           | 0.01       | 42 | 0.82 (0.01) | 0.30            |
| δDARS1 CAG18499::DARS2::cat      | 0.02       | 41 | 1.02 (0.03) | 0.17            |
| δDARS1 CAG18496::DARS2::cat      | 0.09       | 39 | 0.92 (0.04) | 0.08            |
| δDARS1 CAG12135::DARS2::cat      | 0.21       | 40 | 0.80        | 0.08            |
| δDARS1 CAG12173::DARS2::cat      | 0.22       | 38 | 0.86        | 0.08            |
| δDARS1 CAG18493::DARS2::cat      | 0.33       | 41 | ND          | ND              |
| Wild-type DARS1::DARS2::cat      | 0.33       | 36 | 1.00 (0.00) | 0.10            |
| δDARS1 CAG18461::DARS2::cat      | 0.48       | 40 | 0.93 (0.03) | 0.07            |
| δDARS1 CAG12151::DARS2::cat      | 0.46       | 39 | 0.93        | 0.06            |
| δDARS2 CAG18499::DARS1::cat      | 0.01       | 38 | 1.05 (0.01) | 0.52            |
| δDARS2 CAG18496::DARS1::cat      | 0.02       | 42 | 1.06        | 0.27            |
| δDARS2 CAG18488::DARS1::cat      | 0.09       | 39 | 0.99 (0.00) | 0.44            |
| δDARS2 CAG12135::DARS1::cat      | 0.21       | 38 | 1.00 (0.00) | 0.28            |
| δDARS2 CAG12173::DARS1::cat      | 0.22       | 37 | 0.99        | 0.28            |
| δDARS2 CAG18493::DARS1::cat      | 0.33       | 42 | 1.09 (0.07) | 0.65            |
| δDARS2 CAG18461::DARS1::cat      | 0.48       | 41 | 0.97        | 0.50            |
| δDARS2 CAG12151::DARS1::cat      | 0.46       | 39 | 0.97 (0.00) | 0.49            |

* Relative distance from oriC to transposon insertion (see Materials and Methods).
* Doubling time in minimal medium supplemented with glucose and casamino acids grown at 37°C
* Determined as average light scatter from flow cytometric analysis. Numbers are normalized to 1 for wild-type. Standard error is indicated in the brackets.
* Asynchrony index; calculated as described in Materials and Methods.

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Fig 2. Southern blot probed for NKBOR. It shows number of estimated generations of direct competition. 0* is NKBOR into wild-type at t = 0 in both 3A and 3B. 3A shows NKBOR into wild-type, 3B shows DARS1 insertion into DARS1 deficient cells and DARS2 into DARS2 deficient cells, while 3C shows DARS1 into DARS1 DARS2 deficient cells and DARS2 into DARS1 DARS2 deficient cells. Selected representative bands are highlighted: 1. NKBOR Clone fimE (#1–#4); 2. DARS1 Clone fimE; 3. DARS1 Clone ydeS; 5. DARS2 Clone rppH/rrf; 6. DARS2 Clone lgo/lgoR; 7. DARS2 Clone tdrR; 8. DARS1 Clone fimE (#5–#6); 9. DARS1 Clone tomB; 10. DARS1 Clone gsp. See Materials and Methods for details.

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Fig 3. Graphic representation of resolved transposon insertions sites in wild-type, ΔDARS1, ΔDARS2, and ΔDARS1 ΔDARS2. The position of oriC, datA, DARS1, DARS2, and terC is indicated in the individually figures. The number of insertion sites is displayed in percent for insertions resolved at t = 700. (A) NKBOR insertion sites in the wild-type at t = 0 (black bars) and t = 700 (green bars) resolved by full genome sequencing. Exact insertions sites are listed in S1 Table. (B) DARS1 insertion sites in ΔDARS1 at t = 0 (black
Selection of DARS1 insertions from a random pool in DARS1 or DARS1 DARS2 deficient cells resulted in fewer bands over time on a Southern Blot; i.e., six and five separate band at t = 700, respectively (Fig 2B and 2C). By genome sequencing 71.4% of all DARS1 insertions in DARS1 deficient cells mapped to four different insertions in the fimE gene (termed DARS1 Clone fimE #1, #2, #3, and #4, Fig 3B, S2 Table). DARS1 was also inserted in the ydeS gene close to terC (DARS1 Clone ydeS) and in the intergenic region between yfbN and yfbO (DARS1 Clone yfb), that of interest has the same gene dosage as the wild-type DARS1 copy. The functions of putative proteins encoded by ydeS, yfbN and yfbO are not known. Also, DARS1 in DARS1 deficient cells was found twice in yghG (termed DARS1 Clone yghG #1 and #2) and in adeQ (termed DARS1 Clone adeQ). In enterotoxigenic E. coli YghG is an outer membrane lipoprotein that is required for the correct localization of the GspD secretin in the outer membrane [38], while AdeQ is a high-affinity adenine transporter in E. coli K-12 [39]. These results indicate that the gene dosage of DARS1 does not specify its location; i.e., no unique location of DARS1 was selected for.

Transposon insertions sites for DARS1 into DARS1 DARS2 deficient cells were resolved using easy gene walking only. We isolated 20 single clones and mapped their transposon insertion sites. Here, two different insertions were mapped to the fimE gene (termed DARS1 Clone fimE #5 and #6, Fig 3C, S3 Table). DARS1 was also inserted in the intergenic region between gspA and gspC (DARS1 Clone gsp) and in the intergenic region between tomB and acrB (DARS1 Clone tomB). TomB is in a toxin-antitoxin operon with hha, where expression of TomB diminish the toxicity of Hha expression [40]; while the gspCDEFGHJKLMO (gspC-O) and gspAB operons encode homologs of type II secretion machinery involved in extrusion of folded proteins [41].

Selection of DARS2 insertions from a random pool in DARS2 deficient cells resulted in the selection of only one band at t = 700 (Fig 2B), indicating a single optimal chromosomal position for DARS2. This correlates well the data above; i.e. movement of DARS2 had a larger effect on the control of the cell cycle than movement of DARS1. Genome sequencing matched 97.8% of the DARS2 insertions to two locations approximately 650 bp from the wild-type DARS2 position, and separated by only 8 bp (S4 Table; Fig 3D). One insertion was located in the intergenic region between the ptsP and rppH gene (DARS2 Clone IR). This clone was already present at high frequency after 500 generations of growth (S4 Table). The second DARS2 proximal insertion was located inside the rppH gene (DARS2 Clone rppH), which encodes for RppH an RNA pyrophosphohydrolase that initiates mRNA decay [42]. The remaining 2.2% of the mapped inserts were found in the intergenic region between nanS and nanM (termed DARS2 Clone nan). nanS and nanM are transcribed in an operon with nanC, which supports the efficient use of α-N-acetylneuraminic as the sole source of carbon [43]. The Southern Blot for DARS2 into ΔDARS2 showed an abrupt changed between 400 and 500 generations of direct completion (Fig 2B), therefore both time points were resolved by full genome sequencing (S4 Table). Several interesting DARS2 insertions were found in t = 400, which were not present when the experiment was terminated after 700 generations. One DARS2 insertion was found in chromosomal position 2.316.202 bp (S4 Table), which is close to the tested DARS2 mirror
position on opposite replication arm (yafJ; Table 1). This suggest that these and other may have a fitness advantage over the majority of the initial 70,000 insertions, but that they were overall less fit than the insertions immediately flanking DARS2 strongly suggesting that the wild-type position is optimal for DARS2 function.

Two pairs of insertion sites for DARS2 into ΔDARS1 ΔDARS2 were resolved after 700 generations of direct competition. One pair included closely spaced insertions in the lgo gene (DARS2 Clone lgoR) and in the intergenic region between lgoR and lgorT (DARS2 Clone IR lgor) and the other were two insertions in the gene ttdR (DARS2 Clone ttdR #1 and #2) (Fig 3E). lgoR is a predicted transcriptional regulator that is essential for growth on L-galactonate as the sole carbon source [44], while ttdR is an LysR-type transcriptional regulator for L-tartrate fermentation that is induced under anaerobic growth [45]. The two DARS2 insertions, lgoR and ttdR, have almost the same distance to oriC albeit located on each replication arm (S5 Table). Thus, indicating that the optimal position of DARS2 in ΔDARS1 ΔDARS2 cells could be linked to gene dosage. The abrupt change between t = 300 and t = 400 for DARS2 into ΔDARS1 ΔDARS2 (Fig 2C) was also investigated by full genome sequencing (S5 Table). Of interest we find none of the selected end-point at t = 300, but DARS2 Clone ttdR #1, DARS2 Clone lgo, and DARS2 Clone lgoR was found at t = 400.

Cell cycle and fitness of selected transposon insertions

Representative transposon insertions identified by easy gene walking were moved into a fresh background by P1 transduction and analyzed by flow cytometry (S6 Table). None of the selected clones had a different doubling time compared to wild-type (+/- 2 minutes). All DARS1 insertions in ΔDARS1 cells (fimE #1, fimE #2, ydeS, and yfb) restored cell cycle parameters to those of wild-type cells, and all DARS1 insertions in ΔDARS1 ΔDARS2 cells (fimE #5, fimE #6, gsp, and tomB) restored the phenotype to that of DARS2 deficient cells. The DARS2 insertions next to the deleted DARS2 locus (IR and rppH) fully complemented loss of DARS2. Furthermore, the DARS2 insertions selected in ΔDARS1 ΔDARS2 cells (lgoR and ttdR) restored the low origin/mass ratio to levels close to a cell deficient in only DARS1.

We proceeded to investigate the fitness of representative selected clones. Here the DARS2 Clone IR, which was predominant after 700 generations culture evolution, was tested against wild-type and ΔDARS2 cells. The wild-type was found to be dominant to ΔDARS2 (1.5 LOG differences after 80 generations of competition) (S4A Fig) as expected [19]. The wild-type was also slightly dominant over the DARS2 Clone IR (0.7 LOG differences) (S4B Fig) whereas DARS2 Clone IR was slightly dominant to ΔDARS2 (0.8 LOG differences) (S4C Fig). Thus, although DARS2 Clone IR did not have an identical fitness to the wild-type it was more fit than ΔDARS2 as expected. The pattern seen for DARS2 Clone IR was also observed for DARS1 Clone ydeS tested against the wild-type and ΔDARS1, while DARS1 Clone fimE #3 was dominant to both the wild-type and ΔDARS1; probably due to the fitness advantage of mutating fimE in the used competition experimental setup.

The activity of DARS1, DARS2, and data is affected by transcription

Gene expression is known to be affected by local chromosomal context [23]. The effect of transcription on activity of cis-acting regions such as data, DARS1 and DARS2 is not known. The three regions were cloned into the R1 based plasmid pNDM220 [46], downstream on the IPTG inducible pA1/O4/O3 promoter. The three resultant plasmids were transformed into wild-type cells and their effect on the initiation of replication, in the presence or absence of IPTG, assessed by flow cytometry (Fig 4). The vector plasmid pNDM220 did not alter synchrony or origin concentration irrespective of IPTG addition (compare Fig 4A and 4E). In the absence of
IPTG, plasmid pNDM220::datA reduced the cellular origin content and concentration without affecting synchrony (compare Fig 4A, 4B and 4I). Plasmid-carried DARS1 and DARS2 increased the cellular origin content and concentration (compare Fig 4A and 4C for DARS1, Fig 4D for DARS2, Fig 4I) as previously observed [16]. Only the presence of pNDM220::DARS2 resulted in asynchronous initiations (Fig 4D).

Strong transcription through either datA, DARS1 or DARS2, by addition of IPTG, restored both origin concentration and synchrony to wild-type level (Fig 4F, 4G and 4H). Therefore, transcription through datA, DARS1, and DARS2 is detrimental to their function and further enforce that the activity of either region could be affected by local chromosomal context.

Discussion

We have previously found a conserved distance from oriC to the non-coding regions DARS1, DARS2 and datA in E. coli [19]. Site-directed translocation of DARS1, DARS2, and datA showed perturbed regulation of initiation when the regions were relocated away from their wild-type position; highlighting the importance of their natural position.

Position of datA

There are at least two explanations for the over-initiation resulting from datA relocation to terC; i.e., gene dosage and local access to DnaA<sub>ATP</sub> (compare Fig 5A to 5B). The chromosomal datA position is close to oriC and this was proposed to enhance interaction with DnaA<sub>ATP</sub>...
Fig 5. A schematic model of effect of DARS1 and datA relocation. (A) Wild-type; oriC and the dnaA gene are sequestered by the SeqA/Dam system following initiation of replication ([68]; not on figure). The sequestering period allows RIDA and the early duplication of datA to decrease the DnaAATP/DnaAADP ratio to a level, which counters unwanted re-initiation. (B) Relocation of datA close to terC; only RIDA and the single copy of datA next to terC acts to lower the DnaAATP/DnaAADP ratio during the sequestering period, resulting in higher concentration of DnaAATP compared to the wild-type. In addition, datA is speculated to have increased interaction with DnaAATP released from the dnaA gene due to the close proximity of the two [15]. Therefore the relocation of datA to terC would lead to increased inactivation of DnaAATP, a higher DnaAATP/DnaAADP ratio compared to the wild-type. In addition, datA is speculated to have increased interaction with DnaAATP released from the dnaA gene due to the close proximity of the two [15].

(C) Relocation of DARS1 close to oriC; RIDA and datA acts to lower the DnaAATP/DnaAADP ratio similar to the wild-type. However, the early duplication of DARS1 results in an earlier rejuvenation of DnaAATP from DnaAADP. Thus, with DARS1 close to oriC, the DnaAATP level increases faster than for wild-type cells, which leads to the following initiation at a reduced mass. (D) Relocation of DARS2 close to terC; DARS2 is cell cycle regulated; i.e. needs to be bound by IHF to be active [17]. IHF will only bind and activate DARS2 just prior to initiation [17]. Thus, moving DARS2 close to oriC will not alter the origin concentration because IHF availability does not change; i.e. no early rejuvenation activity. However, moving DARS2 close to terC lowers its gene dosage, which may be
insufficient to generate the preinitiation burst in DnaA\textsuperscript{ATP} necessary for initiation at all cellular origins; i.e. 
synchrony. oriC, terC, DARS1, DARS2, data, the dnaA gene, and RIDA are indicated in the figure. Red 
circle = DnaA\textsuperscript{ADP}, green circle = DnaA\textsuperscript{ATP}. The size of the filled green or red circles is proportional to the 
cellular amounts of DnaA\textsuperscript{ATP} and DnaA\textsuperscript{ADP}, respectively.

released from oriC at initiation, resulting in a high DDAH activity [15]. Thus, when data is 
relocated to terC, both gene dosage and interaction with DnaA\textsuperscript{ATP} is diminished resulting in an 
increased DnaA\textsuperscript{ATP}/DnaA\textsuperscript{ADP} ratio and increased origin concentration (Fig 5B). The Gram-
positive bacteria Bacillus subtilis also contains DnaA box clusters (DBC) that analogue to data can 
repress untimely initiation [47]. The DBC in B. subtilis is however, unlike data, only 
shown to titrate DnaA\textsuperscript{ATP} [47]. Of interest, the genomic position of the DBC is important for 
regulation of initiation; i.e. relocation from close to oriC (wild-type position) to terC reduces its 
function [47], as shown here and previously [28] for data. In addition, we find that the diminished 
data function in terC can be complemented to wild-type by a second data copy in the 
terminus, suggesting that gene dosage is an important parameter for an optimal data function.

The optimal positions of DARS1 and DARS2

Directed translocation of DARS1 indicated little preference for location, and only insertions very 
close to oriC resulted in an increased origin concentration, possibly due to a gene dosage effect or 
increased interaction with oriC associated DnaA (Fig 5C). In agreement with this, culture evolution 
revealed no optimal chromosomal position for the DARS1 region, although it’s presence is 
important [19]. The strong selection for loss of fimE in otherwise wild-type cells, suggest that the 
DARS1 insertions into fimE does not indicate the optimal position of DARS1 but results from a 
fitness advantage of disrupting fimE. The level of fimA transcription was increased in fimE 
mutants relative to the wild-type. As fimA encode the Type I major fimbrial subunit, fimE 
mutants are likely to carry an elevated number of fimbria. fimE mutant cells formed a pellicle at 
the top of the culture tubes suggesting, that increased fimbriation facilitates migration towards 
more aerobic conditions, which in turn could lead to a small growth advantage relative to wild-
type cells. The selected DARS1 insertions into ydeS, yghG, adeQ or yfb in DARS1 deficient cells or 
in tomB or gsp in DARS1 DARS2 deficient cells does not immediately suggest why these locations 
should provide a fitness advantage, and it may well be that these would be outcompeted by fimE 
insertions had competition proceeded longer. As the majority of the insertions are distant from 
oriC, it may be a fitness disadvantage to have DARS1 next to the origin.

Directed translocation of DARS2 indicated that many positions (CAG18499, 18496, 18488, 
12135, 18493, and yaff) restored origin concentration and synchrony to wild-type levels. Yet, 
only two DARS2 insertions were selected in ΔDARS2 cells after 700 generations of growth; 
97.8% were located next to the wild-type DARS2 position, while 2.2% were located in the inter-
genic region between nanS and nanM. At t = 400, an insertion was found on the opposite repli-
cation arm with an almost identical distance to oriC as the wild-type DARS2 position, but this 
insertion was not recovered after 700 generations. Thus, gene dosage cannot be the single 
determinant for optimal position. This is corroborated by the inability of a low-copy DARS2 
plasmid to complement DARS2 deficiency on the chromosome. The present data therefore 
implies that DARS2 and the immediate surroundings at its wild-type position specify the optimal 
genomic position. Even though we only investigate the effects of DARS2 translocation on the 
regulation of initiation of replication, we cannot exclude that the optimal DARS2 position, 
either directly or indirectly, contributes to fine tune other DnaA functions in the cell.

A cell with two functional DARS2 regions only resembled wild-type cells when the addi-
tional DARS2 region was located close to oriC (high gene dosage) or directly exchanged the
wild-type DARS1 region. This suggests that the local genomic context at DARS1 also provide an ideal setting also for DARS2 activity. Interestingly, RNA sequencing reveals very low transcriptional activity at the genomic DARS1 and DARS2 locations [48], and we observed that both DARS1 and DARS2 lost activity by transcription though the regions (Fig 4). Therefore both gene dosage and local transcriptional activity contribute to DARS activity. Recent reports also highlight the importance of a conserved genomic position for the function of key regulatory genes [49–51]; i.e. the local genomic environment is important for function. Thus, the importance of the local genomic environment may very well contribute to the selected positions of DARS1 and DARS2.

**DARS1 and DARS2 play different roles for replication control**

The activity of DARS1 is neither cell cycle nor growth phase regulated; i.e. always active [16]. Thus, de novo synthesis of DnaA will along with the constitutive DnaAADP rejuvenation at DARS1 ensure a steady DnaAATP increase throughout the cell cycle of wild-type cells. Therefore increased DARS1 gene dosages lead to an increased origin concentration (Fig 5C).

Rejuvenation at DARS2 is dependent on the binding of both Fis and IHF [17]. Therefore the activity at DARS2 is growth phase regulated by Fis, i.e. is only active during exponential growth [17], and cell cycle regulated by IHF [17]. Maximal IHF binding to activate DARS2, immediately precedes initiation [17]. Initiation of all cellular origins in synchrony was explained by a mechanism where DnaAATP released from the first origin initiated will trigger initiations on fully methylated not yet initiated origins, by a cascade-like mechanism [52]. However, new initiations will inevitably lead to more DNA loaded β-clamps that are instrumental in RIDA [12] which is therefore expected to accelerate. We propose that timely duplication and activation of DARS2 raises the DnaAATP/DnaAADP ratio to a sufficient high level at the onset of initiation, to ensure that all origins present in the cell are initiated in virtual synchrony even though RIDA is increased during the initiation period (for review see [53]). When DARS2 is located near terC the gene dosage may be too low to result in a sufficient pre-initiation burst in DnaAATP to ensure synchronous initiation at all cellular origins despite of its activity remaining cell cycle regulated (Fig 5D). This explains why a cell with two DARS2 loci will initiate in synchrony but fail to obtain a wild-type like origin/mass, while a cell with two DARS1 loci will be asynchronous (Table 2). Thus, DARS1 is primarily responsible for coupling replication initiation to cell mass increase, whereas DARS2 is primarily important for maintaining synchronous initiation of all origins contained within a single cell.

**Materials and Methods**

**Growth conditions**

Cells were grown in Luria–Bertani Broth (LB) medium or AB minimal medium supplemented with 0.2% glucose, 10 μg/ml thiamine, and 0.5% casamino acids. Cells were cultured at 37°C.
When necessary, antibiotics were added to the following concentrations: kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml; ampicillin, 150 μg/ml; tetracycline, 10 μg/ml.

**Bacterial strains**

All strains are found in Table 3.

**Plasmids**

**Plasmids for movement of DARS1, DARS2, and datA.** The chromosomal DARS1, DARS2, and datA locus were PCR amplified from MG1655 using primers DARS1_pKD3_FW and DARS1_pKD3_RV, DARS2_pKD3_FW and DARS2_pKD3_RV, and datA_pKD3_FW and datA_pKD3_RV (S7 Table), respectively. The PCR products were cut with NotI and PvuI and ligated with NotI- and PvuI-cut pKD3 [55], resulting in pKD3::DARS1 (pJFM5), pKD3::DARS2 (pJFM6), and pKD3::datA (pJFM9). Each plasmid was confirmed by sequencing.

**DARS2 on low-copy number plasmid.** The chromosomal DARS2 region was PCR amplified from MG1655 using primers DARS2_pALO277_FW and DARS2_pALO277_RV (S7 Table). The resultant PCR products was cut with SalI and BstBI and ligated with SalI- and BstBI cut pALO277 [30], resulting in pALO277::DARS2 (pJFM7). The plasmid was confirmed by sequencing.

**Plasmids for transposon-mediated random insertion of DARS1 and DARS2.** The chromosomal DARS1 and DARS2 loci were PCR amplified from MG1655 using primers DARS1_pNKBOR_FW and DARS1_pNKBOR_RV and DARS2_pNKBOR_FW and DARS21_pNKBOR_RV (S7 Table), respectively. The resultant PCR products were cut with BamHI and PstI and ligated with BamHI- and PstI cut pNKBOR [35]. Each PCR product was inserted between the two IS10 IR elements, resulting in NKBOR::DARS1 (pJFM3) and NKBOR::DARS2 (pJFM1). Each plasmid was confirmed by sequencing.

**Plasmids for investigating the effect of transcription through DARS1, DARS2, and datA.** The chromosomal DARS1 and DARS2 and datA locus was PCR amplified from MG1655 using primers DARS1_pNDM220_FW and DARS1_pNDM220_RV, DARS2_pNDM220_FW and DARS2_pNDM220_RV, and datA_pNDM220_FW and datA_pNDM220_RV (S7 Table), respectively. The PCR products were cut with BamHI and EcoRI and ligated with BamHI- and EcoRI-cut pNDM220 [46], resulting in pNDM220::DARS1 (pJFM10), pNDM220::DARS2 (pJFM11), and pNDM220::datA (pJFM12). Each plasmid was confirmed by sequencing.

**Relocation of datA, DARS1, and DARS2**

A set of strains containing the transposon Tn10 (encoding tetracycline resistance) at known positions on the chromosome were described previously [27] and generously provided by Dr. Martin G. Marinus (Fig 1A). None of the Tn10 insertions from the Singer library altered origin concentration (origins/mass), synchrony of initiation of replication, or doubling time of otherwise wild-type cells.

The Tn10 insertions CAG18499, CAG18496, CAG18488, CAG12173, CAG1315, CAG18493, CAG12151, and CAG18461 were individually moved into BW25113 [55], MG1655 strR (ALO4292), MG1655 strR ΔDARS1 (ALO4314), MG1655 strR ΔDARS2 (ALO4312), and MG1655 strR ΔdatA::kan (ALO4331) [19] by P1 transduction using established procedures [56] and by selection for tetracycline resistance.

In the individual Tn10 transformed strains the tetC gene on the Tn10 was replaced by either the datA, DARS1, or DARS2 region linked to the cat gene by the lambda red procedure [55].
Table 3. Bacterial strains.

| Strain      | Genotype                          | Abbreviated form | Reference/Source |
|-------------|-----------------------------------|------------------|------------------|
| BW25113     | lacF, rrnBT14ΔlacZWJ16 hsdR514ΔaraBADΔraBADΔrhaBADΔD78 |                  | [55]             |
| MG1655      | F' λ ph−1                         |                  | [66]             |
| ALO4292     | MG1655 strRa                      | Wild-type        | [19]             |
| ALO4254     | ΔDARS2::cat                       | ΔDARS2           | [19]             |
| ALO4310     | ΔDARS2::cat strRa                 | ΔDARS2           | [19]             |
| ALO4312     | ΔDARS2 strRa                      | ΔDARS2           | [19]             |
| ALO4313     | ΔDARS1::cat strRa                 | ΔDARS1           | This work        |
| ALO4314     | ΔDARS1 strRa                      | ΔDARS1           | This work        |
| ALO4331     | ΔdataA::kan strRa                 | ΔdataA           | [19]             |
| CAG12135    | MG1655 recD1901::Tn10a            |                  | [26]             |
| CAG12151    | MG1655 zdh-925::Tn10a             |                  | [26]             |
| CAG12173    | MG1655 cysC95::Tn10a              |                  | [26]             |
| CAG18488    | MG1655 z/d-2231::Tn10a            |                  | [26]             |
| CAG18493    | MG1655 zbi-29::Tn10a              |                  | [26]             |
| CAG18496    | MG1655 faAB101::Tn10a             |                  | [26]             |
| CAG18499    | MG1655 z/d-501::Tn10a             |                  | [26]             |
| ALO4412     | ΔDARS1 CAG18499::DARS1::cat tetR strRa | This work        |                  |
| ALO4413     | ΔDARS1 CAG18499::DARS2::cat tetR strRa | This work        |                  |
| ALO4414     | ΔDARS2 CAG18499::DARS1::cat tetR strRa | This work        |                  |
| ALO4415     | ΔDARS2 CAG18499::DARS2::cat tetR strRa | This work        |                  |
| ALO4475     | ΔdataA CAG18499::dataA::cat tetR strRa | This work        |                  |
| ALO4440     | ΔDARS1 CAG18496::DARS1::cat tetR strRa | This work        |                  |
| ALO4441     | ΔDARS1 CAG18496::DARS2::cat tetR strRa | This work        |                  |
| ALO4442     | ΔDARS2 CAG18496::DARS1::cat tetR strRa | This work        |                  |
| ALO4443     | ΔDARS2 CAG18496::DARS2::cat tetR strRa | This work        |                  |
| ALO4476     | ΔdataA CAG18496::dataA::cat tetR strRa | This work        |                  |
| ALO4378     | ΔDARS1 CAG18488::DARS1::cat tetR strRa | This work        |                  |
| ALO4379     | ΔDARS1 CAG18488::DARS2::cat tetR strRa | This work        |                  |
| ALO4380     | ΔDARS2 CAG18488::DARS1::cat tetR strRa | This work        |                  |
| ALO4381     | ΔDARS2 CAG18488::DARS2::cat tetR strRa | This work        |                  |
| ALO4477     | ΔdataA CAG18488::dataA::cat tetR strRa | This work        |                  |
| ALO4585     | ΔDARS1 CAG12135::DARS1::cat tetR strRa | This work        |                  |
| ALO4586     | ΔDARS1 CAG12135::DARS2::cat tetR strRa | This work        |                  |
| ALO4463     | ΔDARS2 CAG12135::DARS1::cat tetR strRa | This work        |                  |
| ALO4464     | ΔDARS2 CAG12135::DARS2::cat tetR strRa | This work        |                  |
| ALO4419     | ΔDARS1 CAG12173::DARS1::cat tetR strRa | This work        |                  |
| ALO4420     | ΔDARS1 CAG12173::DARS2::cat tetR strRa | This work        |                  |
| ALO4421     | ΔDARS2 CAG12173::DARS1::cat tetR strRa | This work        |                  |
| ALO4478     | ΔdataA CAG12173::dataA::cat tetR strRa | This work        |                  |
| ALO4431     | ΔDARS1 CAG18493::DARS1::cat tetR strRa | This work        |                  |
| ALO4432     | ΔDARS1 CAG18493::DARS2::cat tetR strRa | This work        |                  |
| ALO4433     | ΔDARS2 CAG18493::DARS1::cat tetR strRa | This work        |                  |
| ALO4434     | ΔDARS2 CAG18493::DARS2::cat tetR strRa | This work        |                  |
| ALO4479     | ΔdataA CAG18493::dataA::cat tetR strRa | This work        |                  |
| ALO4390     | ΔDARS1 CAG12151::DARS1::cat tetR strRa | This work        |                  |
| ALO4608     | ΔDARS1 CAG12151::DARS2::cat tetR strRa | This work        |                  |
| ALO4391     | ΔDARS2 CAG12151::DARS1::cat tetR strRa | This work        |                  |

(Continued)
Table 3. (Continued)

| Strain   | Genotype                                      | Abbreviated form | Reference/Source |
|----------|-----------------------------------------------|------------------|------------------|
| ALO4392  | ΔDARS2 CAG12151::DARS2:cat tetR strR<sup>a</sup> |                  | This work        |
| ALO4480  | Δdata CAG12151::dataA:cat tetR strR<sup>a</sup>|                  | This work        |
| ALO4328  | ΔDARS1 CAG18461::DARS1::cat tetR strR<sup>a</sup>|                  | This work        |
| ALO4329  | ΔDARS1 CAG18461::DARS2::cat tetR strR<sup>a</sup>|                  | This work        |
| ALO4591  | ΔDARS2 CAG18461::DARS1::cat tetR strR<sup>a</sup>|                  | This work        |
| ALO4330  | ΔDARS2 CAG18461::DARS2::cat tetR strR<sup>a</sup>|                  | This work        |
| ALO4481  | Δdata CAG18461::dataA::cat tetR strR<sup>a</sup>|                  | This work        |
| ALO5041  | Δdata CAG12151::dataA CAG18461::dataA::cat tetR strR<sup>a</sup>|                | This work        |
| ALO5042  | ΔDARS2 yafJ::DARS2::cat strR<sup>a</sup>       |                  | This work        |
| ALO5043  | ΔDARS1::DARS2::cat strR<sup>a</sup>            |                  | This work        |
| ALO4255  | MG1655 tonA                                    |                  | This work        |
| ALO4256  | ΔDARS1::cat tonA<sup>a</sup>                   |                  | This work        |
| ALO4257  | ΔDARS2::cat tonA<sup>a</sup>                   |                  | This work        |
| ALO4259  | ΔDARS1::cat ΔDARS2 tonA<sup>a</sup>            |                  | This work        |

<sup>a</sup> Genotype otherwise as *E. coli* MG1655

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Briefly, *data* was PCR amplified from pJFM9, *DARS1* from pJFM5, and *DARS2* from pJFM6 using primers *tetC<sub>FW</sub>* and *tetC<sub>RV</sub>* (S7 Table). *tetC<sub>FW</sub>* and *tetC<sub>RV</sub>* will PCR amplify both the cloned locus into pKD3 (see above) along with the *cat* cassette including the two FRT-styes.

CAG18499::*dataA::cat*, CAG18496::*dataA::cat*, CAG18488::*dataA::cat*, CAG12173::*dataA::cat*, CAG18493::*dataA::cat*, CAG18461::*dataA::cat*, and CAG12151::*dataA::cat* were individually moved from BW25113 into MG1655 strR Δ*dataA::kan* by P1 transduction and by selection for *cat* and *kan* resistance. Hence, derivatives of the wild-type strain devoid of *dataA* at the original locus and instead carrying Tn<sub>10</sub>::*dataA* at indicated chromosomal loci were created. In addition, a strain with two Tn<sub>10</sub>::*dataA* loci in the terminus region were created by removing the *cat* gene from CAG18461::*dataA::cat*, by pCP20, according to a method described previously [57]. Thereafter the CAG12151::*dataA::cat* were moved from BW25113 into MG1655 strR Δ*dataA::kan* CAG18461::*dataA* by P1 transduction, selecting for *cat* resistance, resulting in a strain with *dataA* inserted in both CAG18461 and CAG12151 (AL05041).

CAG18499::*DARS1::cat*, CAG18496::*DARS1::cat*, CAG18488::*DARS1::cat*, CAG12135::*DARS1::cat*, CAG12173::*DARS1::cat*, CAG18493::*DARS1::cat*, and CAG18461::*DARS1::cat*, CAG12151::*DARS1::cat* were individually moved from BW25113 into MG1655 str<sup>R</sup> Δ*DARS1* and MG1655 str<sup>R</sup> Δ*DARS2* by P1 transduction and by selection for *cat* resistance. Hence, derivatives of the wild-type strain devoid of *DARS1* at the original locus and instead carrying Tn<sub>10</sub>::*DARS1* at indicated chromosomal loci were created (Table 2). Similarly, derivatives of the wild-type strain devoid of *DARS2* at the original locus and instead carrying Tn<sub>10</sub>::*DARS1* at indicated chromosomal loci were created.

CAG18499::*DARS2::cat*, CAG18496::*DARS2::cat*, CAG18488::*DARS2::cat*, CAG12135::*DARS2::cat*, CAG18493::*DARS2::cat*, CAG18461::*DARS2::cat*, and CAG12151::*DARS2::cat* were individually moved from BW25113 into MG1655 str<sup>R</sup> Δ*DARS1* and MG1655 str<sup>R</sup> Δ*DARS2* by P1 transduction and by selection for *cat* resistance. Hence, derivatives of the wild-type strain devoid of *DARS2* at the original locus and instead carrying Tn<sub>10</sub>::*DARS2* at indicated chromosomal loci were created (Table 2). Also derivatives of the wild-type strain devoid of *DARS1* at the original locus and instead carrying Tn<sub>10</sub>::*DARS2* at indicated chromosomal
loci were created. Furthermore, DARS2 were relocated to yafJ. yafJ was replaced with DARS2 amplified from pJFM6 using primers yafJ_FW and yafJ_RV (S7 Table) in MG1655 StrR ΔDARS2 harboring pKD46, resulting in the MG1655 StrR ΔDARS2 yafJ::DARS2::cat mutant (ALO5042). DARS2 was also relocated to DARS1 in MG1655 StrR. DARS1 was replaced with DARS2 amplified from pJFM6 using primers D2toD1_FW and D2toD1_RV (S7 Table) in MG1655 StrR harboring pKD46, resulting in the MG1655 StrR ΔDARS1::DARS2::cat mutant (ALO5043).

**Transposon-mediated random insertion of DARS1 and DARS2**

A spontaneous tonA mutant of *E. coli* MG1655 was isolated by Dr. Stanley Brown, resulting in *E. coli* MG1655 tonA (ALO4255).

The DARS1 region was replaced with the cat gene in MG1655 harboring pKD46, resulting in the ΔDARS1::cat mutant (ALO4075). Briefly, the cat gene was PCR amplified using primers DARS1_KO_FW and DARS1_KO_RV from pKD3. The resultant DNA fragments were introduced into ALO1825 bearing pKD46. Each deletion was verified by PCR. The DARS1 deletion was moved from ALO4075 to ALO4255 by P1 transduction, selecting for chloramphenicol resistance, resulting in MG1655 tonA ΔDARS1::cat (ALO4256). The DARS2 deletion was moved from ALO4254 into ALO4255 by P1 transduction, selecting for chloramphenicol resistance, resulting in MG1655 tonA ΔDARS2::cat (ALO4257). The cam cassette was removed from ALO4257 and the DARS1 deletion was moved from ALO4075 into the chloramphenicol sensitive MG1655 tonA ΔDARS2 by P1 transduction, selecting for chloramphenicol resistance, resulting in MG1655 tonA ΔDARS1::cat ΔDARS2 (ALO4259).

**Replication-associated gene dosage**

The replication-associated gene dosage was estimated for every position for growth in ABTG + CAA and LB. Eq (1) was used to calculate the replication-associated gene dosage Here the average no. of copies per chromosome of a gene with position x on the chromosome (x = 0 at the origin and x = 1 at the terminus), C is the replication period in minutes, D is the time following termination of replication until cell division, and \( \tau \) is the doubling time [58].

\[
X = 2^{\left(\frac{C \times (1-x) + D}{C \times \ln(2)}\right)}
\] (1)

\[
G = \frac{\tau}{C \times \ln(2)} \left[2^{\frac{C \times \tau}{D}} - 2^{D/\tau}\right]
\] (2)

At generation times below 60 min the C- and D period has been shown to be constant in *E. coli* K-12 strains; i.e. 42 minutes and 33 minutes, respectively [59]. Generation time for the wild-type grown in ABTG + CAA was shown to be 40 minutes. By Eq (1) the gene dosage of DARS2 is calculated to be 1.66 copies/cell at the given growth rate. Hence, by Eq (2), the cellular DNA content (G) was found to be 1.82 genome equivalents per cell. As the copy number of plasmid F is 1–2 per genome equivalent [31] it follows that the copy number per cell of the F plasmid pALO277 and derivatives is 1.82–3.64.

**In culture competition experiment for transposon-mediated random insertion of DARS1 and DARS2**

pNKBOR is a R6K-based suicide vector that permits the random insertion of a mini-transposon (NKBOR) into a \( \pi \) protein deficient *E. coli* chromosome [35]. Here we transformed pJFM1 (NKBOR::DARS2) into ΔDARS2 and ΔDARS1 ΔDARS2, pJFM3 (NKBOR::DARS2) into
ΔDARS1 and ΔDARS1 ΔDARS2, and pNKBOR (NKBOR) into wild-type selecting for kan resistance. Approximately 70,000 random insertions were obtained for each transformation. The 70,000 strains were pooled and inoculated in the same tube. They were grown in LB aerated by continuous shaking at 37°C. The populations were propagated by continuously transfers after estimated 10 generations. Samples for genomic DNA from each population were taken at 100-generation intervals, until direct competition for estimated 700-generations (used for southern blot; Fig 2). After 700-generations of direct competition 10 single clones was isolated from NKBOR::DARS2 into ΔDARS2, 20 single clones were isolated from NKBOR::DARS1 into ΔDARS1, from NKBOR::DARS1 into ΔDARS1 ΔDARS2, and from NKBOR::DARS2 into ΔDARS1 ΔDARS2, and 15 single clones were isolated from NKBOR into wild-type.

Southern blot analysis
Total cellular DNA was prepared according to Løbner-Olesen and von Freiesleben [60]. DNA was digested with *Pvu*I, and fragments were separated on a 0.7% agarose gel, transferred by capillary transfer to a Hybond-N+ membrane (Amersham Pharmacia Biotech), and probed with an approx. 1 kb NKBOR fragment, which hybridize to NKBOR. The probe was prepared by PCR amplification using primers NKBOR_Probe_FW and NKBOR_Probe_RV (S7 Table) using pNKBOR as template and labeled with [α-32P]dATP (Amersham Pharmacia) using the Random Primer system (DECAprime II DNA Labeling Kit; Life Technologies).

Easy gene walking
The chromosomal position of the NKBOR, NKBOR::DARS1, and NKBOR::DARS2 insertion was found as described by Harrison et al. [36], using nested primers specific to NKBOR (NKBOR_N3, NKBOR_N2, and NKBOR_1) and random primers Random_HindIII and Random_Sau3AI (S7 Table).

Whole-genome sequencing
Whole-genome sequencing was performed at the University of Copenhagen on an Illumina MiSeq benchtop sequencer. A total of 6 million paired-end reads were generated, with read length of 35 to 300 nucleotides. Reads were aligned to 150 N’s contiguous to NKBOR, AF310136.1 1,904…2,204 using Bowtie2 [61] with interval between seed substrings = S,1,1.15 and maximum number of ambiguous characters = L,0,0.9. Aligned reads were then aligned to MG1655 ref|NC_000913.3 and NKBOR gb|AF310136 using blastN [62] and sorted for contiguous alignment MG1655-NKBOR. Note that the coverage in the present deep sequencing was insufficient for a complete mapping of insertion sites. The data presented in S1–S5 Tables therefore contains a representative subset of the total number of insertions.

RNA isolation and cDNA synthesis
Total RNA from bacterial samples was extracted using the GeneJET RNA Purification Kit (Thermo Fisher Scientific) according to the manual. Following treatment of RNA with TURBO DNase (Ambion), cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit for reverse transcriptase PCR according to the manufacturer’s protocols (Thermo Fisher Scientific). In parallel, RNA samples were subjected to agarose gel electrophoresis and NanoDrop (Thermo Fisher Scientific) to verify quality and yield. qPCR with primers specific to the *rpoA* gene (the α subunit of the RNA polymerase core enzyme) (S7 Table) [63] was performed on cDNA samples prepared with and without reverse transcriptase to confirm no genomic DNA contamination of the RNA preparations following DNase treatment.
Quantitative polymerase chain reaction (qPCR)

**fimA.** Primers designed to amplify *fimA* ([S7 Table](#)) were targeted to regions of unique sequence within the gene. The qPCR was performed using Takara SYBR Premix Ex Taq II (RR820A) in a BioRAD CFX96 (95°C 30 s, 39×(95°C 5 s + 60°C 30 s), 95°C 15 s, 60°C 60 s). All data were normalized to the endogenous reference gene *rpoA* [63] with primers taken from the same article. These data were transformed to log2 to obtain a change difference (n-fold) between strains.

**F plasmid to ter.** F plasmid copy number quantified relative to *ter* was performed as previously described [64].

Relative distance

The relative distance between *oriC* and transposon insertions sites were calculated as described previously [19].

Flow cytometry

Flow cytometry was performed as described previously [65] using an Apogee A10 instrument. For each sample, a minimum of 30,000 cells were analyzed. Numbers of origins per cell and relative cell mass were determined as described previously [65].

Asynchrony index

Asynchrony was calculated as described by Løbner-Olesen *et al.* [65]. Initiations were considered asynchronous when A > 0.1.

Competition experiment in LB

The fitness of *DARS2* Clone *rrpH*, *DARS1* Clone *fimE* #3, and *DARS1* Clone *ydeS* were compared to the wild-type and either Δ*DARS1* or Δ*DARS2* (indicated in the text) during direct competition in LB medium. The competing strains were inoculated pairwise at an approximate concentration of (10⁷ CFU/mL) each. The populations were propagated by continuously transfers in LB medium. Samples from each population were taken at 10-generation intervals. Each sample was diluted in 0.9% NaCl and plated on LB plates with appropriate antibiotics. All plates were incubated for 18–24h at 37°C prior to counting.

Supporting Information

**S1 Fig. Effect of *DARS2* complementation on a low-copy number plasmid.** (A) The ter/F ratio was determined by qPCR from wild-type cell with either pALO277 or pALO277::*DARS2* (as indicated). B-G: Flow cytometric analysis of wild-type and *DARS2* deficient cells with or without the plasmids. Cells were grown in AB minimal medium supplemented with 0.2% glucose, 10 μg/ml thiamine, and 0.5% casamino acids at 37°C. Wild-type (MG1655) or Δ*DARS2* carried no plasmid, pALO277, or pALO277::*DARS2* as indicated in individual panels. Details are given in the table on the right ([H](#)), wherein numbers are normalized to 1 for wild-type. (TIF)

**S2 Fig. Representative flow cytometry histograms of *DARS* complementation.** Representative flow cytometry histograms of *DARS1/DARS2* insertions close to *oriC* (CAG18499), a Tn10 insertion close to *data* (CAG18488), and one close to *terC* (CAG12151). Cells were grown in AB minimal medium supplemented with 0.2% glucose, 10 μg/ml thiamine, and 0.5% casamino acids at 37°C. Wild-type, Δ*DARS1*, and Δ*DARS2* are shown in A, B, and F, respectively.
Derivatives of the wild-type strain MG1655 devoid of DARS1 at the original locus and instead carrying an additional copy of DARS2 close to oriC, close to datA, or close to terC are shown in C, D, and E, respectively. Derivatives of the wild-type strain MG1655 devoid of DARS2 at the original locus and instead carrying an additional copy of DARS1 close to oriC, close to datA, or close to terC are shown in G, H, and I, respectively. See Table 2 for details.

**S3 Fig. Insertions in fimE leads to increased fimbriation.** (A) Quantification of the fimA mRNA level. Quantitative PCR was performed as described in Materials and Methods. Relative fimA mRNA levels in strains ΔDARS1, ΔDARS1 ΔDARS2, NKBOR Clone fimE, DARS1 Clone fimE #1, DARS1 Clone fimE #2, and DARS1 Clone fimE #3 were determined. In this experiment, the rpoA mRNA was used as an internal control. Three biological measurements were performed, and standard deviations are shown. (B) Remnants of pellicle formation. Wild-type (1.) and NKBOR Clone fimE (2.) grown for 10 hours in LB at 37°C. Framed is the presumed remnant from the pellicle in NKBOR Clone fimE. The culture had been shaking (diagonally) in a shaker overnight. Thus when hold diagonally the pellicle remnant would align with the surface of the culture. The picture was however taken with the tubes more or less hold vertical to better show the difference in pellicle formation between fimE+ (wild-type) and fimE- (NKBOR Clone fimE).

**S4 Fig. Fitness of selected DARS2 insertions.** Competition experiment in LB medium. A: Wild-type vs. ΔDARS2; B: Wild-type vs DARS2 Clone rppH; C: ΔDARS2 vs DARS2 Clone rppH. Bars represent the standard error of the log10 mean number of CFU per ml. For details see Supporting Information.

**S1 Table. NKBOR into wild-type.** a Chromosomal position of transposon insertion site on the E. coli MG1655 genome at = 0 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 50 insertions were resolved for this time point. b Chromosomal position of transposon insertion site on the E. coli MG1655 genome at = 700 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 228 insertions were resolved for this time point.

**S2 Table. DARS1 into ΔDARS1.** a Chromosomal position of transposon insertion site on the E. coli MG1655 genome at = 0 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 14 insertions were resolved for this time point. b Chromosomal position of transposon insertion site on the E. coli MG1655 genome at = 700 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 227 insertions were resolved for this time point.

**S3 Table. DARS1 into ΔDARS1 ΔDARS2.** a Chromosomal position of transposon insertion site on the E. coli MG1655 genome at = 700 resolved by easy gene walking.

**S4 Table. DARS2 into ΔDARS2.** a Chromosomal position of transposon insertion site on the E. coli MG1655 genome at = 0 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 39 insertions were resolved for this time point. b Chromosomal position of transposon insertion site on the E. coli MG1655 genome at = 400 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 54 insertions were resolved for this time point. c Chromosomal position of transposon insertion site on the
DNA Replication Control Is Linked to Genomic Positioning of Control Regions in *Escherichia coli*  

*E. coli* MG1655 genome a t = 500 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 13 insertions were resolved for this time point. Chromosomal position of transposon insertion site on the *E. coli* MG1655 genome a t = 700 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 278 insertions were resolved for this time point.

(S5 Table. *DARS2 into ΔDARS1 ΔDARS2.*  

a Chromosomal position of transposon insertion site on the *E. coli* MG1655 genome a t = 0 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 9 insertions were resolved for this time point. b Chromosomal position of transposon insertion site on the *E. coli* MG1655 genome a t = 300 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 20 insertions were resolved for this time point. c Chromosomal position of transposon insertion site on the *E. coli* MG1655 genome a t = 400 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 52 insertions were resolved for this time point. Chromosomal position of transposon insertion site on the *E. coli* MG1655 genome a t = 700 resolved by full genome sequencing. Percent of aligned reads is given in the brackets. A total of 275 insertions were resolved for this time point.

(S6 Table. Flow cytometric characterizing of NKBOR, NKBOR::DARS1, and NKBOR::DARS2 insertions.  
a Transposon sites found be Easy-Gene Walking at t = 700. b The transposon insertions selected from the five set-ups were moved into a fresh background by P1 transduction; NKBOR Clone *fimE* into wild-type, *DARS1* Clone *fimE* #1, *fimE* #2, *ydeS*, and *yfb* into *DARS1* deficient cells, *DARS1* Clone *fimE* #5, *fimE* #6, *tomB*, and *gsp* into *DARS1 DARS2* deficient cells, *DARS2* Clone IR and *rppH* into *DARS2* deficient cells, and *DARS2* Clone *tddR* and *lgoR* into *DARS1 DARS2* deficient cells. c Doubling time in LB grown at 37°C. d Determined as average light scatter from flow cytometric analysis. Numbers are normalized to 1 for wild-type. e Asynchrony index; calculated as described in Methods.

(S7 Table. Primers.

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