The Escherichia coli datA site promotes proper regulation of cell division

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In Escherichia coli inhibition of replication leads to a block of cell division. This checkpoint mechanism ensures that no cell divides without having two complete copies of the genome to pass on to the two daughter cells. The chromosomal datA site is a 1 kb region that contains binding sites for the DnaA replication initiator protein, and which contributes to the inactivation of DnaA. An excess of datA sites provided on plasmids has been found to lead to both a delay in initiation of replication and in cell division during exponential growth. Here we have investigated the effect of datA on the cell division block that occurs upon inhibition of replication initiation in a dnaC2 mutant. We found that this checkpoint mechanism was aided by the presence of datA. In cells where datA was deleted or an excess of DnaA was provided, cell division occurred in the absence of replication and anucleate cells were formed. This finding indicates that loss of datA and/or excess of DnaA protein promote cell division. This conclusion was supported by the finding that the lethality of the division-compromised mutants ftsZ84 and ftsI23 was suppressed by deletion of datA, at the lowest non-permissive temperature. We propose that the cell division block that occurs upon inhibition of DNA replication is, at least in part, due to a drop in the concentration of the ATP–DnaA protein.

INTRODUCTION

In Escherichia coli cell division involves the formation of a septum that separates the cell into two daughter cells. It is crucial that the two daughter nucleoids are segregated into each half of the cell, so that each daughter cell receives a copy of the genetic material and that none of the nucleoids are trapped in the septum. To ensure this, cell division and chromosome segregation are highly regulated processes involving a number of different proteins (see Egan & Vollmer, 2013; Harry et al., 2006; and Weiss, 2004, for reviews), the most studied being FtsZ. This protein polymerizes to form a ring-like structure at the site of division and serves as a scaffold for recruitment of other division proteins (Bi & Lutkenhaus, 1991). How the timing of the formation of the FtsZ ring is regulated is not clear. It is known that the chromosome replication cycle is independent of the cell division cycle, but that cell division does not occur in the absence of replication and nucleotide bound to the protein (the ATP-form of the DnaA is the active form for initiation of replication (see Haeusser & Levin, 2008, for a review)). This regulation involves the datA site. The datA site is a 1 kb DNA sequence with five well conserved DnaA binding sites (DnaA-boxes) (Kitagawa et al., 2007). Recently it was shown that the datA site, together with the IHF protein, stimulates hydrolysis of the ATP bound to the DnaA protein and can therefore contribute to the inactivation of the DnaA protein in a process called DDAH (datA-dependent DnaA–ATP hydrolysis) (Kasho & Katayama, 2013). The hydrolysis of the ATP bound to DnaA is also stimulated by the Hda protein together with the β-clamp of the DNA

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Abbreviation: RIDA, regulatory inactivation of DnaA.

One supplementary figure is available with the online version of this paper.
polymerase (Katayama & Sekimizu, 1999). This process, called regulatory inactivation of DnaA (RIDA), has been shown to be dependent on ongoing replication (Kurokawa et al., 1999).

The presence of excess datA leads to a delay in cell division (Kitagawa et al., 1998; Morigen et al., 2003) and deletion of the datA site results in cell division at a smaller cell mass relative to wild-type (Morigen et al., 2005). This indicates that the datA site also affects cell division.

Here, we used a dnaC2 mutant (Carl, 1970; Withers & Bernander, 1998) to investigate the influence of the datA site or an elevated level of the DnaA protein on the checkpoint that inhibits cell division after a blockage of chromosome replication. We found that both lack of the datA site and an increase in DnaA concentration led to a bypass of this checkpoint in some cells, indicating that the level and activity of DnaA in the cells might be a factor in the regulation of cell division.

METHODS

Bacterial strains, plasmids and growth conditions. All strains used are Escherichia coli K-12 and are listed in Table 1. Cells were grown in AB minimal medium (Clark & Maaloe, 1967) supplemented with 10 µg thiamine ml⁻¹, 0.4 % glucose and 0.5 % Casamino acids (Glu–CAA medium) at 37 °C. Twenty-five micrograms ampicillin ml⁻¹, 25 µg kanamycin ml⁻¹, 30 µg chloramphenicol ml⁻¹ or 10 µg tetracycline ml⁻¹ was added when necessary. The growth rates were determined by measuring the optical density of the cultures at 450 nm. The thr::Tn10datA2 (Withers & Bernander, 1998), datA::Kan (Kitagawa et al., 1998) and recA938::Cam (Zyskind et al., 1992) alleles were transferred by P1 transduction from MG1655datA2, RSD48 and AL5972, respectively, to the recipient strains listed in Table 1. W3110datA2 and MOR165 (W3110::datAdnaC2) were transformed with MiniR1 or MiniR1::datA (Morigen et al., 2001) by electroporation. To induce overexpression of the dnaA gene, six groups of W3110datA2::dnaC2dnaA16 cells (Krause et al., 1997) growing exponentially (OD600 0.1) at 30 °C were treated with different concentrations of IPTG (0–0.4 mM) and immediately shifted to 42 °C. Growth of the fisA and fisD strains and their datA counterparts was performed on LB plates, while the fisZ mutant and its datA counterpart were grown on low salt LB (containing 0.02 g NaCl ml⁻¹ instead of 0.04 g NaCl ml⁻¹) on which the temperature sensitivity of fisZdatA can be detected.

Microscopy. E. coli dnaC2 cells were grown to early exponential phase (optical density at 450 nm of 0.1) at a permissive temperature (30 °C) and then shifted up to a non-permissive temperature (42 °C) and grown for a further 4 h. Samples for microscopy were taken at 0, 1, 2, 3 and 4 h after the shift to the non-permissive temperature. Cell culture (1.5 ml) was harvested, washed once in ice-cold, filtered TE buffer, resuspended in 100 µl of the same buffer and then fixed in 1 ml of ice-cold, filtered 77 % ethanol. Fixed cells were mounted on a poly-L-lysine coated microscope slide and the DNA was stained with Hoechst 33258 (5 µg ml⁻¹, Sigma) in mounting medium (40 % glycerol in PBS pH 7.5). Visualization of stained cells was performed using a Leica DM6000B phase-contrast/fluorescence microscope equipped with a × 63 objective and a BF340–380 excitation filter. Pictures were taken using a Leica DFC350FX digital camera that was connected to a computerized image analysis system (LAS AF software, version 2.0.0, Leica). The fluorescence image was merged with the phase-contrast image and the number of anucleate cells counted.

Flow cytometry. Cells were harvested at 0, 1, 2, 3 and 4 h after the shift to the non-permissive temperature, washed and resuspended in 100 µl TE buffer and fixed by adding 1 ml 77 % ethanol. The cells were washed in 0.1 M phosphate buffer, pH 9.0, and total protein content was stained overnight using 1.5 µg FITC ml⁻¹ (Sigma) in the same buffer (Wold et al., 1994). The cells were washed in 0.02 M Tris buffered saline, pH 7.5, and resuspended in the same buffer. The DNA was stained for 30 min in 1.5 µg Hoechst 33258 ml⁻¹ (Sigma) in the same buffer (Torheim et al., 2000). The samples were analysed on an LSR II flow cytometer (BD Biosciences) as mentioned previously (Morigen et al., 2009). When anucleate cells were to be detected with flow cytometry, the DNA content per cell was determined at a high voltage of UV to detect such cells. The average DNA content per cell ratio was determined as average Hoechst fluorescence per cell.

Western blotting. Cells were harvested by centrifugation and SDS samples of cell extracts and purified proteins were prepared as previously described (Fossum et al., 2003). Two micrograms of the cell extract (total protein) was subjected to 12 % SDS-PAGE (40 mA, 2–3 h) and Western blotting was performed using an anti-DnaA antibody and an ECF fluorescence kit (GE Healthcare). Quantification of the amount of DnaA in each sample relative to the control cells at the zero time point was performed using Image Quant software (Molecular Dynamics).

RESULTS AND DISCUSSION

Anucleate cells accumulate in the absence of the datA site in replication arrested cells

To block replication, we used a dnaC2 mutant, which cannot initiate chromosomal replication at a non-permissive temperature (Carl, 1970; Withers & Bernander, 1998). New initiations are then blocked, but ongoing replication and cell division continue until all cells contain one fully replicated chromosome after approximately 120 min. At this time point, further cell division as well as initiation of replication should be blocked (Withers & Bernander, 1998). Exponentially growing dnaC2 and dnaC2datA cells were shifted from a permissive temperature (30 °C) to a non-permissive temperature (42 °C). The behaviour of the cells in the absence of replication over a time-course at the non-permissive temperature was investigated by microscopy and flow cytometry to see whether cell division was inhibited. The appearance of DNA-free (anucleate) cells was taken as a readout of cell division occurring even though the chromosome had not been replicated (Fig. 1a–f).

In the control cells (dnaC2), a few anucleate cells appeared after 2 h of incubation at the non-permissive temperature. After 4 h at 42 °C, 6 % of cells were anucleate (Fig. 1b, g), showing that a few cells had divided in the absence of replication and that the inhibition of cell division under these circumstances is not absolute. This phenotype has also been observed in other temperature-sensitive mutants where replication is blocked (Hirota et al., 1968). Also, in the dnaC2datA cells, anucleate cells started to appear after 2 h at the non-permissive temperature. However, in this case the frequency of anucleate cells increased significantly more than in the control cells. After 4 h at the
non-permissive temperature, 25% of the cells were anucleate (Fig. 1d, g). Approximately 3% of the control cells and 15% of the ΔdatA cells were found to be anucleate after 4 h at the non-permissive temperature, as measured by flow cytometry (Fig. S1, available in the online Supplementary Material).

An excess of datA sites would be expected to have the opposite effect of its deletion. We investigated dnaC2 cells containing MiniR1–datA (Morigen et al., 2001), which have about a fourfold excess of datA (Morigen et al., 2003). In this case, no anucleate cells appeared during the incubation at 42 °C (Fig. 1f, g). This indicates that the extra datA loci aid the division block in replication-arrested cells.

The change in the number of two-chromosome cells in the period of incubation at the non-permissive temperature was also investigated by flow cytometry in the three strains (Fig. 1h). Approximately 55% cells from each of the three strains (dnaC2, dnaC2ΔdatA and dnaC2 with MiniR1–datA) had two chromosomes after 1 h at 42 °C. The proportion decreased to 22% in control cells and 15% in ΔdatA cells after 4 h, but was still approximately 50% in the cells with excess datA. This demonstrates that the presence of extra datA inhibits cell division in the absence of chromosome replication. The fact that the number of two-chromosome cells does not reach zero during incubation at a non-permissive temperature in the control could be a result of incomplete replication in some of the cells. It has previously been shown that lack of active DnaC during elongation leads to a failure to complete replication in 18% of cells, because of the lack of loading of DnaC by the primosomal proteins (Maisnier-Patin et al., 2001).

To test whether the accumulation of anucleate cells was a result of an increase in the amount of available active (ATP-form) DnaA protein in the cells lacking datA, we investigated control cells (dnaC2) with an increasing concentration of DnaA by introducing the dnaA gene on a plasmid under control of an inducible promoter (Fig. 1i). We then observed that anucleate cells also accumulated when extra DnaA was produced (Fig. 1i). This result indicates that both deletion of datA and excess DnaA promotes cell division in the absence of DNA replication.

The cellular DnaA concentration was also measured in the dnaC2, dnaC2ΔdatA and dnaC2 with MiniR1–datA cells over a time-course of incubation at 42 °C. We found that the DnaA concentration decreased gradually during incubation at the non-permissive temperature and was about 50% compared to the zero time point in all three strains after 4 h (Fig. 1j). The decrease in DnaA concentration was not a result of DnaA degradation at 42 °C, as the concentration of DnaA was not decreased

### Table 1. Bacterial strains and plasmids

<table>
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<tr>
<th>Strain or plasmid</th>
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Fig. 1. Anucleate cells accumulate in ΔdatA cells in the absence of replication. Cells of W3110dnaC2 (a, b), W3110dnaC2ΔdatA (c, d) and W3110dnaC2MiniR1–datA (e, f) were exponentially grown in Glu–CAA medium at 30 °C. The cells at an OD 450 of 0.1 were shifted to a non-permissive temperature (42 °C), sampled at the time points indicated, fixed in 70% ethanol, stained in Hoechst 33258 and analysed by microscopy to visualize cells with or without nucleoid and determine the number of anucleate cells. Arrows indicate anucleate cells. The percentage of anucleate cells in the control cells (W3110dnaC2), ΔdatA cells and cells with MiniR1–datA was determined by microscopy (g). (h) The number of two-chromosome cells during incubation at 42 °C was measured by flow cytometry. (i) An exponentially growing culture (at 30 °C) of W3110dnaC2/pdnaA116 cells was treated with different concentrations of IPTG to achieve different amounts of the DnaA protein and shifted to 42 °C. The number of anucleate cells after 4 h at 42 °C was determined by flow cytometry and plotted as a function of DnaA concentration relative to the cells with 0 mM IPTG. The DnaA concentration was measured by immunoblotting using an anti-DnaA antibody. (j) The DnaA concentration in W3110dnaC2, W3110dnaC2ΔdatA and W3110dnaC2MiniR1–datA cells during the incubation at 42 °C was measured by immunoblotting using an anti-DnaA antibody. The values are relative to that at the zero time of the control and are the mean of three experiments. Squares represent the control (dnaC2), filled triangles represent ΔdatA and filled squares represent cells with MiniR1–datA.
when protein synthesis was inhibited by chloramphenicol at this temperature (data not shown). This result indicates that the expression of the DnaA protein does not keep up with bulk protein synthesis when chromosome replication is inhibited and that this might be part of a possible checkpoint mechanism to inhibit cell division when replication is blocked.

The *datA* site has recently been discovered to have (together with the IHF protein) DnaA-inactivating activity (Kasho & Katayama, 2013). In a wild-type scenario, the RIDA system is active. Thus, major changes in the level of ATP–DnaA after deletion of the *datA* site would not be expected (Katayama *et al.*, 2001). However, it has been shown that the RIDA system is dependent on ongoing replication (Kurokawa *et al.*, 1999). In the *dnaC2* cells, ongoing replication forks can finish but new initiations are blocked when the cells are shifted up to the non-permissive temperature. Thus, after a certain time-period (approximately 2 h; Withers & Bernander, 1998) there should be no active replication forks. This corresponds to the time point when the anucleate cells start to appear (Fig. 1g). It might therefore be that the DnaA-inactivating activity of the *datA* site becomes necessary for the *dnaC2* cells under these circumstances. Deletion of *datA* could lead to a higher level of active DnaA or a shift in the balance between the ATP-form and ADP-form of DnaA in these cells, which again could lead to cell division in the absence of replication.

Overexpressed DnaA is likely to be in the ATP-form, as the level of ATP in the cell is many times higher than the level of ADP (Bochner & Ames, 1982). Anucleate *dnaC2* cells were also found to accumulate at the non-permissive temperature after overproduction of DnaA, although to a lesser extent than in the *ΔdatA* mutant. This result supports the above assumption that an increased level of active DnaA, or a shift in a critical balance between ATP- and ADP-form DnaA in the cells, can trigger cell division in the absence of replication. The reason why fewer anucleate cells are observed after overproduction of DnaA than after deletion of *datA* is not known, but it could be that the overproduction of DnaA leads to a different balance between the ATP- and ADP-form DnaA compared to the *ΔdatA* cells, or that other mechanisms come into play after such a substantial overproduction (3–16-fold).

Altogether our results suggest that a net increased activity of DnaA, or an increase in the ratio of ATP- to ADP-form DnaA, is responsible for triggering cell division in replication-arrested cells.

**The inhibitory effect of *datA* is not dependent on the SOS response**

If replication is blocked due to DNA damage, induction of the SOS response leads to expression of the SfiA protein, which in turn inhibits cell division by interacting with FtsZ (D’Ari & Huisman, 1983; Huisman & D’Ari, 1981). It has been shown that a moderate SOS response is induced in *dnaC2* mutant cells at the non-permissive temperature (Løbner-Olesen *et al.*, 2008). To see whether the effect of *datA* on cell division was dependent on the SOS response, we transferred a *recA938* allele to the *dnaC2*, *dnaC2ΔdatA* and *dnaC2MiniR1ΔdatA* cells. The RecA protein is an obligatory factor for induction of the SOS response and the *dnaC2recA938* mutant was confirmed to be deficient in SOS induction by measurement of transcription from a *sfiA-lacZ* operon fusion (data not shown). To stop replication, exponentially growing cells were shifted from 30 °C to 42 °C and cell division (anucleate cells) was subsequently measured by flow cytometry (Fig. 2a). As shown in Fig. 2b, 6–8% cells were anucleate in all three mutants at the time of shift-up. This was not observed in cells with wild-type RecA (Fig. 1g) suggesting some DNA degradation in the *recA938* mutant, a phenotype that has also been shown previously (Capaldo & Barbour, 1975; Skarstad & Boye, 1993; Zahradka *et al.*, 2009). In agreement with previous findings (Skarstad & Boye, 1993), the anucleate cells that occurred due to DNA degradation in the *recA938* mutant were larger in size, and the frequency of these cells was about 5–9% of the whole population (data not shown). The frequency of anucleate cells in the control (*dnaC2recA938*) started to increase after about 2 h and reached about 23% after 4 h, while in the culture of *ΔdatA* cells 33% of the cells were anucleate after 4 h at 42 °C (Fig. 2b). In the presence of excess *datA*, the number of anucleate cells (8%) was not changed during incubation at the non-permissive temperature (Fig. 2). The results indicate that the *datA* locus is in fact an inhibitor of cell division and that the effect is independent of the SOS response.

**Deletion of *datA* partially suppresses the temperature sensitivity of *ftsI23* and *ftsZ84* mutants**

FtsZ is a key protein for cell division, forming a Z-ring at the division site (Bi & Lutkenhaus, 1991) and FtsA and FtsI are essential components of the division apparatus (Bi & Lutkenhaus, 1991; Margolin, 2000). To investigate whether the effect of *datA* on cell division was connected to any of the components of the division apparatus, we investigated whether deletion of *datA* could suppress the temperature sensitivity of four different *fts* (Ts) mutants; *ftsA12*, *ftsA27*, *ftsI23* and *ftsZ84* (Dai & Lutkenhaus, 1992). The *fts* mutants and their *ΔdatA* derivatives were grown on LB agar at 30 °C, 37 °C and 42 °C. None of these cell division deficient strains could grow at 42 °C. We found that deletion of *datA* led to growth of *ftsI23* cells at 37 °C (no growth at 42 °C) and growth of *ftsZ84* cells at 42 °C (fewer colonies relative to the number of colonies at 30 °C), but did not affect the temperature sensitivity of the *ftsA* mutants. The deletion of *datA* did not affect the growth rate of any of these strains (data not shown). These result show that deletion of *datA* partially
suppresses the growth defect of ftsZ84 and ftsI23 at a non-permissive temperature. This indicates that the activity of the compromised cell division proteins was increased enough at the lowest non-permissive temperature to allow division. These results support the above suggestion that loss of datA or the subsequent changes in the concentrations of ATP- or ADP-forms of the DnaA protein promote cell division in cells where division would otherwise not occur, due to either a block in the replication or defects in the cell division apparatus.

Since loss of datA could suppress the growth defect of the ftsZ mutant, we investigated whether there was a direct interaction between the DnaA protein and the FtsZ protein. We could not show any specific interaction between the two proteins, neither with cross-linking of the two proteins by disuccinimidyl suberate (DSS) nor ELISA (data not shown).

Another explanation for the effect of DnaA (or datA) on cell division could be that DnaA, being a transcription factor, affects transcription of cell division genes (Messer & Weigel, 1997). There are several putative DnaA boxes in the fisQAZ operons (Robinson et al., 1984), but DnaA control of fis expression has been controversial and different experimental setups have given different results (Garrido et al., 1993; Masters et al., 1989; Smith et al., 1996). To investigate whether deletion or the presence of extra copies of the datA site affected the transcription of genes involved in cell division, we compared gene expression in exponentially growing ΔdatA cells and cells with MiniR1-datA to that of wild-type cells by microarray analysis. However, expression measurements by microarray did not detect changes in transcription of cell division genes (data not shown). This indicates that the changes in expression are either too small to be detected in a microarray analysis, or that the effect of the DnaA protein on cell division does not involve changes in the expression of the proteins involved in cell division.

CONCLUSIONS

We have shown that when the level of active DnaA is increased, both by deletion of datA and by overproduction of DnaA, the frequency of production of anucleate cells is increased when DNA replication is inhibited. We found that inhibition of replication in itself led to a reduced DnaA concentration. This reduction in the concentration of DnaA could be a part of the checkpoint that operates upon inhibition of replication to ensure that no cell divides without having two fully replicated chromosomes.

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Fig. 2. The inhibitory effect of datA is not dependent on the SOS response. Cells (MOR259, MOR260 and MOR261) were grown exponentially in Glu–CAA medium at 30 °C, and treated, sampled and analysed as outlined in the Fig. 1 legend. (a) Dual parameter (FITC and Hoechst) histograms for each strain incubated at 42 °C for 4 h (left panel) are shown. The peak labelled ‘a’ at the leftmost corner represents anucleate cells. (b) The numbers of anucleate cells for each sample were found by quantifying peak ‘a’ in the flow cytometry histograms (right panel) and double-checked by microscopy. Squares represent the control (dnaC2recA938), filled triangles represent ΔdatA and filled squares represent cells with MiniR1–datA.


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