Absence of the BaeR protein leads to the early initiation of DNA replication in *Escherichia coli*

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ABSTRACT. *Escherichia coli* cells have dozens of two-component systems to sense and respond to various stimuli, and thereby cope with changing environments. BaeS/BaeR is one such two-component system, and it deals with a variety of envelope stresses. Interestingly, the ArcA/ArcB and TorS/TorR two-component systems are known to be associated with initiation of DNA replication; however, the effects of BaeS/BaeR on initiation of DNA replication remain unknown. Flow cytometry analysis revealed that the average number of replication origins (*oriC*s) per cell in Δ*baeR* mutants was approximately 30% higher than that in wild-type cells. So was the growth rate of Δ*baeR* cells. Ectopic expression of BaeR from the p*baeR* plasmid reversed the Δ*baeR* mutant phenotypes. The results indicate that absence of BaeR leads to the early initiation of DNA replication. Further, deletion of BaeR caused an increase in the amount of DnaA per cell, but did not change concentration of DnaA, which is the initiator protein.The average number of *oriC*s per cell in Δ*spy* mutants was the same as that found in the wild-type cells although *spy* gene expression is controlled by BaeR. These results suggest that BaeR may indirectly affect initiation of replication by controlling expression of the *dnaA* gene.

Key words: Response regulator BaeR; DNA replication; *Escherichia coli*

INTRODUCTION

Bacterial cells have dozens of two-component systems (TCS) that sense and respond to specific stimuli and subsequently allow the cells to cope with a variety of environmental conditions. A classical TCS includes a histidine kinase (HK) on the membrane and a response regulator in the cytoplasm. HK can be phosphorylated because of environmental stimuli, and the phosphate group can be transferred to its response regulator. The *Escherichia coli* envelope (inner membrane, periplasm, outer membrane, and bacterial appendages) is exposed to a variety of environmental stresses, including changes in temperature, pH, and osmolarity, toxic compounds, and oxidative stress (Leblanc et al., 2011). *E. coli* cells possess different kinds of stress signaling systems, one of which, BaeS/BaeR, senses and defends against divalent ion toxicity (Ruiz and Silhavy, 2005). BaeS/BaeR is a TCS consisting of a sensor HK, BaeS, and a response regulator (RR), BaeR (Raffa and Raivio, 2002). When BaeS senses changes in the concentration of divalent ions, a phosphotransfer from the conserved histidine residue of BaeS to the aspartate residue in the cognate response regulator BaeR occurs after a second round of autophosphorylation (Nagasawa, et al., 1993; Raffa and Raivio, 2002). Phosphorylated BaeR as a dimer actively binds to its target promoters and subsequently regulates expression of a number of genes (*mdtABCD*, *acrD*, *baeSR*, *spy*, *tolC*, *ygiABC*, *ynjABCD*, and *yeeN*) (Oshima et al., 2002; Nishino et al., 2005). These genes are associated with cell adaptation to envelope stress.

It has been shown that the CtrA, ArcB, PhoQ, and TorR RRs are associated with the initiation of DNA replication (Soncini and Groisman, 1996; Colloms et al., 1998; Jacobs et al., 2003; Yuan et al., 2015) . Here we show that the absence of BaeR leads to the early initiation of DNA replication.

MATERIAL AND METHODS

Bacterial strains and plasmids

The *E. coli* K-12 bacterial strains and the plasmids used in this study are listed in Table 1. The *baeR* gene was amplified by polymerase chain reaction (PCR), using the chromosomal DNA of strain BW25113 (Baba et al., 2006) as a template and the primers 5'-CGGGATCCGAGAGAAG TATGACCGAGTTAC-3' and 5'-CCCAAGCTTCTAAACGATGCGGCAGGCG-3'. The resultant fragment was inserted at the *Bam*HI and *Hin*dIII sites in pUHE21, where the expression of *baeR* is under the control of the *lac* promoter (Soncini et al., 1995), leading to the construction of the p*baeR* plasmid. The plasmid was introduced into competent cells by $CaCl_2$ transformation.

Table 1. Bacterial strains and plasmids.

Strains and plasmids	Relevant genotype	Source or reference
BW25113	Wild-type *rrnB3 ΔlacZ4787 hsdR514* Δ(*araBAD*)567Δ(*rhaBAD*)568 *rph-1*	(Baba et al., 2006)
MOR393	BW25113 *spy::kan*	(Baba et al., 2006)
MOR452	BW25113 *baeR::kan*	(Baba et al., 2006)
MOR1195	BW25113/p*baeR*	This study
MOR1196	BW25113/pUHE21-2*lac*q	This study
MOR1199	MOR452/p*baeR*	This study
MOR1209	MOR452/pUHE21-2*lac*q	This study
pUHE21-2*lac*q	rep$_{pMB1}$Ap^R*lacI*q	(Soncini et al., 1995)
p*baeR*	*baeR* gene on pUHE21-2*lac*q	This study

Growth media and conditions

Cells were exponentially grown in ABTGcasa medium (Morigen et al., 2005) at 37°C. Ampicillin (50 μg/mL), kanamycin (50 μg/mL), and chloramphenicol (30 μg/mL) were added when required for selection. To induce the expression of BaeR from the p*baeR* plasmid at 37°C, 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the exponentially growing cells in ABTGcasa medium.

Flow cytometry

Exponentially growing cells in ABTGcasa medium at 37°C were treated with 300 μg/mL rifampicin and 10 μg/mL cephalexin for 3-4 generations. Rifampicin prevents the initiation of replication by inhibiting transcription, which is required for the initiation of replication but allows the completion of ongoing replication. Cephalexin blocks cell division at the time of addition of the drug (Skarstad et al.,1986; Boye and Løbner-Olesen, 1991). The rifampicin- and cephalexin-treated cells were fixed in 70% ethanol. The cells were stained with Hoechst 33258 for 30 min after one wash in Tris-HCl buffer (pH 7.5), and subsequently analyzed using an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA). Preparation of standard samples and post analysis were as described previously (Morigen et al., 2003).

Determination of total protein per cell

Exponentially growing cells in ABTGcasa at 37 °C were collected on ice. 9 mL of the cell culture were harvested by centrifugation at 4 °C, washed in 1 mL of TE buffer, and resuspended in 200 μL TE buffer containing 1% SDS and glycerol and then boiled for 5 min. Total protein amount in a fixed volume of the cell extract mentioned above was determined by a colorimetric assay (BCA kit, pierce) as described previously (Morigen et al., 2003). The number of cells in a certain volume of the cell culture and the protein amount per cell was dermined as described previously (Liu et al., 2014).

Western blotting

Determination of the DnaA concentration in the cell extract mentioned above as mentioned previously (Morigen et al., 2001).

RESULTS

Deletion of the *baeR* gene leads to the early initiation of DNA replication

To investigate whether the BaeR response regulator from BaeS/BaeR also affects the initiation of DNA replication, we analyzed the replication patterns of the Δ*baeR* mutant and wild-type cells by flow cytometry. In the wild-type cells 2-, 4- and a small proportion of 8-origin cells were detected, whereas only 4- and 8-origin cells were found in the Δ*baeR* mutant (Figure 1). The average number of origins for replication (*oriC*s) per cell was 4.4 for the wild-type cells and 5.7 for the Δ*baeR* mutant (Figure 1, Table 2), an increase of 30% in the Δ*baeR* mutant relative to the wild-type cells. Concomitantly, the growth rate increased slightly (the doubling time was 34 min for the wild-type cells and 31 min for the mutant) (Table 2). The results indicate that the absence of BaeR leads to the early initiation of DNA replication relative to that in the wild-type cells.
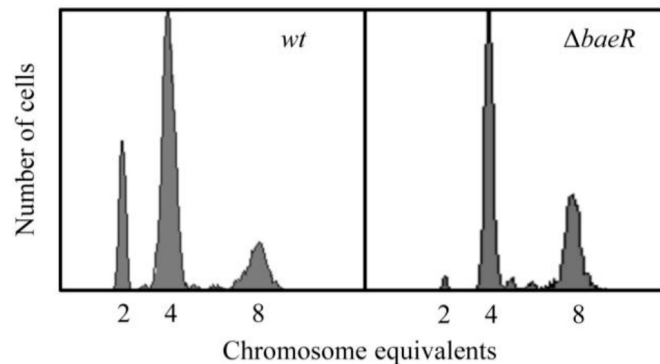
Figure 1. Deletion of the *baeR* gene results in the early initiation of replication. Exponentially growing cells in ABTGcasa medium at 37°C were treated with rifampicin and cephalexin for 3-4 generations. Subsequently, cells were fixed in 70% ethanol and analyzed by flow cytometry, as described in the Material and Methods section. The number of fully replicated chromosomes per cell represents the number of origins present at the time of drug addition. For each analysis, 10,000 cells were included.
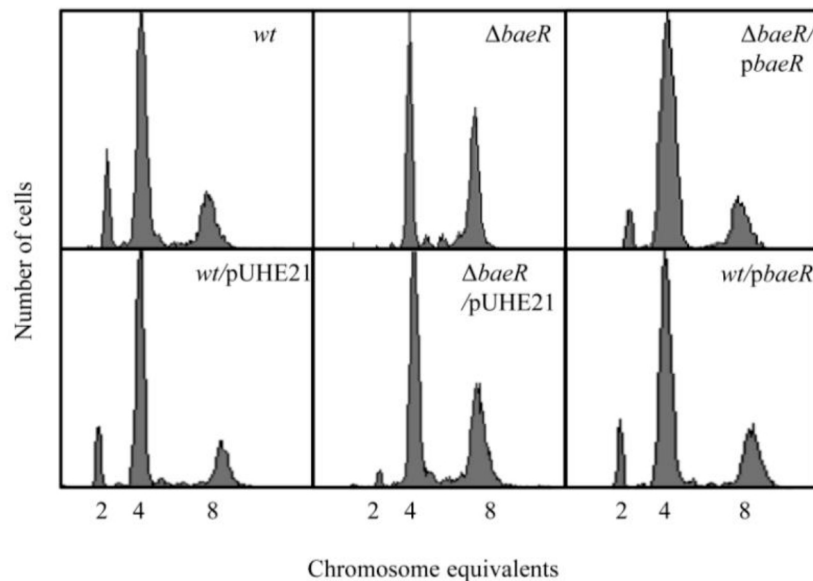
Table 2. Absence of BaeR leads to an increase in the number of origins per cell.

Strains	Genotype	A.O.	Doubling time (min)
BW25113	Wild-type	4.4 ± 0.1	34 ± 0.6
MOR393	Δ*spy*	4.3 ± 0.1	34 ± 0.4
MOR452	Δ*baeR*	5.7 ± 0.7	31 ± 1.2
MOR1195	BW25113/p*baeR*	4.1 ± 0.2	38 ± 0.6
MOR1196	BW25113/pUHE21-2*lac*q	4.3 ± 0.1	35 ± 0.2
MOR1199	Δ*baeR*/p*baeR*	4.2 ± 0.1	37 ± 0.6
MOR1209	Δ*baeR*/pUHE21-2*lac*q	5.4 ± 0.0	32 ± 0.1

Exponentially growing cells in ABTGcasa medium were treated with rifampicin and cephalexin, fixed in 70% ethanol, and then analyzed by flow cytometry, as described in the Material and Methods section. The average number of origins per cell (A.O.) was calculated using software provided by BD Biosciences. Each experiment was repeated three times and standard errors are given.

Ectopically expressed BaeR reverses the replication pattern of the Δ*baeR* mutant

To ascertain whether ectopically expressed BaeR can reverse the replication pattern of the Δ*baeR* mutant, we constructed the p*baeR* plasmid, which expresses BaeR under the control of the *lac* promoter. The wild-type and Δ*baeR* mutant cells were transformed with the plasmid p*baeR* or the control vector. Exponentially growing cells containing p*baeR* or the control vector in ABTGcasa medium with IPTG to induce BaeR expression, were treated with rifampicin and cephalexin, stained with Hoechst, and analyzed by flow cytometry, as mentioned above. The Δ*baeR* mutant expression of BaeR from p*baeR* produced cells with 2, 4, or 8 origins and an extended doubling time (37 min compared with 31 min for cells with the control vector) (Figure 2, Table 2). The average number of origins per cell decreased from 5.7 in the Δ*baeR* cells carrying the control vector to 4.2 in the Δ*baeR* cells containing p*baeR* (Figure 2, Table 2). When the BaeR protein was ectopically overexpressed in the wild-type cells, the DNA replication pattern was similar to that in the wild-type cells without excess BaeR, containing 2, 4, and 8 origins (Figure 2), and the average number of origins per cell decreased slightly (Table 2). The results indicate that ectopically expressed BaeR reverses the Δ*baeR* mutant phenotype.

**Figure 2.** Ectopically expressed BaeR reverses the ∆*baeR* mutant phenotype. Exponentially growing cells in ABTGcasa medium containing isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.25 mM) to induce expression of BaeR from the p*baeR* plasmid at 37°C were treated with rifampicin and cephalexin for 3-4 generations and then analyzed by flow cytometry as described in the Material and Methods section.

BaeR affects the amount of DnaA per cell

The DnaA protein is the initiatior for DNA replication (Braun et al., 1985; Løbner-Olesen et al., 1989). Therefore, the DnaA protein amount and/or concentration have been known as a limiting factor for initiation of replication. It is likely that BaeR affects initiation of replication by altering the amount or concentration of DnaA. In order to detect this posibility, we measured the amount of DnaA protein per cell. Wide type and ∆*baeR* cells with or without overexpressed BaeR were exponentially grown in ABTGcasa medium at 37°C and the total amount of protein per cell was measured as described in Material and Methods. Also the DnaA concentration in these cell extracts were measured by Western blotting. The amount of DnaA protein per cell in ∆*baeR* cells was increased relative to that in wide-type (Figure 3A). In addition, we found that the total amount of protein per cell in ∆*baeR* cells was also observed to be increased compared with that in wide-type (Figure 3B). The results indicate that BaeR affects the initiation by changing the amount of DnaA per cell.

Absence of the *spy* gene does not affect initiation of DNA replication

The BaeR protein regulates expression levels of a number of genes including the *spy* gene. BaeR may indirectly affect the initiation of replication by controlling the expression of its target genes. To test this possibility, we analyzed the replication pattern of the ∆*spy* mutant cells, as described above, since expression of the *spy* gene is controlled by BaeR. Flow cytometry analysis showed that the ∆*spy* mutant cells had the wild-type DNA replication pattern with a doubling time of 34 min (Figure 4, Table 2). The results suggest that BaeR does not affect initiation of DNA replication by regulating expression of the *spy* gene.
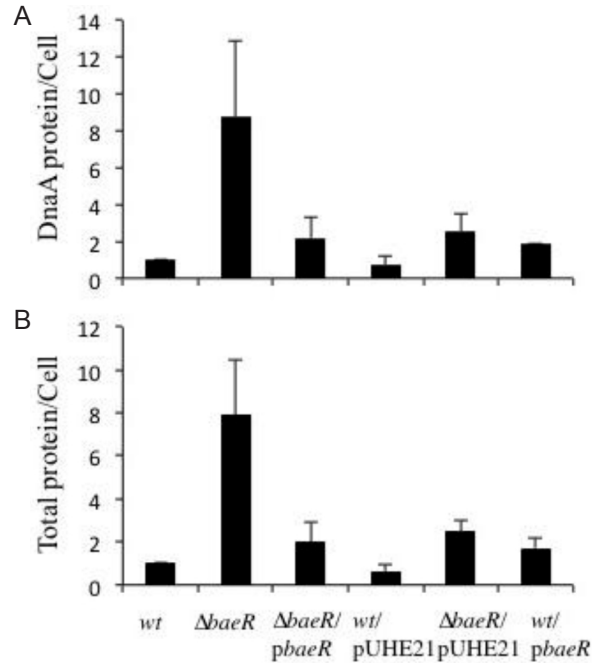
Figure 3. A. BaeR affects the amount of DnaA per cell. **A.** The amount of DnaA protein per cell is increased in Δ*baeR* cells. Exponentially growing cells in ABTGcasa at 37°C were harvested by centrifugation at 4°C. **B.** Total protein amount in a fixed volume of cell extract was determined by a colorimetric assay (BCA kit) as described previously (Liu et al., 2014). The DnaA concentration was determined by immunobloting. The DnaA protein amount per cell was then estimated through counting the number of cells used in the measurement.
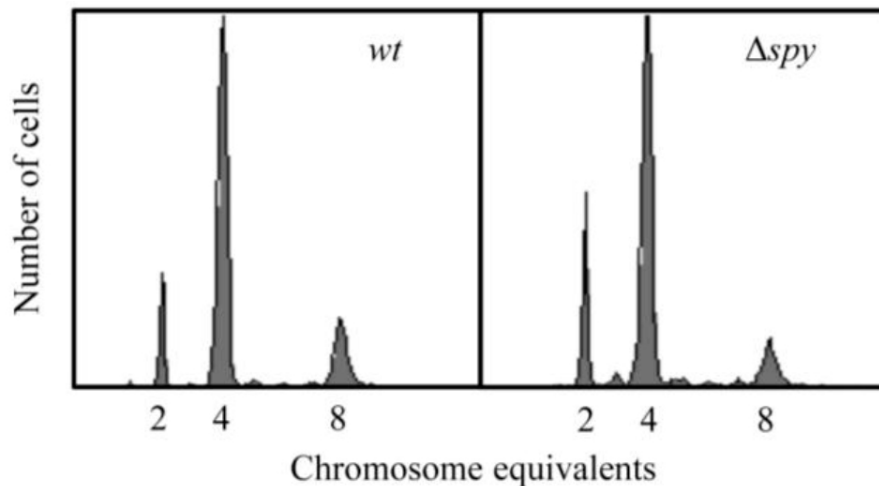
Figure 4. DNA replication pattern is not changed in the Δ*spy* mutant. Exponentially growing cells in ABTGcasa medium at 37°C were treated with rifampicin and cephalexin for 3-4 generations and then analyzed by flow cytometry as described in the Material and Methods section.

DISCUSSION

In this study, we found that the absence of the BaeR protein led to the early initiation of DNA replication. Furthermore, ectopically expressed BaeR restores the wild-type replication pattern in the ΔbaeR mutant cells. BaeR affects the initiation of DNA replication either directly or indirectly. It is likely that BaeR may indirectly affect the initiation of replication by regulating expression of its target genes such as the *spy* gene (Oshima et al., 2002; Nishino et al., 2005). However, deletion of the *spy* gene results in a wild-type replication pattern , suggesting that the Spy protein is not associated with initiation of replication. It could also be possible that the products from *mdtABCD*, *acrD*, *baeS*, *tolC*, *ygiABC*, *ynjABCD*, and *yeeN* may affect the initiation of replication since the expression of these genes is controlled by BaeR (Oshima et al., 2002; Nishino et al., 2005). Alternatively, the expression of the initiator protein DnaA could increase in the absence of BaeR. Indeed, the amount of DnaA protein was increased in ΔbaeR cells.

Moreover, bacterial cells have dozens of TCS; the *E. coli* ArcA/ArcB and TorS/TorR TCS are associated with the initiation of DNA replication (Soncini and Groisman, 1996; Colloms et al., 1998; Jacobs et al., 2003; Yuan et al., 2015), although their underlying mechanisms remain elusive.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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