Absence of RstA Results in Delayed Initiation of DNA Replication in *Escherichia coli*

Yuan Yao 1,2, Cuilan Lv 1 and Runxiu Zhu 1,*

1 Department of Neurology of Inner Mongolia People’s Hospital, Hohhot 010017, China; yuanyao129@imu.edu.cn
2 School of Life Sciences, Inner Mongolia University, Hohhot 010010, China
* Correspondence: zhjyq129@163.com; Tel.: +86-18047191885; Fax: +86-471-3286206

**Abstract:** Bacterial two-component systems are responsible for both sensing and initiating a response to a wide range of environmental changes. RstB/RstA is an uncharacterized *Escherichia coli* two-component system, the regulatory effects of which on the *E. coli* cell cycle remain unclear. We found that while the doubling time and average number of replication origins per cell in an ΔrstB mutant were the same as the wild-type, the average number of replication origins in an ΔrstA mutant was 18.2% lower than in wild-type cells. The doubling times were 34 min, 35 min, and 40 min for the wild-type, ΔrstB, and ΔrstA strains, respectively. Ectopic expression of RstA from plasmid pACYC-rstA partly reversed the ΔrstA mutant phenotypes. The amount of initiator protein DnaA per cell was reduced by 40% in the ΔrstA mutant compared with the wild-type, but the concentration of DnaA did not change as the total amount of cellular protein was also reduced in these cells. Our results suggest that deletion of RstA leads to delayed initiation of DNA replication, and that RstA may indirectly affect initiation of replication by controlling expression of *dnaA*.

**Keywords:** Response regulator; RstA; two-component system; DNA replication; *Escherichia coli*

**Introduction**

Two-component systems are major signal transduction mechanisms in bacteria that are used to sense and respond to a huge variety of environmental stimuli. The two components that characterize these systems consist of a histidine kinase and a response regulator. The two-component system autophosphorylates at the histidine kinase, generating a high-energy phosphoryl group that is subsequently transferred to an aspartate residue in the response regulator.

RstB/RstA composes a two-component system in bacteria in which the histidine kinase, RstB, senses an extracellular signal. RstB autophosphorylates at a conserved
histidine residue in the transmitter domain, and then transphosphorylates an invariant aspartate residue located in the receiver domain of RstA. Phosphorylated RstA can activate or repress target genes, thereby initiating a response to the extracellular stimulus. Previous studies have shown that response regulators TorR and BaeR are associated with the initiation of DNA replication (Yao et al. 2015; Yuan et al. 2015). However, until now, it has not been known how the RstB/RstA system affects DNA replication. In the current study, we determined that the absence of RstA leads to delayed initiation of DNA replication.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The *E. coli* K-12 bacterial strains and the plasmids used in this study are listed in Table 1. The *rstA* gene, along with its native promoter, was amplified by polymerase chain reaction from the genomic DNA of *E. coli* strain BW25113 (Baba et al. 2006) using primers 5ʹ-ccctcgagccagttgctttgtcaccggac-3ʹ and 5ʹ-ccaagcttcgcttattcccatgcatgagg-3ʹ. The resultant fragment was inserted at the *Xho*I and *Hind*III sites of pACYC177 (Chang and Cohen 1978), generating plasmid pACYC-*rstA*, within which the expression of *rstA* was under the control of its native promoter. pACYC-*rstA* was introduced into competent cells by CaCl₂ transformation.

*E. coli* cultures were grown to an optical density at 450 nm (OD₄₅₀) of 0.15 in ABTGcasa medium (Morigen et al. 2005) at 37°C. Ampicillin (50 μg/mL) and kanamycin (50 μg/mL) were added when required for selection.

Flow cytometry

Cells were grown to OD₄₅₀=0.15 in ABTGcasa medium supplemented with 300 μg/mL rifampicin and 10 μg/mL cephalexin at 37°C for three to four generations. Rifampicin inhibits 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 (Boye and Løbner-Olesen 1991; Skarstad et al. 1986). Cells treated with rifampicin and cephalexin were fixed in 70% ethanol. Following one wash in Tris-HCl buffer (pH 7.5), cells were stained with Hoechst 33258 for 30 min, and then analyzed by flow cytometer (BD Biosciences, San Jose, CA, USA). A total of 10,000 cells were included for each analysis. Preparation of standard samples and analysis methods were as described previously (Morigen et al. 2003).

Determination of total protein per cell

Cells were grown to OD₄₅₀=0.3 in ABTGcasa medium at 37°C and then placed on ice. A 9-mL aliquot of the cell culture was harvested by centrifugation at 12000 rpm for 8 minutes at 4°C, washed in 1 mL of Tris-EDTA buffer, resuspended in 200 μL of Tris-EDTA buffer containing 1% SDS and glycerol, and then boiled for 5 min.
The total amount of protein in the fixed volume of cell extract (9 mL) was determined using a colorimetric assay (BCA kit, Pierce Chemical, Rockford, IL, USA) as described previously (Morigen et al. 2003). The number of cells in the initial volume of culture and the protein amount per cell was determined as described previously (Liu et al. 2014).

Western blotting

The DnaA concentration in the cell extract was determined as previously described (Morigen et al. 2001).

Results and Discussion

Deletion of rstA results in delayed initiation of DNA replication

To investigate whether the RstB/RstA two-component system affects the initiation of DNA replication, we analyzed the replication patterns of wild-type, ΔrstB, and ΔrstA mutant E. coli cells by flow cytometry. Differences in cell cycle parameters were also compared between the ΔrstB and ΔrstA mutants and wild-type cells (Fig. 1, Table 2). Fewer cells in the B period and more cells in the D period were observed for the ΔrstB mutant compared with the wild-type. In the ΔrstA mutant, 24% of cells were in the B period, 75% were in the C period, and 1% were in the D period, whereas the wild-type had 20% of cells in the B period, 58% in the C period, and 22% in the D period. In addition, both the wild-type and ΔrstB mutant cultures contained 2-, 4-, and a small proportion of 8-origin of replication cells, whereas only 2- and 4-origin cells were found in the ΔrstA mutant cultures (Fig. 1). The average number of origins of replication (oriC) per cell was 4.4 for the wild-type cells, 4.3 for the ΔrstB mutant, and 3.6 for the ΔrstA mutant (Table 2). Concomitantly, the growth rate of the ΔrstA mutant also decreased slightly, with doubling times of 34 min, 35 min, and 40 min recorded for the wild-type, ΔrstB, and ΔrstA strains, respectively (Table 2). These results indicate that the absence of RstA leads to delayed initiation of DNA replication relative to wild-type cells.

Ectopically-expressed RstA partly reverses the delayed replication of the ΔrstA mutant

To determine whether ectopically-expressed RstA could reverse the delayed replication in the ΔrstA mutant, the pACYC-rstA plasmid was constructed to express RstA under the control of its native promotor. Over-expression of RstA in the ΔrstA mutant strain resulted in fewer cells in the C period (67% of cells) and more cells in the B (28%) and D (5%) periods compared with the ΔrstA strain (Table 2). In addition, the average number of origins of replication per cell increased from 3.6 in the ΔrstA mutant to 4.0 in the ΔrstA mutant containing pACYC-rstA, and the doubling time
decreased from 40 min for the mutant to 37 min for ΔrstA::pACYC-rstA cells (Fig. 2, Table 2). When the RstA protein was ectopically over-expressed in the wild-type, fewer cells were observed in the B period and more cells were in the D period compared with the wild-type (Fig. 2), and the average number of origins of replication per cell increased from 4.4 in the wild-type to 5.0 in the wt::pACYC-rstA strain. Further, the doubling time of the over-expression strain decreased to 32 min (Fig. 2, Table 2). Expression of the control plasmid in wild-type or ΔrstA cells did not affect replication. These results indicate that ectopically-expressed RstA could partially reverse the ΔrstA mutant phenotype. However, this in trans complementation would likely lead to other phenotypes, including bacterial filamentation and defects in septation.

**RstA affects the amount of DnaA per cell**

DnaA is the initiator of DNA replication. Therefore, the amount and/or concentration of DnaA is a limiting factor for the initiation of replication. It is likely that RstA affects initiation of replication by altering the amount or concentration of DnaA. To investigate this, we measured the amount of DnaA per cell in the wild-type and ΔrstA strains, both with and without pACYC-rstA. In addition, the DnaA concentration in these cell extracts was measured by western blotting. The amount of DnaA per cell in the ΔrstA strain was reduced by 40% relative to the wild-type (Fig. 3A). In addition, we found that the total amount of protein per cell in the ΔrstA strain also decreased to 71% of that of the wild-type (Fig. 3B). These results indicated that the deletion of RstA delayed the initiation of replication by decreasing the total amount of protein, including DnaA, in the cell. RstA may indirectly affect the initiation of replication by controlling the expression of dnaA. In agreement with these findings, the expression of dnaA was decreased by 25% in ΔrstBΔrstA E. coli cells relative to the wild-type (Oshima et al. 2002).

In conclusion, the deletion of rstA delayed the initiation of replication by decreasing the amount of DnaA and the total cellular protein levels in *E. coli*.

**Competing interests**

Authors declare no competing interests in regards to this project.

**Author contribution statement**

Y.Y. and R.Z., designed research, Y.Y., performed research, C. L., and Y.Y., analyzed data, Y.Y., prepared the figures, and Y.Y., wrote the manuscript. All authors reviewed the manuscript.

**Acknowledgments**

The work was supported by grants from the Natural Science Foundation of Inner Mongolia (Grant No. 2014MS0803 to Runxiu Zhu) and the Natural Science Foundation of Inner Mongolia People’s Hospital (Grant No. 2016105 to Yuan Yao).
References


Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW25113</td>
<td>Wild type rrnB3 ΔlacZ4787 lsdR514 Δ(araBAD)567Δ(rhaBAD)568 rph-1</td>
<td>(Baba T et al.2006)</td>
</tr>
<tr>
<td>MOR309</td>
<td>BW25113 rstA::kan</td>
<td>(Baba T et al.2006)</td>
</tr>
<tr>
<td>MOR457</td>
<td>BW25113 rstB::kan</td>
<td>(Baba T et al.2006)</td>
</tr>
<tr>
<td>YY6</td>
<td>MOR309/pACYC-rstA</td>
<td>This work</td>
</tr>
<tr>
<td>YY7</td>
<td>BW25113/pACYC-rstA</td>
<td>This work</td>
</tr>
<tr>
<td>YY8</td>
<td>MOR309/pACYC177</td>
<td>This work</td>
</tr>
<tr>
<td>YY10</td>
<td>BW25113/pACYC177</td>
<td>This work</td>
</tr>
<tr>
<td>pACYC177</td>
<td>repD58sApR (bla) KmR</td>
<td>(Chang and Cohen 1978)</td>
</tr>
<tr>
<td>pACYC-rstA</td>
<td>rstA gene on pACYC177</td>
<td>This work</td>
</tr>
</tbody>
</table>

Table 2. Absence of RstA leads to a decrease in the number of origins per cell.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell cycle distribution (%)</th>
<th>A.O.</th>
<th>Doubling time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B-period</td>
<td>C-period</td>
<td>D-period</td>
</tr>
<tr>
<td>BW25113</td>
<td>20±1.5</td>
<td>58±1.5</td>
<td>22±1.0</td>
</tr>
<tr>
<td>MOR457</td>
<td>11±1.0</td>
<td>60±1.5</td>
<td>29±1.5</td>
</tr>
<tr>
<td>MOR309</td>
<td>24±1.0</td>
<td>75±1.0</td>
<td>1±0.5</td>
</tr>
<tr>
<td>YY6</td>
<td>28±1.5</td>
<td>67±1.5</td>
<td>5±0.5</td>
</tr>
<tr>
<td>YY7</td>
<td>2±1.0</td>
<td>58±1.5</td>
<td>40±1.0</td>
</tr>
<tr>
<td>YY8</td>
<td>29±1.5</td>
<td>70±1.0</td>
<td>1±0.5</td>
</tr>
<tr>
<td>YY10</td>
<td>11±1.0</td>
<td>60±1.0</td>
<td>29±1.5</td>
</tr>
<tr>
<td>MOR309(acidic condition)</td>
<td>41±1.0</td>
<td>58±1.0</td>
<td>1±0.5</td>
</tr>
<tr>
<td>YY6 (acidic condition)</td>
<td>18±1.5</td>
<td>80±1.0</td>
<td>2±1.0</td>
</tr>
</tbody>
</table>

Exponentially growing cells in ABTGcasa medium were treated with rifampicin and cephalaxin, 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.) and the number of cells in B-, C- and D-period were calculated using software provided by BD Biosciences. Each experiment was repeated three times and standard errors are given.
Figure legends

**Fig. 1** Deletion of *rstA* results in delayed initiation of replication. Cultures were grown to OD_{450}=0.15 in ABT.Gcasa medium at 37°C and treated with rifampicin and cephalexin for three to four generations. Subsequently, cells were fixed in 70% ethanol and analyzed by flow cytometry. The number of fully replicated chromosomes per cell represents the number of origins of replication present at the time of antibiotic addition. A total of 10,000 cells were used for each analysis.

**Fig. 2** Ectopically-expressed RstA partially reverses the ΔrstA mutant phenotype. Cultures were grown to OD_{450}=0.15 in ABT.Gcasa medium at 37°C to express RstA from the pACYC-rstA plasmid. Cells were treated with rifampicin and cephalexin, and then fixed using ethanol after three to four generations. The chromosome number per cell was measured by flow cytometry.
Fig. 3 RstA affects the amount of DnaA per cell. **A** The amount of DnaA per cell was increased in the ΔrstA mutant. Cultures were grown to OD$_{450} = 0.3$ in ABTGcasa at 37°C, and then harvested by centrifugation at 4°C. **B** The total amount of protein in a fixed volume of cell extract was determined by a colorimetric assay (BCA kit). The DnaA concentration was determined by immunoblotting. The amount of DnaA per cell was then estimated by counting the number of cells used in the measurement.

© 2016 by the authors; licensee *Preprints*, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license (http://creativecommons.org/licenses/by/4.0/).