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The QseB/QseC Signaling Affects Initiation of Chromosomal Replication through Regulating Expression of the DnaA Protein in *E.coli*

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Abstract

The *Escherichia coli* two-component system, QseB/QseC signaling regulates expressions of more than 50 genes encoding flagellar proteins and proteins associated with virulence. In this work, we found that absence of the *qseB* gene led to an early initiation of chromosomal replication in a manner of medium-independent, the early initiation was shown to be reversed by ectopic expression of QseB from a plasmid p*qseB*. Further, absence of the *qseB* gene resulted in an increase of DnaA concentration and the purified QseB protein did not interact with the *oriC* DNA *in vitro*. The early initiation was also found in the *ΔqseC* mutant cells. These results indicate that absence of the QseB/QseC signaling increases concentration of DnaA and the later subsequently triggers the early initiation of replication. Flourescene microscope analysis showed that the QseC protein localized at cell membrane while QseB was in cytosol.

Keywords: QseB/QseC signaling, QseB protein, initiation of DNA replication, DnaA protein, *E. coli*.

1. Introduction

Quorum sensing is via the production of compounds called as autoinducers that involved in pathogenesis and nonpathogenic, including flagellation, motility, shiga toxin production, cell growth (Sperandio et al., 2001; Liu et al., 1995) and two-component signaling system (Sharma et al., 2010; Kostakioti et al., 2009: Sperandio et al., 2004, 2003); as well as genes involved in bacterial metabolism, nucleotide and protein biosynthesis, DNA repair, cell growth division and DNA replication (Matthew et al., 2006; Helen et al., 1998) in *E.coli*.

The QseB/QseC (Quorum sensing *E. coli* regulators B and C) signaling is responsible for cell-to-cell communication, quorum sensing, controlling bacterial behavior by various signal molecules. QseC is a transmembrane protein that interacts through its periplasmic sensory domain with AI-3 and the stress hormones epinephrine and norepinephrine to undergo autophosphorylation at its cytoplasmic domain (Hughes et al., 2009; Marcie et al., 2005). The phosphorylated QseC transfers the phosphate to its cognate response regulator QseB, which activates transcription of FlhDC, the master transcriptional regulator of the flagellar gene complex, and autoregulates its own

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transcription by binding to the *flhDC* and QseBC promoters, respectively (Clarke et al., 2005; Kendall et al., 2007).

Few proteins participated in the initiation of chromosomal replication process (Zyskind and Smith, 1986) including DnaA (initiator), DnaB (helicase), DnaC (laoder) and DnaG (primase). In the first event of initiation, several DnaA - ATP molecules bind to specific 9 bp sites in *oriC* (Funnell et al., 1987; Fuller et al., 1984). An architectural protein IHF binds *oriC* and bends the DNA to help the interaction between the DnaA proteins and the AT rich region (Messer, 2002; Hwang and Kornberg, 1992), and DNA strands is separated at the AT rich region. (Messer, 2002; Hwang and Kornberg, 1992), and DNA strands is separated at the AT rich region. The DnaB/DnaC complex interacts with an open complex, DnaC loader loads a DnaB helicase onto each of the single strands generated by the melting process (Funnel et al., 1987). DnaC leaves the complex, the DnaB helicase lengthenes the single stranded region and then loads DnaG primase, which synthesizes RNA primer. Finally, the DNA polymerase III holoenzyme is the single stranded DNA binding (SSB) protein and DNA gyrase are loaded (Morigen et al., 2005; 2003).

We investigated the influence of the QseB/QseC signaling on initiation of DNA replication, and its localization on cell.

2. Materials and Methods

Bacterial Strains and Plasmids

All bacterial strains used were K12 listed in Table 1. PCR fragment of the aspC gene with its native promoter region using a pair of primers of 5′ GCGGATCCCCAGTCTTTATCGACTTCACCC 3′ and 5 ′CGAAGCTTGATTAGCGTCAGCCTGACGCGCAG 3′ was inserted into plasmid pACYC177 at BamHI and HindIII sites, resulting in a plasmid over-expresssing QseB, namely pACYC177- *qseB*. The plasmid was introduced into competent cells by eletroporation. Growth Media and Condition Cells were grown in LB or ABTGcasa medium (Morigen et al., 2005) at 37 °C. 50 mg/ml of kanamycin, 30 mg/ml of chloramphenicol and 50 mg/ml of ampicillin were added when required for selection.

Table 1. Strains and plasmids used in this study

Strains	Relevant genotype	Reference
BW25113	Wild type	Baba *et al* (2006)
MOR318	*BW25113/qseB::Kan^R*	Yixin.Shi (2011)
B1	*BW25113 /pACYC177-qseB*	This work
B2	*MOR318 /pACYC177-qseB*	This work
MOR578	*BW25113 /qseC::Kan^R*	Yixin.Shi (2013)
MOR541	*DH5a GFP-qseC*	Yixin.Shi (2012)
B3	*MOR578 / GFP qseC*	This work
B4	*MOR318 / GFP qseC*	This work
MOR611	*DH5a GFP-qseB*	Yixin.Shi (2013)
B5	*MOR318 / GFP qseB*	This work
B6	*MOR578 / GFP qseB*	This work
Plasmids		
pACYC177	repp15AApR (bla) KmR	Chang (1978)
pMD™19	Cloning vector	Takara
pET-28a	*repColE1 neo lacI PT7*	EMD Biosciences
p*qseB*	*qseB* gene on pACYC177	this work
pCA24N	rep pMB1CmRlacIqppT5-lacthisGFP	Kitagawa (2006)
pQseB-GFP	*qseB* gene on pCA24N (pCA24N derivative)	Kitagawa (2006)
pQseC-GFP	*qseC* gene on pCA24N (pCA24N derivative)	Kitagawa (2006)

Flow Cytometry Analysis

Exponentially growing cells in LB, ABTGcasa medium were collected at OD600/450= 0.15, and then treated with 300 mg/ mL of rifampicin and 10 mg/mL of cephalexin for 3–5 generations. Rifampicin inhibits initiation of replication through preventing transcription which is required for replication initiation but allows ongoing rounds of replication to finish, whereas cephalexin blocks cell division (Boye et al., 1999; 1986). After treatment with these drugs, cells end up with an integral number of chromosomes, representing the number of origins per cell at the time of drug addition (Skarstad et al., 1986). Cells treated with rifampicin and cephalexin were fixed in 70 % ethanol. Slowly growing cells in medium of ABTGcasa were collected by centrifugation at OD450= 0.15 and fixed in 70 % ethanol. Following one wash in Tris-HCl buffer (pH7.5), cells were stained in Hoechst33258 for 30 min, and analyzed by flow cytometer (BD LSRFortessa). Preparation of standard sample and post analysis were as described previously (Morigen et al., 2005; Wold et al., 1994).

Determination of Total Protein per Cell

Exponentially growing cells in ABTGcasa at 37 ^{o}C were collected on ice. 9 mL of the cell culture were harvested by centrifugation at 4 ^{o}C, washed in 1 ml of TE buffer, and resuspended in 200 ml 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). To measure the number of cells in a certain volume of the cell culture, 10 mL of the cells mentioned above were diluted 104 and 105 times and then plated on LB agar plates with required antibiotics. After incubation at 37 ^{o}C overnight, the number of the colonies were counted. Using the amount of protein in a certain volume of cell extract from 9 ml of the culture and the number of cells in 10 mL of the culture, the protein amount per cell was calculated.

Western Blotting

The cell extract mentioned above was also used to determine the DnaA concentration by Western blotting. Fixed amounts of cell extracts were subjected to SDS-PAGE (12 %). The protein was transferred to a polywinylidene difluoride (PVDF) membrane by semi-dry blotting. The membrane was probed with anti-rabbit antibody for DnaA and secondary antibody which was also antirabbit IgG (TransGen Biotec) as described previously (Morigen et al., 2001).

3. Results

1. The QseB protein does not directly interacts with oriC region

Our previously study resulted shown that the absence of QseB/QseC signaling leads to early initiation of DNA replication but mechanism behind is elusive (Fig. 1) (Baigalmaa, Morigen, 2016). It could be possible the QseB protein may interact with *oriC* region to assist initiation of replication at *oriC*.

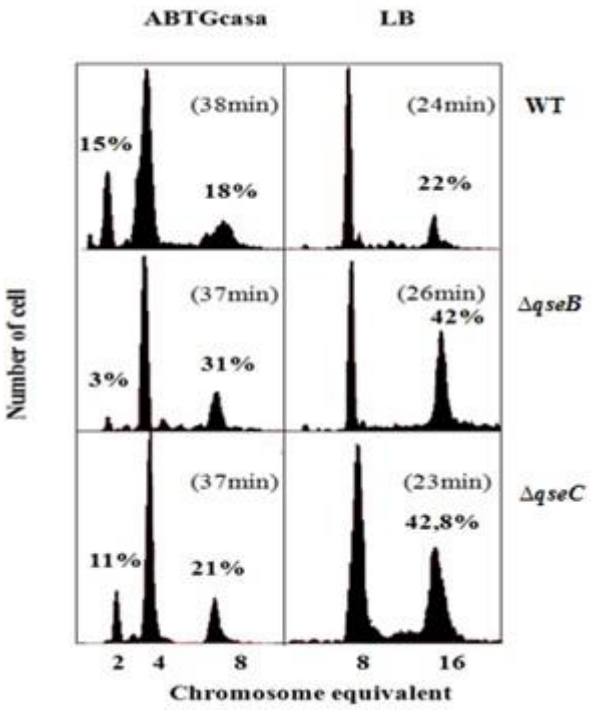

Fig. 1. Absence of QseB/QseC signaling results in early initiation of replication. Exponentially growing cells in media indicated were treated with rifampicin and cephalexin for 3-5 generations, and fixed in 70 % ethanol. Cells were analyzed by Flow cytometer after staining in Hoechst 33258 for 30 min. Y-axis represents number of cells measured while X-axis indicates chromosome equivalents per cell. 10000 cells were included in each measurement, other information are indicated

To test the possibility, we purified the QseB-His protein. For purification, the *E. coli* strain containing pET28a-*qseB* was grown at 37 °C in LB to an OD_{600} of 0.6, at which point arabinose was added to a final concentration of 0.2 % and allowed to induce for three hours. Protein purification was then performed using nickel columns according to manufacturer's instructions (Qiagen). The purifed protein was checked on an SDS-PAGE gel (Fig. 2).

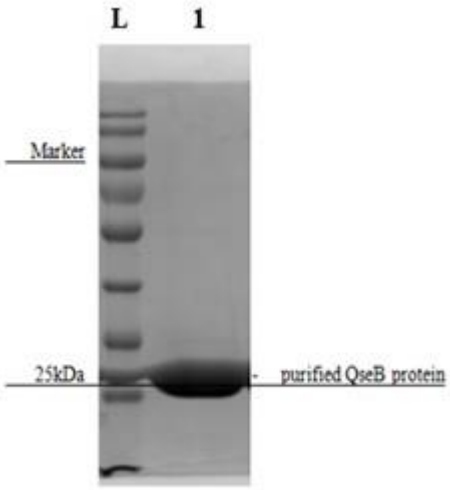

Fig. 2. Purification of the QseB protein. Purified the QseB-His protein and test the production with SDS-PAGE. L represents protein ladder; while 1 represent the QseB protein, whose molecular weight is 25kDa

To test interaction between the QseB protein and *oriC*, the QseB protein was incubated with the *oriC* DNA (PCR amplified fragment, about 1.1 kb), and then loaded on agarose gel for gel-shift assay. When the *oriC* DNA is bound with QseB, the *oriC* fragment would migrate slower than one that is not bound with the protein in gel-shift assay. As shown in fig. 3, the *oriC* DNA was not found to be shifted. The BSA protein was included in the experiment as a negative control. The result suggests that QseB does not directly interact with the *oriC* region.

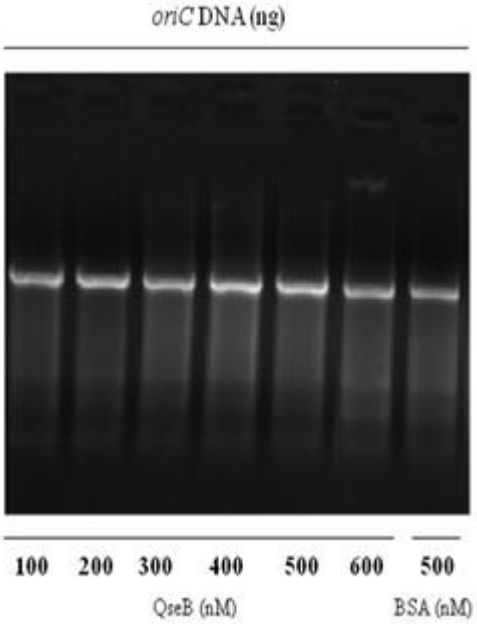

Fig. 3. The QseB protein does not interacts with *oriC*

For Gel shift assay, the *oriC* DNA was incubated with QseB at different concentrations as indicated at bottom. Then the proposed complex was loaded on agarose gel and analyzed be gel-shift assay. The gel was then stained in Safe-green nucleic acid dye. The BSA protein was included in the experiment as a negative control.

2. *Absence of QseB enhances concentration of the DnaA protein*

In the previous section, we showed that QseB did not interact with the *oriC* region, then how does the QseB protein affect initiation of replication? It could be possible that the QseB/QseC signaling would change concentration of the DnaA protein. The DnaA protein is is essential for initiation of replication at the *Escherichia coli oriC* both *in vivo* (Tomizawa and Selzer, 1979) and *in vitro* (Fuller et al., 1984). To check the possibility of changes in DnaA concentration, western blotting technique was used to detect concentration of the DnaA protein (Fig. 4) as described previously (Liu et al., 2014). Interestingly, concentration of the DnaA protein in *ΔqseB* mutant was increased significantly relative to that of wild-type (WT) cells (Fig. 4) as we expected. The results indicate that the QseB protein affects initiation of replication through regulating expression of the DnaA protein since DnaA directly initiates replication by interacting with the *oriC* DNA.

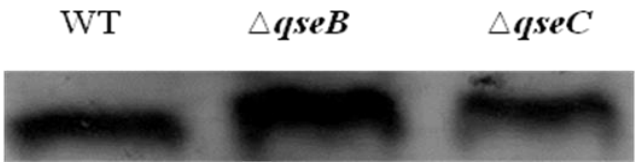

Fig. 4. The QseB protein enhances the DnaA protein amount per cell

Absence of the QseB protein enhances concentration of the DnaA protein. The DnaA protein concentration was determined by Western blotting as described previously (Liu et al., 2014).

3. The QseC protein localizes at cell membrane while QseB in cytosol

As mentioned above, the sensor protein, QseC, localizes at cell membrane. To detect subcellular localization of the QseB protein. The *ΔqseB*/pQseB-GFP and *ΔqseC*/pQseC-GFP cells were exponentially grown in ABTGcasa medium with induction of QseB-GFP and pQseC-GFP fusion by IPTG. Cells were collected, fixed in 70% ethanol and then visualized under fluorescent microscope. We found that the QseC-GFP protein was clearly on membrane while QseB-GFP was at two poles in some cells or all over in cytosol (Fig. 5). The results confirm that QseC-GFP is on membrane while QseB-GFP is cytosol.

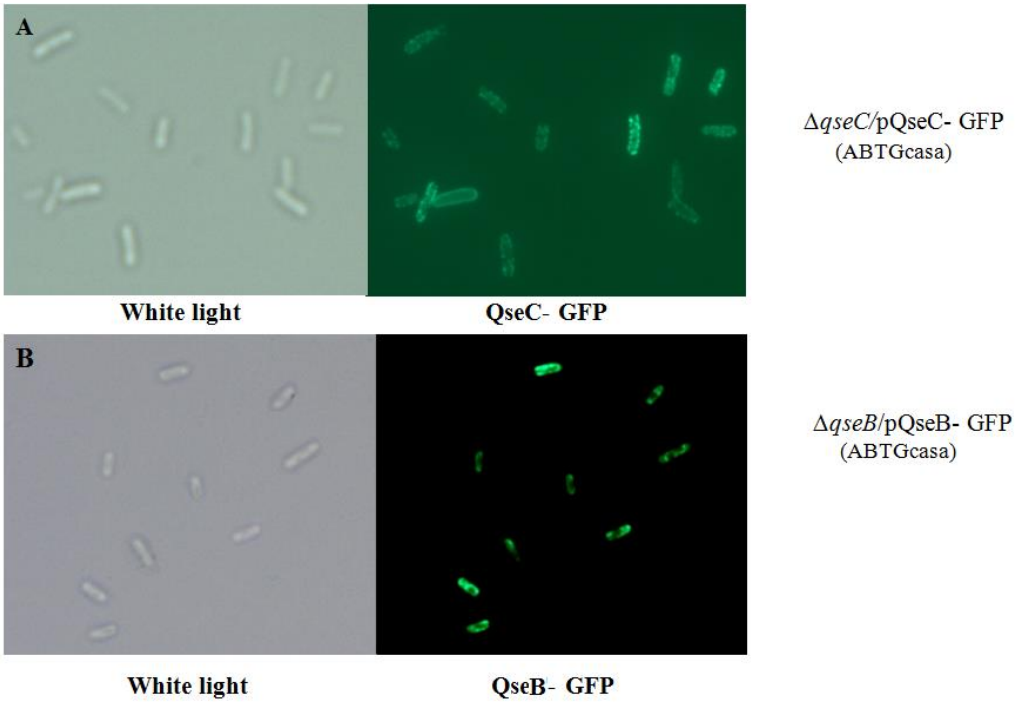

Fig. 5. The QseC protein localizes at cell membrane while QseB in cytosol. Exponentially growing cells were fixed in 70 % ethanol and then visualized by fluorescent microscope. The QseC- GFP (A) or QseB- GFP fusion (B) is in green

4. Conclusion

The Quorum-sensing QseB/QseC two-component pathway regulates bacterial motility in response to bacterially created quorum-sensing signals called autoinducers (AI) and the human hormone, epinephrine (Sperandio et al., 2005; Sperandio et al., 2002). Here we showed that the QseB/QseC signaling influenceed intiation of chromosomal replication.

The DnaA protein exerts a positive regulation on initiation of replication (Løbner-Olesen et al., 1989; Braun et al., 1985). The amount or concentration of DnaA protein is thought to be a limiting factor for initiation of replication. We showed that the absence of both *qseB* and *qseC* genes led to the early initiation of chromosomal replication (Fig. 1), indicating that QseB/QseC signaling indeed affects initiation of replication. To understand the mechanism behind, the purified QseB protein (Fig. 2) was incubated with the *oriC* DNA fragment, but no interaction between QseB and *oriC* was found (Fig. 3). Interestingly, however, DnaA concentration was dramatically increased in the absence of *qseB* gene (Fig. 4). The result can explain why initation occurs early in Δ*qseB* cells since the increased initiator protein DnaA concentration might change the timing of replication initiation. In accordance with the observation, initiation of replication was found to be affected by changing the amount of DnaA per cell in *aspC* mutant which is defective in AspC-mediated metabolism of aspartate (Liu et al., 2014). The sensor protein, QseC, localizes at cell membrane. To detect subcellular localization of the QseB protein (Fig. 5).

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