ACTA ABP BIOCHIMICA POLONICA

Vol. 65, No 3/2018
425–429
https://doi.org/10.18388/abp.2017_2360

Regular paper

Chlamydial genomic MinD protein does not regulate plasmid-dependent genes like Pgp5

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***Chlamydia* has a unique intracellular developmental cycle, which has hindered the single protein function study of *Chlamydia*. Recently developed transformation system of *Chlamydia* has greatly advanced the chlamydial protein's function research and was used to find that a chlamydial plasmid-encoded Pgp5 protein can down-regulate plasmid-dependent genes. It is assumed, that chlamydial genomic MinD protein has a similar function to Pgp5. However, it is unknown whether MinD protein regulates the same plasmid-dependent genes. We replaced pgp5 gene in the shuttle vector pGFP::CM with minD gene of *C. trachomatis* (CT0582) or *C. muridarum* (TC0871). The recombinant plasmid was transformed into plasmid-free organisms-CMUT3 and qRT-PCR was used to detect the transcription level of plasmid-encoded and -dependent genes in these pgp5 deficient organisms. As a readout, GlgA, one of the plasmid-regulated gene products was detected by immunofluorescence assay. After recombination, transformation and plaque purification, the stable transformants CT0582R and TC0871R were generated. In these transformants, the plasmid-dependent genes were up-regulated, alike in the pgp5 premature stop mutant and pgp5 replacement with mCherry mutant. GlgA protein level was also increased in all pgp5 mutants, including CT0582R and TC0871R. Thus, our study showed that genomic MinD protein had different function than Pgp5, which was useful for further understanding the chlamydiae.**

Key words: *Chlamydia*, MinD, Pgp5, Transformation system

Received: 17 November, 2017; revised: 27 June, 2018; accepted: 03 July, 2018; available on-line: 15 September, 2018

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Abbreviations: EB, Elementary body; RB, Reticular body; PCR, polymerase chain reaction; C. *trachomatis*, *Chlamydia trachomatis;* C. *muridarum*, *Chlamydia muridarum;* qRT-PCR, quantitative real-time polymerase chain reaction; GFP, green fluorescent protein; CPAF, chlamydial protease-like activity factor; WT, wild type; PBS, phosphate buffer saline; CT0582R, CMUT3-pGFP::CM CT0582Rpgp5; TC0871R, CMUT3-pGFP::CM TC0871Rpgp5; pgp5S, the pgp5 premature stop mutant organism; mCherryR, the pgp5 replacement with mCherry mutant organism

INTRODUCTION

Chlamydia trachomatis (*C. trachomatis*) is an obligate intracellular bacteria with a highly specialized biphasic developmental cycle, including elementary body (EB) and reticular body (RB) stages. The EB is the infectious form and can bind to the susceptible host cells. After entering into the host cell, EBs differentiate into RBs and the RBs are the metabolically active, replicative forms of the organisms, which revert to EBs accumulating within the inclusion until they are released from the host cell.

C. trachomatis can cause many human infections and diseases, including trachoma, urethritis, prostatitis, epididymitis, and cervicitis, hydrosalpinx, infertility, etc. (Sherman *et al*., 1990; Kari *et al*., 2011; Dong *et al*., 2014; Kari *et al*., 2014; Chen *et al*., 2015). However, the pathogenic mechanism of *C. trachomatis* remains unknown. The lack of dedicated genetic technologies is the main reason. In 2011 a chlamydial plasmid shuttle vector-based transformation system was developed by Wang and coworkers (Wang *et al*., 2011). This technology has offered an opportunity for chlamydiologists to define the functions of single proteins encoded by the chlamydial plasmid (Wang *et al*., 2013). Among these plasmid-encoded proteins, Pgp4 is a positive regulator of many plasmid-dependent genes, while the deficiency in Pgp3, 5 or 7 failed to significantly affect the expression of the investigated plasmid- dependent genes (Gong *et al*., 2013; Song *et al*., 2013). It was also reported that *Chlamydia muridarum* (*C. muridarum*) plasmid-encoded Pgp5 exhibited negative regulation, since the deficiency in Pgp5 resulted in up-regulation of plasmid-dependent genes (Liu *et al*., 2014). It was demonstrated in *in vitro* characterization experiments, that Pgp4 or Pgp5 might have an important role in the function of the plasmid (Zhong, 2017). *In vivo* characterization experiments of these *C. muridarum* transformants indicated that the plasmid-encoded Pgp3 was a key virulence factor in chlamydia-induced hydrosalpinx, and Pgp5 also contributed to chlamydial pathogenicity, although not as robustly as Pgp3 (Liu& Huang *et al*., 2014; Ramsey *et al*., 2014; Huang *et al*., 2015).

Pgp5 is predicted to be a homolog of MinD protein with 239 amino acids. It is known that MinD protein can bind to ATP and participate in plasmid/chromosome segregation (Stephens *et al*., 1998; Read *et al*., 2000). However, it is unknown whether MinD protein could regulate the plasmid-regulated chromosomal genes similar to Pgp5. In the current study, the *C. muridarum* transformation system was used to construct the Pgp5 replacement transformants with MinD (CT0582 or TC0871), quantitative real-time polymerase chain reaction (qRT-PCR) and indirect immunofluorescence were used to investigate the expression of the plasmid-regulated genes after pgp5 replacement with minD (CT0582 or TC0871).

MATERIALS AND METHODS

Cell lines and *Chlamydia organisms*. *C. muridarum* strains including the wild type (WT), the plasmid free (CMUT3), the intact plasmid transformant (Intact), the pgp5 premature stop mutant (pgp5S), the pgp5 replacement with mCherry mutant (mCherryR) organisms [from Dr. Guangming Zhong's lab at the University of Texas Health Science Center at San Antonio, USA] were propagated, purified, aliquoted and stored as described previously (Chen *et al*., 2015). The new pgp5 replacement mutants created from the intact plasmid transformant are described below. HeLa (human cervical epithelial carcinoma cells) cells used in this study were purchased from the Institute of Dermatology (PUMC, Nanjing, PRC). For chlamydial infection in cell culture, cells were grown in 6-well plates or 24-well plates with or without coverslips containing DMEM (Gibco, New York, USA) with 10% fetal bovine serum (FBS, Institute of Hematology, CAMS &PUMC, Tianjin, China) in 37°C, 5% CO_2, and were inoculated with chlamydial organisms as described previously (Zhong *et al*., 2001).

Constructing recombinant plasmids of pgp5 gene replacement mutants. For making pgp5 gene replacement mutants, we used primer pairs of our own design (shown in Supplementary Table 1 at www.actabp.pl) to amplify DNA fragments lacking pgp5 gene from the plasmid pGFP::CM, and to produce DNA fragments containing CT0582 or TC0871 genes from *C. trachomatis* or *C. muridarum* genome DNA, respectively, using AccuPrime pfx SuperMix (Life technologies, Grand Island, NY). The obtained PCR products were fused to produce the appropriate plasmids using an in-fusion HD cloning kit (Clontech Laboratories Inc, Mountain View, CA) as described (Liu *et al*., 2014). Bacterial colonies with positive green fluorescence were screened with PCR using the forward primer designed for the CT0582 or TC0871 and the reverse primer designed for the plasmid pGFP::CM (Supplementary Table 2 at www.actabp.pl). Screen PCR with a part of bacterial colony as a template was carried out as following: initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 1 min, 55°C for 30 s and 72°C for 1 min, followed by a final elongation at 72°C for 8 min. PCR products were visualized on a 1.0% agarose gel. Plasmids were isolated from positive bacterial colonies identified after PCR screening, and subsequently partially digested by BamHI and XhoI (New England Biolabs, Ipswich, MA). Plasmids with the desired fragments confirmed with restriction digestion were fully sequenced and transformed into *E. coli* K12 ER2925 (Dam- Dcm- strain, New England Biolabs, Ipswich, MA) for amplification. The newly constructed pGFP::CM CT0582Rpgp5 and pGFP::CM TC0871Rpgp5 plasmids were used for transforming chlamydial organisms.

Transforming plasmid-free C. muridarum CMUT3 organisms. The pGFP::CM CT0582Rpgp5 or pGFP::CM TC0871Rpgp5 plasmids were introduced into the plasmid-free *C. muridarum* strain CMUT3 in the form of a purified EB according to a previously published protocol (Liu *et al*., 2014). Briefly, CMUT3 organisms (1×10^7 IFU) and plasmid DNA (7 μg) were mixed in a total volume of 200 μl $CaCl_2$ buffer and incubated for 45 minutes at room temperature. Freshly trypsinized HeLa cells (6×10^6 cells) were re-suspended in 200 μl $CaCl_2$ buffer and added to the EB/plasmid mixture for a further 20 min incubation at room temperature. The final mixture was plated into a six-well plate together with pre-warmed DMEM+10% FBS without cycloheximide or ampicillin. The cells were allowed to adhere to the culture plate at 37°C in 5% CO_2 for 12 h. Then, cultures were replenished with fresh DMEM+10% FBS containing cycloheximide (2 mg/ml) and ampicillin (5 mg/ml) (Sigma, St. Louis, MO) and incubated for additional 24h. Inclusions positive for green fluorescence protein (GFP) were identified under a fluorescence microscope (Olympus, Center Valley, PA) and subsequently transferred to fresh monolayers of HeLa cells, cultured in the presence of ampicillin (10 μg/ml). The resultant GFP-positive inclusions were defined as generation two and were passaged for 2 to 3 additional generations. The *C. muridarum* organisms of CMUT3-pGFP::CM CT0582Rpgp5 (CT0582R) or CMUT3-pGFP::CM TC0871Rpgp5 (TC0871R) were plaque-purified as described previously (Chen *et al*., 2015) for *in vitro* characterization as described below.

Reverse transcription and quantitative Real-Time PCR. To quantify transcripts, HeLa cells grown in 6-well plates (1×10^6/well) were infected with EBs containing WT, CMUT3, Intact, pgp5S, mCherry, TC0871R and CT0582R organisms at a multiplicity of infection (MOI) of 2. Twenty hours after infection, cells were harvested using TRIzol reagent (Life technologies, Grand Island, NY) and total RNA from each sample was extracted according to the manufacturer's instructions. RNA preparations were used for cDNA synthesis with random hexamer primers using a ThermoScript Reverse Transcription System (Life technologies, Grand Island, NY). Then, qRT-PCR were performed using a Cobas Z480 Real-Time PCR Detection System (Roche, Basel, CH). We used gene-specific primers for plasmid-encoded and -regulated genes described in (Liu *et al*., 2014), and they included unlabeled primers and Double-Quenched Probe (5'FAM/ZEN/3'IBFQ; Integrated DNA Technologies, Coralville, Iowa). In addition, TC0052 (ompA, independent of plasmid regulation) and TC0248 (chlamydial protease-like activity factor, cpaf) were included as controls. The qRT-PCR conditions were as follows: initial denaturation at 95°C for 3 min followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. Transcript copy numbers for a given gene were calculated based on triplicate samples and a standard plasmid DNA prep, and further normalized to the copy numbers of chlamydial *lpdA* mRNA in the corresponding samples.

Indirect immunofluorescence assay. For immunofluorescence imaging, HeLa cells infected with EBs containing WT, CMUT3, Intact, pgp5S, mCherry, TC0871R and CT0582R organisms were fixed at 30 hours post infection with 2% paraformaldehyde in PBS for 45 min at room temperature, and permeabilized with 0.1% Triton X-100 for an additional 6 min. After blocking, cell samples were subjected to antibody and chemical staining. A rabbit anti-chlamydial organism antibody plus a goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (green; Jackson ImmunoResearch, West Grove, PA) were used to visualize chlamydial organisms. Mouse anti-GlgA (glycogen synthase A) antibodies (from Dr. Guangming Zhong' Lab) plus a goat anti-mouse IgG conjugated with Cy3 (red; Jackson ImmunoResearch) were used to visualize GlgA. Hoechst 33258 (blue, Sigma) was used to visualize DNA (blue). Immunofluorescence images were acquired using an Olympus fluorescence microscope equipped with multiple filter sets.

Statistical analysis. Data analysis was performed using the student's *t*-test. Data is expressed as mean ± S.E. Differences among means were considered to be significant at $p<0.05$.

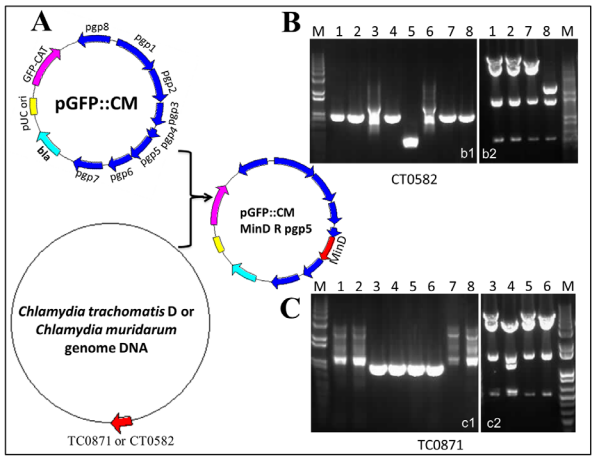

Figure 1. Construction of the *C. muridarum* plasmid-based shuttle vectors of pgp5 replacement with CT0582 or TC0871.
(**A**) The pGFP::CM CT0582 R pgp5 or the pGFP::CM TC0871 R pgp5 shuttle vectors were constructed by replacing the pgp5 gene of the pGFP::CM with the *C. trachomatis* CT852 or *C. muridarum* TC0871, using an in-fusion cloning technique. (**B**) Agarose electrophoresis of screen PCR (b1) and endonuclease digestion (b2) of the recombinant plasmid pGFP::CM CT0582 R pgp5; (**C**) Agarose electrophoresis of screen PCR (c1) and endonuclease digestion (c2) of the recombinant plasmid pGFP::CM TC0871 R pgp5.

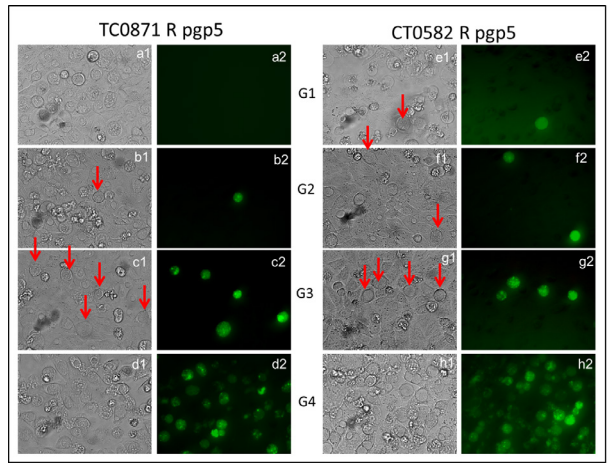

Figure 2. Selection and propagation of the new transformants CT0582R & TC0871R.
The plasmids pGFP::CM CT0582 R pgp5 or the pGFP::CM TC0871 R pgp5 were transformed into plasmid-free (pf) CMUT3 organisms in the form of purified elementary bodies (EBs). HeLa cells with or without GFP positive inclusions (defined as generation #1) were scraped and inoculated in the presence of ampicillin. Selection and passage were repeated for four rounds (generation #4). Most inclusions were GFP positive, and a single clone was isolated *via* a plaque assay and purified for further studies.

RESULTS

Construction of *C. muridarum* of pgp5 replacement with minD (CT0582 or TC0871)

To create pgp5 gene replacement plasmid with minD (CT0582 or TC0871), named pGFP::CM CT0582 R pgp5 or pGFP::CM TC0871 R pgp5, the pgp5 gene in the shuttle vector pGFP::CM was replaced with CT0582 or TC0871 (Fig. 1A). The PCR screen identified the expected genes in the recombinant plasmids: pGFP::CM CT0582 R pgp5 (Fig. 1.b1) and pGFP::CM TC0871 R pgp5 (Fig. 1.c1). The correct plasmids were further identified by endonuclease digestion, the plasmid pGFP::CM CT0582 R pgp5 was digested into 6061bp, 3924bp, 1409bp, 503bp fragments (lane 1 and 2 in Fig. 1.b2), while the pGFP::CM TC0871 R pgp5 was digested into 4377bp, 3924bp, 1409bp, 1133bp, 551bp, 503bp fragments (lane 4 in Fig. 1. c2).

The pGFP::CM CT0582 R pgp5 and pGFP::CM TC0871 R pgp5 plasmids were subsequently transformed into the CMUT3 organisms. GFP positive inclusions (CT0582R or TC0871R) in generation 1 were passaged to newly cultured cells in the presence of ampicillin. After repeating the selection and passage for four rounds, when most of the inclusions remained GFP positive (Fig. 2), a single clone was finally isolated using a plaque assay and it was purified for subsequent experiments.

Effect of pgp5 replacement with minD (CT0582 or TC0871) on the expression of plasmid-encoded and -regulated genes

The expression levels of 16 plasmid-encoded and -regulated genes were determined in wild type and six plasmid-free *C. muridarum* (CMUT3, Intact, pgp5S, mCherryR, CT0582R & TC0871R). The transcript copy numbers normalized to lpdA mRNA for each of sixteen measured genes are shown along the Y-axis in Fig.3. The six plasmid-dependent genes were: TC0181 and hypothetical genes: TC0319, TC0357, TC0419, TC0420 and TC0421. We focused on differences in expression levels of these six plasmid-dependent chromosomal genes between pgp5S, mCherryR, CT0582R and TC0871R transformants. Surprisingly, the expression levels of the six plasmid-dependent genes were similar in the transformants of pgp5S, mCherry R, CT0582R &TC0871R, but much higher in CMUT3, WT& Intact (Fig. 3). Also, TC0052 and TC0248 controls were not affected by any pgp5 mutants (Fig. 3).

Effect of pgp5 replacement with minD (CT0582 or TC0871) on GlgA protein expression

We further monitored GlgA protein expression in these stable pgp5 replacement transformants (Fig. 4). As described previously, GlgA expression was not detected in the plasmid-free CMUT3-infected cultures, and was restored in CMUT3 organisms transformed with the parent plasmid pGFP::CM. We found that GlgA expression was increased in cultures infected with pgp5S, mCherryR, CT0582R and TC0871R. These results were consistent with the changes of transcription level of TC0181.

DISCUSSION

Chlamydiae are a causative agent of numerous diseases in human and animals. They are obligate intracellular parasites with a unique biphasic developmental cycle. Because of the specialized developmental cycle, there were no suitable genetic tools for chlamydial research until 2011, and the functions of only a few chlamydial proteins were predicted, whereas most of them remained unknown. The transformation system developed for *Chlamydia* has since facilitated research that was not previously feasible.

In the current study, we used the *C. muridarum* transformation system to study the phenotype of pgp5 replacement with minD (CT0582 or TC0871). We showed that after pgp5 replacement with minD (CT0582 or TC0871), some plasmid-dependent chromosomal genes, including TC0181 (GlgA, homolog of ct798)

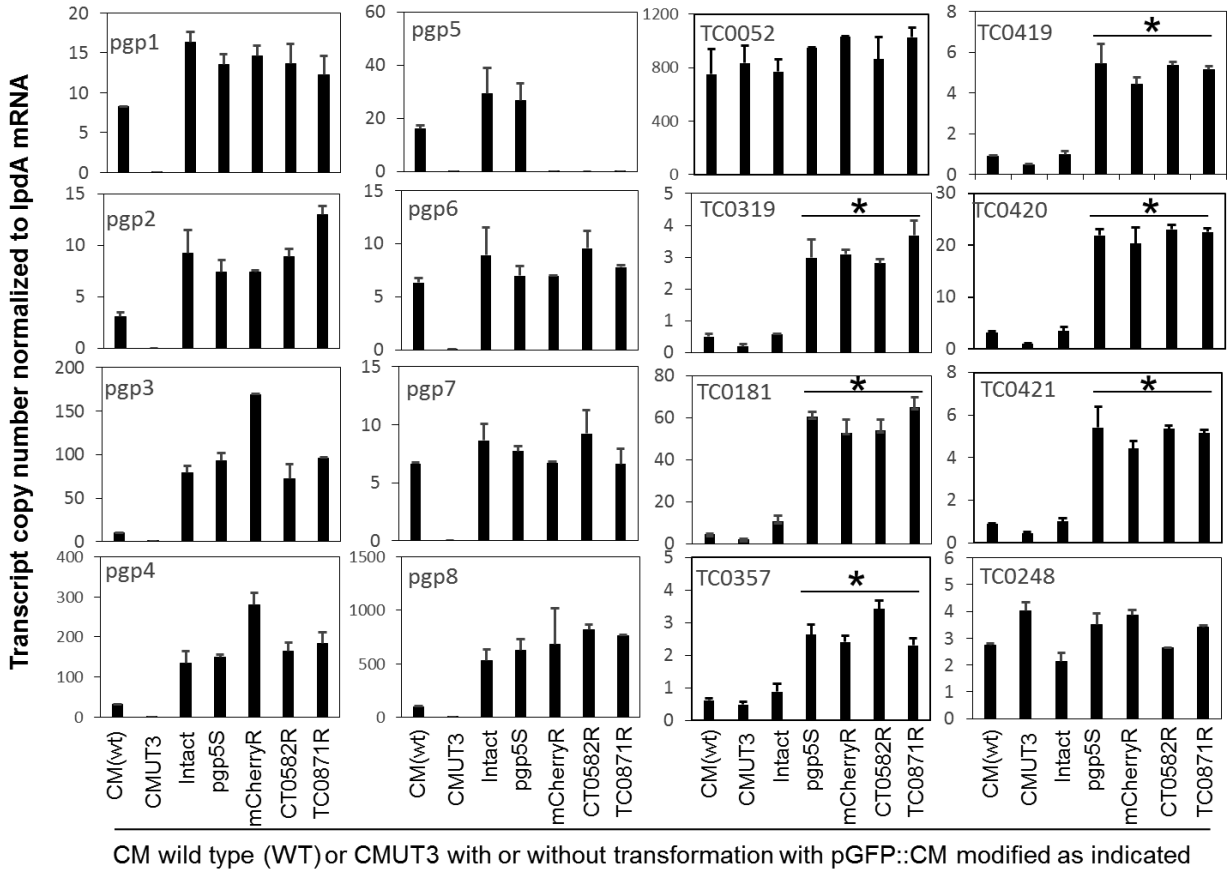

Figure 3. Effect of pgp5 replacement with MinD (CT0582 or TC0871) on the expression of plasmid-encoded and -regulated genes. Wild type (wt) *C. muridarum* and six plasmid-free *C. muridarum* organisms (CMUT3, Intact, pgp5S, mCherryR, CT0582R and TC0871R) were used to infect HeLa cells, as listed along the X-axis. The transcript copy numbers normalized to lpdA mRNA for each of 16 measured ORFs are shown along the Y-axis. Note that the expression levels of TC0319, TC0181, TC0419, TC0420, TC0421 and TC0357 were significantly increased in the transformants of pgp5S, mCherryR, CT0582R & TC0871R. Stars (*) indicate significant increase of the gene expression when compared to transformants that carry the intact plasmid pGFP::CM.

and TC0319 (homolog of ct049), TC0357 (homolog of ct084), TC0419 (homolog of ct142), TC0420 (homolog of ct143) and TC0421 (homolog of ct144) were clearly up-regulated. The indirect immunofluorescence assay also indicated that the expression of GlgA protein was markedly increased. Our previous study found that deletion of pgp5 gene or premature termination of pgp5 also up-regulated the mentioned plasmid-dependent chromosomal genes (Liu *et al.*, 2014). Taken together, the findings implicate that neither *C. trachomatis* MinD (CT0582) nor *C. muridarum* MinD (TC0871) has the function of suppressing these plasmid-regulated genes' expression.

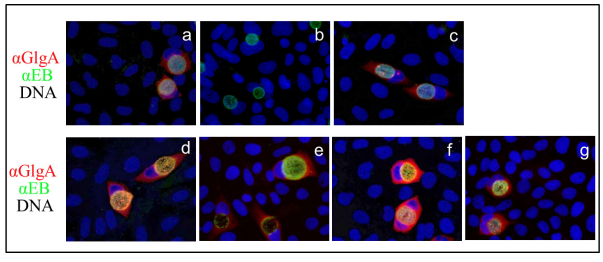

Figure 4. Effect of replacement of pgp5 with minD (CT0582 or TC0871) on GlgA protein expression. The pgp5-mutant transformants and WT, CMUT3, Intact organisms were used to infect HeLa cells. The infected cells were used for triple immunofluorescence labeling for GlgA proteins (**red**), chlamydial organisms (**green**) and DNA (**blue**). Note that GlgA expression was increased in the cultures infected with pgp5S (**panel d**), mCherryR (**panel e**), CT0582R (**panel f**) and TC0871R (**panel g**).

Pgp5 is predicted to be a homolog of MinD that participates in genome segregation/partitioning (Stephens *et al.*, 1998; Read *et al.*, 2000). Our previous study showed, that after deletion or premature termination of Pgp5, chlamydial plasmid can still replicate and segregate, which indicated that Pgp5 did not participate in the plasmid/genome segregation (Liu *et al.*, 2014). In the current study we found that genomic MinD did not have the Pgp5's function of suppressing genes expression. So we conclude that Pgp5 is not be MinD, and genomic MinD has different functions from Pgp5. The protein sequence comparisons of genomic MinD and Pgp5 in *C. trachomatis* or *C. muridarum* also supported this conclusion. The Pgp5 from *C. trachomatis* and *C. muridarum* shared ~80% identity, and the genomic MinD proteins from both species also shared ~80% amino acid sequence identity. These results indicated that both the genomic MinD and the plasmid-encoded Pgp5 are highly conserved proteins. However, the homology between any genomic MinD protein and any Pgp5 is only 30%, which supported our experimental results that genomic MinD protein may have the different function from plasmid-encoded Pgp5, for example, in suppressing the chromosomal genes expression.

Identification of the exact function of genomic MinD protein requires further experimentation. The transfor-

mation system allows for generation of plasmid gene knockouts and plasmid-based gene knock-ins, but it is not suitable for creating targeted, selectable chromosomal mutations. Fortunately, TargeTron™ system employed in a variety of Gram positive and negative bacteria was adapted for *Chlamydia* (Johnson, Fisher *et al*., 2013; Weber *et al*., 2016; Key & Fisher, 2017). According to this research, this system can inactivate targeted chromosomal genes and can be a suitable method for manipulating the chlamydial genome (Thompson *et al*., 2015). In the future, the *C. muridarum* mutant of minD gene would be constructed used theTargeTron™ system and the function of genomic MinD protein could be further identified.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgements of Financial Support

This work was supported by the National Natural Science Foundation of China (Grant Nos. 31570178, 31100138), the Natural Science Foundation of Tianjin (Grant No. 16JCQNJC11100) and Science & Technology Foundation for Selected Overseas Chinese Scholar, Burea of Personnel of China, Tianjin(Grant No: 2016007).

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