J. Microbiol. Biotechnol. (2018), 28(5), 816–820
https://doi.org/10.4014/jmb.1712.12040

jmb

Effects of *flaC* Mutation on Stringent Response-Mediated Bacterial Growth, Toxin Production, and Motility in *Vibrio cholerae*

Hwa Young Kim[1], Sang-Mi Yu[2], Sang Chul Jeong[2], Sang Sun Yoon[1], and Young Taek Oh[2]*

[1]*Department of Microbiology and Immunology, Brain Korea PLUS Project for Medical Science, Institute for Immunology and Immunological Diseases, Yonsei University College of Medicine, Seoul 03722, Republic of Korea*
[2]*Freshwater Bioresources Utilization Division, Nakdonggang National Institute of Biological Resources, Sangju 37242, Republic of Korea*

Received: December 18, 2017
Revised: February 25, 2018
Accepted: February 26, 2018

First published online
March 15, 2018

**Corresponding author*
Phone: +82-54-530-0932;
Fax: +82-54-530-0949;
E-mail: ohyt@nnibr.re.kr

pISSN 1017-7825, eISSN 1738-8872

The stringent response (SR), which is activated by accumulation of (p)ppGpp under conditions of growth-inhibiting stresses, plays an important role on growth and virulence in *Vibrio cholerae*. Herein, we carried out a genome-wide screen using transposon random mutagenesis to identify genes controlled by SR in a (p)ppGpp-overproducing mutant strain. One of the identified SR target genes was *flaC* encoding flagellin. Genetic studies using *flaC* and SR mutants demonstrated that FlaC was involved in bacterial growth, toxin production, and normal flagellum function under conditions of high (p)ppGpp levels, suggesting FlaC plays an important role in SR-induced pathogenicity in *V. cholerae*.

Keywords: *Vibrio cholerae*, stringent response, (p)ppGpp, *flaC*, cholera toxin, motility

The stringent response (SR) is a bacterial defense mechanism that is activated in response to various growth-inhibiting stresses, by accumulation of the small nucleotide regulator (p)ppGpp, and induces global changes in bacterial transcription and translation [1, 2]. In *Vibrio cholerae* (the causative agent of pandemic disease cholera), the *relA* and *relV* genes are involved in the production of (p)ppGpp, whereas the *spoT* gene encodes an enzyme that hydrolyzes it [3].

Recent work from our group showed that the SR is activated by the alternative electron acceptor trimethylamine oxide (TMAO) in *V. cholerae*, stimulating anaerobic and steady-state growth and modulating cholera toxin (CT) production [4, 5]. A mutant strain defective in (p)ppGpp production (*i.e.*, Δ*relA*Δ*relV*Δ*spoT*; (p)ppGpp0) lacked the ability to produce CT and exhibited excessive growth under anaerobic TMAO respiration. In contrast, the Δ*relA*Δ*spoT* mutant overproducing (p)ppGpp generated high levels of CT and exhibited growth retardation [5]. Thus, accumulation of intracellular (p)ppGpp results in inverse regulation of bacterial cell growth and CT production.

In this study, we carried out a genome-wide screen for genes related to SR-mediated regulation of virulence and growth. Transposon (Tn) random mutagenesis was used as a global genetic screening system to identify genes controlled by (p)ppGpp [6]. We constructed a random Tn mutant library based on the Δ*relA*Δ*spoT* mutant using the plasmid pBTK30, which contains a gentamicin resistance-marked transposable element and mariner C9 transposase. *Escherichia coli* strain SM10/λ pir harboring pBTK30 and the *V. cholerae* recipient strain (Δ*relA*Δ*spoT* mutant) were mixed and spread onto Luria-Bertani (LB) agar plates followed by incubation for 6 h at 37°C. The cells were then resuspended in LB medium, and dilutions were spread on LB plates containing 50 μg/ml gentamicin and 200 μg/ml streptomycin to select Tn insertion mutants. A total of 1,500 bacterial colonies were screened for mutants exhibiting growth retardation. Bacterial colonies were inoculated in a 96-well plate containing LBT (LB medium containing 50 mM TMAO) broth and were grown under anaerobic conditions for 16 h. Bacterial growth was assessed spectrophotometrically by measuring the optical density at 600 nm. A total of 42 candidate mutants that exhibited higher growth rates than the Δ*relA*Δ*spoT* mutant were obtained in the initial screen.

The 42 candidate mutants were tested for their capacity for CT production, which was measured in culture

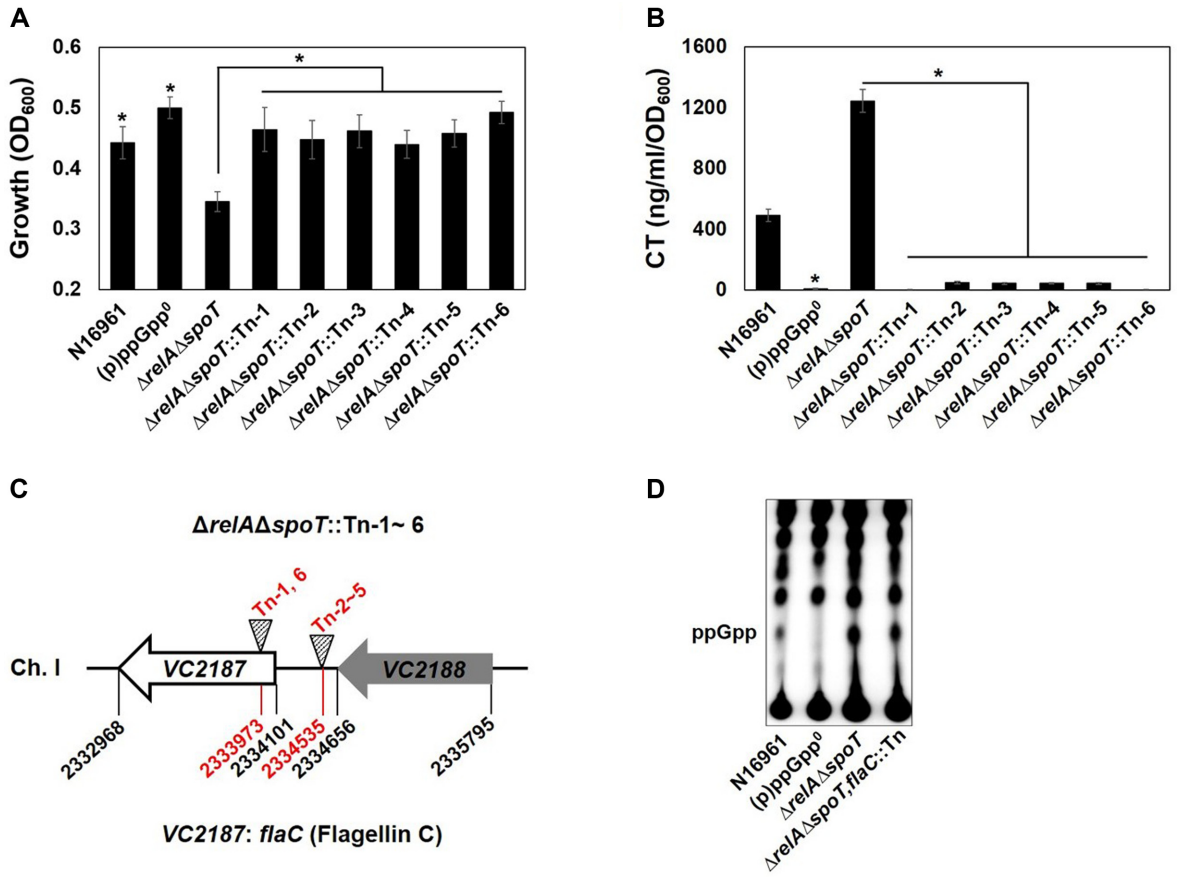

Fig. 1. Selection of transposon (Tn) insertional mutant strains derived from the Δ*relA*Δ*spoT* mutant strain that reverse (p)ppGpp-induced growth and virulence phenotypes.

(**A**) Changes in the growth of wild-type strain N16961 and (p)ppGpp^0, Δ*relA*Δ*spoT*, and six selected mutant strains are shown. Bacterial cells were inoculated in LB broth containing trimethylamine oxide (LBT) and cultured under anaerobic conditions for 16 h. OD_{600} values were determined as a measure of relative growth. Values represent the mean ± SD of three independent experiments. *P < 0.03 vs. Δ*relA*Δ*spoT* mutant strain. (**B**) Cholera toxin (CT) production in bacterial cells grown in LBT under anaerobic conditions. The culture supernatant was harvested and CT levels were determined by enzyme-linked immunosorbent assay. Values represent the mean ± SD of three independent experiments. *P < 0.001 vs. Δ*relA*Δ*spoT* mutant strain. (**C**) Schematic depiction of the *V. cholerae VC2178* locus. Arrowheads indicate the position of Tn insertions. (**D**) Intracellular ppGpp was detected by TLC analysis. Bacterial cells were anaerobically grown in LBT with [^{32}P]-orthophosphate for overnight. Cellular extracts were prepared and analyzed by TLC.

supernatants by GM1 enzyme-linked immunosorbent assay as previously described [7]. We ultimately selected six candidate mutants (Δ*relA*Δ*spoT*::Tn-1–6) that showed decreased CT production and recovery of (p)ppGpp-induced growth retardation (Figs. 1A and 1B). Compared with the parental strain, the OD_{600} value of the six candidate mutants was higher than that of wild-type strain N16961 after 16 h of culture (Fig. 1A). On the other hand, the mutants produced low levels of CT (Fig. 1B). Consistent with our previous observation that CT production and growth in response to (p)ppGpp accumulation are inversely regulated, we found an inverse relationship between CT production and growth in mutants with the Tn insertion. The site of Tn insertion in the mutants was determined by arbitrary PCR as described elsewhere [5] using the specific primer BTK30-Tnp1 (CACCGCTGCGTTCGGTCAAG) and the two random primers RP-1 (CTTACCAGGCCACGC GTCGACTAGTACNNNNNNNNNGATAT) and RP-2 (CTTACCAGGCCACGCGTCGACTAGTACNNNNNN NNNNACGCC). This was followed by a second round of PCR using the product from the first round as a template and the primers BTK30-Tnp2 (CGAACCGAACAGGCC TTATGTTCAATTC) and RP-3 (CTTACCAGGCCACGC GTCGACTAGTAC). The product was sequenced using the

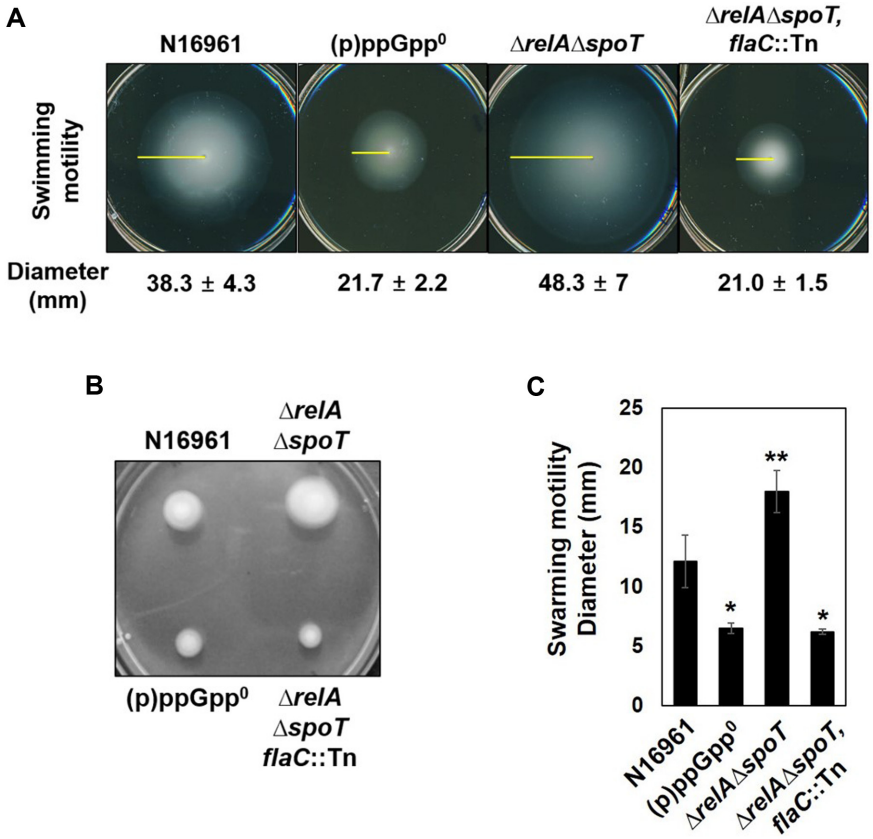

Fig. 2. FlaC is involved in regulation of stringent response-mediated cell motility in *Vibrio cholerae*.
Cells were cultured in LB broth containing trimethylamine oxide under anaerobic conditions for 16 h and used for motility assays. (**A**) Bacterial strains were spot-inoculated on a 0.3% (w/v) agar LB plate to evaluate swimming motility. (**B**, **C**) Swarming motility was assessed on 0.5% (w/v) agar LB plates.

primer BTK30-Tnp3 (TGGTGCTGACCCCGGATGAAG). The Tn insertion site was identified by comparison with the genome sequence of *V. cholerae* 7th pandemic strain N16961 available in public databases. Interestingly, the genes carried by Δ*relA*Δ*spoT*::Tn-1 and 6 and Δ*relA*Δ*spoT*::Tn-2, 4, and 5 that were disrupted by Tn insertion were identified as *VC2187*(*flaC*) and its promoter region, respectively (Fig. 1C).

To confirm whether the accumulation of (p)ppGpp was reduced by *flaC* mutation, we next measured intracellular (p)ppGpp levels of bacterial cells under growth by anaerobic TMAO respiration. Intracellular (p)ppGpp concentration was measured as previously described [5]. The Δ*relA*Δ*spoT*, *flaC*::Tn mutant accumulated high levels of intracellular (p)ppGpp, which was not significantly different from the Δ*relA*Δ*spoT* mutant (Fig. 1D). These results indicate that the mutation of *flaC* was not affected by the accumulation of (p)ppGpp.

V. cholerae has five flagellin genes arranged at two loci, *flaA, C* and *flaE, D, B*. The *flaA*, a major flagellin coding gene, is associated with flagellum formation and motility; however, the precise function of the other flagellar filament-coding genes, including *flaC*, is not clearly defined [8]. Previous reports have shown that the gene expression of *relA*, which encodes an enzyme involved in the synthesis of (p)ppGpp, is correlated with that of *flaC* under starvation environment conditions in *V. cholerae* [9]. Furthermore, some reports indicate that flagellin-coding genes play an essential role in bacterial growth and virulence [10–13]. Here, we found that the Tn insertion in *flaC* in the Δ*relA*Δ*spoT* mutant reduced CT production, which reversed SR-induced growth retardation. Together, our results suggest that FlaC has potential role as a regulator of (p)ppGpp-mediated toxin production and cell growth. However, there is no report that FlaC can act as a transcriptional factor, and we do not yet know the mechanistic relationship between flagellin and the SR, so further studies are needed.

Interestingly, the Δ*relA*Δ*spoT*, *flaC*::Tn mutant strain

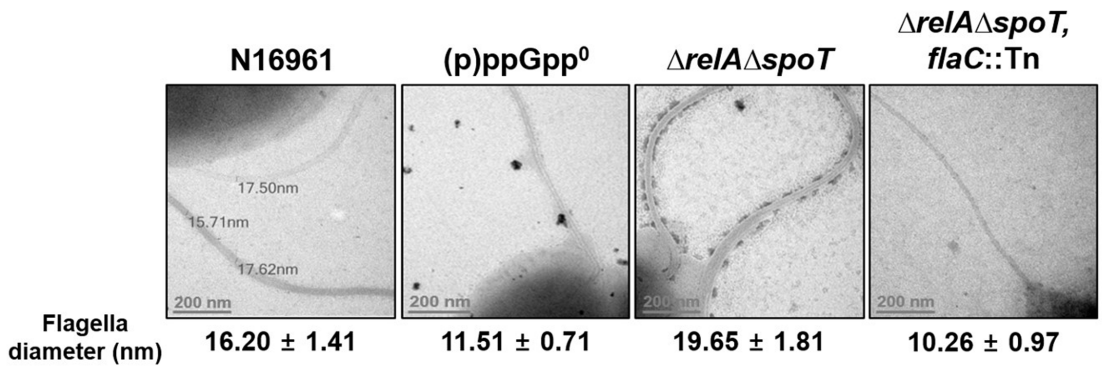

Fig. 3. Transmission electron microscopy analysis of *V. cholerae* strains.

Representative transmission electron micrographs of flagella from wild-type *V. cholerae* strain N16961 and (p)ppGpp^0, Δ*relA*Δ*spoT*, and Δ*relA*Δ*spoT*, *flaC*::Tn mutant strains. Scale bar, 200 nm. The flagellar diameter was measured using iTEM acquisition and analysis software (Olympus Soft Imaging Solutions GmbH, Germany).

showed a severe decrease in cell motility compared with the parent strain (Fig. 2A). Swimming and swarming assays were performed to measure bacterial cell motility as described previously [14]. Bacterial cells grown anaerobically in LBT were spot-inoculated on 0.3% (w/v) agar LB plate for 12 h at 37°C, with the diameter of the circular halo measured as an estimate of swimming motility; 0.5% (w/v) agar LB plates were used for the swarming assay. It is worth noting that bacterial cell motility is (p)ppGpp dependent. We observed that motility was decreased in the (p)ppGpp^0 mutant, whereas the Δ*relA*Δ*spoT* mutant was hypermotile as compared with wild-type strain N16961 (Fig. 2). These results confirm that (p)ppGpp is involved in the motility of *V. cholerae*. Furthermore, the Δ*relA*Δ*spoT*, *flaC*::Tn mutant showed a similar degree of motility as the (p)ppGpp^0 mutant, indicating that FlaC is associated with the SR-induced hypermotile phenotype.

We next compared the morphology of the flagellum of wild-type strain N16961 and mutant strains. Cells were anaerobically cultured overnight in LBT and examined by transmission electron microscopy (JEM 1010; JEOL, Japan) as previously described [15], after negative staining with a 2% aqueous solution of phosphotungstic acid (pH 7.4). Interestingly, we found that the Δ*relA*Δ*spoT*, *flaC*::Tn mutant strain had thinner flagella than the parent mutant strain Δ*relA*Δ*spoT*. Furthermore, the diameter of the flagellum was correlated with the ability of (p)ppGpp production (Fig. 3). These results indicate that (p)ppGpp alters bacterial cell motility by modulating flagellar thickness and that FlaC plays an important role in this process.

In conclusion, the mutation in *flaC* in the Δ*relA*Δ*spoT* mutant strain reduced CT production and abolished (p)ppGpp-induced growth retardation in *V. cholerae*. In addition, the Δ*relA*Δ*spoT*, *flaC*::Tn mutant showed decreased flagellar diameter as compared with the parent strain, which was associated with reduced motility. This report describes a potential function for FlaC that involves the regulation of SR-induced toxin production, growth, and hypermotility.

Acknowledgments

This work was supported by a grant from the National Research Foundation (NRF) of Korea (Grant 2014R1A1A2056139) funded by the Korean government. This work was also supported by a Nakdonggang National Institute of Biological Resources (NNIBR) grant funded by the Ministry of Environment, Republic of Korea.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Dalebroux ZD, Swanson MS. 2012. ppGpp: magic beyond RNA polymerase. *Nat. Rev. Microbiol.* **10:** 203-212.
2. Srivatsan A, Wang JD. 2008. Control of bacterial transcription, translation and replication by (p)ppGpp. *Curr. Opin. Microbiol.* **11:** 100-105.
3. Das B, Pal RR, Bag S, Bhadra RK. 2009. Stringent response in *Vibrio cholerae*: genetic analysis of *spoT* gene function and identification of a novel (p)ppGpp synthetase gene. *Mol. Microbiol.* **72:** 380-398.

4. Lee KM, Park Y, Bari W, Yoon MY, Go J, Kim SC, *et al*. 2012. Activation of cholera toxin production by anaerobic respiration of trimethylamine *N*-oxide in *Vibrio cholerae*. *J. Biol. Chem.* **287:** 39742-39752.
5. Oh YT, Park Y, Yoon MY, Bari W, Go J, Min KB, *et al*. 2014. Cholera toxin production during anaerobic trimethylamine *N*-oxide respiration is mediated by stringent response in *Vibrio cholerae*. *J. Biol. Chem.* **289:** 13232-13242.
6. Cameron DE, Urbach JM, Mekalanos JJ. 2008. A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **105:** 8736-8741.
7. Gardel CL, Mekalanos JJ. 1994. Regulation of cholera toxin by temperature, pH, and osmolarity. *Methods Enzymol*. **235:** 517-526.
8. Klose KE, Mekalanos JJ. 1998. Differential regulation of multiple flagellins in *Vibrio cholerae*. *J. Bacteriol*. **180:** 303-316.
9. Mishra A, Taneja N, Sharma M. 2012. Viability kinetics, induction, resuscitation and quantitative real-time polymerase chain reaction analyses of viable but nonculturable *Vibrio cholerae* O1 in freshwater microcosm. *J. Appl. Microbiol*. **112:** 945-953.
10. He Y, Xu T, Fossheim LE, Zhang XH. 2012. FliC, a flagellin protein, is essential for the growth and virulence of fish pathogen *Edwardsiella tarda*. *PLoS One* **7:** e45070.
11. Dingle TC, Mulvey GL, Armstrong GD. 2011. Mutagenic analysis of the *Clostridium difficile* flagellar proteins, FliC and FliD, and their contribution to virulence in hamsters. *Infect. Immun*. **79:** 4061-4067.
12. Magnusson LU, Gummesson B, Joksimović P, Farewell A, Nyström T. 2007. Identical, independent, and opposing roles of ppGpp and DksA in *Escherichia coli*. *J. Bacteriol*. **189:** 5193-5202.
13. Ramos HC, Rumbo M, Sirard JC. 2004. Bacterial flagellins: mediators of pathogenicity and host immune response in mucosa. *Trends Microbiol*. **12:** 509-517.
14. Partridge JD, Nieto V, Harshey RM. 2015. A new player at the flagellar motor: FliL controls both motor output and bias. *MBio* **6:** e02367.
15. Bari W, Lee KM, Yoon SS. 2012. Structural and functional importance of outer membrane proteins in *Vibrio cholera* flagellum. *J. Microbiol*. **50:** 631-637.