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Dissection of ToxR-dependent and ToxR-independent stress-regulated pathways in *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus is a seafood-borne Gram-negative bacteria causing diarrheal diseases in humans world wide. ToxR is a membrane-associated transcriptional factor which plays an important role in acid stress tolerance and regulates the expression of virulence genes including type III secretion system 1 (T3SS1) and type VI secretion system 1 (T6SS1) in *V. parahaemolyticus*. However, possible mechanisms of ToxR mediating virulence gene expression have not been fully understood. In this study, we demonstrated that ToxR is essential for *V. parahaemolyticus* to tolerate acid stress by constructing a ToxR deletion mutant (Δ*toxR*) and its complemented strain (*toxR*$^{+}$). Quantitative PCR showed that the expression of *toxR* was up regulated under acid stress condition. RNA-seq analysis showed that *ompU* encoding one of outer membrane proteins was dramatically down regulated in Δ*toxR*. Furthermore, the mutation of *ompU* also led to a significant reduction in tolerating acid stress indicating that ToxR mediated acid stress through regulating *ompU* expression. RNA-seq results further confirmed that acid stress condition could alter multiple signaling pathways either depending on ToxR (e.g., quorum sensing, fatty acid metabolism) or independent of ToxR (e.g., biosynthesis of secondary metabolites, microbial metabolism in diverse environment, biosynthesis of antibiotics, biosynthesis of amino acids and carbon metabolism pathways). We also for the first time demonstrated that ToxR positively regulated the expression of T6SS2 gene and the interbacteria killing activity. Our study provides comprehensive understanding of signaling pathways which are regulated by both acid stress and ToxR.

1. Introduction

Vibrio parahaemolyticus is a seafood borne Gram-negative pathogen which causes acute gastrointestinal diseases including abdominal pain, diarrhea, nausea, vomiting, and fever (Austin, 2010; Baker-Austin et al., 2017). In immunocompromised patients, *V. parahaemolyticus* can lead to systemic infection, such as sepsis (Baker-Austin et al., 2018). *V. parahaemolyticus* was initially reported to cause an outbreak of gastrointestinal diseases with 272 illness and 20 deaths due to the consumption of sardines in Osaka, Japan in 1951(Fujino et al., 1953). In the subsequent several decades, 20%–30% of the food outbreaks in Japan were caused by *V. parahaemolyticus* each year (Alam et al., 2002). In the United States, over 40 of food poisoning events were caused by *V. parahaemolyticus* between 1973 and 1998 (Daniels et al., 2000). In China, *V. parahaemolyticus* is one of the leading causes of foodborne illnesses, which accounts for 33.7% of foodborne pathogen infections. More importantly, the incidence of *V. parahaemolyticus* infection has recently been increased significantly, while the incidence of other foodborne pathogens remains stable (Baker-Austin et al., 2018), which highlights the importance of establishing effective strategies to control and prevent *V. parahaemolyticus* infection.

V. parahaemolyticus harbors two chromosomes encoding two type 3 secretion systems (T3SS1 and T3SS2) (Calder et al., 2014). Initial studies suggested that thermostable direct hemolysin (TDH) was a virulence factor (Osawa et al., 2002; Baker-Austin et al., 2010). Later studies demonstrated that T3SS2 encoded by a cluster of over 25 genes on chromosome 2 was critical for *V. parahaemolyticus* to cause gastrointestinal diseases by using an infant rabbit infection model (Hubbard et al., 2016), which recapitulated many features of infection in humans (Herrington et al., 1988). T3SS2 is not active under the environmental

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growth condition and can be activated upon stimulation by bile salts (Kodama et al., 2010; Li et al., 2016). T3SS2 secretes and translocates effectors directly into host cells to hijack the signaling pathways for the benefits of bacterial colonization in the small intestine (Trosky et al., 2004; Zhang et al., 2012; Zhou et al., 2013). For example, as one of the effectors of T3SS2, VopV could directly bind to both actin and filamin of intestinal epithelial cells to elongate the microvilli (Hiyoshi et al., 2011), which is essential for *V. parahaemolyticus* colonization. The effector VopZ could target to TAK1 and thus inhibit NF-kB activity, and cause diarrheal disease (Zhou et al., 2013).

Recent transposon inserting sequencing analysis revealed a transmembrane transcriptional factor (ToxR), which is responsible for bile-mediated activation of T3SS2 and the virulence of *V. parahaemolyticus* in the infant rabbit model (Hubbard et al., 2016). Under the bile salt condition, T3SS2 expression requires the presence of ToxR, which the deletion of ToxR completely eliminated the expression T3SS2 (Hubbard et al., 2016). ToxR was initially identified in *V. cholera* as an important regulator for the virulence (Krukonis et al., 2000; Provenzano et al., 2000). ToxR is localized on the bacterial inner membrane and is composed of cytoplasmic DNA binding region, transmembrane region, and the periplasmic signal recognition region (Miller et al., 1987). In *V. cholera*, ToxR senses the extracellular signal, e.g., organic acid, bile salt, which leads to the activation of the DNA-binding region and alteration of gene expression (Midgett et al., 2017; Lembke et al., 2018). Studies have also shown that ToxR in *V. cholera* can directly regulate expression of outer membrane protein U (OmpU) and OmpT in response to bile salt stimulation (Provenzano and Klose, 2000; Provenzano et al., 2000; Provenzano et al., 2001). In *V. parahaemolyticus*, ToxR plays an important role in regulating virulence, and resisting to stress conditions, particularly under acid stress condition (Zhang et al., 2017, 2018). *V. parahaemolyticus* can survive and replicate in the acidic environment, e.g., gastrointestinal tract. *Salmonella* and *E. coli* were also able to resist the acidic condition (Merrell and Camilli, 2002) suggesting that acid resistant mechanisms are widely present in enteric pathogens. However, the possible mechanisms of acid resistance remain unclear. Considering the importance of ToxR in pathogenesis and acid resistance of *V. parahaemolyticus*, we performed transcriptomic analysis to reveal global gene expression on neutral and acidic conditions in WT and *toxR* mutant strains. We provided a comprehensive view on how acidic condition and ToxR shape the global gene expression in *V. parahaemolyticus*. For the first time, we demonstrated that ToxR positively regulated type VI secretion system 2 (T6SS2) gene expression and bacterial killing activity.

2. Materials and methods

2.1. Strains, plasmids and materials

The bacterial strains and plasmids used in this experiment were shown in Table 1. *V. parahaemolyticus* and the corresponding in-frame deletion mutant or complement strains were cultured at 37 °C in LB medium (containing 1% NaCl) as described previously (Zhou et al., 2013). *Escherichia coli* was cultured in an LB medium at 37 °C. The final concentrations of antibiotics and other inducers were as follows: carbenicillin 50 μg/mL, chloramphenicol 25 μg/mL, kanamycin 50 μg/mL, IPTG 500 nmol/L, and L-arabinose 0.04% (w/v).

2.2. Construction of the pair of toxR deletion mutant and complement strains

The genome of the *V. parahaemolyticus* strain RIMD2210633 was used as a template to design primers (Table 2). The upstream and downstream sequences of *toxR* were amplified and cloned into the suicide plasmid pDM4 as described previously (Zhou et al., 2008, 2010). The recombinant plasmid was transferred to the wild type strain (WT) by conjugation. After double cross-over, *toxR* deletion mutant (Δ*toxR*) was isolated and verified by PCR. To construct the complement strain, *toxR* gene was amplified and cloned into pMMB207 as described previously (Zhou et al., 2008, 2010). Subsequently, pMMB207-*toxR* was transferred to Δ*toxR* to form *toxR*$^+$ strain. Deletion of *ompU* was constructed similarly.

2.3. Growth curve analysis

The WT, Δ*toxR* and *toxR*$^+$ were respectively measured for growth curve under LB culture condition. Overnight culture was separated into fresh LB medium at 1% inoculation. Subsequently, 100 μl culture was taken every 1 h to measure OD_{600} and obtain growth curve for all the strains.

2.4. Acid stress assay

The WT, Δ*toxR* and *toxR*$^+$ were streaked onto TCBS plate and grown overnight. Individual colony were picked and inoculated into fresh LB medium. After 6–8 h growth at 37 °C, fresh culture was separated into fresh LB at 1% inoculation and grown until OD_{600} reached 0.4 − 0.6. In the medium, culture to OD_{600} to 0.4 − 0.6. One milliliter of such culture was centrifuged and the bacterial cell pellet was resuspended with 1 mL LB medium of pH 7.0 and pH 4.5, respectively, and adapted for 1 h. CFU of the survivals under both pH7.0 and pH.4.5 was determined by plate counting.

2.5. Quantitative PCR assay

RNA samples from WT, Δ*toxR* and *toxR*$^+$ were extracted by using Trizol, and quantitative PCR was performed as described previously (Gu et al., 2016). Briefly, RNA (1 μg) was reverse transcribed to cDNA by using random primers. Three independent experiments were performed with specific primer pairs list in Table 2 on Applied Biosystems 7500 (Applied Biosystems, Foster City, CA, USA), and the *gyrB* was used as a negative control.

2.6. RNA-seq analysis

RNA samples from WT and Δ*toxR* were obtained after both strains were grown in LB medium for 4–5 h. WT culture was transferred to LB at pH4.5 for 2 h and RNA samples were also extracted. All the samples contain three biological replicates. RNA samples were then submitted to Genewiz for RNA-seq analysis. EdgeR was used for data analysis. Based on the cutoff of 2-fold, differentially expressed genes (WT vs Δ*toxR* and WT pH4.5 vsWT pH7.0) were obtained. KEGG analysis was performed using KOBAS 3.0 program (Xie et al., 2011).

2.7. Western blotting

Overnight culture of WT, Δ*toxR* and *toxR*$^+$ were inoculated onto fresh LB and grown for 5–6 h. Protein samples were obtained and submitted for SDS-PAGE. Following transfer onto PVDF membrane and blocking, primary antibody (anti-Hcp2) was added and incubated for 2 h. Subsequently, secondary HRP-conjugated goat anti-rabbit antibody was added and developed with ECL chemiluminescence.

2.8. Bacterial killing assay

V. parahaemolyticus (intrinsically resistant to carbenicillin) culture and *E. coli* (MC4100, which carries a tetracycline resistance plasmid) were mixed at the ratio of 4:1 and 50ul of the mixture was spotted on LBS (LB containing 3% NaCl) for 4 h at 30 °C. Subsequently, the mixture was serially diluted to determine the CFU of *V. parahaemolyticus* on LB plate containing carbenicillin and *E. coli* on LB containing tetracycline.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
E. coli		
DH5α λ*pir*	Host for π requiring plasmids	Labortaory collection
SM10 λ*pir*	Host for π requiring plasmids, conjugal donor	Liang et al. (2003)
V. parahaemolyticus		
RIMD 2210633	Clinical isolate. $Carb^r$	Makino et al. (2003)
Δ*toxR*	RIMD2210633, in-frame deletion in *toxR*, $Carb^r$	This study
toxR$^+$	Δ*toxR*, pMMB207 expressing the *toxR-his* gene, $Carb^r$, Cm^r	This study
Δ*ompU*	RIMD2210633, in-frame deletion in *ompU*, $Carb^r$	This study
Plasmids		
pDM4	Suicide vector, *pir* dependent, R6K, *SacBR*, Cm^r	Wang et al. (2002)
pMMB207	IncQ $lacI^q$ Δ*bla* $P_{tac\text{-}lac}$ *lacZa*, Cm^r	Morales et al. (1991)
pDM4::*toxR*	Plasmid for deletion of *toxR*, Cm^r	This study
pDM4::*ompU*	Plasmid for deletion of *ompU*, Cm^r	This study
pMMB207::*toxR*	RBS and *toxR* sequences clones into pMMB207, Cm^r	This study

2.9. Statistical analysis

GraphPad Prism was used for statistical analysis. Student *t*-test was used to determine the significance of differential gene expression. $P < 0.05$ was considered as statistical significance.

3. Results

3.1. ToxR mediates tolerance to acid stress in V. parahaemolyticus

Studies have shown that ToxR is involved in multiple stress responses (Mathur and Waldor, 2004; Merrell et al., 2001). To understand the underlying mechanisms of ToxR-mediated stress response, we constructed *toxR* mutant (Δ*toxR*) and complemented (*toxR*$^+$) strains. Growth curve results showed that there was no growth defect for Δ*toxR* and *toxR*$^+$ compared to the WT under neutral (pH7.0) LB condition (Fig. 1A) (Fig. 1B, top panel). In contrast, under acid stress condition (pH4.5), the survival of Δ*toxR* was significantly reduced by comparing to the WT, and the complementation of Δ*toxR* with wild type *toxR* (*toxR*$^+$) restored the survival ability (Fig. 1B, lower panel), which verified the mediation of the tolerance to acid stress by *toxR* in *V. parahaemolyticus*.

3.2. Transcription of toxR is up regulated under acid stress condition

To further investigate if *toxR* mediating acid stress response was due to its up regulation, we performed qRT-PCR to determine the transcription of *toxR* under neutral and acid stress conditions. The results first showed that there was undetectable *toxR* transcription in Δ*toxR* (Fig. 1C, second bar), verifying that *toxR* was completely knocked out in Δ*toxR*. The results further showed that *toxR* transcription under acid stress condition was significantly higher than that under neutral condition (Fig. 1C, compared the 1st bar to the 4th bar), suggesting that acid stress condition could enhance *toxR* transcription. Transcription of *toxR* in *toxR*$^+$ was much higher than that in WT under neutral or acidic conditions (Fig. 1C, compare the 3rd bar to the 1st and 4th bars). These results indicated that *toxR* could mediate acid tolerance through its own up regulation.

3.3. Overview of global gene expression that are regulated by ToxR and acid stress

The results described above showed that ToxR could mediate *V. parahaemolyticus* stress tolerance (Fig. 1B). To further elucidate the possible mechanisms of ToxR to the acid stress tolerance, we performed

Table 2
Primers used in this study.

Primers	Sequence (5'-3')
ToxRup-F	GAGCGGATAACAATTTGTGGAATCCCGGGAATCAAATGACAGCTTAACGCCAAGA
ToxRup-R	CAGAAGTCGTTCATTTAGTTCTTCTTAGATGGATT
ToxRdown-F	GAACTAAATGAACGACTTCTGACGCAATCGTTGAAC
ToxRdown-F	AGCGGAGTGTATATCAAGCTTATCGATACCGACCTGCGAAAATACGCAATGTCACGT
ToxRout-F	GCCTAAATGTTCGATAACGTCA
ToxRout-R	TTGCTCAACTTTAAGGTCACTG
OmpUup-F	GAGCGGATAACAATTTGTGGAATCCCGGGACCAGTCGTAGGACTAGGTAGTGCGG
OmpUup-R	ACTGGAATTACATCTTATTTGTCCTAGTTAGCTGT
OmpUdown-F	CAAATAAGATGTAATTCCAGTCTTTAGACTTGAATA
OmpUdown-F	AGCGGAGTGTATATCAAGCTTATCGATACCATAACGGCATCGGAACTCGAGGTTT
OmpUout-F	TCGCCACGTAGGGTCATTGGA
OmpUout-R	TTTTCGCCAATGACAGCAACG
ToxR-com-F	TCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGACTAACATCGGCACCAA
ToxR-com-R	TCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGTTTGCAGATGTCTGTTGGA
ToxRRT-F	CTCAAAACCTTGCTCACGCC
ToxRRT-R	CAAAGCGGCAACGAAGTTGT
OmpURT-F	AAAGATGGCGACGCTCAAGA
OmpURT-R	ACACCACCTTGTTCGTTGGT
VPA1027-RT-F	TAGCGCTGCATCCATACGAG
VPA1027-RT-R	GGTACAGCAATGGACCGTGA
VPA1028-RT-F	AAACTCCCCTTTCATCCCCG
VPA1028-RT-R	GTGAGGCAGGCGTAGGTAAA
*gyrB*RT-F	TTACCGTCATGGTGAGCCTG
*gyrB*RT-R	CACGCAGACGTTTTGCTAGG

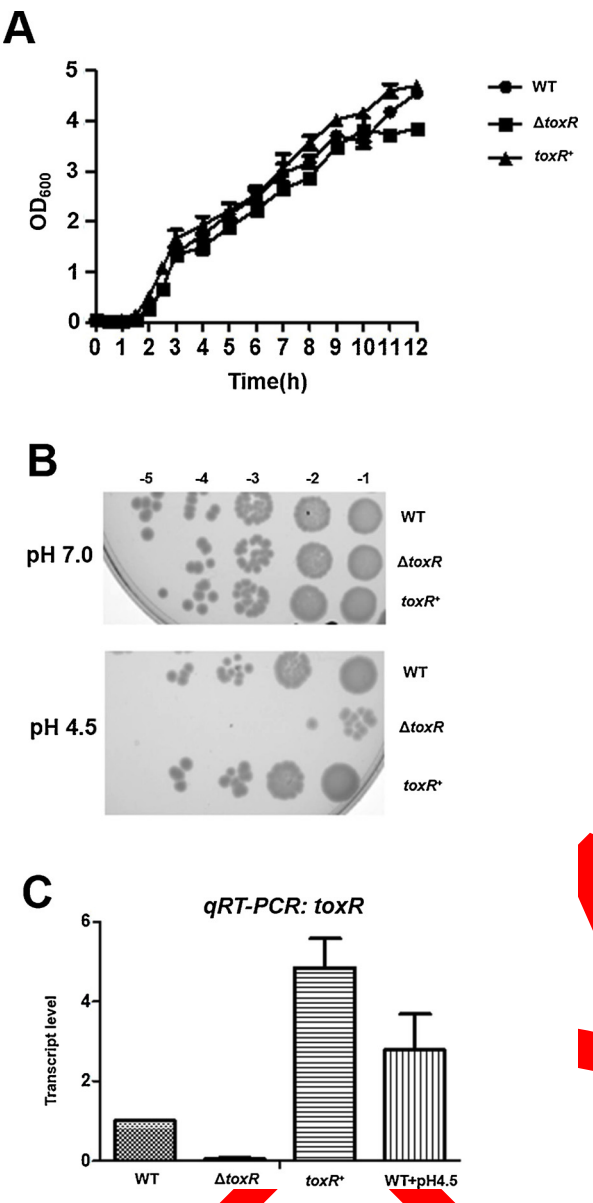

Fig. 1. ToxR regulates acid tolerance. Growth curve of WT, Δ*toxR* and *toxR*$^+$ (A). Growth of WT, Δ*toxR* and *toxR*$^+$ under pH7.0 and pH4.5 (B). qRT-PCR analysis of toxR gene expression (C).

RNA-seq for WT under neutral and acid conditions and Δ*toxR* under neutral condition. We were unable to conduct RNA-seq for Δ*toxR* under acid condition as it was sensitive to acid stress. Under neutral condition, the expression of 279 genes was altered in Δ*toxR* compared to WT (Fig. 2A). Among them, 145 genes were up regulated, while 134 genes were down regulated at the cutoff of 2-fold (Fig. 2A). We then compared genes expression between neutral and acid conditions for WT and the results showed that expression of 757 genes was altered under acid condition by comparing to the neutral condition (Fig. 2B). Among those of 757 genes, 285 genes were up regulated, while 472 genes were down regulated under the acid condition compared to the neutral condition (Fig. 2B). Further analysis showed that 74 genes were regulated by both ToxR and acid stress (Fig. 2C). These results indicated that acid condition affected the expression of these 74 genes potentially through ToxR.

3.4. KEGG analysis of genes that are regulated by acid condition

We performed KEGG analysis to further elucidated the pathways, which were altered under acid stress condition. The results showed that a total of 82 genes in the biosynthesis pathway of secondary metabolites were altered (46 genes were down regulated and 36 genes were up regulated) under acid condition by comparing to neutral condition (Fig. 3A). A total of 82 genes in the pathway of microbial metabolism in diverse environment (43 genes were down regulated and 39 genes were up regulated) was also altered under acid condition (Fig. 3A). Carbon metabolism pathway included 27 down regulated genes and 22 up regulated genes under acid condition (Fig. 3A). Biosynthesis of antibiotics pathway included 36 down regulated genes and 30 up regulated genes under acid condition (Fig. 3A). Fatty acid biosynthesis and metabolism pathways were up regulated under acid condition (Fig. 3A). Majority of genes (n = 19) in the quorum sensing (QS) pathway were down regulated, whereas a little number of genes (n = 3) in the QS were up regulated under acid condition (Fig. 3A). Overall, these results indicated that acid stress condition could alter multiple metabolic and QS pathways in *V. parahaemolyticus*.

3.5. KEGG analysis of genes that are regulated by ToxR under neutral condition

Since acid stress condition up regulated ToxR expression (Fig. 1C), we determined if alteration of pathways under acid condition would be potentially dependent on ToxR. We performed KEGG analysis to reveal the pathways, which were altered in Δ*toxR* by comparing to WT. The most prominent pathway for the majority of genes were co-regulated by the acid condition and ToxR was by QS (12 genes in QS were up regulated, 1 gene in QS was down regulated in Δ*toxR* compared to WT) and fatty acid metabolism (5 genes in fatty acid metabolism pathway were down regulated in Δ*toxR* compare to WT) (Fig. 3B). These results indicated that acid stress condition could regulate QS and fatty acid metabolism potentially through ToxR. There were significantly lower number of genes in the biosynthesis and metabolisms pathways that were altered in Δ*toxR* when compared to those altered under acid condition. For example, only 5 genes (two were up regulated and three were down regulated) in the pathway of microbial metabolism in diverse environment were altered in Δ*toxR* comparing to WT (Fig. 3B). Only 6 genes (three were up- and three were down regulated) in the biosynthesis of secondary metabolism were altered Δ*toxR* comparing to WT. These results indicated that the regulation of biosynthesis and metabolism pathways under acid condition were independent of ToxR. The most significant pathways altered in Δ*toxR*, but not under acid condition were cationic antimicrobial peptide (CAMP) resistance, which 14 genes were down regulated and 14 genes were up regulated, while the bacterial chemotaxis were 9 genes down regulated and 2 genes up regulated (Fig. 3B). Flagella assembly and bacterial chemotaxis pathways were up regulated in Δ*toxR* compared to WT (Fig. 3B). These results indicated that CAMP, chemotaxis and flagella could be regulated by ToxR, but not by acid stress condition.

3.6. Regulation of T3SS1 and T6SS2 by ToxR and acid condition

In addition to the KEGG analysis, we also examined the distribution of the altered genes in the chromosomes of *V. parahaemolyticus*. There were a cluster of genes (encoding T3SS1) that were up regulated in Δ*toxR* compared to WT, but shown no significant difference in the acid stress condition (Fig. 4A). These results indicated that T3SS1 genes were regulated by ToxR, but not by acid stress condition. Besides, the ORFs in the T6SS2 that are regulated by *toxR* and acid condition were shown in Fig. 4B. These results indicated that T6SS2 genes are not only regulated by ToxR, but also by acid stress condition.

3.7. ToxR regulates acid stress potentially through OmpU

In addition to T3SS1, T6SS2 and other KEGG pathways, RNA-seq also revealed a gene encoding outer membrane protein U (*ompU*) which

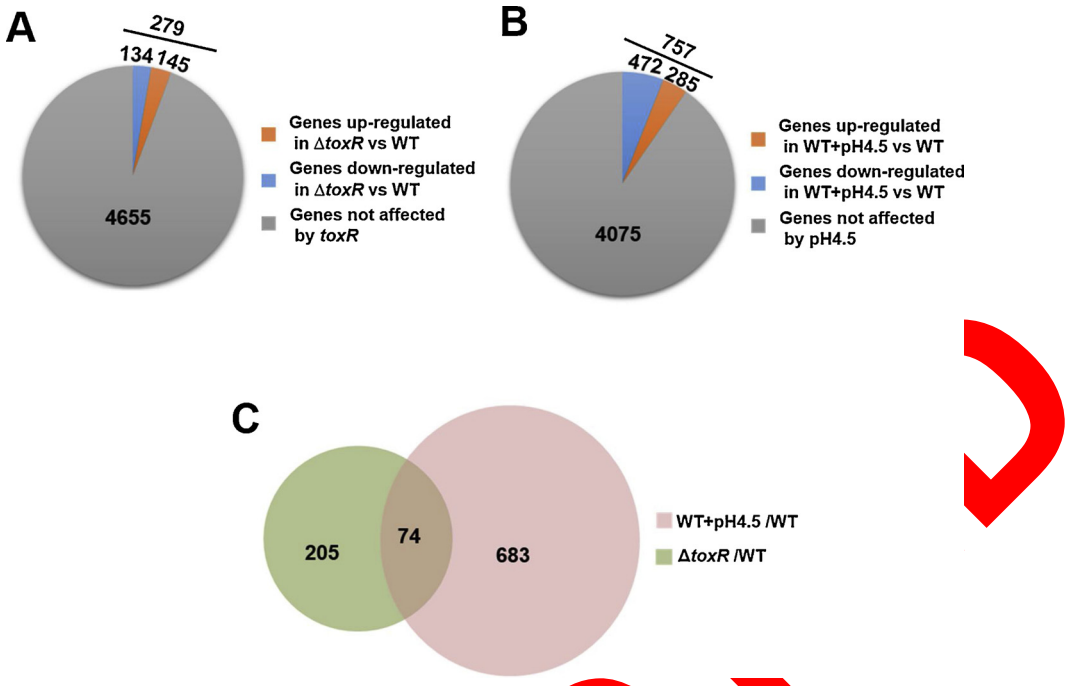

Fig. 2. Regulation of gene expression by ToxR and acid stress condition. Number of genes that was regulated ToxR (A). Number of genes that was regulated by acid stress condition (B). Number of genes that was co-regulated by ToxR and acid stress condition (C).

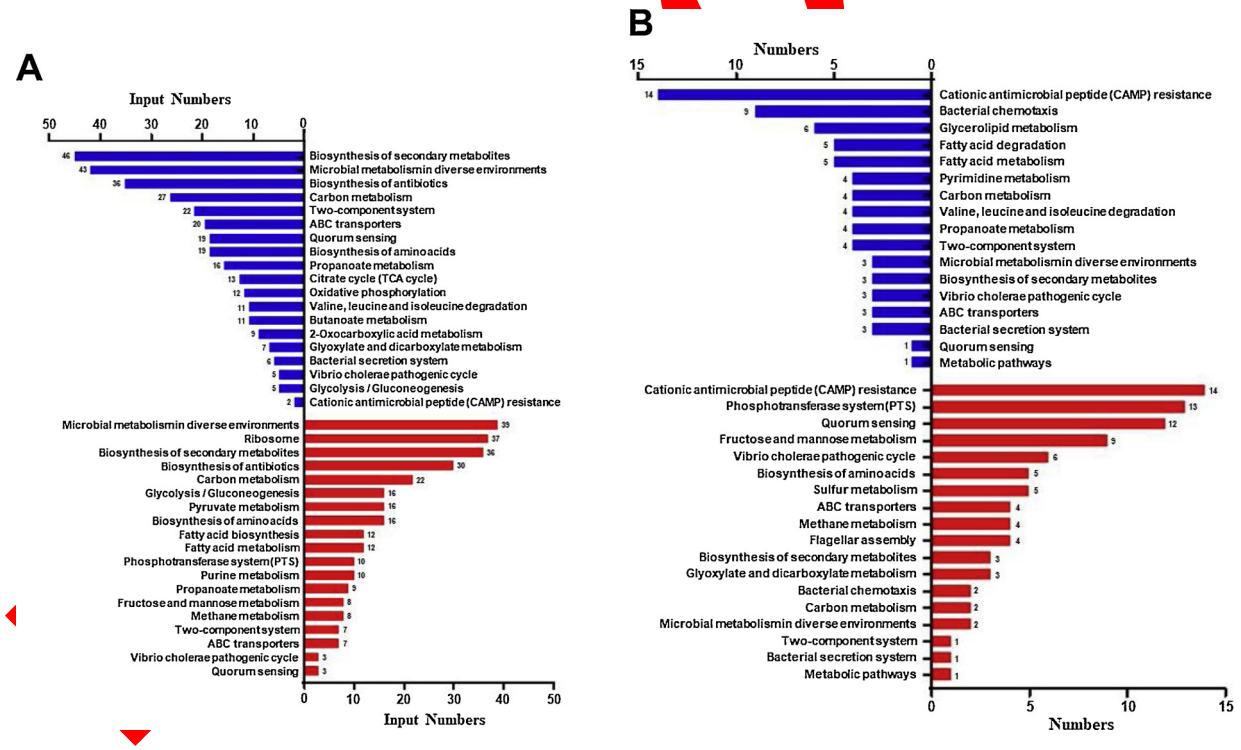

Fig. 3. KEGG analysis of pathways that are regulated by acid stress condition (A) and ToxR (B). Blue represents pathways that are down regulated and red represents pathways that are up regulated. Number on each bar represents the number of genes that was altered in each pathway.

was significantly down regulated in Δ*toxR* by comparing to WT (Fig. 5A). Furthermore, *ompU* was up regulated under acid stress condition when compared to neutral condition (Fig. 5A, compare the 1st and 3rd bars). Quantitative RT-PCR results demonstrated that *ompU* was significantly down regulated in Δ*toxR* by comparing to WT, while the complementation of Δ*toxR* with a wild type *toxR* gene fully restored the expression of *ompU* (Fig. 5B). Similarly, qRT-PCR showed that *ompU* expression was up regulated under acid condition when compared to the neutral condition (Fig. 5B). These results indicated that *ompU* could be co-regulated by ToxR and acid stress condition. These results also indicated that reduction of acid tolerance in Δ*toxR* could be due to the reduced expression of *ompU*. Thus, we constructed *ompU* deletion mutant Δ*ompU*. Under neutral LB condition, there was no significant growth difference between WT and Δ*ompU* (Fig. 5C, upper panel). However, under acid stress condition, the growth of Δ*ompU* was reduced by 10-fold when compared to that of WT (Fig. 5C). In additional,

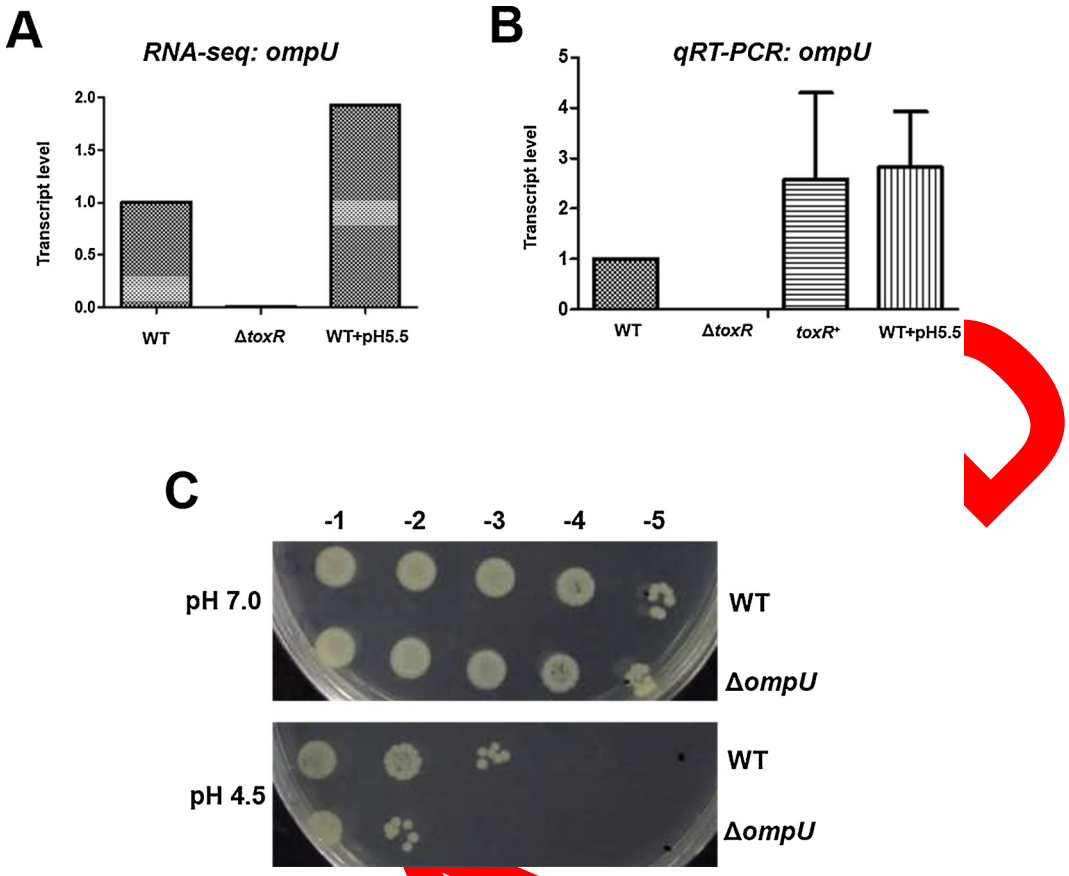

Fig. 4. Genes in T3SS1 (A) and T6SS2 (B) cluster regulated by ToxR and acid stress condition. In panel A, 1–47 represents ORF Vp1656-Vp1702; In panel B, 1–24 represents ORFs Vpa1024-Vpa1047.

the previous study demonstrated that the complementation of *toxR* mutant with *ompU* would fully restore its acid tolerance (Whitaker et al., 2012). These results indicated that ToxR could regulate acid tolerance potentially through OmpU.

3.8. ToxR regulates bacterial killing activity of T6SS2

RNA-seq revealed that ToxR could regulate the expression of T6SS2 gene cluster (Fig. 5B). We further measured Hcp2 (one of the T6SS2 components) expression in both WT and Δ*toxR* by western blot, and the results showed that Hcp2 was expressed in WT, but not in Δ*toxR*. Complementation of Δ*toxR* with a wild type *toxR* restored Hcp2 expression to WT level (Fig. 6A). Quantitative PCR also showed that expression of *vpa1027* and *vpa1028* (two ORFs in T6SS2) was reduced in Δ*toxR* compared to WT (Fig. 6B). As one of the important functions of T6SS is interbacterial killing, we determined if ToxR could affect bacterial killing of *V. parahaemolyticus* against *E. coli*. The results showed that the killing effect of Δ*toxR* was reduced by 10-fold compared to the WT (Fig. 6C). These results indicated that ToxR could mediate interbacterial killing through regulating the expression of T6SS2 genes. Interestingly, Δ*toxR* complement strain (*toxR*$^+$) had even more reduced killing effect when compared to WT even though Hcp2 expression was fully restored to the WT level (Fig. 6A).

4. Discussion

To cause gastrointestinal diseases, enteric pathogens have to survive the extreme acidic environment in the stomach and replicate in the mildly acidic environment in the small intestine. In *V. cholera*, ToxR, a membrane-bound transcriptional factor, is essential for the survival in the acidic condition (Provenzano et al., 2000). In *V. cholerae*, ToxR is also critical for bacterial survival under stress conditions including acidic and bile salt conditions (Goulart et al., 2010; Midgett et al., 2017). Studies have shown that ToxR mediates tolerance to acid through the outer membrane protein U (Merrell et al., 2001) as over expression *ompU* in *toxR* mutant fully restores the defects of *toxR* mutant in acid tolerance (Merrell et al., 2001). Our RNA-seq and qRT-PCR results were consistent with these previous studies (Fig. 5A and B). We also demonstrated that ToxR-mediated acid tolerance might be due its own and OmpU up regulation. In addition to mediating acid tolerance, ToxR was shown to play a critical role in activating the expression of T3SS2 (Hubbard et al., 2016), a main virulence factor, under bile salt condition. Thus, *toxR* mutant was dramatically attenuated in both colonization and gastrointestinal symptoms in infant rabbit model (Hubbard et al., 2016). These studies indicated that ToxR is an important virulence factor in *V. parahaemolyticus*. However, the role of ToxR in regulation of global gene expression in *V. parahaemolyticus* has not been elucidated. Therefore, in this study, we examined transcriptome of both WT and *toxR* mutant, and also compared the transcriptomic alteration under acid stress condition with an expectation to provide comprehensive understanding of ToxR in mediating signaling pathways in *V. parahaemolyticus*.

Our RNA-seq analysis showed that multiple biosynthesis and metabolism pathways were altered under the acid stress condition compared to the neutral condition. For example, fatty acid metabolism pathway was down regulated under acid stress condition. RNA-seq analysis also showed that fatty acid metabolism pathway was up regulated in *toxR* mutant compared to the WT. Coupling of the results that ToxR was up regulated under acid stress condition, it could be that the down regulation of fatty acid metabolism under acidic condition could be due to the up regulation of ToxR. Thus, ToxR could be a negative regulator for fatty acid metabolism. These results also suggested

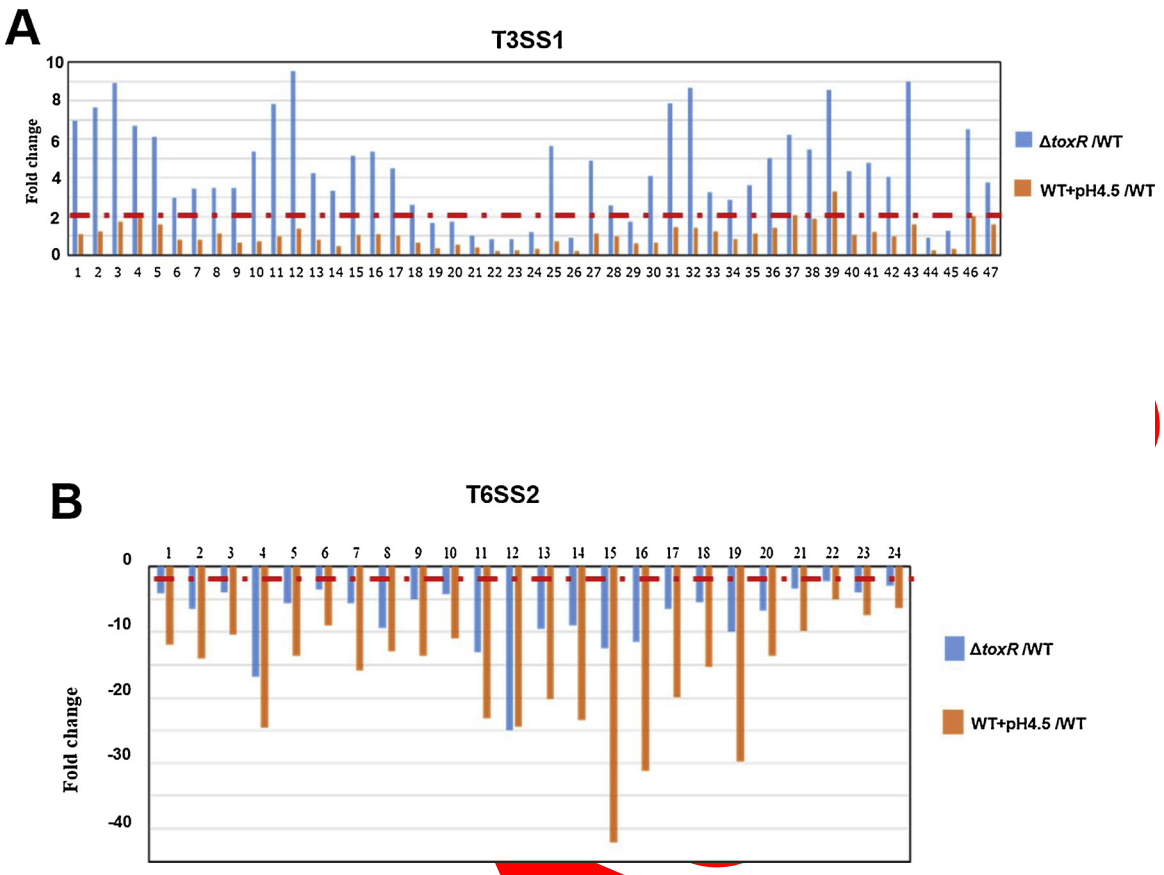

Fig. 5. ToxR and acid stress condition regulate OmpU expression. OmpU is regulated by ToxR and acid stress condition as revealed by RNA-seq (A). OmpU is regulated by ToxR and acid stress condition as revealed by qRT-PCR. OmpU is important for acid stress tolerance.

that reduction of fatty acid metabolism might be beneficial to protect bacterial cells under acid stress condition. The majority of QS genes could be co-regulated by acid stress condition and ToxR indicating that acid-mediated regulation of QS was dependent on ToxR. Biosynthesis of secondary metabolites pathway could be both up regulated and down regulated under acidic condition, indicating that synthesis of some secondary metabolites might be increased while synthesis of other secondary metabolites was reduced. Regulation of biosynthesis of secondary metabolites pathway by ToxR was not evident as there are only three genes in this pathway were altered after *toxR* knockout. These results indicated that acid-mediated regulation of biosynthesis of secondary metabolites pathway might be independent of ToxR. Similarly, the regulation of microbial metabolism in diverse environment, biosynthesis of antibiotics, biosynthesis of amino acids and carbon metabolism pathways by acid stress condition could also likely be independent of ToxR, as there were few or no genes in these pathways that were altered in Δ*toxR* compared to WT. Previous studies showed that ToxR mutation led to increased expression T3SS1 (Osei-Adjei et al., 2017), which was consistent with our results that the entire operon of T3SS1 was up regulated in Δ*toxR* compared to WT (Fig. 5A). These results indicated that ToxR could negatively regulate T3SS1 gene expression. However, under acid stress condition in which ToxR was up regulated, T3SS1 gene expression was not further decreased (Fig. 5A). This is probably because T3SS1 was very minimally expressed under regular LB condition (Zhou et al., 2008) and thus the negative effect on T3SS1 by up regulated ToxR under acidic condition was not evident.

V. parahaemolyticus harbors two T6SSs (T6SS1 and T6SS2) encoded on chromosome 1 and chromosome 2, respectively. Previous studies have shown that deletion of ToxR can lead to the up regulation of T6SS1 gene expression (Zhang et al., 2017). However, in our RNA-seq analysis, we did not observe up regulation of T6SS1 genes in Δ*toxR* compared to WT. This is probably because our RNA-seq studies have used LB medium containing 1% salt, while previous studies have used high salt condition to culture *V. parahaemolyticus*. It is known that high salt conditions can activate T6SS1, while low salt represses T6SS1 gene expression (Salomon et al., 2013). Thus, low salt culture condition used in this study might not permit the up regulation of T6SS1 in Δ*toxR*. Interestingly, we observed a dramatic down regulation of T6SS2 gene expression in Δ*toxR* when compared to WT. More importantly, we observed that bacterial killing effect of Δ*toxR* was decreased by approximately 10-fold when compared to WT (Fig. 6C), demonstrating that ToxR positively regulated T6SS2. This is the first time that T6SS2 gene expression and interbacteria killing activity have been demonstrated to be regulated by ToxR. Surprisingly, we found that complement of Δ*toxR* with a wild type *toxR* gene did not restore the bacterial killing effect although expression of key T6SS2 gene was restored. We speculated that over expression of *toxR* in *toxR*$^{+}$ strain may alter other signaling circuit resulting in the repression of interbacteria killing effect. In addition to metabolism, T6SS2 and T3SS1, we also observed that ToxR could regulate bacterial chemotaxis, cationic antimicrobial peptide resistance and flagella assembly (Fig. 7), highlighting a multiple role of ToxR in *V. parahaemolyticus*. Interestingly, these pathways are not regulated by acid stress condition (Fig. 3A and B).

In summary, transcriptomic analysis revealed that acid stress mediates multiple signaling pathways that could be dependent and independent of ToxR. We also for the first time showed that ToxR positively could regulate T6SS2 and over expression ToxR further decreases T6SS2 bacterial killing effect of *V. parahaemolyticus*. We also revealed previously unappreciated signaling pathways that were regulated by ToxR (e.g., bacterial chemotaxis, flagella assembly and T6SS2), which would open up new directions for future research.

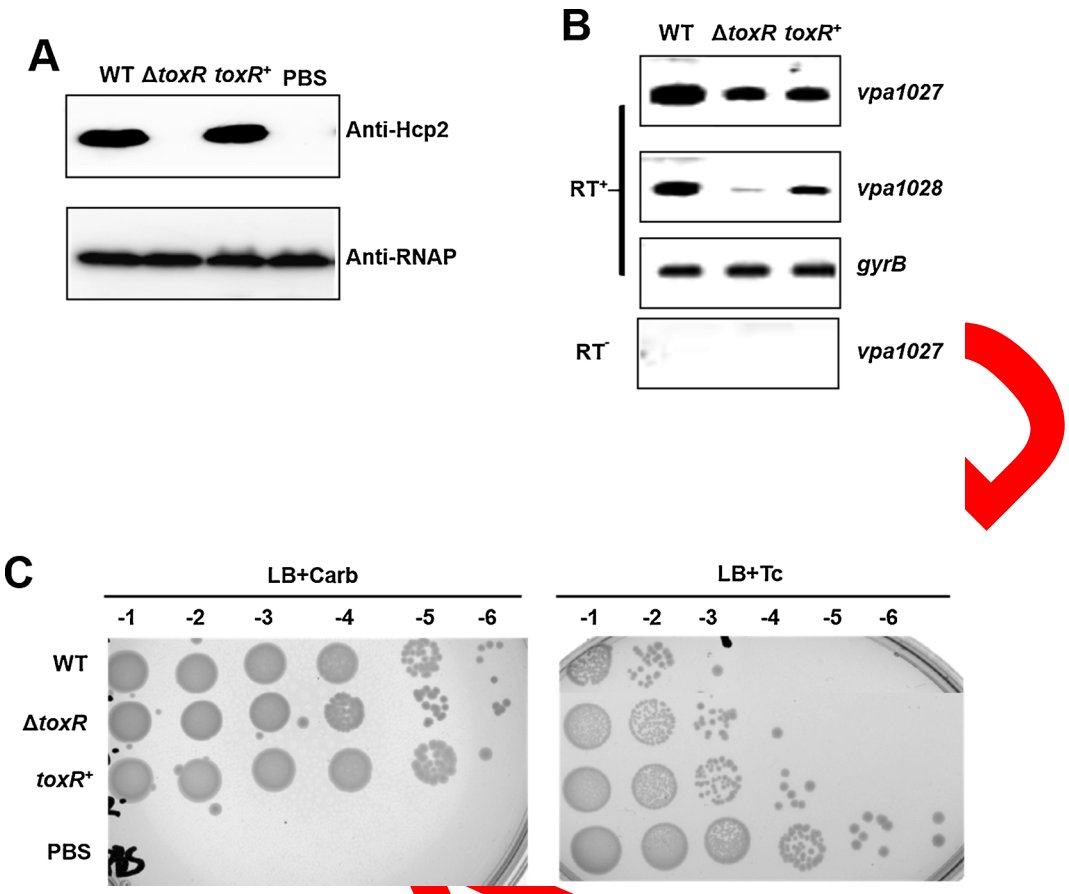

Fig. 6. ToxR regulates T6SS2 gene expression and bacterial killing effect. Western blot shows that ToxR positively regulates Hcp2 expression (A). qRT-PCR shows that ToxR positively regulates vpa1027 and vpa1028 gene expression (B). ToxR plays an important role in bacterial killing (C).

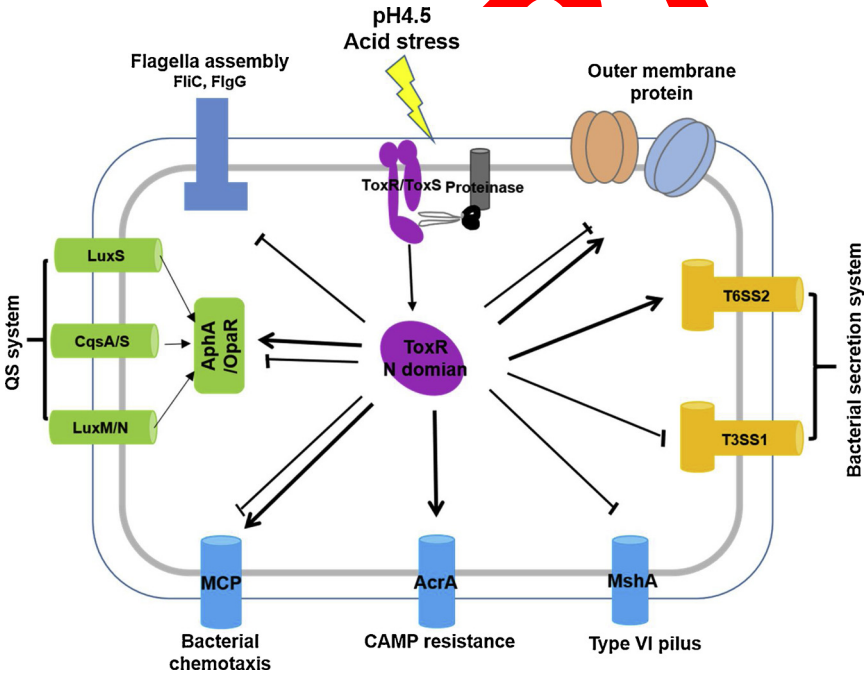

Fig. 7. Schematic model of ToxR and acid stress regulatory network. Under acid stress condition, ToxR is cleaved and the functional N-terminal domain is released to regulate gene expression in the signaling pathways including quorum sensing, bacterial chemotaxis, CAMP resistance, flagella assembly, type VI pilus, T3SS1, T6SS2 and outer membrane proteins. Acid stress condition regulates QS through ToxR. Flagella, CAMP, chemotaxis, T3SS1 are regulated by ToxR, but not by acid stress.

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