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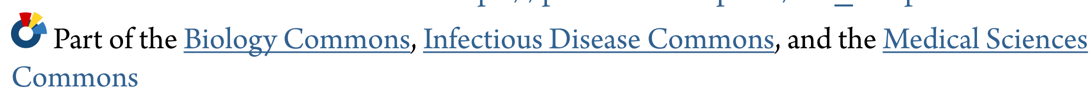
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Defining the role of two newly identified proteins in the *Vibrio vulnificus* TonB2 system

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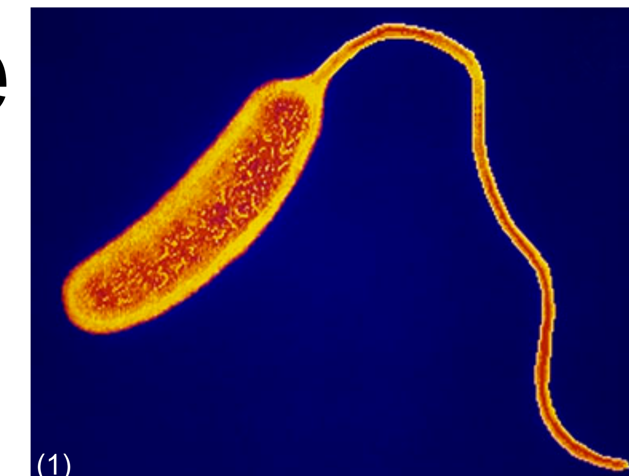

INTRODUCTION

Vibrio vulnificus is a Gram-negative marine bacteria pathogen that preferentially resides in warm saltwater environments, flourishing on coastal areas during summer months (2). As the most fatal foodborne pathogen in the USA, 1 in 7 of about 200 annual cases result in death (2,7). Infection by *Vibrio vulnificus* can be caused by sea water contact with open wounds or ingestion of raw shellfish, which bio-accumulate *V. vulnificus* to high toxicity levels. Rising seawater temperatures may provide potential conditions for an increased infection rate of *V. vulnificus* in the future, as 85% of cases occur in seasonally warm waters (2). While most healthy individuals experience diarrhea, vomiting, and abdominal pain, infected individuals with elevated serum iron levels due to pre-existing chronic conditions can result in fatal septicemia (3).

Vibrio vulnificus, like most life, is dependent on the cell's uptake of iron for survival and full pathogenicity, linking elevated iron levels to fatality in infections. Within the cell, iron plays important roles across a variety of biological processes such as redox reactions, signaling, and metabolism (4). This highly significant process of iron uptake has lead to organismal production of specialized siderophore chelators, which retrieve external iron from the environment with high affinity.

In gram-negative bacteria, siderophores return to the cell through outer membrane receptors powered by the TonB energy-transduction system. The TonB system is typically made up of three transmembrane proteins—TonB, ExbB, and ExdB (6). *Vibrio* species are unique in containing three variations of TonB, two of which contain three additional proteins: TtpC, Orf1, and Orf6 (6). The precise location, structure, and function of Orf1 and Orf6 are still being investigated. The TonB1 and TonB2 systems are preferentially transcribed *in vivo* vs *in vitro*, indicating that transcription levels may change in different environments (10). In a microassay of TonB systems in *V. vulnificus*, TonB2 appeared to obtain nearly an 8-fold increase in transcription when grown in low iron conditions, further support of the hypothesis that transcription levels of TonB are responsive to iron levels of the environment, and can be further analyzed through reverse-transcriptase PCR of the genes within the system (12).

Research has indicated that the primary siderophore uptake pathway may utilize the TonB2 system and may have individual effects on other cellular functions, such as motility. In *Pseudomonas aerigunosa,* mutants with impaired TonB3 systems also displayed motility defects, while TonB1 and TonB2 had little to no effect on motility (9). Research has shown motility defects in *V. vulnificus* when all three TonB systems were impaired, but the systems have yet to be assessed independently (10).

The TonB systems may also play a role in the vulnerability to antibiotics, due to it's control in bringing siderophores in and out of the cell. In *Pseudomonas aerigunosa*, mutants with ineffective TonB systems showed hypersusceptibility to antibiotics (11). In this case, the TonB system also functioned as an efflux pump, driving antibiotics out of the cell, allowing higher levels of survival in the presence of antibiotics, which could also be the case for *V. vulnificus.*

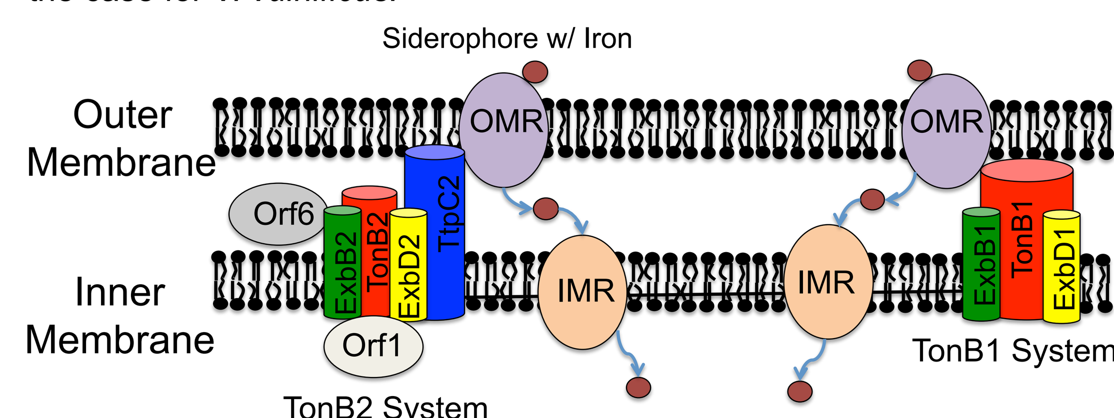

Figure 1. The organization of the TonB1 and predicted organization of the TonB2 systems in *Vibrio vulnificus.*

Table 1. TonB2 system homology among other *Vibrio* species

Vibrio species	Orf1	TtpC2	ExbB2	ExbD2	TonB2	Orf6
V. parahaemolyticus	51	58	61	86	42	36
V. alainolyticus	49	60	60	86	43	39
V. cholerae	53	58	68	86	47	35
V. anguillarum	49	58	62	85	47	37

Basic Local Alignment Search Tool (BLAST)

Orf1 Conserved Domain:
•DUF3450 = Domain of unknown function

Orf6 Conserved Domain:
•YfgC = TRP repeats, suggests protein-protein interactions.

HYPOTHESIS

Orf1 and Orf6 are essential components of the TonB2 iron uptake system of *Vibrio vulnificus.*

MATERIALS AND METHODS

Reverse Transcription PCR (RT-PCR)

The *Vibrio vulnificus* wild type strain was grown in tryptic soy broth with 2.5% NaCl (TSBS). Dipyridyl (Dip) and ferric ammonium citrate (FAC) were added to create an iron poor and an iron rich environment, respectively. The cultures were grown until they reached an Optical Density (OD) at 600nm of about 0.3 (about 2 hours) and were harvested.

RNA was harvested following Qiagen RNAprotect and RNeasy protocols. The cells were lysed enzymatically and treated with a Qiagen DNase to ensure only RNA was harvested. RNA was purified over a spin column and eluted in nuclease free water. Samples were also treated with Turbo DNase following ThermoFisher Turbo DNA-free protocol to ensure degradation of any DNA that may be in the sample.

Purified RNA samples underwent reverse transcription to synthesize cDNA using the Qiagen QuantiTect Reverse Transcription protocol. For each reaction, there was a control reaction run that underwent the same protocol, but in the absence of reverse transcriptase.

Samples of cDNA underwent PCR treated with primers for *orf1*, *orf6*, and *tonB2* genes. As a control, primers for 16s rRNA and glyceraldehyde 3-phosphate dehydrogenase (*gapDH*) housekeeping genes were used. All PCR products were run in gels. Pictures of the gels were taken under UV light with a GelLogic camera at exposures of 0.1, 0.2, 0.5, and 1.0 seconds.
All images of gels were analyzed using imageJ software. To quantify the brightness of the bands, equal areas in gel lanes were analyzed for the changes in pixel color. Curves that were representative of these changes were integrated to achieve a numerical value. These values were then compiled based upon media condition and primer set used.

Antibiotic Disc Diffusion Assays

Vibrio vulnificus strains were grown in nutrient rich media, TSBS, overnight and then plated onto large petri dishes containing nutrient poor media, CM9. Antibiotic discs (Hardy Diagnostics) were placed on top of bacterial lawns and allowed to grow at 37°C for 24 hours. Clearings were then measured and average. Duplicate plates were created each day and the experiments were repeated on multiple days.

Motility Assays

Vibrio vulnificus strains were grown in nutrient rich media, TSBS, overnight and then subcultured to an OD_{600} of 0.02 into fresh TSBS. Cultures were allowed to grow until mid-Log phase (OD_{600} ~0.7) and were then normalized to to an OD_{600} of 0.6 using TSBS. Cultures were then spotted (2µl) onto large petri dishes containing Heart Infusion agar with a NaCl concentration of 2.5% and an agar concentration of 0.3% (to allow for motility on solid surfaces). After 24hrs at 37°C, the diameters of each colony was measured. Duplicate plates were created each day and the experiments were repeated on multiple days.

RESULTS

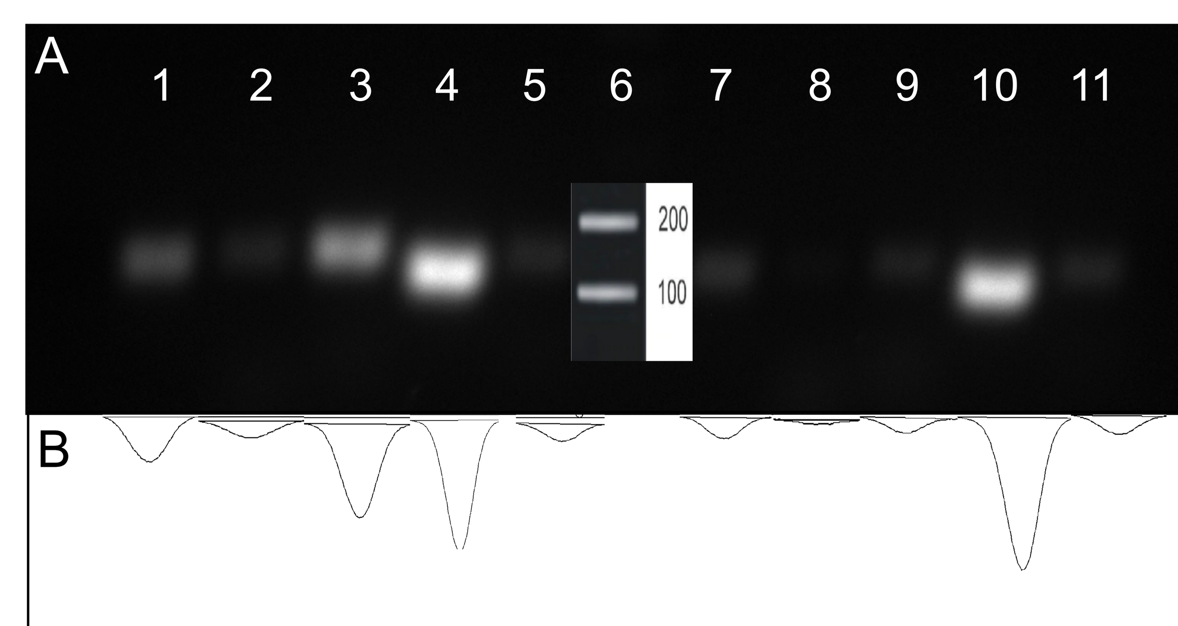

Figure 2. Reverse Transcription PCR (RT-PCR) analysis of Wild Type *V. vulnificus* grown in iron-deficient and iron-rich conditions. (A) Lanes 1-5=RT-PCR samples containing the iron chelator Dipyridyl (Dip) (50mg/mL); Lane 6=Molecular 1kb ladder (Invitrogen); Lanes 6-11=RT-PCR samples containing Ferric Ammonium Citrate (FAC) (250mg/mL). The order of genes tested under each condition: *orf1, orf6, tonB2, 16s rRNA, gapDH*. (B) ImageJ plot analysis with peaks representing band intensity. Values are shown in Figure 3.

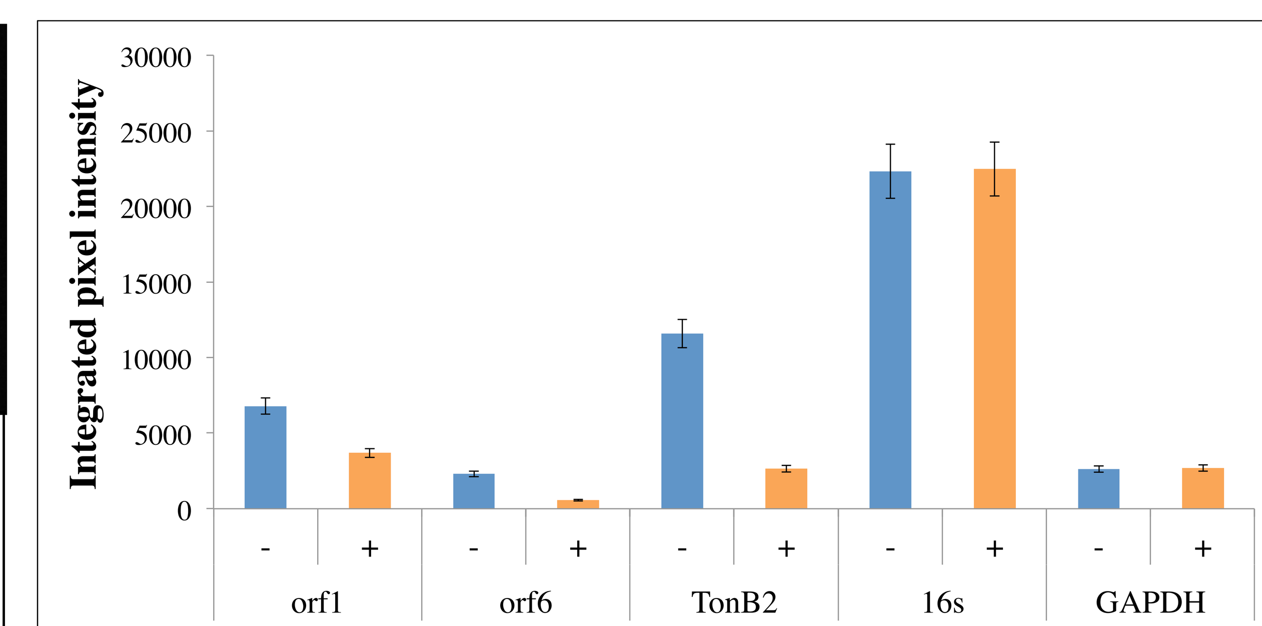

Figure 3: RNA expression of *V. vulnificus* genes in iron-rich (+) compared to iron-deficient (-) conditions. Housekeeping genes 16s rRNA and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as controls.

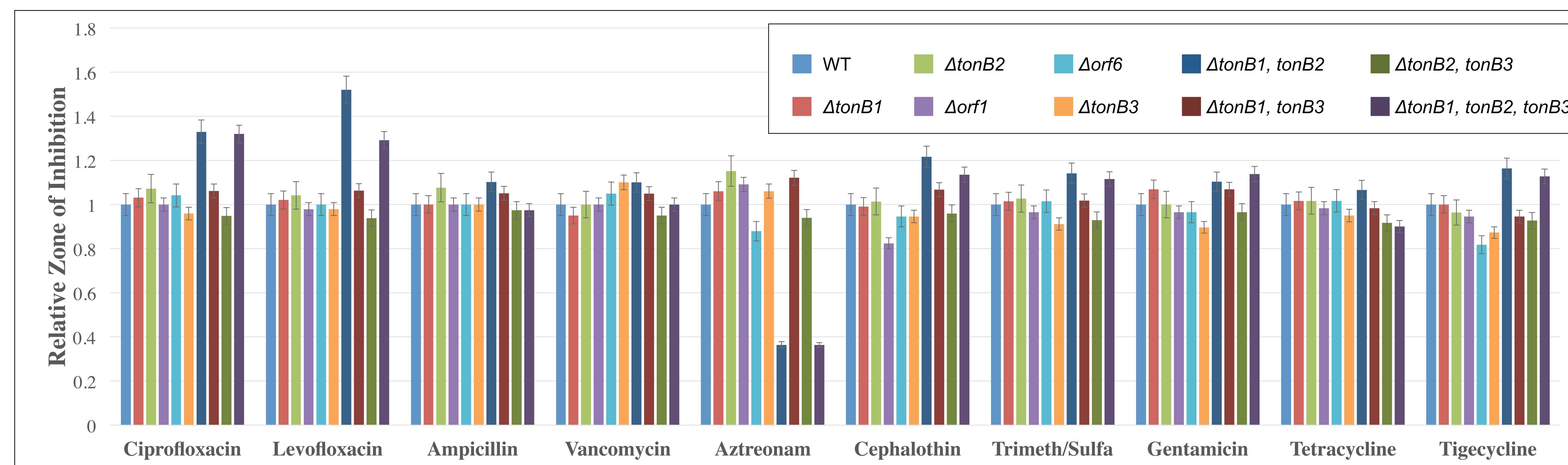

Figure 4. Antibiotic disc diffusion assays of various mutant strains of *V. vulnificus*.

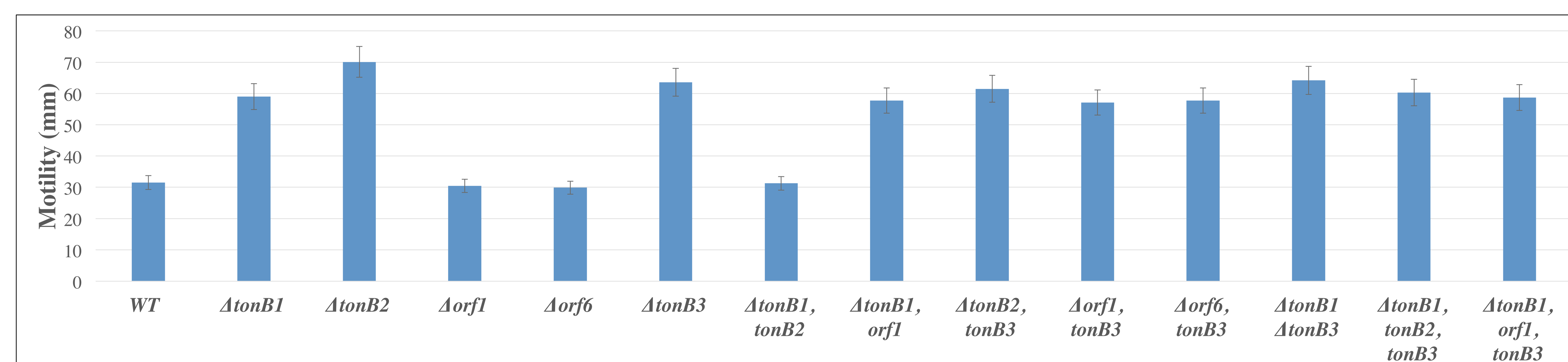

Figure 5. Motility assays of various mutant strains of *V. vulnificus.*

CONCLUSION

- RNA expression of *orf1*, *orf6*, and *TonB2* in iron-deficient conditions is significantly greater than iron-rich conditions.
- RNA expression for the housekeeping genes 16s rRNA and GAPDH did not vary significantly between iron-rich and iron-deficient conditions.
- Both the TonB1 and TonB2 system must be compromised to see a change in drug sensitivity.
- The TonB1 and TonB2 system appear to power a drug efflux system for the export of Fluoroquinolones (Ciprofloxacin, Levofloxacin, and others not shown).
- TonB1 and TonB2 appear to power an uptake system of Aztreonam.
- All mutations within the TonB systems, with the exception of *orf1, orf6,* and the double mutant *tonb1/tonb2,* allow the cells to move more quickly over surfaces.

FUTURE DIRECTIONS

•Complementation studies to look at motility when gene function has been restored.
•Protein localization of Orf1 and Orf6 using tagged versions of each protein and visualization through Western Blots.
•*In vivo* formaldehyde crosslinking followed by Western Blots to determine potential complexes with other TonB2 system proteins.
•Determine specific interaction sites of Orf1 and Orf6 in the TonB2 system.
•Virulence assays to determine if either Orf1 or Orf6 effect overall pathogenicity.

ACKNOWLEDGEMENTS

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