Protozoan predation drives the adaptive evolution of *Vibrio cholerae*

by

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Certificate of original authorship

I, Md Mozammel Hoque declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Other Publications

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Abbreviations

AI	Autoinducer
ANOVA	Analysis of variance
ARMS	Amplification refractory mutation system
ATCC	American type culture collection
BLAST	Basic Local Alignment Search Tool
C	Celsius
cAMP	Cyclic adenosine monophosphate
CI	Competition index
COGs	Cluster of orthologous groups
c-di-GMP	Cyclic di-guanosine monophosphate
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
cm	Centimetre
CT	Cholera toxin
DNA	Deoxyribonucleic acid
g	Gravitational force
GFP	Green fluorescent protein
h	Hour
HAP	Hemagglutinin protease
HMDS	Hexamethyldisilazane
HTH	Helix turn helix
INDELS	Insertion and Deletions
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Lysogeny Broth
min	Minute
ml	Millilitre
µl	Microlitres
mm	Millimetre
mM	Millimolar
µM	Micromolar
MOI	Multiplicity of infection

NCBI	National Center for Biotechnology Information
nM	Nanomolar
NO	Nitric oxide
NSS	Nine salts solution
nsSNPs	Non-synonymous single nucleotide polymorphisms
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffer saline
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PYG	Proteose yeast extract
QS	Quorum sensing
qRT-PCR	Quantitative real-time polymerase chain reaction
rpm	Revolutions per minute
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SEM	Scanning Electron Microscopy
SNP	Single nucleotide polymorphisms
sSNPs	Synonymous single nucleotide polymorphisms
T6SS	Type VI secretion system
TCP	Toxin-coregulated pili
VPS	Vibrio polysaccharide
WHO	World Health Organization
WT	Wild type

Abstract

Protozoa are unicellular eukaryotic organisms that play an important role in controlling bacterial population structure and composition in the environment. Heterotrophic protozoa survive by feeding on bacteria. Many pathogenic bacteria are capable of resisting predation and some are able to multiply inside of these hosts. To resist predation, bacteria have evolved many mechanisms or defensive traits and often these traits contribute to the persistence of the pathogen in the environment and give rise to virulence upon encounter with human and animal hosts.

The waterbourne bacterium, *Vibrio cholerae,* is the etiological agent of the disease cholera and shares an ecological niche with the free-living amoeba, *Acanthamoeba castellanii*. Here, the experimental evolution of the model pathogen *V. cholerae* with *A. castellanii* was performed for three months with the aim to increase our understanding of the effects of long-term protozoan predation on the evolution of virulence-related traits and how that impacts environmental persistence.

Long-term adaptation with the amoeba host leads to phenotypic and genetic variability in *V. cholerae*. Late-stage amoeba adapted *V. cholerae* showed trade-offs among multiple phenotypic traits that contribute to their enhanced intracellular survival and fitness in amoeba. Whole genome sequencing and mutational analysis revealed that these altered phenotypes and improved fitness were linked to non-synonymous mutations in conserved regions of the flagellar transcriptional regulator, *flrA.* Transcriptomic analysis of the Δ*flrA* mutant revealed that increased iron acquisition, oxidative stress resistance and metabolic co-ordination are also associated with improved intracellular survival and fitness. Additionally, adaptation with the amoeba host result in *V. cholerae* isolates that exhibited an increased capacity to colonise

zebrafish, establishing a connection between protozoan predation and enhanced environmental persistence.

The results presented here highlight multiple adaptation strategies acquired by the pathogen when under intense grazing pressure. Predation pressure drives the accumulation of beneficial mutations that serve as key drivers of the adaptation process and enhance commensalism with the host protozoa. Further, this study provides an important contribution to the understanding of the adaptive traits that evolve in pathogens under predatory pressure, and how these adaptive traits impact colonisation of eukaryotic hosts.