The Role of YeaZ in the VBNC state of *Vibrio parahaemolyticus* NCTC 10884

Shih-Hsun Chen, M.Sc (National Taiwan Ocean University)

A thesis submitted for the Degree of Doctor of Philosophy

School of Molecular and Biomedical Science

Faculty of Sciences,

The University of Adelaide

Adelaide, South Australia, Australia

10 July 2012

Presentation of Figures and Tables

All figures and tables referenced in this thesis are placed at the end of relevant chapters. This has been done to minimize the impact of large numbers of figures and tables on document flow and as an aid to interpretation of results and discussion sections of each chapter.

Table of Contents

Chapter 1: Literature review 1

1.1 The Genus *Vibrio* 1

1.2 Ecology of Vibrio Species 1

1.2.1 The Effect of Seasonal Temperature and Salinity in Vibrios..................................2

1.2.2 Vibrios in Aquatic Wildlife and Zooplankton..4

1.2.3 Bacteriophages and Vibrios...5

1.3 Pathogenesis of *Vibrio parahaemolyticus*...6

1.4 The Viable But Non-Culturable State...8

1.4.1 Induction of the VBNC State 10

1.4.2 Characterization of VBNC State Cells...12

1.4.3 Virulence of Cells in the VBNC State..14

1.4.4 Gene Expression in the VBNC State..15

1.4.5 Antibiotic Resistance of VBNC Cells 16

1.5 Resuscitation from the VBNC State 17

1.5.1 Resuscitation following a Temperature Upshift..17

1.5.2 Resuscitation Promoting Factors...17

1.5.3 Biological Mediators of the VBNC State..18

1.5.4 Autoinducers and the VBNC State..19

1.6 The YjeE/YeaZ/YgjD Signal Transduction Network 20

1.7 YeaZ Protein-Protein Interactions...21

1.7.1 YeaZ 21

1.7.2 Structure of YeaZ 21

1.7.3 YgjD 22

1.7.4 YjeE...24

1.8 Summary 25

1.9 Hypothesis and Aims 25

1.9.1 Overall Aims and Hypothesis...25

1.9.2 Specific Hypothesis 26

1.9.3 Specific Aims 26

Chapter 2: Materials and Methods 43
2.1 Chemicals and Reagents 43
2.2 Buffers and Solutions 43
2.3 Bacteria and Yeast Strains 43
2.4 Growth Media 43
2.5 DNA and Protein Molecular Weight Markers 44
2.6 Plasmids used in this Study 44
2.7 Oligonucleotide Primers 44
2.8 Antibodies 44
2.9 Molecular Biology Techniques 44
2.9.1 Preparation of Chromosomal DNA 44
2.9.2 Preparation of Plasmid DNA 44
2.9.3 Polymerase Chain Reaction (PCR) 44
2.9.4 Restriction Enzyme Digestion 45
2.9.5 Extraction of DNA from Agarose Gel 45
2.9.6 DNA Ligation 45
2.9.7 Transformation of Chemically Competent *E. coli* Cells 45
2.9.8 Electro-transformation of *V. parahaemolyticus* Cells 46
2.9.9 DNA Sequencing 46
2.9.10 Agarose Gel Electrophoresis 47
2.10 RNA Transcript Analysis 47
2.10.1 RNA Isolation 47
2.10.2 RNA Quantification 48
2.10.3 RNA Quality Confirmation 48
2.10.4 Quantitative Reverse Transcription PCR 48
2.11 Protein Techniques 48
2.11.1 Protein Quantification 48
2.11.2 SDS-PAGE 49
2.11.3 Western Blot Analysis 49
2.11.4 Expression and Purification of His-tagged fusion Protein 50
2.11.5 Production of a YeaZ specific Polyclonal Antiserum 50
2.11.6 Co-immunoprecipation 51

2.11.7 Immunofluoresence Microscopy 51
2.11.8 Extraction of Outer Membrane Protein 52
2.11.9 Trichloroacetic Acid (TCA) Precipitation 52
2.11.10 Formaldehyde Cross-linking 53
2.11.11 MALDI-TOF Mass Spectrometry 53
2.11.12 Bacterial Two-Hybrid Analysis 54
2.11.13 Yeast Two-Hybrid Analysis 55
2.11.14 Transmission Electron Microscopy (TEM) 56
2.12 Site-directed Mutagenesis by Overlap Extension PCR 56
Chapter 3: Factors affecting Resuscitation and Entry to the VBNC State 67
3.1 Introduction 67
3.2 Experimental Approach 68
3.3 Results 68
3.3.1 Effect of Initial Cell Concentration and NaCl Concentration on Culturability 68
3.3.2 Effect of Initial Cell Concentration and pH on Culturability 71
3.3.3 Effect of Cell Concentration and NaCl Concentration on Resuscitation 72
3.3.4 Effect of Cell Concentration and pH on Resuscitation 73
3.4 Discussion 74
Chapter 4: The role of YeaZ in the VBNC state 92
4.1 Introduction 92
4.2 Experimental Approach 93
4.3 Results 94
4.3.1 Characterization of $yeaZ_{Vp}$ Nucleotide and Amino Acid Sequence 94
4.3.2 Cloning the *yea*Z gene from *V. parahaemolyticus* strain NCTC 10884 94
4.3.3 Mutagenesis of $yeaZ_{Vp}$ 94
4.3.4 Construction of a *V. parahaemolyticus yeaZ*::*kan* Mutant 95
4.3.5 Complementation Analysis 96
4.3.6 Overexpression and Purification of YeaZ_{Vp} 96
4.3.7 Generation of a Polyclonal Antiserum for YeaZ_{Vp} 97
4.3.8 Characterisation of the $yeaZ_{Vp}$ Mutant 98
4.3.9 The Effect of YeaZ_{Vp} Fusion Protein on Resuscitation 99
4.3.10 Cellular Location of YeaZ in *V. parahaemolyticus* NCTC 10884 99

4.3.11 $YeaZ_{Vp}$ Levels during VBNC Induction and Resuscitation 100
4.3.12 Transcriptional Analysis of VBNC State and Resuscitating Cells 101
4.3.13 TEM of *V. parahaemolyticus* NCTC 10884 Cells in the VBNC State 102
4.4 Discussion 103
Chapter 5: YeaZ Protein-Protein Interactions 132
5.1 Introduction 132
5.2 Aims and Approaches 133
5.3 Results 134
5.3.1 Characterization of the $YgjD_{Vp}$ and $YjeE_{Vp}$ Nucleotide and Amino Acid Sequences 134
5.3.2 Protein-protein Interactions in Bacterial Two-hybrid Analysis 135
5.3.3 Protein-protein Interactions in Yeast Two-hybrid Analysis 138
5.3.4 Protein-protein Interactions in Pull-down Analysis 141
5.3.5 Co-immunoprecipitation of $YeaZ_{Vp}$ Associated Proteins 144
5.3.6 $YeaZ_{Vp}$ Domains involved in Protein-Protein Interactions 144
5.4 Discussion 148
Chapter 6: Conclusions 179
6.1 Introduction 179
6.2 Conclusions 180
6.3 Significance and Future Work 182
Chapter 7: References 184
Appendices 200

List of Figures

Figure 1.1: Counts of *Vibrio* species from the east coast of North Carolina, USA. 27
Figure 1.2: Counts of *Vibrio* species(■) and estuarine bacteria (●) in sea sediments. 28
Figure 1.3: Counts of Vibrios and TVC within oysters from South Australia. 29
Figure 1.4: Counts of Vibrio and TVC within oyster grow seawater in South Australia. 30
Figure 1.5: Entry of *V. vulnificus* into the VBNC state. 31
Figure 1.6: Morphological characteristic of *V. alginolyicus* cells analysed by scanning electron microscopy. 32
Figure 1.7: Proposed interaction of regulatory factors involved in the VBNC state. 34
Figure 1.8: Time required for resuscitation of VBNC *V. vulnificus* cells. 35
Figure 1.9: Effect of purified RPF on stimulation of growth of washed *M. luteus* cells. 36
Figure 1.10: Alignment of Rpf amino acid sequences. 37
Figure 1.11: Dose-dependency of recombinant Rpf protein in *Salmonella* Oranienburg. 38
Figure 1.12: Alignment of YeaZ amino acid sequences. 39
Figure 1.13: Model of YjeE, YeaZ and YgjD essential signal transduction network. 40
Figure 1.14: Structure of YeaZ from *S.* Typhimurium. 41
Figure 1.15: The potential YeaZ biological dimer of *S.* Typhimurium. 42
Figure 3.1: Counts of *V. parahaemolyticus* cells incubated in ASW containing 2.5% NaCl at 4°C and 20°C. 78
Figure 3.2: Counts of *V. parahaemolyticus* cells incubated in ASW containing 5.0% NaCl at 4°C and 20°C. 79
Figure 3.3: Counts of *V. parahaemolyticus* cells incubated in ASW containing 10% NaCl at 4°C and 20°C. 80
Figure 3.4: Time to loss of culturability of *V. parahaemolyticus* cells suspended in ASW containing different levels of NaCl. 81
Figure 3.5: Plots of $\ln(N/N_0)$ *vs.* time at 4°C in ASW with different levels of NaCl. 82
Figure 3.6: Counts of *V. parahaemolyticus* incubated at 4°C and 20°C in ASW at pH 5........84
Figure 3.7: Counts of *V. parahaemolyticus* incubated at 4°C and 20°C in ASW at pH 8........85
Figure 3.8: Counts of *V. parahaemolyticus* incubated at 4°C and 20°C in ASW at pH 9.5.....86
Figure 3.9: Effect of pH on time to loss of culturability of *V. parahaemolyticus* cells in ASW adjusted to different pH. 87

Figure 3.10: Plot of ln(N/N_0) versus time at 4°C in ASW adjusted to different pH. 88
Figure 3.11: Counts of *V. parahaemolyticus* during resuscitation from the VBNC state from 4°C upshift to 20°C in ASW containing 2.5% NaCl and 5% NaCl 90
Figure 3.12: Counts of *V. parahaemolyticus* NCTC 10884 during resuscitation from the VBNC state from 4°C upshift to 20°C in ASW at pH 8. 91
Figure 4.1: DNA and amino acid sequence for $YeaZ_{Vp}$. 109
Figure 4.2: PCR amplification of the 1561bp fragment *yeaZ*$_{Vp}$..110
Figure 4.3: Construction of plasmid pCTX100. 111
Figure 4.4: Construction of plasmid pCTX101. 112
Figure 4.5: PCR confirmation of the 1.9 kbp *yeaZ::kan* fragment of pCTX101. 113
Figure 4.6: Construction of plasmid pCTX102. 114
Figure 4.7: PCR confirmation of the 1.9 kbp fragment of the pCTX102..............................115
Figure 4.8: PCR confirmation of the *V. parahaemolyticus yeaZ*$_{Vp}$ mutant. 116
Figure 4.9: Construction of plasmid pCTX103. 117
Figure 4.10: PCR amplification of the 3 kbp *yeaZ*$_{Vp}$ fragment...118
Figure 4.11: Construction of pCTX104...119
Figure 4.12: Construction of pCTX105...120
Figure 4.13: Purification of $YeaZ_{Vp}$ protein. 121
Figure 4.14: Western blot analysis of $YeaZ_{Vp}$ proteins. 122
Figure 4.15: Effect of a *yeaZ* mutation on entry into the VBNC state. 123
Figure 4.16: Effect of exogenous $YeaZ_{Vp}$ protein on resuscitation from the VBNC state.....124
Figure 4.17: SDS-PAGE separation of proteins from *V. parahaemolyticus*. 125
Figure 4.18: Detection of cell surface associated $YeaZ_{Vp}$ by IF..126
Figure 4.19: $YeaZ_{Vp}$ prepared from VBNC state cells and Resuscitated Cells. 127
Figure 4.20: Expression of the *rpoS* and *yeaZ* genes in different states................................128
Figure 4.21: TEM images of sections of wild type *V. parahaemolyticus* cells. 129
Figure 4.22: TEM images of sections of *V. parahaemolyticus yeaZ* mutant cells. 130
Figure 5.1: Nucleotide and deduced amino acid sequence for $YgjD_{Vp}$. 155
Figure 5.2: Nucleotide and deduced amino acid sequence for $YjeE_{Vp}$.................................156
Figure 5.3: Construction of plasmid pCTX106. 157
Figure 5.4: Construction of plasmids pCTX107, pCTX108 and pCTX109. 158
Figure 5.5: $YeaZ_{Vp}$ interaction with $YeaZ_{Vp}$, $YgjD_{Vp}$ and $YjeE_{Vp}$. 159

Figure 5.6: Construction of plasmid pCTX110. 160
Figure 5.7: Construction of plasmids pCTX111 and pCTX112. 161
Figure 5.8: Protein interaction in yeast two-hybrid. 162
Figure 5.9: Construction of plasmid pCTX113. 163
Figure 5.10: Construction of plasmids pCTX114 and pCTX115. 164
Figure 5.11: Confirmation of pCTX114 and pCTX115. 165
Figure 5.12: Co-expression of $YeaZ_{Vp}$ with $YgjD_{Vp}$ or $YjeE_{Vp}$ in *E. coli* BL21DE3. 166
Figure 5.13: The His-tag pull down assay of $YeaZ_{Vp}$ complexes. 167
Figure 5.14: Western blot detection of cross-linked $YeaZ_{Vp}$ complexes. 168
Figure 5.15: Immuno-precipitation of $YeaZ_{Vp}$ associated protein. 169
Figure 5.16: YeaZ amino acid sequence alignments. 170
Figure 5.17: The structure of $YeaZ_{ST}$ and predicted structure of $YeaZ_{Vp}$. 171
Figure 5.18: Potential interaction domains and amino acids in $YeaZ_{ST}$ dimers. 172
Figure 5.19: Construction of plasmids pCTX116, pCTX117 and pCTX118. 173
Figure 5.20: Interaction of $YeaZ_{Vp}$ mutants with $YgjD_{Vp}$. 174
Figure 5.21: Interaction of I75A, L83A and F85A $YeaZ_{Vp}$ mutants with $YeaZ_{Vp}$. 175
Figure 5.22: Western blot analysis of interacting $YeaZ_{Vp}$ and $YgjD_{Vp}$ protein. 176
Figure 5.23: Western blot analysis of interacting $YeaZ_{Vp}$ mutants and $YeaZ_{Vp}$ wt proteins. 177

List of Tables

Table 1.1: Bacterial known to enter a VBNC state....33

Table 2.1: Bacteria and Yeast strains used in this study....58

Table 2.2: Composition of SD medium....59

Table 2.3: Plasmids used in this study....60

Table 2.4: Primer pairs used in this study....63

Table 4.1: Effect of a *V. parahaemolyticus yeaZ* mutation on resuscitation from the VBNC state....131

Table 5.1: Protein mass spectrometry sequencing results for potential $YeaZ_{Vp}$ associated proteins....178

Abstract

Vibrio parahaemolyticus is an important food-borne pathogen known to enter a Viable But Non-Culturable (VBNC) state that increases the survival of *V. parahaemolyticus* during stress eg. starvation and low temperature. The purpose of this study was to better characterize the impact of environmental factors on loss of culturability associated with entry into a cold induced VBNC state by *V. parahaemolyticus* NCTC 10884 and undertake an analysis of the role of resuscitation promoting factors in the molecular mechanism that underpins this physiological phenomenon.

Entry to the VBNC state was examined for high (10^9 and 10^7 cells mL^{-1}) and low (10^5 and 10^3 cells mL^{-1}) concentration cell suspensions in artificial seawater (ASW) adjusted to different pH and salinity, with incubation at 4^oC. Salinities (5 and 10% NaCl) and pH (5 and 9.5) considered suboptimal for growth resulted in short incubation periods required for entry to the VBNC state. Interestingly, the time to loss of culturability of all suspensions tested was found to be dependent on cell suspension concentration. Furthermore, low density cell suspensions, but not high density suspensions that had achieved complete loss of culturability, could not be resuscitated following a temperature upshift to 20^oC. This novel result indicated that survival of VBNC state cells is likely to require maintenance nutrients from dead cells within the suspension.

To investigate the role of potential resuscitation factor proteins in establishment of the VBNC state and subsequent recovery, a *V. parahaemolyticus* NCTC 10884 $yeaZ_{Vp}$ mutant was constructed. Compared to the wild type parent, suspensions of the $yeaZ_{Vp}$ mutant took substantially longer to lose culturability under conditions required to achieve a VBNC state. Furthermore, this mutant was unable to be resuscitated from the VBNC state. Complementation with $yeaZ_{Vp}$ returned the mutant phenotype to a wild type phenotype. These results suggested that $YeaZ_{Vp}$ plays an integral role in establishment of the VBNC state and subsequent resuscitation for *V. parahaemolyticus* NCTC 10884.

To determine the patterns of expression and cellular distribution of $YeaZ_{Vp}$ in *V. parahaemolyticus* NCTC 10884, a recombinant, His-tagged $YeaZ_{Vp}$ protein (*ca.* 25 kDa) was over expressed from *E. coli* BL21DE3 and purified using affinity chromatography. Purified protein was used to raise a polyclonal antibody of $YeaZ_{Vp}$ protein in a rabbit. This specific

antiserum was used to detect presence of $YeaZ_{Vp}$ in the VBNC state cells and during resuscitation. Western analysis and immunofluorescence microscopy was used to demonstrate $YeaZ_{Vp}$ protein is located in the cytoplasm of cells as well as outer-membrane fractions of *V. parahaemolyticus* NCTC 10884.

As YeaZ has been shown to interact with proteins that are considered essential proteins involved in viability (YgjD and YjeE), interaction of the $YeaZ_{Vp}$ with these proteins in *V. parahaemolyticus* NCTC 10884 was examined. Protein-protein interactions were determined using bacterial and yeast two-hybrid analysis, pull down analysis and immunoprecipitation analysis. Two-hybrid analysis showed that $YeaZ_{Vp}$ was able to interact strongly with other $YeaZ_{Vp}$ monomers as well as $YgjD_{Vp}$, but only weakly with $YjeE_{Vp}$. These results confirmed previous work that showed that YeaZ homologs interact with YgjD, and form dimeric structures. His tag technologies were also used to demonstrate that $YeaZ_{Vp}$ natively interacts with $YjeE_{Vp}$ and $YgjD_{Vp}$ when pairs of these three proteins were expressed in *E. coli*. Furthermore, the ability of $YeaZ_{Vp}$ to form dimers was separately demonstrated following cross-linking of $YeaZ_{Vp}$ natively expressed in *E. coli*. Attempts to demonstrate this in *V. parahaemolyticus* NCTC 10884 were not successful. However, the immunoprecipitation of *V. parahaemolyticus* NCTC 10884 whole cell lysates with a $YeaZ_{Vp}$ specific antiserum indicated that $YeaZ_{Vp}$ is also associated with OmpU. This suggested that $YeaZ_{Vp}$ that decorates the cell surface of this organism may interact with OmpU.

To determine amino acid residues involved in interaction of $YeaZ_{Vp}$ monomer that lead to formation of dimeric forms, site-directed mutagenesis of $YeaZ_{Vp}$ was used together with two-hybrid analysis. The results showed that the YeaZ L83A and F85A substitutions significantly affected interactions between $YgjD_{Vp}$ and $YeaZ_{Vp}$. However, a YeaZI75A substitution only affected interaction between monomers of $YeaZ_{Vp}$. These results suggested that L83 and F85 residues of $YeaZ_{Vp}$ play important roles in the $YeaZ_{Vp}$ and $YgjD_{Vp}$ protein-protein interactions.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Shih-Hsun Chen and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

____

Shih-Hsun Chen

Date: Tuesday, July 10, 2012

Abbreviations

~	Approximately
°C	degrees Celsius
μF	Microfarad/s
μg	Microgram
μL	Microlitre
g	Relative centrifugal force
A_{600}	Absorbance at 600 nm
aa	Amino acid/s
Amp	Ampicillin
bp	Base pairs
ca.	Circa
CFU	Colony forming units
cm	Centimeters
Cm	Chloramphenicol
d	days
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic-acid disodium salt
Erm	Erythromycin
g L^{-1}	Grams per litre
h	hour/s
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPK	Histidine kinase
IPTG	Isopropyl-β-D-thio-galactopyranoside
Kb	Kilobase/s
kDa	Kilodalton/s
kg	Kilogram/s
kV	Kilovolts/s
L	Litre/s

LB	Luria Bertani broth
M	Molar
mg	Milligram/s
min	Minute/s
mL	Millilitre/s
mM	Millimolar
MQ	MilliQ grade pure water
mRNA	Messenger RNA
ms	Milliseconds
nm	Nanometres
OD_{600}	Optical density of 600 nm
O/N	overnight
ONPG	O-nitrophenyl-β-D-galactopyranoside
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative reverse-transcription PCR
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RR	Response regulator
RT	Room temperature
SDS	Sodium Dodecyl Sulphate
sec	Second/s
SOC	Super Optimum broth with Catabolite repression
spp	Species
TAE	Tris-acetate EDTA buffer
TTSS	Type three secretion system
TVC	Total viable count (= Total Plate Count)
vol	Volume/s

v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Acknowledgements

I would like to thank my supervisor Dr. Connor Thomas. Thank you for giving me the opportunity to do my PhD with you and for teaching me so much in research. Under his patience, encouragement and guidance, I have grown and developed much as a scientist. I am grateful for his patience and understanding. I also like to thank my co-supervisor Dr. Renato Morona for advice and help on my research.

Thanks are also due to Dr. Keith Shearwin and Dr. Grant Booker for advice on bacterial two-hybrid analysis and Site-directed mutagenesis analysis. To Miss Lyn Waterhouse and Ruth Williams, Adelaide Microscopy Center, for their patient and advice to solve my problem in TEM experiment. To all my lab members Rethish, Caroline, Johnson, Ann and Mei Mei, thanks for your advice, support and friendship. I appreciate all the joy and fun with you guys in the lab. To all the people from other labs Jennifer, Rachelle, and Mei Zhi, thanks for the joy brought to us during the break time. I also like to thank all the people from Morona, Paton, McColl lab and Grant Booker, especially Dr. David Ogunniyi, Dr. Layla Mahid, Min Yan and Ethan Chen for their help, support and advice in my research. To other members in the school, including CUS stuffs, Sergei Volgin and Garry Penney, thank you for being there to help and provide advice.

Finally, I would like to thank my parents and my wife for all your encouragement, love and wonderful support to me in many ways. Especially to my wife, thank you to being here with me for all your love, support and encouragement over the years of study. I could not have done this without you, and I love you very much.