Molecular Factors Involved in Suppression of Pathogenic *Vibrio owensii* by Potential Probiotic Bacteria

Balkeas Abd Ali Abd Aun Jwad

B. Sc. (University of Baghdad, Iraq)

M. Sc. (Babylon University)

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School of Science and Technology

University of New England

Armidale, New South Wales, 2351,

Australia

Declaration of Originality

I declare that the substance of this thesis is my own work.I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that the best of my knowledge any help received in the preparation of this thesis, and all sources used, have been acknowledged in this thesis.

Balkeas Jwad

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Abstract

In recent decades a rapid increase in the market demand for seafood has led to a significant decline in wild seafood populations. Therefore, the aquaculture industry now represents the best alternative seafood provider. In Australia, the spiny lobster *Panulirus ornatus* has the greatest market demand. However, production from the aquaculture hatcheries together with continuous use of antibiotics has led to an increase in the resistance of bacterial pathogens and the development of particularly resistant strains, thereby rendering antibiotic treatment ineffective. Alternative strategies to confine the spread and virulence of bacterial diseases are in demand due to the risk of further resistance development and the possibility of resistant pathogens evolving into human pathogens. Therefore, in recent years, the research focus has broadened to include probiotics, defined by Fulleret al. (1989) as a live microbial feed supplement that has beneficial affects to the host animals by improving intestinal microbial balance. The use of probiotics as a means of disease control and an alternative to antibiotics has shown to be promising in aquaculture, particularly in fish and shellfish larva-culture.

The current study aimed to identify probiotic bacteria that are antagonistic to the development of Vibriosis, which is a bacterial disease widely spread in Australian's lobster aquaculture industry, caused by members of the genus *Vibrio*. *Vibrio ownsii* is one of the most virulent pathogens, causing severe losses of lobster larva cultures in lobster hatcheries. In the current study we isolated and identified beneficial bacteria (potential probiotics) from a culture collection from corals and shrimps. These potential probiotic strains suppress the lobster pathogen *V. owensii* DY05, as determined in well diffusion assays.

In total, 400 isolates were tested from different species of the corals *Turbinaria* and *Acropora*. A total of 99 isolates were tested from stressed shrimp, both male and female and from the ambient seawater. Of all 499 isolates, 111 were identified as antagonistic to DY05, 80 of them from coral (20% of all isolates) and 31 from shrimp (31% of all isolates). Most of these antagonistic (potential probiotic) isolates produced at least 10 mm in diameter inhibition zone in well diffusion assays.

According to the 16S rRNA gene fragment sequences the coral isolates were assigned to nine genera in four classes. Most of the isolates belong to Gammaproteobacteria (89%), with the other three classesbeingBacilli, Actinobacteria and Alphproteobacteria. The isolates from shrimp that demonstrated antagonism against the pathogen were affiliated with three classes and six genera, with the Gammaproeobacteria predominating (81%) and the other classes beingBacilli and Actinibacteria.On the genes level *Vibrio* species were the dominant phyla, accounting for 62.5% of coral isolates and 74% of shrimp isolates. *Psychrobacter* spp.

were isolated from coral at only 23.7%. Other less common bacterial isolates included *Staphylococcus, Bacillus, Micrococcus, Pseudovirio, Pseudoalteromonas*, *Acinetobacter* sp., *Photobacterium* sp*., Pseudovibrio* sp.and *Brachybacterium* sp. Due to the high occurrence of isolates that are antagonistic against the lobster pathogen *V. owensii* DY05, it can be deduced that coral and shrimp marine environments represent promising sources for isolation of probiotic bacteria.

In carrying the study further, the three bacterial isolatesthat expressed strong and fast antagonistic activity against DY05 were chosen for preliminary characterisation of the observed antagonistic activity. These bacteria were identified to the genus level as *Pseudoalteromonas* sp. strain 80, *Psychrobacter* sp. strain 62 and *Vibrio* sp. strain 34. Preliminary characterisation of antagonistic activity involvedproteolysis assays, which indicated that the three isolates demonstrated activities consistent with gelatinase, caseinase and amylase enzymatic breakdown. Such proteases and small molecules may be responsible of antimicrobial activity.Several methods were used to concentrate and fractionate filtered supernatants to identify or characterise the active molecules. In addition, zymography was used to identify the active fractions of culture supernatants. These fractions were subject to analysis by liquid chromatography / mass spectrometry (LC/MS) to partly identify active antimicrobial proteins. The studied strainswere found to producenumerous protein compounds, some of which were specifically proteolytic enzymes. *Vibrio* sp. strain 34 was found to produce VtpA metalloprotease. *Pseudoalteromonas* sp. strain 80 was found to produce several proteases include metallopeptidase, prolyloligopeptidase, zinc metallopeptidase, iron superoxide dismutase and serine endoprotease. Lastly, *Psychrobacter* sp. strain 62 was found to produce superoxide dismutase and inorganic pyrophosphatase. Cultures were also examined for small active molecules using gas chromatography / mass spectrometry (GC-MS). Several alkenes volatiles were identified; however these volatile alkenes were also present in non-active strains. Therefore, it was concluded that these volatiles were not related to the antimicrobial activity.

Transposon mutagenesis was undertaken in an attempt to identify the genesrelated to biosynthesis of metabolites responsible for pathogen suppression. Successful mutagenesis of *Pseudoalteromonas* sp. strain 80 yielded a mutant, named 7Km, which lacked inhibitory activity against the pathogen and also lost pigmentation. Southern blot analysis confirmed an insertion of the mini-Tn10 transposon in one location in the genome. The gene affected in the mutant was recovered using plasmid rescue and was found to share 100% homology with a

membrane bound transporter proteinof the resistance-nodulation-division (RND) family. The closest homology was to RND protein of *Pseudoalteromonas flavipulchra* JG1, a marine antagonistic bacterium with abundant antimicrobial metabolites. RND proteins were implicated in drug resistance of important pathogens and in the transport of substances from the cell, suggesting that a mutationin such a transporter probably abolished the excretion of pathogen-suppressive substances from *Psedoalteromonas* sp. strain 80 cells.

The current study confirms that the shrimp and coral microbial community represent good sources for the isolation of beneficial bacteria, which may be promising candidates for application as probiotics in aquaculture hatcheries, in particular *Psedoalteromonas* sp. strain 80, *Psychrobacter* sp. strain 62 and *Vibrio* sp. strain 34. The use of a diversity of probiotics theoretically proves advantageous as they have different mechanisms of pathogen inhibition.

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Abbreviations

bp	base pairs
DNA	deoxyribonucleic acid
Kb	kilobase
MA	marine agar
MB	marine broth
LB10	Luria Broth 10 (marine)
µL	microlitre
µg	microgram
mg	milligram
mL	millilitre
hr	hour
rpm	revolution per minunte
NMSC	National Marine Science Centre
NSW	New South Wales
NSWMPA	New South Wales Marine Parks Authority
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SIMP	Solitary Islands Marine Park
SWST	South-West Solitary Island
HL-ASWS	healthy-looking tissue
M-ASWS	disease margin
D-ASWS	disease tissue
BLAST	basic local alignment search tool
MS	male stressed
FS	female stressed
sp.	specie
spp.	species
SUPA	Sydney University Prince Alfred Macromolecular Analyses Centre
SW	seawater
UV	ultraviolate
Amp	ampicillin
Sm	streptomycin
Tc	tetracyclin
Cm	chloramphenicol

Km	kanamycin
Rif	rifampicin
TLC	thin layer chromatography
X-gal	5-bromo-4-chloro-3-indoyl β-D-galactopyranoside
GC-MS	gas chromatography mass spectrometry
LC-MS	liquid chromatography mass spectrometry
TCBS	thiosulfate-citrate-bile salts-sucrose agar
SDS	sodium dodecyl sulphate
SDS- PAGE	sodium dodecyl sulphate-polyacrilamide gel electrophoresis
gDNA	genomic DNA
M	molar
V	volts

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