Bacteriophages and bacteriocins: agents for biocontrol of the fish pathogen *Streptococcus iniae*

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ABSTRACT

Streptococcus iniae is one of the most widespread and costly pathogens in fish aquaculture. Bacteriophages (phages) and bacteriocins may be the optimal new therapeutic agents for *S. iniae* that lack many of the problems associated with current control measures. This study aims to discover and characterise phages and bacteriocins with activity against *S. iniae*.

Cross spotting assays and mitomycin C inductions were conducted to screen for prophage within the JCU library of 48 *S. iniae* isolates. Phages were induced with 150-350 ng ml^{-1} mitomycin C, isolated through filtration and concentrated by ultracentrifugation. Phages were characterized by plaque assays on *S. iniae*, transmission electron microscope analysis, and gel electrophoresis of *Eco*RI digested phage DNA. Mitomycin C inductions revealed 20.83% of *S. iniae* isolates show growth curves indicative of lysogeny. Plaque assays confirmed inducible prophages in 14.58% of isolates. Each of the induced phages displayed distinctive host ranges within the *S. iniae* library. Four phages, vB_SinS-44, vB_SinS-45, vB_SinS-46 and vB_SinS-48 lysed over 78% of isolates, and were highly concentrated. Phage vB_SinS-45 was extracted at the highest concentration of 4.2×10^{11} PFU ml^{-1} following ultracentrifugation. Transmission electron microscope analysis revealed phage particles from these four samples that exhibited long, non-contractile tails and isometric heads consistent with morphology of *Siphoviridae*. Restriction digests of phage DNA revealed two distinct cutting patterns, indicating similarity between phages in bacteria isolated from different hosts and geographic regions. The genome sizes estimated from the restriction digest are 28.5 kb and 66 kb for vB_Sin-45 and vB_Sin-46, respectively. The phages isolated and characterized in this study are the first described lysogenic phages associated with *S. iniae*, and are excellent candidates for genetic modification for use in therapy, prevention or research of *S. iniae* infections in fish.

To isolate lytic phages, environmental samples were collected from fish and prawn farms in Queensland. Numerous methods to expose *S. iniae* lytic phages were trialed,

including enrichments in various sets of *S. iniae* isolates, filter-free isolation techniques to minimize damage to phages, and concentration of samples using ultracentrifugation and polyethylene glycol before and after enrichment processes. No lytic phages specific for *S. iniae* were successfully isolated in this study. Spatial and seasonal distribution of the host bacteria, as well as farm vaccination programs may have prevented the collection of phages from environmental samples. Lytic phages will likely be found on farms experiencing current outbreaks of *S. iniae*, none of which occurred during this study.

During the course of the project, a bacteriocin-like inhibitory substance (BLIS) with strong antibacterial activity against *S. iniae* was discovered. This BLIS, found to be produced by *Lactococcus lactis* ssp. *lactis*, was characterized through antagonism assays on the *S. iniae* library, plasmid curing tests, protein isolation, concentration, and SDS-PAGE analysis. The kinetics of production, effects on cell viability, heat, pH and enzyme tolerance of the BLIS were also determined. The BLIS was found to be a protein bacteriocin, termed BacL49, that is heat and pH stable (100°C for 60 min, pH 2.5-9.5), and sensitive to proteinase K, α-chymotrypsin, trypsin and papain. BacL49 active proteins were found to be 5 kDa and 54 kDa in size. The bacteriocin appeared not to be plasmid regulated. BacL49 is produced at the end of the log phase and antagonistic activity decreases following early stationary phase. The bacteriocin displays antagonism against 93.75% of *S.iniae* isolates. Though BacL49 is susceptible to trypsin, unlike most forms of nisin, sequencing of the protein needs to be completed before confirming that this bacteriocin is unique. BacL49 should be considered as a therapeutic agent against *S. iniae*, due to its heat and pH stability and bactericidal activity against the majority of *S. iniae* strains. BacL49 has the potential to be delivered to fish as a purified peptide or in the form of a live probiotic as *L. lactis* ssp. *lactis* L49.

Phage therapy remains an excellent potential tool for control of *S. iniae* in aquaculture, despite the inability of this study to isolate a lytic phage against the bacterium. Further study, *in vivo* trials, and manipulation of both the bacteriocin and phages isolated in this study could produce novel, effective biocontrol agents in the fight against *S. iniae*.

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ABBREVIATIONS

ADH	arginine dehydrolase
ATP	adenosine triphosphate
AU	arbitrary units
BA	blood agar
BLIS	bacteriocin-like inhibitory substance
cfs	cell-free supernatant
CFU	cell forming units
CNS	central nervous system
CSOA	colistin sulphate and oxolinic acid
DNA	deoxyribonucleic acid
ECP	extracellular products
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FKC	formalin-killed cells
H_2O	water
HCl	hydrochloric acid
HIA	heart infusion agar
HIB	heart infusion broth
IFAT	indirect fluorescent antibody technique
JCU	James Cook University
MMC	mitomycin C
OD_{540}	optical density at 540 nm
OD_{600}	optical density at 600 nm
$OD_{260/280}$	ratio of optical density at 260 nm and 280 nm
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed-field gel electrophoresis
PFU	plaque forming units

PGM	phosphoglucomutase
QLD DPI	Queensland Department of Primary Industries
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl suphate
SDS-PAGE	sodium dodecyl suphate polyacrilamide gel electrophoresis
SLS	Streptolysin S
PC	Private Company
TEM	transmission electron microscope
TE	tris-EDTA
VLP	virus-like particles
WA DA	Western Australia Department of Agriculture