Endawoke Hailu

Characterization of *Janthinobacterium* strains isolated from the skin and rearing water of Atlantic salmon (*Salmo salar*) fry

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
Co-supervisor: Eirik D. Lorentsen
July 2023

Master's thesis

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Abstract

Mucosal surfaces cover the skin, gills, and gut of fish. It serves as a barrier between the body and the environment. Fish larvae are hatched germ-free, but immediately after hatching, the mucosal surface of the larvae is colonized by microbes from the surrounding environment. This initial colonization is known to be important in shaping fish health and development. However, little is known about host-microbe interactions and its effects on the fish. The Atlantic salmon has a long yolk sac fry period which makes it a preferable choice to study the interaction between host and microorganism. Recently, the Analysis and Control of Microbial Systems (ACMS) group developed a novel gnotobiotic experimental model system, where salmon fry can stay germ-free for its entire yolk sac fry stage.

The aim of this study was to investigate the initial colonization of Atlantic salmon yolk sac fry by five *Janthinobacterium sp. s*trains called PBA, PBB, 3.108, 3.109, and 3.116. Originally, PBA and PBB were isolated from a rearing flask with yolk sac fry, while 3.108, 3.109, and 3.116 were isolated from the skin of salmon fry grown in a commercial Recirculating Aquaculture Systems (RAS). The growth nature on TSA/TSB general growth media and the enzymatic profiles of the strains was characterized prior to investigating their ability to colonize salmon yolk sac fry.

The 16S rRNA gene sequences were highly similar for the five isolates. However, morphology and enzyme assay profiles were different between them. The growth of the isolates on TSA/TSB with 0.1% Tween-80 revealed that PBA and PBB have purple colonies indicating violacein production, whereas 3.108, 3.109, and 3.116 have white phenotypes. The strains grew planktonic in TSB with 0.1% Tween-80 shaken vigorously at room temperature. Furthermore, the enzymatic activity test conducted to test the 19 enzymes in the strains showed a significant difference in their enzymatic profile among some strains.

The initial colonization of Atlantic salmon yolk sac fry by the individual strains, as well as a mixture of all five *Janthinobacterium* strains, was investigated. The experiments for studying the colonization of the yolk sac fry had to be repeated because contamination and fish death occurred in the first experiments. Hence, a total of four fish experiments were carried out. In two the final, successful fish experiments, the colonization of the fry by the *Janthinobacterium sp.* isolates was studied by exposing eggs and yolk sac fry to a mixture of the five strains (Exp. 3), and by exposing the eggs to each of the strains separately (Exp. 4).

The colonizing ability, as well as possible interaction between the strains in Experiment 3, was investigated by Illumina sequencings of *jqsA* (encoding the autoinducer synthetase JQSA) amplicons. As the strains had highly similar 16S rRNA sequences, we chose to use another marker gene (*jqsA*) which could better differentiate between the strains.

The Illumina sequencing results showed that all of the strains except 3.116 were able to colonize the salmon fry. The 3.109 strain, originally isolated from salmon skin, appeared to have the highest relative abundance in yolk sac fry, while PBA and PBB, originally isolated from the rearing water of flasks with yolk sac fry, had the highest relative abundance in the water. The CFU analyses of the samples taken from the mono-associations corroborated well with the results from mixed experiments.

Sammendrag

Slimhinner dekker skinnet, gjellene og tarmen til fisk. Den virker som en barriere mellom kroppen og omgivelsene. Plommesekkyngel er bakteriefrie når de klekker, men umiddelbart etter klekking blir slimhinnene kolonisert av mikroorganismer fra omgivelsene. Denne umiddelbare koloniseringen er viktig i å forme fiskens helse og utvikling. Utover dette er lite kjent om effektene på fisken som kommer av vert-mikrobe interaksjoner. Atlanterhavslaksen har en lang plommesekkyngel fase, noe som gjør det godt egnet til å studere interaksjoner mellom vert og mikroorganismer. Nylig har gruppen Analyser og kontroll av mikrobielle system (AKMS) utviklet et gnotobiotisk eksperimentell modell system, hvor lakseyngel kan holdes bakteriefrie gjennom hele plommesekk stadiet.

Målet med dette prosjektet var å studere den første koloniseringen av plommesekkyngel for fem *Janthinobacterium sp.* Stammer kalt PBA, PBB, 3.108, 3.109, og 3.116. Opprinnelig ble PBA og PBB isolert fra vann fra fiskeflasker med plommesekkyngel, mens 3.108, 3.109, og 3.116 ble isolert fra skinnet til lakseyngel fra et kommersielt resirkulerende akvakultur system (RAS). Vekst karakteristikker på det generelle dyrkingsmediet TSA/TSB og den enzymatiske profilen til de fem stammene ble studert før dere evne til å kolonisere plommesekkyngel ble undersøkt.

16s rRNA genesekvensene til de fem stammene var svært like. Derimot var både morfologien og de enzymatiske profilene deres forskjellige mellom stammene. Veksten til stammene på TSA/TSB med 0.1% Tween-80 viste at PBA og PBB har lilla kolonier, noe som indikerer violacein produksjon, mens 3.108, 3.109 og 3.116 har hvite fenotyper. Stammene vokste planktonisk i TSB med 0.1% Tween-80 med kraftig risting ved romtemperatur. Videre viste testen av 19 ulike enzymatiske aktiviteter en signifikant forskjell mellom den enzymatiske profilen til stammene.

Den første koloniseringen av plommesekkyngel for de fem *Janthinobacterium sp. s*tammene ble undersøkt både for individuelle stammer, og en blanding av alle fem. På grunn av kontaminering og døde disk måtte eksperimentene for å studere koloniseringen av plommesekkyngel repeteres. Derfor ble totalt fire fiskeforsøk gjennomført. I det to siste, suksessfulle fiskeeksperimentene, ble de fem stammenes evne til å kolonisere yngel undersøkt ved å eksponere egg og plommesekkyngel for en blanding av de fem stammene (Exp. 3), og ved å eksponere (egg og (Om du eksponerte både egg og yngel)) yngelen for individuelle stammer (Exp. 4)

Evnen til å kolonisere og mulige interaksjoner mellom stammene i eksperiment 3 ble undersøkt ved hjelp av Illumina sekvensering av *jqsA (*koder for autoinducer syntethasen JQSA) amplikonsekvensering. På grunn av svært like 16s rRNA sekvenser valgte vi å bruke et annet gen (*jqsA*) for å skille mellom stammene.

Resultatene fra Illumina sekvenseringen viste at alle stammene, unntatt 3.116, kunne kolonisere lakseyngelen. Stamme 3.109, som tidligere har blitt isolert fra lakseyngelskinn, viste seg å være mest tallrik på plommesekkyngelen, mens PBA og PBB, isolert fra vann fra fiskeflasker, var mest tallrike i vann. CFU analysene av prøvene fra forsøkene med enkelt stammer stemte godt over ens med resultatene fra eksperimentene med ben blanding av alle stammene.

Abbreviations

3.108	*Janthinobacterium* strain
3.109	*Janthinobacterium* strain
3.116	*Janthinobacterium* strain
ACMS	Analysis and Control of Microbial Systems
CFU	Colony forming unit
CVR	Conventionally raised eggs
DBH	Days before hatching
DNA	Deoxyribonucleic acid
DPE	Day post-exposure
DPH	Days post hatching
EPS	Extracellular polymeric substance
EU	European Union
FAO	Food and Agriculture Organization
GF	Germ free
HGT	Horizontal gene transfer
IBT	Institution for Biotechnology
J.lividum	*Janthninobacterium lividum*
jqsA	Autoinducer gene
J.SP.	*Janthinobacterium* species
NTC	Non-templet control
NTNU	Norwegian University of Sciences and Technology
OD	Optical density
OUT	Operational Taxonomy Unit
PBA	*Janthinobacterium* strain
PBB	*Janthinobacterium* strain
PCR	Polymerase chain reaction
QS	Quorum sensing
RAS	Recirculating Aquaculture System
rDNA	Ribosomal deoxyribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SGM	Salmon gnotobiotic medium
TSA/TSB	Tryptic Soy Agar/Broth

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1 Introduction

1.1 Atlantic salmon (*salmo salar*)

The Atlantic salmon is an anadromous fish, which means that it lives in the ocean for most of its life but migrates into freshwater rivers and streams for spawning(1). They lay their eggs in the same freshwater stream or rivers where they were born, after spending one to five years in the ocean (2). Their migration is thought to be an adaptation that ensures their offspring's survival in freshwater environments while taking advantage of the abundant feeding opportunities the ocean offers (3). Atlantic salmon is found in the rivers and oceans in the Northern hemisphere, including the Northern Atlantic and Arctic Ocean. It is especially common in the Northern Atlantic oceans of North America, Europe, Greenland, and Russia. A large area of the Norwegian Sea is home to Atlantic salmon (4, 5). Salmon migrate upstream and spawn in rivers and streams between September and February, and the eggs hatch the following spring. It is believed that salmon migration may be initiated by circannual rhythms of reproduction hormones synchronized with the photoperiod(6). When the fish reach the spawning ground, the male fish starts building a nest in the gravel, and then the female salmon lays eggs in the nest (7). Once the female salmon has laid eggs, the male salmon fertilizes them (8). The eggs hatch after approximately 500 day degrees(9).

The Atlantic salmon, life cycle, is generally classified into six main stages referred to as egg, alevins, fry, parr (juveniles), smolt, and adult salmon (Fig. 1) (10). Until it develops into a fry, the newly hatched alevin feeds on the yolk sac(9). Depending on temperature and other parameters, it usually takes up to a month for an alevin to become a fry. However, it has been reported that as the water temperature decreases, the duration of the life period of the alevins life stage increases(11). Studies have shown that the alevin stage is the most sensitive stage in the salmon life cycle. It was reported that 14-61% of the alevins die before it develops to fry(12). This is due to the fact that alevins are very sensitive to changes in oxygen as their respiratory system is poorly developed. The alevins typically respire through the cutaneous surface of their body (13). As alevins grow, their yolk sac is consumed, and they develop into fry(10, 14). Depending on temperature and feeding conditions, most of the parr (juveniles) remain in the freshwater for 2-3 years(7, 15). The Parrs become smolts after undergoing various morphological and physiological changes called smoltification. As a smolt, the salmon is prepared for its life in the sea, where most of its feeding and growth occur (15).

Atlantic salmon is one of the most important aquaculture fish species. Salmon ranks among the top 10 species most produced and most valuable fish species(16). According to the World Aquaculture 2020 report, approximately 2.5 million tons of salmon are produced annually(17). However, salmon is susceptible to several diseases caused by viruses (such as salmon louse), bacteria, fungi, and protozoa(18). Saprolegnia, an oomycete, commonly recognized as water molds, is another organism causing disease that affects aquatic organisms, mainly salmon and rainbow trout fish in freshwater.

The organism affects both dead and living fish as well as eggs. Fish with the disease develop cotton-like growths on their skin, fins, and gills(19, 20). Fish can die as a result of the growth extending into the muscle tissue (21). As the disease affects eggs and newly hatched fry, it

has become a major problem in Norwegian Atlantic salmon farming(22). However, little is known about the occurrence of oomycetes in salmon hatcheries(20). Norwegian hatcheries' water sources normally contain Saprolegnia spores, indicating that the fish are continuously exposed to the spores. However, the oomycete infects the fish only when the fish is weakened or, its mucosal surface is damaged (23). Previously, the disease has been effectively controlled by using the organic dye malachite green. It is, however, now prohibited to use the therapeutic chemical in fish produced for human consumption due to its carcinogenic properties. Formalin is the most effective treatment for this disease at present. However, used of formalin is controversial and is under consideration in the European Union (EU) system; therefore it is important to find an alternative way to prevent or treat Saprolegnia(23, 24)

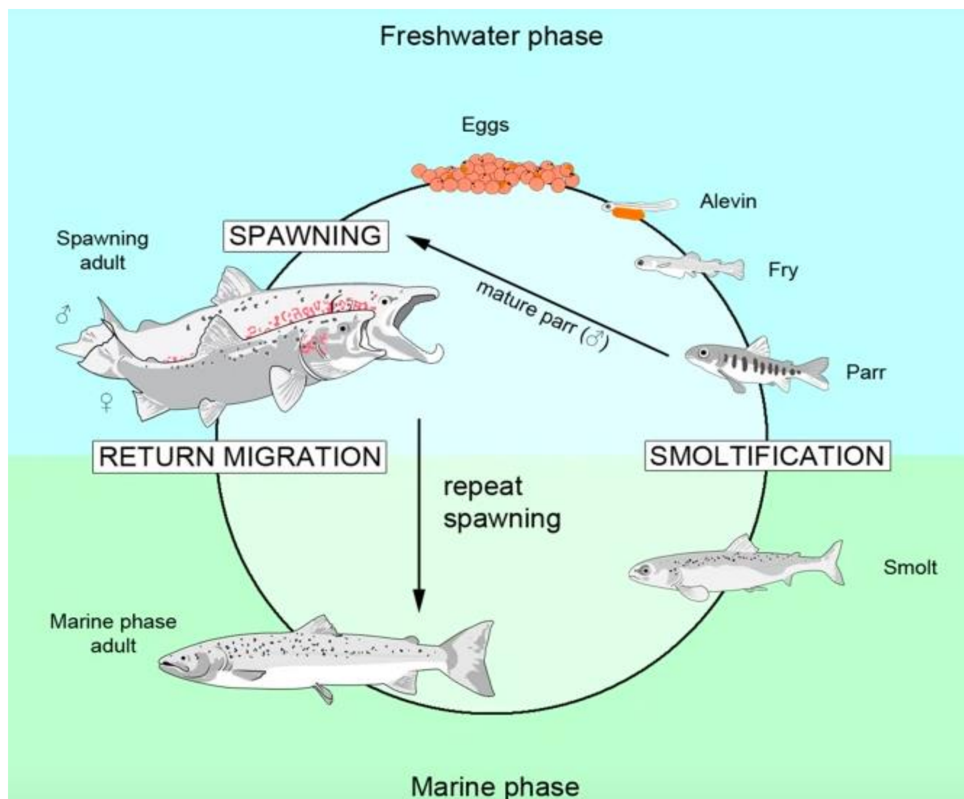

Figure 1: Schematic illustration of Atlantic salmon life cycle.The Atlantic salmon life cycle is divided into six stages where the fish spends the life stages from egg to parr life stages in freshwater and most of its adult life stage in oceans, figure adopted from Mobley et al (14)

1.2 Aquaculture

The practice of aquaculture farming involves the breeding, raising, and harvesting of aquatic organisms, including fish, shellfish, and aquatic plants and algae.The farming is practiced in a controlled environments such as pond or ocean enclosures(25). Aquaculture farming especially fish farming nowadays is one of the fastest growing farming selectors in the world. According to FAO 2012 report, food production by aquaculture accounted for 42% of the global seafood production(26), and global production of fish from aquaculture by 2009

accounted nearly 45% of world's food fish production(27, 28). An increase in population and food consumption has resulted in an increase in aquaculture(29).

The Norwegian aquaculture industry, which is mainly relied on farming Salmon and rainbow trout, is one of biggest exporter of salmon fish in the world(30, 31). The country has complex coastline that extends more than 100,000 km as well as several fjords and islands that make the country an excellent place for marine aquaculture. In 2015 the country produced 1.38 million tons of salmon and trout, making it the second-largest seafood exporter in the world(32, 33).

Even though aquaculture activity is increasing rapidly due to the high market demand for fish, there are several challenges that limit the growth of environmentally sustainable aquaculture (29). Environmental concerns are one of the greatest challenges in aquaculture farming, which have several adverse effects on the environment, such as habitat destruction, water pollution, and other effects on the environment. (27). In addition, sustainable aquaculture farming faces challenges with feeding sustainability, and disease management. (27, 34, 35). Managing diseases can be challenging when fish populations are densely stocked. As a result, the diseases caused by viruses, bacteria, fungi, and parasite infections are adversely affecting the aquaculture industry(36).

Currently, disease management in aquaculture farming is focused on vaccination, antibiotics (very rare in Norway), and using several synthetic chemicals. Therapeutic chemicals kill disease-causing organisms in the water or on the surface of aquatic animals. These methods brought partial success in reducing the disease but with lots of expenses(37, 38). As excessive antibiotic use increases antibiotic resistance, it is therefore very important to have alternative approaches to managing disease that ensures a sustainable aquaculture faring(39, 40).

1.3 Fish mucosal surfaces

Inside the eggs, the alevins are germ-free, but once they hatch, millions of microorganisms from the environment colonize its mucosal layer(41). The mucosal layer is mainly composed of epithelium, lamina propria. and smoothed muscle layers. Additionally, the mucosal system consists of mucus that covers the mucosal surcease and plays an important role in colonizing microorganisms. (42). The mucus acts as a barrier between microorganisms and the host, preventing the pathogen from infection(43). Type 1 mucosal surface is among the most studied mucosal types in mammals. This types of mucosal surface in mammals are mainly found in the intestine, respiratory tract uterus. and it contains mucus-secreting cells and receptors(44). Antigens on pathogen surfaces are recognized by these receptors(45). Even though there are some structural and functional differences, the mucosal surface of fish in the skin, gills, and intestine of teleosts share many characteristics with the type 1 mucosal surface of mammals(46). Figure 2 illustrates the similarities and differences between mammalian mucosal surfaces and teleost mucosae (47).

The mucosal surface covering the fish's skin, gills, and digestive tract plays a crucial role in fish immunity. The fish interacts with the environment through this barrier. It plays a vital role in the beneficial microorganisms' colonization process by providing niches and protection from harmful microbes. Thus, the mucosal tissue is covered by millions of microorganisms (46, 48). The mucosal layers secret different antimicrobial peptides and enzymes that inhibit the entrance of pathogenic microbes into the fish (48). Mucin is a large

glycoprotein secreted by goblet cells and is a major component in mucus. It plays a crucial role in trapping microbes in the mucus layer(49, 50). Thus, it prevents microorganisms from attaching to the fish's surface (42, 47). The mucin on fish surfaces could, however, be a major carbon source for some bacteria. This leads to colonization of the fish surface, such as skin, gills, and intestine, by microbes that use mucin as the source of energy for their growth(51, 52).

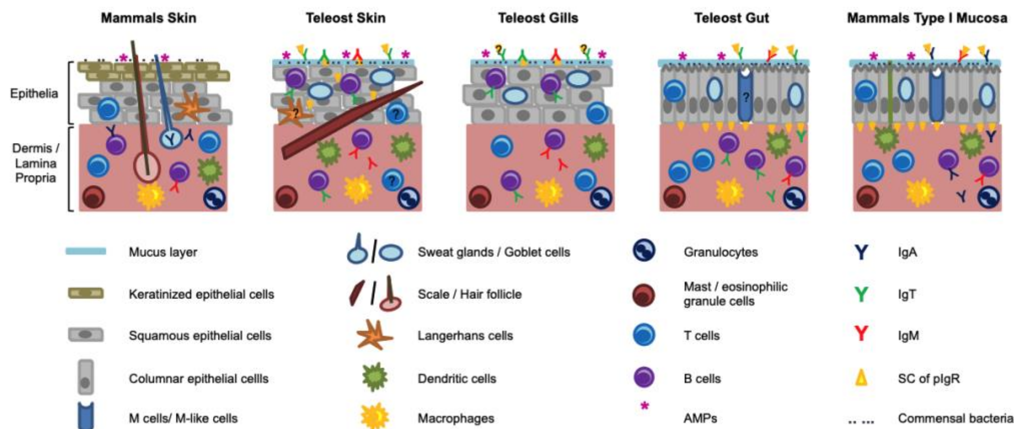

Figure 2: A diagram showing the similarities and differences of structure and cell composition between mammalian skin and type 1 mucosa, and the skin, gills, and gills of teleost fish mucosal surface(47).

1.4 Microorganism-host interactions and its effects

As addressed earlier, the mucosal subrace of the fish is colonized by various bacteria communities once the fish eggs are hatched (41). Therefore, fish mucosal surfaces are colonized by complex microbial communities(53). The microorganism makes a complex and dynamic interaction with the host that can be beneficial or harmful. (54). The health and development of fish are, therefore, largely determined by host-microorganism interaction. This is because the interaction shapes the fish’s immunity as well as metabolism (55). The primary colonizing microbes in the gut initiate the T cells’ response both within and outside the intestine. The T cells play a vital role in orchestrating the adaptive immune system by assisting the recruitment of several immune effectors (56, 57). However, little is known about the fish microbiota compared to understanding human and other mammals’ microbiota. Furthermore, the interaction between fish microbiota and its role needs more understanding(58, 59).

The microbial composition in fish mucosal surfaces is highly dependent on the environment where the fish live and the types of the tissues(47). The fish's external mucosa (skin and gills) are continuously in direct contact with planktonic microorganisms, and the microbial composition in these tissues is highly dependent on the environment which the fish lives(60). On the other hand, the infernal fish mucosa (gut) has direct contact with ingested water and food(61). Studies have shown that microbial composition in skin is more diverse and variable than the microbial composition in the gut as the skin has direct contact with the external environment(61). Most microbial communities in fish’s external mucosal surface, particularly in skin, are aerobic and are important in shaping fish immunity(62, 63).

Most of the studies investigating the host-microbe interaction is focused on gut microorganisms, despite that microbial communities are found in all the mucosal tissue types(61). As addressed in Figure 2, various epithelial cell structures and immunological cell types are present in different mucosal tissues. The tissues also differ in their physio-chemical conditions, such as temperature, pH, and oxygen. Therefore, the fish skin, gill, and gut mucosal surfaces have different roles in fish healthy and development as they differ in structural and functional properties as well as microbial composition (47, 61).

1.5 Salmon fry as a gnotobiotic model

A gnotobiotic study involves the introduction of the microorganism of interest into a germ-free organism and monitoring the effects, for example, on the immune system, digestion as well as metabolic changes for the gnotobiotic animal (64-66).

Using a fish as a gnotobiotic model is advantageous since they release thousands of eggs at once. For statistically verifiable observations, it is helpful to get many offspring from genetically related hosts. In addition, fish are oviparous, which means that their eggs develop outside their bodies. It is, therefore, possible to disinfect eggs at early stages of cell division and monitor development under gnotobiotic conditions. Furthermore, the fish embryo is protected by a chemically resistant chorion that allows eggs to be surface disinfected quickly(67). Zebrafish is the most commonly used gnotobiotic fish model species to study the interaction between fish and microorganisms. This is due to the fact that optical transparency of zebrafish embryos enables researchers to observe cells in development and microbes colonizing the embryos(68). However, the knowledge about fish aquiculture is still limited as the studies done on the area have been using few fish species as gnotobiotic anemias(69)

Recently, the Analysis and Control of Microbial Systems (ACMS) at Norwegian Science and Technology University (NTNU) have designed a novel gnotobiotic experimental model system that allows researchers to study microbial-host interaction in Atlantic salmon. They developed a protocol for the derivation of Atlantic salmon eggs and a system for germ-free husbandry throughout the yolk sac fry (also called alevin) stage. To disinfect eggs' surfaces, suitable chemicals that do not damage salmon mucose were identified. Having this protocol allows researchers to study the interaction between microbiota and host, as well as in developing probiotic treatments, the model plays a pivotal role (66, 70).

1.6 The use of probiotic treatments

Probiotics are living microorganisms that enhances the host immunity by promoting growth and stimulating immunity as well as inhibiting pathogens (71, 72). Probiotic microorganisms should not be harmful to the host, have no antibiotic resistance genes, and be able to colonize the host skin and gut in order to prevent the disease (73). The use of probiotics to boost the immune system in fish, especially in the digestive system, has increased in recent years (74). However, it is important to note that there are several factors that affect the role of the probiotic on the host, including the immunomodulatory potency of probiotics, types of probiotic strains, feeding durations, modes of supplementation, environmental conditions, and doses of probiotics (71, 75).

Nowadays, treating disease using probiotics is a promising approach to secure the sustainability of aquaculture. The probiotics can be used to improve aquatic animals' health as well as water quality (76, 77).

The use of probiotics has been tested in different aquatic organisms, including microalgae. The results showed that the growth of *vibrio alginolyticus* C7b probiotic in the presence of microalgae *Chaetoceros mulelleri*, indicating the organisms can be grown together and can be a good source of food for shrimp(77). In addition, the testes did to improve the growth of Nile tilapia using *Streptococcus* strains as a probiotic microorganism as well as in Swordtail and guppy using *Bacillus subtilis* and *Streptomyces* showed promising results(77).
Several studies have indicated that probiotics are being a promising approach to improve the health of salmon fish. The use of *Lactobacilli* as a probiotic in fish improved fish disease resistance via immune stimulation (78). However, further studies are required on the effects of probiotics in Atlantic salmon (79). Currently, the effects of other potential probiotic candidate, *Janthinobacterium sp.* on Atlantic salmon health is being studied at the Department of Biotechnology and Food science at Norwegian science and technology university (80).

1. 7 The bacterial genus *Janthinobacterium* and its characteristics

Janthinobacterium is a bacterial genus belonging to the phylum proteobacteria, specifically, the family Oxalobacteraceae and is an aerobic, rod-shaped, gram-negative bacteria. Commonly, the strains of this genus have been isolated from various environments such as forests, soil, lakes, and river water (81). Representatives of this genus are also found in extreme environments such as Antarctic snow, oligotrophic groundwater and the Himalayas(82-84). Some studies have reported that *Janthinobacterium lividum* is part of THE skin microbiota of fish and amphibians as well as humans (85-87). Recently, some *Janthinobacterium* strains have been discovered, and their genome has been sequenced(84, 88). It was found that *Janthinobacterium lividum* grows at an optimal temperature, usually between 25-30℃. However, the optimal temperature varies from strain to strain(89). Some psychrophilic *Janthinobacterium sp.* strains grow at 4℃. However, *Janthinobacterium sp.* do not grow at 2 or 37℃ (89-91). Moreover, the studies have shown that the *J.sp.* don not grow below pH 5 or in a media containing 2-9% NaCl(90).

Many *J.sp* strains form biofilms in aquatic ecosystems, and this can help the bacteria to colonize hosts such as fungi and fish skin (92). Biofilm formation is a common response to environmental stress(93, 94). It is a complex structure that is mainly made of an extracellular polymeric substance (EPS) matrix (95). The presence of EPS in biofilms makes the bacteria grow in aggregates (96). The biofilm formation in *J.sp* is high at its early stationary phase, indicating its biofilm formation depends on the growth phase(97). Furthermore, the biofilm formation by *J.sp.* is highly affected by environmental stimuli, suggesting biofilm formation is a response to environmental stress and is a method to survive harish conditions(97).

Some *Janthinobacterium* strains produce the secondary metabolite violacein, which is a purple pigment (81). Violacein is one of the most well-known secondary metabolites produced by *Janthinobacterium sp.* due to its interesting properties. It was reported that the molecule exhibits several biological activities such as antibacterial(98), antifungal(86, 99), antiviral(100), and anticancer activities (101, 102), making it an important candidate molecule in industries such as medicine and cosmetics(102). Surprisingly, it was found that

biofilm formation and violacein production was affected by environmental stimuli such as temperature and glucose presence, suggesting there might be a common metabolic pathway for biofilm formation and violacein production(92, 97).

As stated above, several studies revealed that *Janthinobacterium* exhibits a variety of interesting properties, including antibacterial, antifungal, and antiviral properties(103-105). This is due to the fact that *J.sp.* produces several secondary metabolites that have an interesting properties. For instance, genome analysis of *Janthinobacterium sp.* strain SLBOL showed that, the strain contains conservative cluster of genes for the biosynthesis of secondary metabolites including tropodithietic acid (TDA) and marinocine (106). Tropodithietic acid and marinocine are secondary metabolites that processe potent antibiotic activity against wide ranges of bacteria(107, 108).

Moreover, some studies have shown that *Janthinobacterium* can grow in high levels of CO2 (5%), indicating its capnophilic behavior(91). The study done on *J.lividum* strains also reported that the bacterium contains enzymes that are used to fixe CO_2. In combination with glyoxylate cycle, the ability of *J.lividum* to fix CO_2 could be used to facilitate the glucogenesis, indicating the capnophilic nature of *J.lividum* (109). The capnophilic behavior of the bacterium is one advantage during the colonization of the skin of hosts such as Atlantic salmon yolk sac fry and amphibians as the carbon dioxide secreted by organism's skins serves as a guiding molecule to colonize their skins (91, 110). The study done by the Analysis and Control of Microbial Systems (ACMS) group to compare five different bacteria's ability to colonize the skin of Atlantic yolk sac fry also strengthened the finding. As the experimental group reported, *J.livium'* colonization ability of the skin of Atlantic salmon was better than *Bacillus sp., Pedocacter sp., Arthrobacter sp. and Psychrobacter cibarius* (111). This could be due to the fact that the microorganism thrives in environments with high CO_2 concentrations.

The quorum sensing system in *Janthinobacterium sp* plays a crucial role in survival. Quorum sensing is a cell-to-cell communication process that coordinates their behavior and gene expression according to the population density(112). Some studies suggested that the quorum sensing system in *Janthinobacterium* could be correlated with the regulation and formation of biofilms and of violacein production (97). The study conducted on *Janthinobacterium sp*-HH01 showed that the quorum sensing system in this bacterium is regulated by alpha-hydroxyketone-like autoinducer molecules referred to as JAI-1(113). The regulatory system consists of three genes designated as autoinducer (*jqsA*), sensor kinase gene (*jqsS*), and response regulatory gene(*jqsR*) (99, 114). And it was indicated that the QS system regulates the production of violacein through the jqsA gene (114). There is, however, a need for further studies to identify the structure of the JAI-1 regulatory molecule and the role that it plays in other regulatory circuits in *Janthinobacterium*.

1.8.1 *Janthinobacterium* isolates

During previous project work in the ACMS group, the five *Janthinobacterium sp.* strains referred to as PBB, PBB,3.108,3.109, and 3.116 have been isolated from the skin of the Atlantic salmon fry and the rearing water from flasks with Atlantic salmon yolk sac fry (80, 115). The strains referred to as PBA and PBB were isolated from the rearing water from flasks with Atlantic salmon yolk sac fry, while the remaining strains were isolated from the

skin of Atlantic salmon fry. The violacein operons of these five strains have been successfully sequenced. The results showed that PBA and PBB possessed all five genes in violacein operons. The 3.108 strain did not have a functional VioE gene. The remaining two *J.sp* strains lack the genes of the violence operon(51).
Even though the strains have different phenotypes, the study demonstrated that the strains have closely related 16s rDNA sequences. The strains have been genome sequenced, and their genome analyses are underway (Eirik Degre Lorentsen, oral communication). Furthermore, it was confirmed that all strains grow on both mucin and chitin as the only carbon sources((80). The antagonism towards Saprolegnia of these five strains has been tested.

Strain 3.108 inhibits Saprolegnia growth, while the remaining strains do not(80). However, it was found that the strains' antioomycete properties are not related with violacein production. Currently, the strain's antagonist effects against Saprolegniosis and its potential probiotic candidate on Atlantic salmon health is being studied at the Department of Biotechnology and Food science at Norwegian science and technology university (80).

1.9 Aim of the study

The first main aim of this master thesis was to characterize the growth nature of the five *Janthinobacterium sp.* isolates referred to as PBA, PBB, 3.108,3.109, and 3.116. The second main aim of the study was to investigate the *Janthinobacterium. isolates*' ability to colonize the Atlantic salmon egg and yolk sack fry. The objectives of the study:

- Characterizing the five *Janthinobacterium sp.* strains referred to as PBA, PBB, 3.108,3.109, and 3.116 in general growth media.
- Assessing enzymatic activities of the five strains by performing an enzyme assay.
- Investigating the strains' ability to colonize newly hatched germ-free Atlantic salmon yolk sac fry both when added separately and in a mixture.
- Examine the potential of the isolates to colonize conventionally reared eggs and yolk-sac fry in competition with the commensal microbiota.

The overall aim of this master project was to improve the understanding of the growth characteristics and host colonization ability of *Janthinobacterium sp.* strains.

2 Materials and methods

2.1 Description of *Janthinobacterium sp.* isolates

In this master's thesis, the experiments and analyses conducted to characterize *Janthinobacterium sp.* were performed on five *Janthinobacterium* strains referred to as PBA, PBB, 3.108,3.109, and 3.116. Two of the strains were previously isolated from rearing water with Atlantic salmon yolk sac fry, whereas the remaining three were isolated from the skin of Atlantic salmon yolk sac fry by members of the Analysis and Control of Microbial Systems (ACMS) group (Table 1).

Table 1: An overview of the *Janthinobacterium sp.* isolates and their origins of isolation studied in this master's project.

***J.sp.* strains**	**Origen of isolation**
PBA	Rearing water from flasks with Atlantic salmon yolk sac fry (lab scale culture flasks, eggs from; AquaGen AS
PBB	Rearing water from flasks with Atlantic salmon yolk sac fry (lab scale culture flasks, eggs from; AquaGen AS
3.108	Salmon fry skin (commercial RAS;SalMar)
3.109	Salmon fry skin (commercial RAS;SalMar)
3.116	Salmon fry skin (commercial RAS;SalMar)

2.2 Characterization of five *Janthinobacterium sp.* isolates

The aim of the characterization of the five strains of *J.sp* was to identify and classify the microorganism based on their distinct physical, chemical, and genetic characteristics.

As part of the characterization process, microscopy, morphology, biochemical testing, and 16s RNA sequencing were performed. Biochemical testing was performed to examine different enzymatic activities in the strains. Sanger sequencing was performed to identify the strains and to ensure that we were working with the correct strains.

2.2.1 Growth media and conditions

Liquid and solid media were used to grow the isolates: Tryptic Soya Agar (TSA) and Tryptic Soy broth (TSB) with 0.1% Tween-80 (App. A.). During growth on TSA, the isolates were incubated at room temperature. Overnight cultures of five *J.sp.* isolates were prepared by inoculating a single colony of each isolate from TSA agar plates in 250 ml baffled Erlenmeyer flasks containing 25 ml TSB medium. The 0.1% Tween-80 was added to prevent cells from aggregating and promoting planktonic growth.

The cultures were placed on an orbital shaking table (300 rpm) and incubated under aerobic conditions at room temperature.

In addition, four liquid growth media: Sabourad Dextrose (Sab, Dex), Brian Heart Infusion (BHI), Nutrient Broth (NB), and Glucose Yeast Medium (GY) were used for sterility check testing in the fish experiments (APP. I). Approximately 3 ml of each growth medium was prepared in 13 ml pre-autoclaved tubes. During the fish experiment, to check the sterility status of the rearing water before introducing the *J.sp.* isolates into the fish flasks, 100 μl of rearing water was taken from the fish flasks and added to tubes containing 3 ml of each growth medium. A duplicate of each sample was prepared, and any microbial growth in the samples was visually observed after three weeks of incubation at 7.5℃ and at room temperature.

2.2.2 Growth conditions of *Janthinobacterium sp.* strains

To investigate the growth of the five different *J.sp.* strains, the strains from cryo stocks that have been stored at -80 were streaked on TSA with 1.5% agar. The colony morphology of the isolates was characterized based on a standardized growth pattern and colony characteristics of bacteria and fungi (116). After 24 hours of incubation, colony size was determined by measuring the diameter of each strain's colony in millimeters. Colony characteristics such as color, viscosity, shape, as well as elevation were determined after 5 days of incubation at room temperature. To get fresh bacterial colonies, transferring a single colony from plates to new plates was performed every two weeks throughout the entire project. This further helped to better study the growth of the isolates.

The isolates were grown in TSB to investigate their growth in liquid media. A single colony of each strain from TSA agar was transferred into 13 ml tubes containing 3 ml TSB medium. The colonies were then incubated at room temperature at 300 agitations. Microbial growth conditions were visually inspected after one week of incubation time.

2.2.3 Microscopy

The shape and growing nature of the five *J.sp.* isolates in liquid cultures were studied using the Zeiss Axio VERT. A1 microscope. The overnight cultures (see section 2.2.1) of each strain were fixed with 1% glutaraldehyde and mixed well using a vortex mixer. The samples were then stored at +4℃. The following day, all samples were mixed again in a vortex mixer to disperse the potential aggregation of cells and prevent bacteria from settling at the bottom. Each strain's undiluted and 1:1000 diluted samples were examined under a Zeiss Axio vert.A1 microscope. The unfixed and fixed samples were compared to see the difference in aggregation or clumping of the cells together.

2.2.4 Generating growth curves and establishing the relationship between OD_{600} and cell density

To establish the correlation between the cell counts determined by flow cytometry and OD600 measurements, OD600 and the cell concentration of each isolate measured with flow cytometry were determined. To do this, first an overnight culture for each of the five isolates (described in section 2.2.1) was prepared prior to the experiment. On the morning of the following day, the overnight culture was used to inoculate a new culture from which we measured the OD and took samples. For the first 8 hours, the OD600 of the cultures was measured every 1 and 1/2 hours, then every hour for the next 14 hours. The samples were

then taken from each OD measuring time and fixed to glutaraldehyde for flow cytometry analysis.

A total of 490 ml of each sample from each strain was fixed with 10 ml glutaraldehyde (Glutaraldehyde 1% final concentration). The samples were then stored at +4°C. To analyze the samples in flow cytometry, the samples were diluted 1:100 using 0.2µm filtrated PBS (1x) (App. C) and stained with a working solution of SYBR green I 200x stock to a final concentration of 2x stain to fit with the recommended limit between 100 and 2000 cells/µl. To make SYBR green I 200x stock solution, SYBR green I (10000x) was diluted with a sterile 0.2µm filtrated PBS (1x). The samples were incubated in the dark for 15 minutes at 37°C. After vortexing and mixing, the samples were analyzed with flow cytometry Attune NxT (ThermoFisher) with a flow rate of 100/min for 1.5 minutes. Data collection was carried out using a blue laser (488nm, 50 mW) with detection in BL1 (530/30 nm) and BL3 (695/40) using the following voltage settings; FSC 370V, SSC 440V, BL1 290V, and BL3 440V. 0.2µm. As negative controls and to identify bacterial populations, filtered PBS and SGM were used. Further analysis of the results was performed in Microsoft.

2.2.5 Assessment of enzymatic activity

The enzymatic activities of the five *J.sp.* isolates were examined with the API ZYM test kit following the manufacturer's protocol (App. D). A total of 19 enzymatic activities in the J.*sp.* strains were studied. The five strains were cultivated overnight (see section 2.2.1) prior to the experiment. To test the enzymatic activities of each strain, the overnight culture was diluted to an optical density between 0.5 and 0.6 at 600nm. A total of 65 ml of each diluted culture strain was added to each of the 19 cupules and incubated at 37°C. After 4 hours of incubation, two reagents, ZYM A and B, were added to each cupule in a strip. After five minutes, the color changes from each strip were visually observed and recorded.

2.3 16s rRNA gene sequencing

The 16S rRNA gene sequencing was carried out on each of the five isolates of *J.sp.* to ensure that we were working with the right strains. The DNA of the isolates was extracted and their 16S rRNA genes were amplified for Sanger sequencing as described below. Sanger sequencing results were further analyzed, and the results confirmed that the strains belong to *Janthinobacterium sp*. The overall workflow scheme of the experiment is shown in Figure 3.

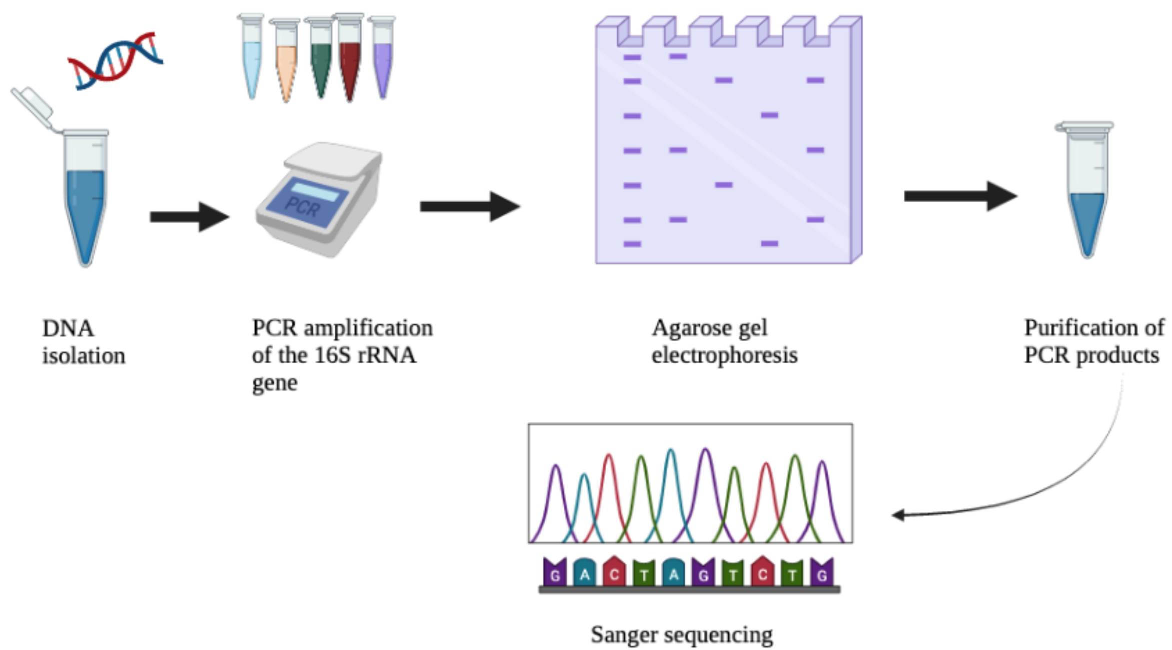

Figure 3: An illustration of the methods used to sequence the 16s rRNA gene in five *Janthinobacterium sp.* isolates are shown in this step-by-step guide.

2.3.1 DNA extraction

To extract the DNA of *J.sp.* strains, overnight cultures of five *J.sp.* isolates were prepared as previously described (section 2.2.1). Then 1 ml of sample from the bacterial cultures were centrifuged for 1 minute at 13,000 x g. The supernatant was removed and the procedure was repeated two times. The DNA was then extracted using the DNeasy PowerSoil kit (Qiagen) protocol following the manufacturer's protocol (App. E.1). The DNA concentration and the purity of the extract was measured using Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometers.

2.3.2 Amplification of 16S rRNA genes

The previously extracted DNA of the *J.sp.* isolates were used as templates to amplify the 16S rRNA genes for Sanger sequencing. The primer combination of EUB8.F and 1492. R with 1500 expected product length was used to amplify the genes whereas the three primers EUB8.F, 1492. R, and 805. R were used in the Sanger sequencing reactions (Table 2). DNA extracts were diluted 1:100 and used as templates in the PCR reactions. For PCR reaction a total of 24 µl master mix solution (Tab. 3) was prepared. A total of 1 µl of DNA diluted extract was added as a template to each master mix solution. The samples were then mixed well using a vortex mixer. Amplification of the samples was performed in a T100™ Thermal cycler (BioRad) as described in Table 4.

Table 2: Primers used for PCR amplification of the 16S rRNA gene (EUB8.F and 1492.R) and as primers in the Sanger sequencing reactions (all primers).

Primers	Target gene	Sequences (5',3')
EU8.F	16s rRNA	AGAGTTTGATCMTGGCTCAG-
1492.R	16s rRNA	TAC GGY TAC CTT GTT ACG ACT T
805R	16s rRNA	ATT ACC GCG GCT G CT GG

Table 3: The components needed to make the master mix (24µl) for PCR amplification of 16s rRNA gene in *J.sp.* strains.

Master mix components	Supplier	Volume 1x (µ*l*)
DNA free H_2O	VWR	16.82
5x Phusion HF Buffer (7.5 mM $MgC1_2$)	Phusion Kit Illumina	5.0
Forward primer (10 mM)	Sigma Aldrich	0.75
Revers primer (10 mM)	Sigma Aldrich	0.75
dDNTP mix (40 mM total)	VWR	0.5
Phusion Hot Strat II DNA polymerase (2 U/ µ*l*)	Phusion Kit Illumina	0.18
Total volume		24

Table 4: PCR cycling conditions used to amplify 16s rRNA gene of five J.*sp.* isolates.

No	Reactions	Temperature (°C)	Time	
1	Warm up	98	∞	
2	Initial denaturation	98	2 minutes	
3	Denaturation	98	15 seconds	36 cycles
4	Annealing	55	2 seconds	36 cycles
5	Elongation	72	2 seconds	36 cycles
6	Final step of elongation	72	5 minutes	
7	Cooling	4	1 minutes	
8	Storage	10	∞	

2.3.3 Gel electrophoresis

Gel electrophoresis was performed to examine the size and quality of the PCR products using 1% agarose gel in Tris-acetate EDTA (TAE) buffer (App. B). Agarose solution (1%) was prepared by dissolving agarose in TAE buffer (1%) by boiling it in a microwave oven. A 250 ml Erlenmeyer flask was filled with 50 ml of 1% agarose and 2.5 ml GelRed was added to stain the gel. The solution was poured into the gel chamber and left for 30 minutes to solidify. To indicate the size of the PCR products, 5 µl of GeneRuler 1 kb plus DNA Ladder (Thermo Scientific) was used as a reference. A total of 5 µl PCR products from each sample were mixed with 1 µl of 6xloading Dye (Thermo Scientific) and loaded onto the gel. The gel was

run at 110 V, 220 mA for 45 minutes. The size and quality of the PCR products were then inspected under UV light using a G: Box HR gel doc (Synegen).

2.3.4 16S rRNA sanger sequencing

Prior to sending the PCR products for Sanger sequencing, purification of the DNA products was performed using the QIAquick® PCR Purification Kit (Qiagen) following the producer's protocol (App. E.3). All procedures were done following the manufacturer's protocol except using 650 µl of PE buffer instead of 750 µl and 22 µl water MilliQ H2O (pH 7-8.5) instead of EB buffer for elution. To send samples for Sanger sequencing, three reactions were prepared for each PCR product using the primers listed (Table.2). For each PCR product, three sequencing reactions were prepared, one for each of the three primers used as sequencing primers (see Table 2). A total of 5µL of purified PCR product was mixed with 5µl sequencing primer (5mM). The samples were then sent to Eurofins Genomic for Sanger sequencing.

The resulting DNA sequences were returned from Eurofins Genomics as FASTA files together with chromatograms. The chromatograms were used to assess the sequence quality. By using Clone Manager 11 (Sci Ed Software) software (117), the resulting sequences were edited, assembled, and aligned. Prior to alignment, DNA sequence regions of poor quality were removed from the sequences. Specifically, regions with poor quality at the beginning and end of the sequence were removed. The reverse sequences were complemented. The resulting assembled sequences were aligned to DNA sequences of *J.sp.* strains previously sequenced. In other words, to confirm the identity of the isolates, an alignment of the exported sequences of each strain with DNA sequencing of the corresponding isolates previously isolated by ACMS groups was performed.

2.4 Experiment on Atlantic salmon fish

2.4.1 Experimental overview

During the fish experiments, the five *J.sp.* isolates were examined for their ability to colonize Atlantic salmon eggs and yolk sac fry. Furthermore, the experiments aimed to examine the interactions between strains during the initial colonization of eggs and yolk sac fry. Upon arrival, the eggs were acclimatized and some of the eggs were surface disinfected to generate germ-free eggs. Those eggs that had been surface disinfected were designated germ-free eggs (GF), while the eggs that had not been surface disinfected were designated conventionally raised eggs (CVR). However, Experiment 1 was repeated due to contamination occurring in the eggs. Additionally, since egg death occurred in Experiment 2, we decided to go for other experimental designs. Therefore, the experiments were conducted in two rounds, Experiment 1 and 2 in the first round and Experiment 3 and 4 in the second round. Duration of egg/yolk sac fry exposure to the isolates varied from experiment to experiment. Samples were collected to investigate colonization success in egg/fish and water using colony-forming unit (CFU) analysis and to determine the relative microbial abundance by amplicon sequencing.

2.4.2 Experimental design

Four fish experiments, where Experiments 1,2 and 3 consisted of two treatments each and Experiment 4 consisted of five treatments were conducted in two rounds. Experiment 1 and 2 were conducted in the first round whereas, Experiment 3 and 4 were performed in the second round.

In Experiment 1, the five *J.sp.* isolates ability to colonize the GF Atlantic salmon eggs and yolk sac fry was investigated. However, Experiment 1 was repeated in Experiment 3 due to contamination. On the other hand, in Experiment 2, the CVR Atlantic salmon eggs were exposed to a mix of the five *J.sp.* isolates to investigate if the isolates could be able to colonize the eggs that already had microbiota. Furthermore, Experiment 4 was performed to investigate if the *J.sp.* isolates could be able to colonize the GF Atlantic salmon yolk sac fry when the isolates were added separately (for detail see each experimental design).

Fish experiments were conducted in two rounds due to contamination and egg death occurred in Experiment 1 and egg death in Experiment 2. The eggs for the first round of the fish experiment, were received from Lerøy within 369-degree days. But for the second round of the fish experiment, eggs were received from Aquagen with 339-degree days. The eggs from Lerøy seemed to be infected. To determine the cell density of each isolate introduced to the flaks containing Atlantic salmon eggs and yolk sac fry, the MacFarland standard based on OD measurements was used.

2.4.3 Fish experiment round one

2.4.3.1 Experiment 1

To investigate the five *J.sp.* isolates ability to colonize germ-free Atlantic Salmon eggs and yolk sac fry, a mix of five *J.sp.* isolates were introduced to the fish flasks-containing germ-free eggs and yolk sac fry in two Treatment conditions. In Treatment 1, the isolates were introduced to the fish flakes containing Atlantic salmon eggs at 12 days before hatch (dbh). Atlantic salmon eggs in Treatment 2 stayed germ-free until the eggs were hatched. In Treatment 2, to examine whether the isolates have different colonization abilities when introduced after hatching, salmon yolk sac fry were exposed to a combination of the isolates at 8 days post hatch (dph) (Fig. 4). The MacFarland standard was used to determine the cell density of bacterial cultures based on OD measurements. The *J. sp.* strains were added to a final concentration of 10^5 of each strain (for detail, see section 2.4.8). Three replicates of tissue flasks were used in each treatment condition and a total of six tissue flasks were utilized to perform the experiment (Fig. 4). Sampling of water, eggs, and fry for microbial analyses was scheduled to be performed at 6 dbh from Treatment 1 and at 31 dph from Treatment 2. However, the experiment was terminated before sample collection due to contamination and egg and yolk sac fry death.

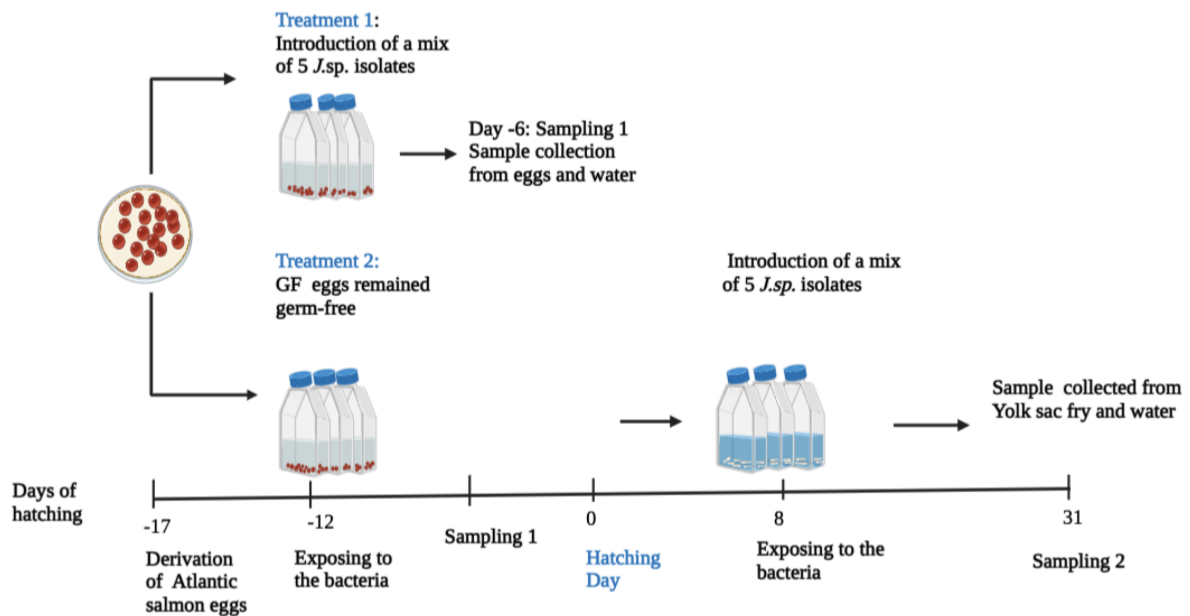

Figure 4: An illustration showing an experimental design of the Atlantic salmon Experiment 1. Figure created with Biorender.com.

2.4.3.2 Experiment 2

To investigate how the *J.sp.* strains colonize the fish skin in competition with other bacteria, pre-acclimatized CVR eggs were exposed to a mix of isolates on 0 Day post-exposure (dpe) in treatment 1, while eggs in treatment 2 remained untreated as a control group (Fig. 5). To reduce microbiota from the egg surface, eggs in Treatment 1 were rinsed twice with sterile Salmon gnotobiotic media (SGM) and 60% of the medium was changed at 6 dpe. In total, six tissue flasks were used in the experiment, three for each treatment condition (Fig. 5).

To determine the relative abundance of the microorganism in samples by amplicon sequencing of 16S rRNA and *jqsA* genes, samples were taken of eggs and water from both treatment groups on 16 dpe.

For egg samples, at 16 dpe, four eggs from each flask were carefully picked up with sterile plastic forceps and transferred to 6-well plates. After the eggs were rinsed twice with a sterile SGM, eggs were then transferred to precellys tubes containing sterile 0.1 mm glass beads (Bertin Technologies). A duplicate of egg samples from each flask from from both treatment conditions was prepared. A total of 6 egg samples, each precellys tube containing four eggs were collected. For water samples, 20ml of rearing water was taken from the flasks and filtered through 0.2 µl nucleopore filter paper (Whatman® Nuclepore™ Track-Etched Membranes). The filter paper was then cut by a sterile blade into several pieces and transferred into the precellys tubes. A duplicate of water samples was collected from each flask from Treatment 1 at 16 dpe. A sample collection from all treatment groups was performed prior to hatching. Both sample types were then stored at -20 for later microbial community analysis.

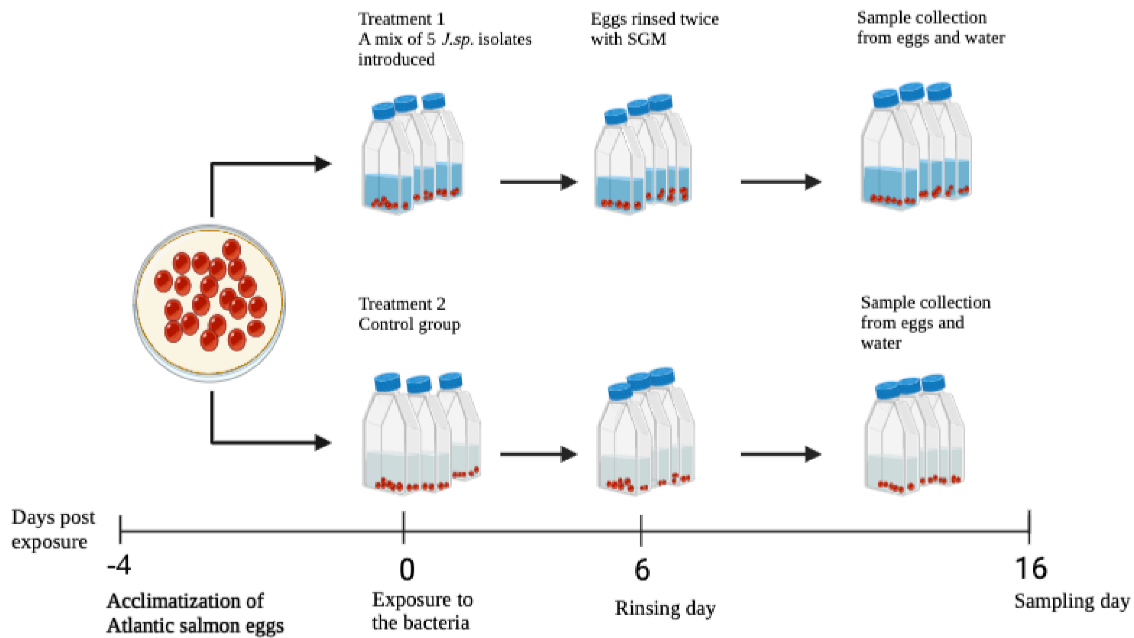

Figure 5: An illustration showing an experimental design of the Atlantic salmon Experiment 2. Treatment 1, Atlantic salmon eggs were exposed with a mix of five *J.sp.* strains and samples were collected before hatching. Figure created with Biorender.com.

2.4.4 Fish experiment round two

2.4.4.1 Experiment 3

Experiment 3 was conducted to investigate whether isolates could colonize the yolk sac and eggs and yolk sac fry of Atlantic salmon. The design for Experiment 3 was similar to Experimental 1 (for details, see 2.4.3.2). The only difference was the exposure date of eggs/yolk sac fry and the types of samples collected. In Treatment 1, a mix of five *J.sp.* isolates were introduced to the tissue flaks containing germ-free Atlantic salmon eggs before hatching, whereas in Treatment 2, the mix of five *J.sp* isolates were introduced to the tissue flaks containing germ-free after hatching, to see whether there is any difference in the colonization abilities of the isolates (Fig. 6). However, samples collected for treatment 1 were collected after all eggs had hatched since eggs hatched earlier than expected. At 27 days of post-hatching, samples were taken from both treatment groups. Yolk sac fry and water samples were taken to determine the relative abundance of the isolates (Fig. 6). Three replicates of tissue flask were used in each Treatment condition, and a total of six tissue flasks were utilized to perform the experiment (Fig. 6)

At the end of the experiment, samples from rearing water and yolk sac fry were collected. For yolk sac fry samples, one fish from each flask from both Treatment was carefully picked up by sterile forceps and transferred to 6-well plates containing sterile filtrated tricaine 52 mg/ml in one plate for euthanizing and sterile SGM in another two plates. The yolk sac fry was euthanized and rinsed twice and transferred into the precellys tubes. Quadruples of the samples from each flask, one fish per sample, were collected from both Treatment 1 and 2 at 27 dph. In total, 24 yolk sac fry were utilized for sampling. For water samples, duplicate water samples were collected from each flask from both Treatment 1 and Treatment 2 at 27

dph. To collected water samples the same technique described in section 2.4.3.2 was used. Both yolks sac fry and water samples were then stored at -20℃ for later microbial analysis.

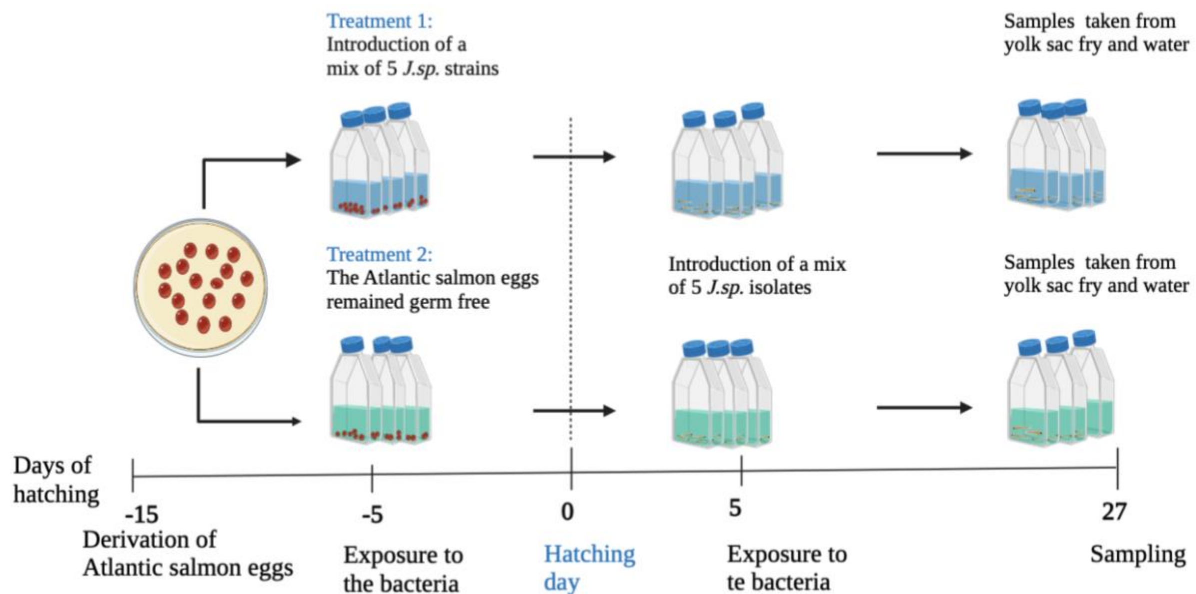

Figure 6: An illustration showing an experimental design of the Atlantic salmon Experiment 3. Figure created with Biorender.com.

2.4.4.2 Experiment 4

This experiment was performed to investigate to which extent the five strains were able to colonize yolk sac fry and rearing water when added separately (mono-associations). Each of the five *J.sp* isolates was added to a separate flask containing SGM and germ-free Atlantic salmon eggs at 10 days pre-hatching. The experiment was performed with five tissue flasks, one for each *J. sp.* isolate (Fig. 7).

Both yolk sac fry and water sample collection was performed at 12 days of post-hatching for CFU analysis. To collect yolk sac fry samples the same, methods described in section 2.4.3.1 were used. The yolk sac fry samples were collected in triplicate from each treatment condition at 12 dph. A total of 15 yolk sac fry (three yolk sacs per treatment) were utilized for sampling. For water samples, duplicate water samples were collected from each flask from each treatment condition at 12 dph. To collect water samples, the same technique described in section 2.4.3.2 was used(Fig. 7).

A total of 400µl SGM was added to each precellys tube containing both yolk sac fry and water samples for homogenization. After homogenization, 50 µl of both sample types were plated on TSA plates for CUF analysis. CFU count was performed after 5 days incubation day.

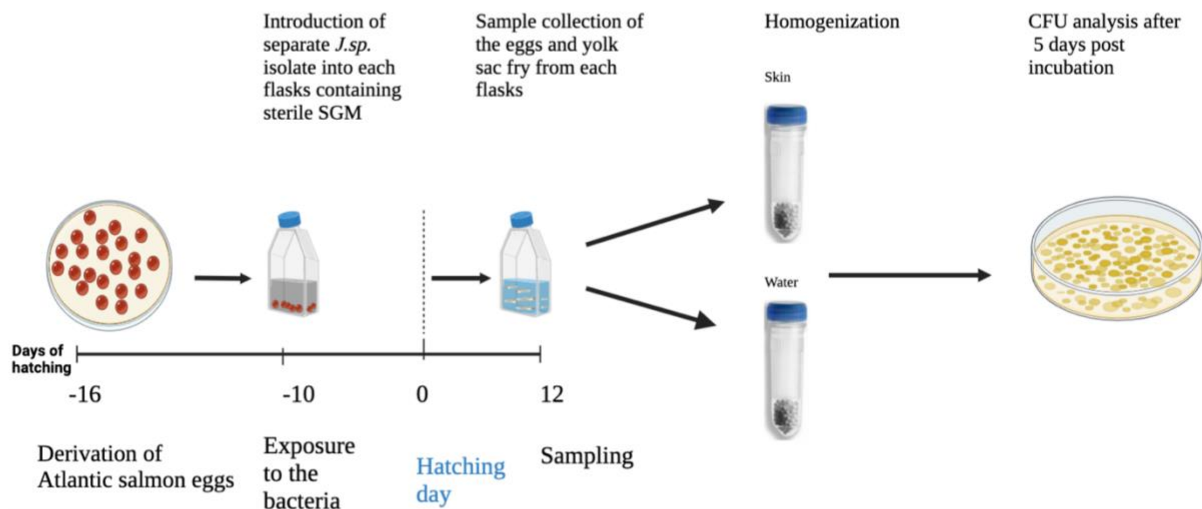

Figure 7: An illustration showing the experimental design of the Atlantic salmon experiment for bacterial colonization with the five *J.sp.* isolates PBA, PBB,3.108,3.109, and 3.116. The same set up per treatment was used for all isolates. Figure created with Biorender.com.

2.4.5 Fish husbandry

As the experiments were conducted in two rounds, Atlantic salmon eggs were obtained from two different companies. In the first round of the experiment, we received eggs from Lerøy Midt Facility in Belsvik in Heim, whereas the second fish eggs were from Aquagen AS from Steigen. Upon arrival, the embryos' health status was visually inspected. Only healthy embryos were used for the experiment. The healthy eggs were distributed into large Petri dishes (140 mm diameter), with approximately 100 eggs in each petri dish. Sterile Salmon gnotobiotic media (SGM, App. G) was then added to the Petri dishes until the eggs were covered. The eggs were then acclimatized for 24 hours at 7.6℃ in a dark room. To generate germ-free eggs, some eggs were treated with antibiotic cocktail solutions and buffodine solution as described in section 2.4.6, while CVR eggs remained untreated. The embryos were then incubated in a dark room at 7.6℃ for the next 24 hours. After surface disinfection and sterilization checks, the eggs were designated germ-free (GF) eggs and those not disinfected were designated conventionally raised (CVR) eggs.

The salmon eggs and yolk sac fry were reared in 250 ml sterilized tissue culture flasks (VWR) in a dark room at 7.6℃ until the end of the experiment. The quality of the rearing water was maintained by changing 60% of the SGM from the flasks three times a week (Monday, Wednesday, and Friday). The husbandry conditions for CVR eggs were the same as for germ-free eggs and yolk sac fry, except for not being disinfected. All procedures were performed in a laminar flow hood and aseptic techniques were used throughout the entire process with UV-disinfected materials.

2.4.6 Generating germ-free Atlantic salmon eggs

To generate germ-free eggs, after a day of acclimatization in SGM, the SGM was removed and the eggs were immersed in approximately 100 ml of the antibiotic cocktail (App. H). The embryos were then incubated in a dark room at 7.6℃ for 24 hours. After 24 hours, the surface of the embryos was disinfected with a Buffodine solution (Fish Tech) of 50 mg/l of available iodine (App. H). The eggs were then transferred into 50 ml conical vials containing Buffodine solution and stayed in the solution for 30 minutes. The eggs were gently agitated during the disinfection process to ensure that the whole surface of the eggs was in contact with the solution. After 30 minutes, the eggs were rinsed four times with sterile SGM by pouring 50 ml SGM into 50 ml conical vials. The SGM medium was poured off each time we added another SGM. The germ-free eggs derived by this protocol were then transferred into 250 ml sterilized tissue culture flasks (VWR) filled with 100 ml of sterile SGM (14 eggs per flask) and placed in a dark room at 7.6℃

The entire procedure was performed under a laminar flow hood after all the materials used to perform the procedure were exposed to UV light for 30 minutes. The materials were disinfected with 70% ethanol before transfer to a laminar flow hood.

2.4.7 Sterility checking

A sterility check was performed prior to exposing the eggs and yolk sac fry to the five *J.sp.* isolates to check the sterility status of surface disinfected eggs. A total of 100 µl of SGM from tissue culture flasks containing Salmon eggs and yolk sac fry was taken and incubated in four different growth mediums (App. I) referred to Sabourad Dextrose (Sab Dex), Brain Heart Infusion (BHI) Nutrient Broth (NB) and glucose yeast Medium (Gy). Before culturing, the growth medium was autoclaved in 13 ml tubes.

A duplicate of samples from each flask was prepared and placed at 7.6℃ and another at room temperature at 23℃ . In addition to this, duplicate of the samples was also prepared by plating 100 µl of SGM taken from the flasks on Tryptic soya (TSA) plates and placed at 23℃ and 7.6℃ where the liquid cultures were placed. There were also negative and positive samples for control. The liquid cultures and the TSA plates were inspected for microbial growth after three weeks of incubation. For treatments where the *J.sp.* isolates were introduced after the eggs were hatched, a second sterility check was performed immediately prior to the addition of the *J.sp.* isolates to ensure that the eggs were still germ-free.

2.4.8 Determining inoculum density

The amount of *J.sp.* strains used to expose the germ-free eggs was determined by measuring OD600 of the overnight cultures of the five *J.sp.* isolates in all experiments. In Experiments 3 and 4 dose determination was also performed by the flow cytometry cell count method to get more reliable bacterial dose. An overnight culture was prepared as described in section 3 the day before the experiment. OD600 of each strain was measured using a spectrophotometer (HITACHI U-5100). The intended final density of bacterial cells in the flasks was 105 CFU/ml for all strains. To obtain a valid OD value (less than 0.3), overnight cultures of the strains were diluted with SGM. Bacterial cell concentration was calculated using the McFarland standard (eq. 1).

$$\text{Bacterial concentration (CFU/ml)} = OD_{600} * 1.2 * 10^9 \qquad [1]$$

The desired volume of the isolates added to the fish flasks was determined by dividing the intended density of bacterial cells in the flasks by bacterial density based on OD and then multiplying by the total volume of SGM in the flask.

2.5 Analytical methods

2.5.1 DNA extraction and PCR amplification of *jqsA* genes in *J.sp.* strains from samples collected from fish experiment

Extraction of DNA from samples collected from fish experiments 2 and 3 was carried out for amplicon sequencing of 16S rRNA and *jqsA* genes in *J.sp.* The DNA extraction was performed using the ZymoBIOMICSTM DNA Miniprep kit (Zymo research) and following the manufacturer's protocol (App. E.2).

There is little variation in the 16S rRNA gene sequence among the strains. To estimate the relative amounts of each strain in colonization experiments with salmon eggs and yolk sac fry, we wanted to use another marker gene that could differentiate between strains more effectively. The sequences for the *jqsA* gene, which encodes for the autoinducer synthetase JQSA, have previously been sequenced by Ph.D. candidate Eirik D. Lorentsen (App. J).

The primers Ill-jqsA_240_2.F and Ill-jqsA-732_2.R, designed by Ph.D. Eirik Degre Lorentsen, were used to amplify a 492 bp fragment of the *jqsA* genes in the *J.sp*. isolates (Table 5). As the primers were new, the PCR runs were tested with different annealing temperatures (gradient PCR) and dilutions to get the highest yield of PCR products.

After identifying the optimal annealing temperature (Fig. 16) and template dilutions (Fig.17) that yield the best PCR products, PCR was conducted for all samples. Aside from the annealing temperature, number of cycles, and primers concentration, the PCR cycling conditions are the same as described in section 2.5.1.2. Here, we used an annealing temperature of 58 °C, 38 PCR cycles, and undiluted DNA extracts as templates.

The amplicon library preparation for Illumina sequencing was performed for PCR products of fish and water samples gained from fish Experiment 3. Since eggs death occurred in Experiment 2 and we did not samples from control group (Treatment 2), we dropped amplicon preparation for samples from Experiment 2. Additionally, there was also time shortages to prepare two different amplicons (based on 16S rRNA and *jqs*A)

Table 5: Primer sets used to amplify a 490 bp region of *jqsA* gene of five *Janthinobacterium sp.* isolates referred to PBA, PBB, 3.108,3.109,3.116.

Primer	Sequences (5'-3')	Melting Point
Ill-JQSA_240_2. F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNN **GGC GSC ATG CTG ATG TC**	51.9
Ill-Jqsa_732_2. R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNN N**CMG TGC GGA AAT GCA CG**	49.5 - 51.9

2.5.1.2 Amplicon library preparation for Illumina sequencing

The *jqsA* amplicons based on water and fish samples from Experiment 3 were further used to make an amplicon library for Illumina sequencing. Bacterial community-based characterization based on 16S rDNA was not done due to time constraints and egg mortality in the CVR flaks in Experiment 2. Therefore, the amplicon library preparation for Illumine sequencing was performed only for PCR products of fish and water samples gained from fish experiment 3. To prepare the amplicon library, the first purification, and normalization of the PCR products were performed using SequalPrep TM Normalization Plate (96) Kit (Invitrogen) following the producer's protocol (App. F.1). A total of 15 µl of PCR products representing yolk sac fry and water samples were used. By using the Nextera XT DNA library preparation kit (Illumina), each PCR product was indexed with unique sequence tags. To run the indexing PCR, a total of 17.5 µL of master mix (Table. 6) was added to each 96-well plate, and then 2.5 µLof each indexing primer and 2.5 µL of the PCR products were added. After indexing, a second PCR run was performed. The PCR run for 12 cycles in a T100TM thermocycler (BioRad) using the PCR cycling conditions shown in Table 7.

Table 6: Master mix per reaction used to prepare amplicon library for illumine sequencing of *jqsA* genes in *J.sp.* isolates.

Reagents	Volume 1x (µl)
DNA-free H20 (	11.8
5x Phusion buffer HF (7,5 mM MgCl2) (Thermo Scientific)	5
10mM dNTP (Thermo Scientific)	0.5
Phusion Hot Start DNA polymerase (Thermo Scientific), 2 U/ µ*l*	0.19
Index 1 (orange lid, N series,)	2.5
Index 2 (white lid, S series,)	2.5
Template (normalized from 1st stage PCR)	2.5

Table 7: PCR cycling condition used to prepare amplicon library for illumine sequencing of *jqsA* genes in *J.sp.* isolates.

Reactions	Temperature (°C)	Time	
Initial denaturation	98	2 minutes	
Denaturation	98	15 seconds	12 cycles
Annealing	55	20 seconds	
Elongation	72	20 seconds	
Final step of elongation	72	5 minutes	
Cooling	4	1 minutes	
Storage	10	∞	

Agarose gel electrophoreses (see section 2.3.3) of the resulting PCR products were then performed to see the quality and size of the second PCR product. Since there were primer dimmers, PCR products were cut out of the gel under UV light in G: Box HR Geldoc (Syngene) by using a sterile scalpel. The DNA fragments were then purified by QIAquick Gel Extraction Kit (Qiagen) by following the manufacturer's (App. E.4) protocol.

The purified, indexed amplicons were pooled together with Stian Stenhaug's amplicons from another project in one 1.5 ml tube, and up-concentrated by using Amicon® Ultra 0.5 mL Centrifugal Filters (fisher scientific) by following the manufacturer's protocol (App. F.2). For step four in the protocol, the samples were spun at 14000 for 10 minutes and washed with 500 µL TE buffer. A total of 17 (9 fish and 8 water) samples represented the final amplicon library. From Treatment 1, two fish samples from each flask (6 fish samples) and four water samples (one sample from each of the two and two samples from one of the flasks) were represented as the final amplicon library. From Treatment 2, three fish samples (two from one of the flasks and one from each of the two flasks) and four water samples (two water samples from one of the flasks and one from each of two flasks) were used for amplicon library preparation.

2.5.1.3 Illumina sequencing data processing

Due to time shortages, the data processing was carried out by Professor Ingrid Bakke (ACMS, IBT NTNU). The data was processed using the USEARCH pipeline (3). Due to poor quality of the reverse reads from the MiSeq sequencing, only the forward reads were used. First, the sequence reads were truncated by 50 nucleotides by using the command "trunclen" to remove poor quality sequence regions in the 3'-ends. Next, the reads were quality filtered by using the command "fastq_filter" with an expected threshold error of 1. OTU clustering was performed at the 99% similarity level by using the command "cluster_otus". Singletons (sequence reads observed only once in the complete data set) were discharges. Finally, the command "usearch_global" was used to generate an OTU table. The OTUs were manually assigned to the five *Janthinobacterium* strains by using the tool "Multi-way" in the program package "Clone Manager" v. 9.51. This is a sequence alignment tool applying a phylogenetic Neighbor-joining approach to identify similar sequences. The representative sequences for four of the OTUs were identical to the corresponding *jqsA* gene sequences, as previously determined in the ACMS group, for the strains PBA, PBB, 3.108, and 3.109. No OTUs with sequences similar to the *jqsA* sequences for strain 3.116 were observed in the data set. Other

OTUs, with low relative abundances, were found to represent Salmo salar sequences and *Janthinobacterium lividum,* and were removed from the ASV table prior to further analyses. The most abundant of these OTUs accounted for 1.3% of the total reads. Three fish samples with between 128 and 242 reads were excluded from further analyses. The final OTU table consisted of 6 fish samples and eight water samples. The number of sequence reads per sample varied from 1514 to 16 589, and the average number of reads was 2561 ±1007 and 4936 ±4936, respectively. For further analyses, the number of reads were normalized to a total of 1 for each sample, and the relative amounts of each of the four OTUs were specified as fractions.

The final OTU table was analyzed using the program package PAST(118). PCoA based on Bray-Curtis similarities was performed to compare the community profiles of the samples. A One-way PERMANOVA test(119) based on Bray-Curtis similarities was performed to test whether the community profiles differed significantly between fish and water samples.

2.5.2 CFU analysis

Yolk sac fry and water samples collected from rearing water and Atlantic salmon yolk sac fry from Experiment 4 were homogenized using Precellys®24 Homogenizer tubes and tissue (Bertin technologies). A total of 400 µl of sterile SGM was added to each individual salmon and water sample before homogenization (see section 2.4.4.2). Both sample types were then homogenized in two rounds for 30 seconds at 2,500 x RPM with 15-second break in between the rounds. The water samples were serially diluted from 1:10 to 1:1000. For the yolk sac fry samples, undiluted, 1:10, and 1:100 diluted samples were used. A total of 50µL of homogenized yolk sac and water samples of each dilution was plated on TSA plates and distributed evenly using sterile glass beads. Plating was carried out in triplicates for each dilution for both sample types. All plates were then incubated at room temperature for 5 days and bacteria colonies from the plates were counted. To obtain reliable results, colonies were only counted for plates between 30 and 300 colonies. For each treatment condition, an average number of CFU was calculated based on a dilution series with a reliable bacterial colony. From one of the replicates, a dilution series with reliable bacterial colonies was selected. In order to calculate an average CFU, we selected dilution series from the rest of the replicates that matched the first replicate. The average CFU was then determined for all replicates.

3 Results

3.1 16S rRNA gene amplification and sanger sequencing results

PCR amplification of the 16S rRNA gene and Sanger sequencing of the five *Janthinobacterium sp.* strains initially isolated from Atlantic salmon skin and rearing water was performed to confirm the strains' identity and ensure that the correct strains were being used. The 16s rRNA gene amplification resulted in the expected PCR products of around 1500 bp (Fig. 8).

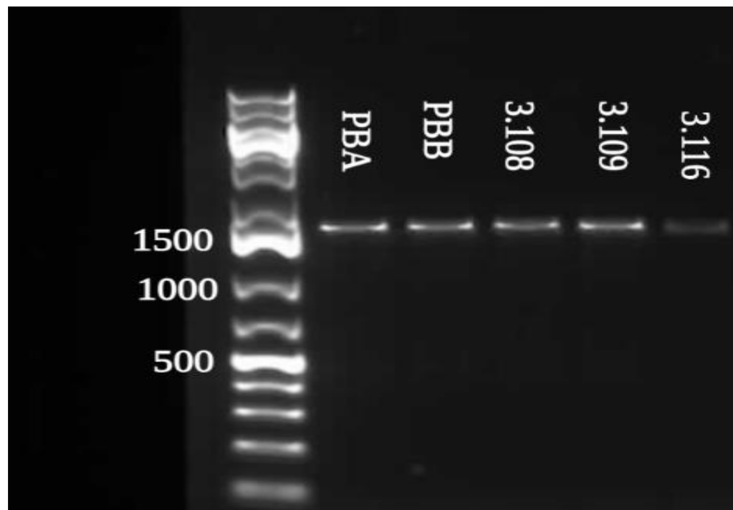

Figure 8: Agarose gel showing PCR products for amplification of 16s rRNA genes in the *J.sp.* strains: PBA, PBB,3.108,3.109, and 3.116. using DNA extraction from an overnight culture of the strains as templates. The genes were amplified by using primer pair EUB8F + 1492R.

The resulting 16S rDNA sequences were returned from Eurofins Genomics as fasta files with chromatograms. Based on the chromatograms, low-quality regions at the beginning of the 16S rDNA sequences were trimmed. The forward and reverse sequences were assembled and then aligned with 16S rRNA gene sequences that had previously been identified for the isolates. The sequencing files that were processed with Clone Manager can be found in the App. K.5. The alignment results showed that for all strains, the 16S rRNA sequencing was identical to those previously identified (App. K.6), confirming the identities of the isolates.

3.2 Characterization of *Janthinobacterium sp.* isolates.

To characterize the five *Janthinobacterium sp.* isolates based on their physical and chemical properties, experiments including culturing, microscopy, and enzyme activity testing were performed.

3.2.1 Colony characteristics of the five strains

To characterize the isolates based on their colony morphology, a single colony of each isolate was streaked on TSA agar plate medium. The colony size inspection after 24 hours of incubation showed some differences among the strains (Table 8). Visual inspection of the colony was performed after 5 days of incubation. The standard bacterial colony

characterization method described in section 2.2.2 was used to characterize the colony morphology of the isolates.

It was found that colonies of PBA and PBB were purple, while 3.108, 3.109, and 3.116 grew as white and viscous slim cells (Fig. 9). The purple-pigmented colonies of PBA and PBB are assumed to be due to the production of the purple pigment violacein. Comparing the PBA and PBB colony colors, the PBA colonies looked darker. This might be due to more violacein production in this strain. It was found that older colonies have more pigmented colonies for both strains. However, the dark purple pigment was sometimes found in newly formed PBB colonies. In other words, although the same growth conditions were used, PBB often grew as less pigmented colonies, but sometimes it grew as dark purple bacterial colonies.

The consistency of colonies for all strains was tested by picking in the colonies with a needle tip. All colonies were viscous; however, colonies of 3.108, 3.109, and 3.116 were more viscous than the violacein-producing strains. And among the non-violacein-producing strains, 3.108 had the highest viscosity.

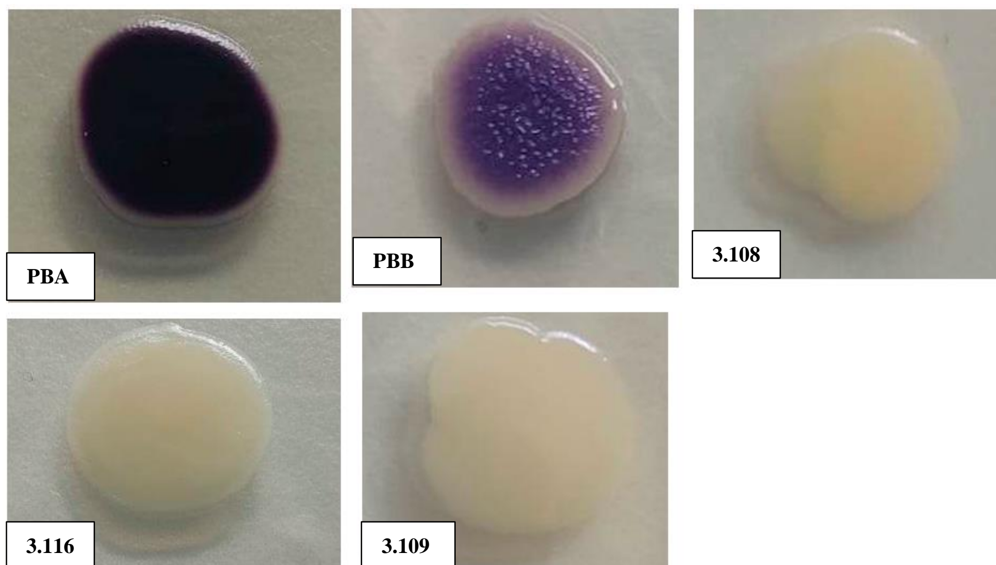

Figure 9: Colony morphology of the five *J.sp.* isolates on TSA agar plates after 5 days of incubation period at room temperature.

Table 8: Colony characteristics of five *J.sp.* isolates: - PBA, PBB,3.108,3.109, and 3.116. After 24 hours of incubation, the diameter of colonies of each strain was measured in millimeters. All other characteristics were performed after 5 days of incubation.

Colony characteristics	**PBA**	**PBB**	**3.108**	**3.109**	**3.116**
Form/shape	Circular	Circular	Circular	Circular	Circular
Elevation	Flat	Flat	Flat	Flat	Flat
Margin	Entire	Entire	Entire	Entire	Entire
Size	Small	Small	pinpoint	pinpoint	Small
Appearance	Shiny/ Smooth	Rough	Shiny/ Smooth	Shiny/ Smooth	Shiny/ Smooth
Consistency /Texture	Viscous	Viscous	Viscous (The most viscous)	Viscous	Viscous
Color	Purple	Purple	cream	White	White
GM-staining	GM-	GM-	GM-	GM-	GM-

3.2.2 Growth nature of the strains in a liquid medium

The nature of the growth of *J.sp.* strains was also examined in TSB liquid medium. Previous work in the ACMS group revealed that strains had different growth patterns in liquid LB medium(51). Hence, we examined their growth in TSB with Tween-80 medium to determine if they grew differently in different types of growth media. Normally, we culture overnight cultures of these strains with vigorous rotation (300 rpm), but here, no rotation is used. All isolates were visually examined after a one-week incubation period at room temperature under aerobic conditions. PBA and PBB are known for their purple phenotypes, but in this experiment, these strains produced less purple-pigmented cells. The PBB, in particular, produces much less purple-pigmented bacterial cells. In tubes containing 3.109, sediments appeared at the bottom of the tube. In tubes containing PBA, 3.108 and 3.116, milky or turbid cells appeared throughout the tubes (Fig. 10).

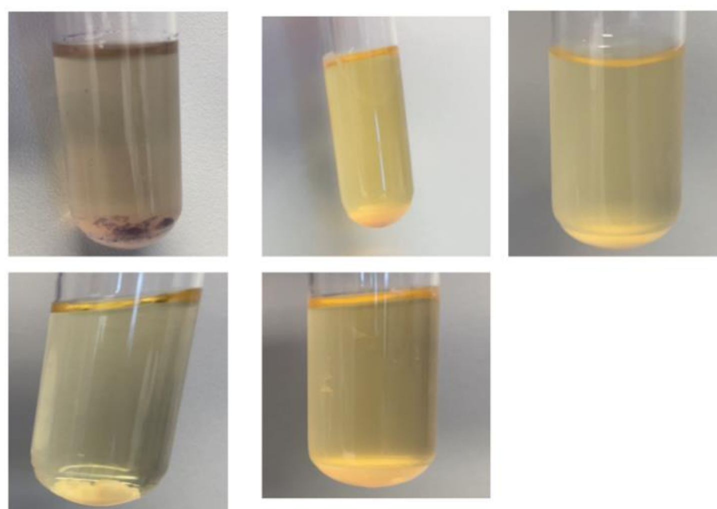

Figure 10: Growth nature of *J.sp.* sprains in TSB-medium with Tween-80 after one week incubation at a room temperature in an aerobic condition.

3.2.3 Microscopy

The microscopic study previously done on five *J.sp.* isolates showed that they had a tendency of growing in aggregates when grown in a liquid culture(51). Therefore, another microscopic study was performed to investigate whether the isolates grow in aggregates when grown in liquid culture with Tween-80 at vigorous shaking (300 rpm). In addition, the isolate's shape and motility were studied. A microscopy study of an overnight culture (described in section 2.2.1) of all isolates was performed using a Zeiss Axio vert. A1 microscope. The isolates were visualized under 40X magnification. The strains seemed to grow planktonic, but some strains (especially PBB) appeared to have cells with minor aggregations (Fig. 11). Moreover, microscopic examination revealed that all isolates were rod-shaped and motile.

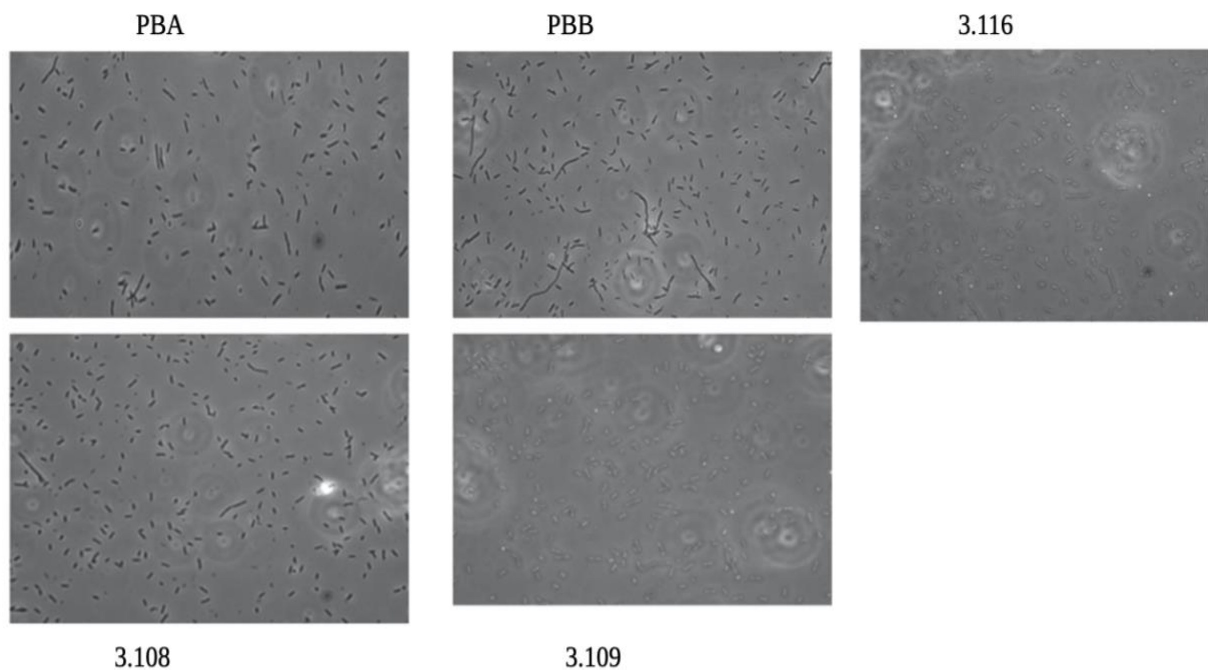

Figure 11: Microscopic pictures showing the five *J.sp.* isolates referred PBA, PBB,3.108,3.109, and 3.116 under a Zeiss Axio vert. A1 microscope with 40X magnification. Overnight cultures (see section 2.2.1) were prepared at room temperature on an orbital table shaker (300 rpm). Samples were diluted 1:100 and vortexed before microscopy study performed.

3.2.4 Growth curves: establishing the relationship between OD and flow cytometry cell counts.

The purpose of the study was to find the correlation between cell number and OD in order to be able to use it to calculate cell number in a culture.

Based on OD600 measurements, growth curves were established for all isolates. Four of the strains, PBA, PBB, 3.109, and 3.116, took seven and a half hours to enter the exponential phase, whereas strain 3.108 took five hours. The exponential part of the growth curves was compared with the corresponding number of bacterial cells counted by flow cytometry. The R^2 indicated that except for 3.109, there was a linear correlation between the two quantification methods, but the strength of the correlation varied among isolates (Fig. 12). However, according to the equations below OD of 1 appears to be less than expected

(compared to the standard assumption of around 1.2^9 CFU/ml) number of bacterial cells, indicating there might have been an error in the diluting process.

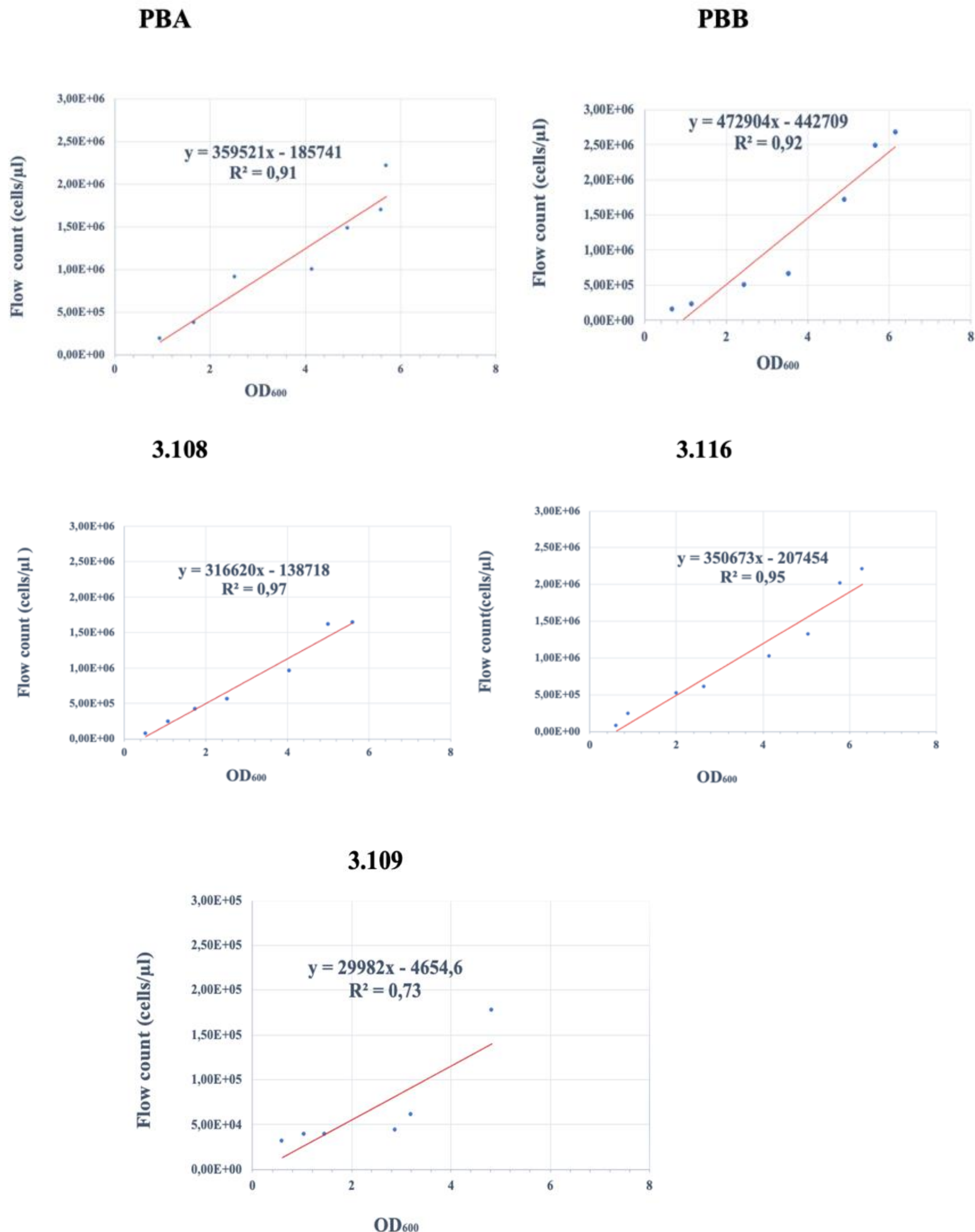

Figure 12: Plots showing a correlation between flow cytometry and OD600 cell quantification methods. Each strain's overnight culture was prepared in TSB (with 0.1% Tween-80) medium at 300 rpm aerobically at room temperature. The OD600 of five *J.sp.* isolates PBA, PBB,3.108,3.109, and 3.116 was measured once every hour for 22 and half hours. The plots were established by generating the growth curves of each isolate based on OD, and then plotting the exponential part of the growth curve with the corresponding number of bacterial cells counted by flow cytometry.

3.2.5 Analysis of enzymatic activity of *Janthinobacterium sp.* strains

To characterize the *J.sp.* isolates based on their biochemical properties, a test of enzymatic activity was performed. The presence or absence of 19 enzymes in each isolate was tested using an analytical profile index (API ZYM) strip test. The isolates seemed to have similar enzymatic activities for most of the substrates tested, except for a few differences (App. K.1). All isolates had activities for the following enzymes: - Alkaline phosphates, leucine arylamidase, Acid phosphatase, and Naphthol-AS-BI-phosphohydrolase. Despite this, each strain had the following unique profile of enzymatic activities: - PBA had enzymatic activities for Cystine arylamidase, and α – chymotrypsin, PBB for Cystine arylamidase, 3.108 for Valine arylamidase, 3.109 for Valine arylamidase, and α -mannosidase and 3.116 for Trypsin.

A principal component analysis based on the table summarizing the results of the enzyme assay (APP. K.1) indicated that in terms of these enzyme activities, the two strains isolated from rearing water, PBA and PBB, were similar to each other, and 3.108 and 3.109 were similar to each other (Fig. 13). However, the analysis indicated that the 3.116 strains had a unique enzymatic profile.

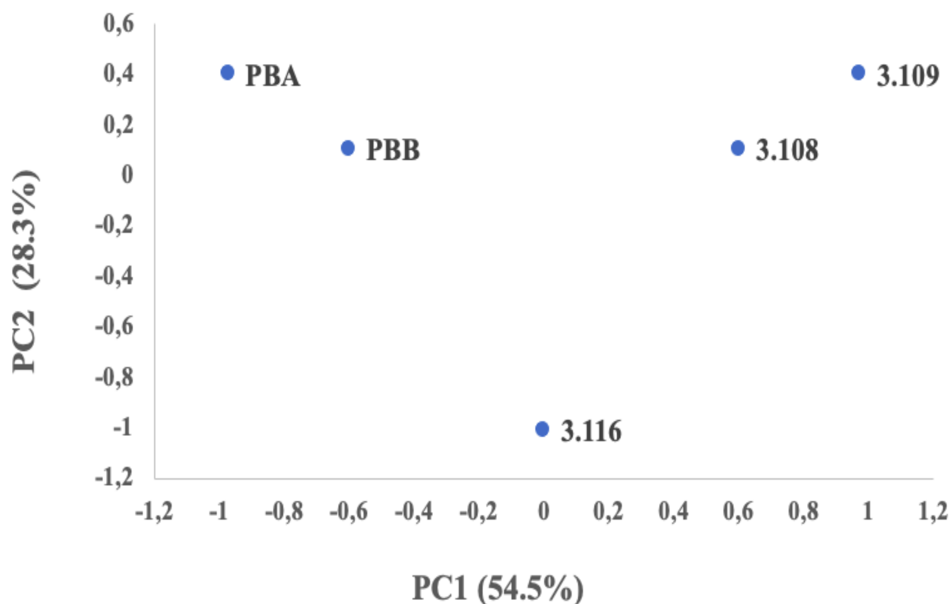

Figure 13: Principal component analysis based on API ZYM enzyme assay test (tested 19 bacterial enzymes) of five *J.sp* strains PBA,PBB,3.108,3.109, and 3.116. Principal component analysis indicated that the PBA and PBB has similar enzyme profile whereas 3.108 and 3.109 has similar enzyme profile, however 3.116 showed different profile of enzymatic activities.

3.3 Fish experiments

The fish experiments were conducted to investigate the five *Jantinobacterium sp.* isolates' ability to colonize the eggs and skin of Atlantic salmon fry. The experiments were also investigating whether some of the isolates were better at colonizing fish, eggs, and rearing water in competition with the others. In addition, it aimed to examine if the isolates could be integrated as a part of the fish microbiota.

3.3.1 Fish Experiment 1

The purpose of this experiment was to examine whether the isolates could be able to colonize the germ-free Atlantic salmon eggs and yolk sac fry. Additionally, it was intended to investigate if any of the strains had a competitive advantage during the colonization of the fish.

Therefore, the germ-free eggs and germ-free yolk sac fry were exposed to a mix of five *J.sp.* isolates and kept in the dark room at 7.5°C. The isolates were added to the fish flasks before hatching in Treatment 1 and after hatching in Treatment 2 (see Fig. 4).

The sterility check for water from fish flasks was conducted right after the surface disinfection of the eggs and prior to exposure to the isolates (for detail, see section 2.4.7). The second sterility check conducted after hatching showed that the eggs were contaminated. Besides contamination, there was also high mortality of egg and yolk sac fry (Table. 9). Therefore, sample collection for this experiment was not conducted. The eggs that appeared to be contaminated were taken from the flasks and plated on TSA agar plates to investigate the cause of the contamination. Visual observations of microbial growth around the eggs after five days of incubation on plates at room temperature showed that there was microorganism growth on the plates, probably Saprolegnia (Fig.14). Other than this, further investigation, such as the sequencing of the samples to identify the cause of the infection was not performed.

Figure 14: Two Atlantic salmon eggs looked infected were taken from different tissue flasks from experiment 1 and incubated on TSA plats to see if there is any microbial growth. The pictures taken after 5 days of incubation indicated saprolegnia like growth.

Egg and yolk sac fry mortality rates differed across tissue flasks. The Table 9 provides a summary of the mortality rate of eggs/yolk sac fry at the end of the experiment. Inspection of any fish death was performed three times per week until the experiment ended.

The sterility tests showed that five of the six rearing flasks were contaminated. Of these, all flasks in treatment group 2 were contaminated, whereas two of the flasks in Treatment group 1 were contaminated (Table 10).

Table 9: Mortality in fish in Experiment 1, *Janthinobacterium sp.* isolates were added to germ free flasks at Day 12 prior to hatching in Treatment group 1, and at Day 8 after hatching in Treatment group 2. There were six fish flasks used in the experiment. Three contained eggs, while the remaining three contained yolk sac fry. The letters E and Y represents eggs and Yolk sac fry respectively. The egg deaths were counted on different days.

Experiment type	Treatment Types	Name of the flasks	Number of egg/yolk sac fry per flasks	Number of dead eggs/fries	Total number of eggs/ fries per Treatment
1	1	E1	14	1	42
		E2	14	5	
		E3	14	3	
	2	Y1	14	0	42
		Y2	14	3	
		Y3	14	4	

Table 10: The sterility status check was conducted on the rearing water contained surface disinfected eggs and also after the eggs were hatched to ensure that the rearing flaks were free from any microorganisms. Rearing water samples from flasks containing Atlantic salmon eggs and yolk sac fry were taken and incubated in four different liquid growth medias referred to Sabourad Dextrose (Sab Dex), Brain Heart Infusion (BHI) Nutrient Broth (NB) and glucose yeast Medium (Gy). Additionally, the samples were also plated on TSA plates. A duplicate of all samples from each flask was prepared and incubated in a dark room at 7.5°C and in a light room at 23°C temperatures. Any microbial growth was then visually inspected after one week incubation period. The five *Janthinobacterium sp.* isolates were added on Day 12 prior to hatching in Treatment group 1, and on Day 8 after hatching in Treatment group 2. There were six fish flasks, three containing eggs, while the remaining three contained yolk sac fry. The "+" indicates microbial growth in samples. The letters E and Y represents eggs and Yolk sac fry respectively.

Sterility status check of surface disinfected eggs/yolk sac fry										
Experiment 1	Sterility check at fish temperature (7.6°C) at dark room					Sterility check at room temperature (23°C)				
Treatment 1	Sab Dex	BHI	NB	GY	TSA Plates	Sab Dex	BHI	NB	GY	TSA plate
E1										
E2					+					
E3		+	+	+			+			+
Treatment 2										
Y1		+	+	+	+	+	+	+	+	+
Y2		+	+	+	+	+	+	+	+	+
Y3		+	+	+	+	+	+	+	+	+

3.3.2 Fish Experiment 2

The aim of this experiment was to examine whether the five isolates were able to colonize the Atlantic salmon eggs and yolk sac fry when they were added to the fish that already had microbiota. In Treatment 1 CVR eggs were exposed to a mix of five *J.sp.* isolates at day 0. A control group that was not exposed to the isolates was included (Treatment 2) in the study to compare any changes in the microbiota (see Fig. 5). After 7 days of exposure to the strains, however, the eggs looked infected and several eggs were dead (Table 11), probably caused by Saprolegnia. Incubating eggs that appeared infected on TSA plates, as described in section 3.3.1, was performed to identify the cause of the infection.

Table 11: Mortality in fish in Experiment 2, the letter E represents flasks to which the five *Janthinobacterium sp.* isolates were added prior to hatching. The experiment was conducted before eggs were hatched. There were a total of six fish flakes used in this experiment. Three of the flasks contained Atlantic salmon eggs exposed to a mix of five *J.sp.* strains, while the remaining three contained CVR. The letter E represents flasks contained eggs exposed to the *J.sp.* strains whereas the letter C represents flasks contained CVR eggs that used as a control group. The egg deaths were counted on different days.

Experiment type	Treatment Types	Name of the flasks	Number of egg/yolk sac fry per flasks	Number of dead eggs/fries	Total number of eggs/ fries per Treatment
1	1	E1	14	3	42
		E2	14	1	
		E3	14	1	
	2	C1	14	0	42
		C2	14	2	
		C3	14	2	

Even though several eggs were dead, samples were collected from flasks referred to as E1-E3 (Table 10) for the potential sequencing of 16S rDNA amplicons to characterize the bacterial communities. However, sample collection from control group (Treatment 2) flasks containing CVR eggs was not performed as all eggs looked infected.

The previous studies done by ACMS groups showed that these five *Janthinobacterium sp.* strains have little variation in their 16S rRNA gene sequences. Thus, an amplicon library based on the *jqsA* gene (codes for autoinducer synthetase JQSA) was scheduled to be prepared in addition to the 16S rDNA amplicon sequencing. The amplicon library based on the *jqsA* gene was designed to estimate the relative amount between the five *J.sp.* strains in the fish and water samples

PCR products representing the 16S rRNA gene were successfully amplified from water and egg samples (Fig. 15). The PCR products representing the *jqsA* gene were included in section 3.3.3.1 since j*qsA* gene amplification was conducted with Experiment 3 samples. Because of time limitations and due to samples lacking from the untreated eggs (control group),

amplicon sequencing of the samples collected from Experiment 2 was not conducted, despite the successful generation of PCR products.

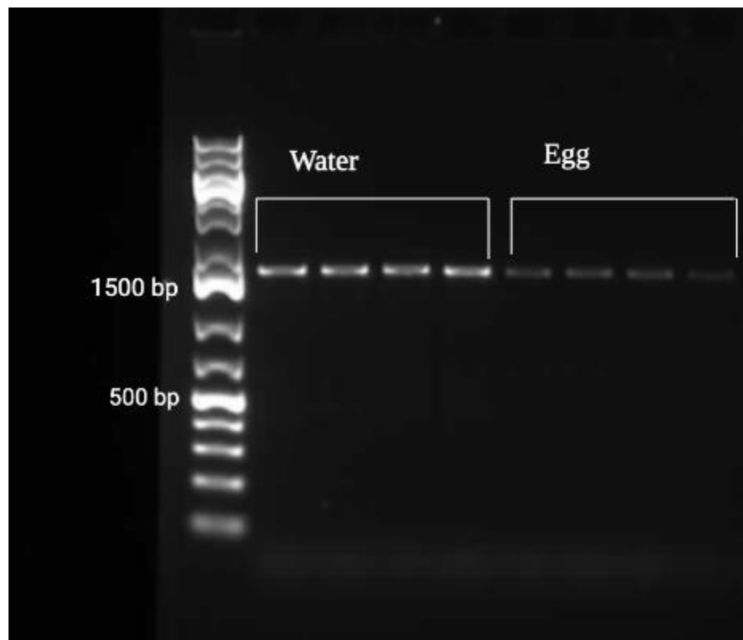

Figure 15: Agarose gel with PCR products representing the v3+v4 region of the bacterial 16S rRNA gene, DNA extraction from homogenized CVR salmon eggs; and from rearing water samples collected from Experiment 2 were used as template. EUB8.F and 1492.R primers were used to amplify the the v3+v4 region of the bacterial 16S rRNA gene (Table 2).

3.3.3 Fish experiment 3

This experiment was conducted to examine if any of the five isolates had a competitive advantage over the others when added in a mix and they try to colonize the same egg/fish (for detail see section 2.4.4.1). There was only one fish that died during this experiment. Second sterility checks conducted before exposing the eggs and yolk sac fry revealed the surface of egg and yolk sac fry was free from microbial growth.

Samples were collected to perform sequencing of the *jqsA* amplicons. This gene was used as a marker gene to assess the relative abundances of the added *Janthinobacterium sp.* strains, since their 16S rRNA gene sequences could not distinguish them. Since the primer sets used to amplify the gene were newly designed, several PCR runs were needed to optimize the PCR products.

3.3.3-1 PCR amplification of *jqsA* gene in *Janthinobacterium sp.*

Primer combinations (primer sequence, see Table 5) were designed to amplify parts of the *jqsA* gene in the isolates. First, it was investigated which annealing temperature gave the best PCR results. As a template, we used DNA extracts from eggs and fry from Atlantic Salmon that had been exposed to the five *Janthinobacterium sp*. strains in Experiment 3. Analyzes of the PCR products on agarose gel (Fig. 16) showed that an annealing temperature of 58 and 54 worked well. The results showed that PCR products of expected lengths (around 500 bp) were obtained for most of the egg and water samples and that some unspecific PCR products

were produced. It was also investigated whether the PCR could be improved by diluting the DNA extracts 1:10 before they were used as templates. Better PCR outcomes were obtained when undiluted DNA extracts were used as templates (Fig. 17). However primer formation was a major challenge when attempting to amplify the *jqsA* fragments from fish samples.

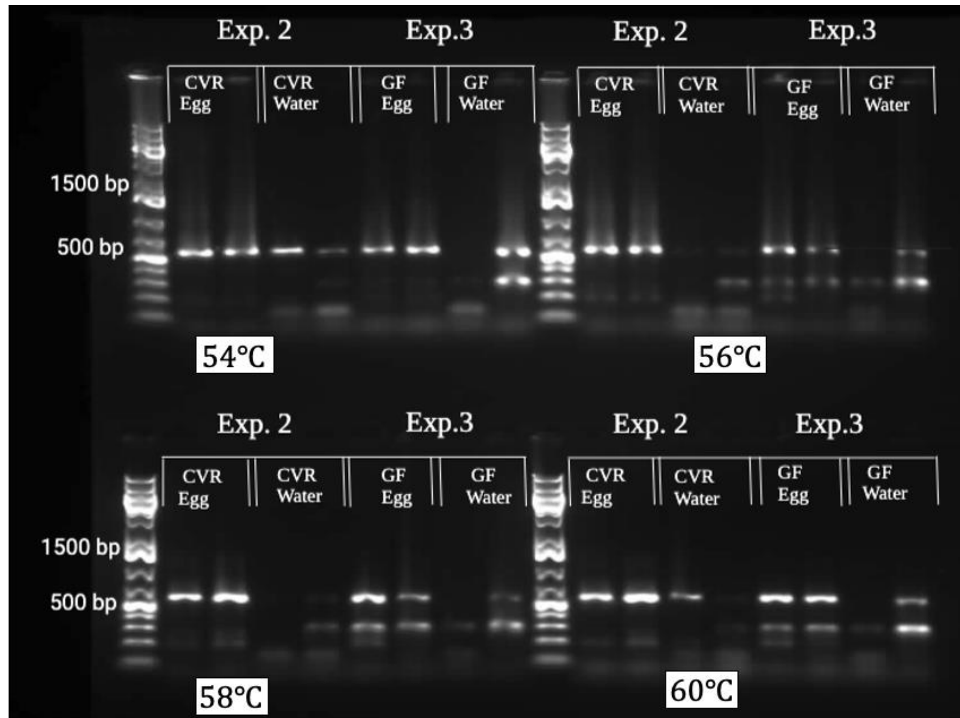

Figure 16: Agarose gel picture showing PCR products of the amplified parts of *jqsA* gene. DNA extracts from samples of homogenized Atlantic salmon egg and yolk sac fry, and rearing water system exposed by the five *J.sp.* isolates were used as a template. Lanes representer by CVR Egg and CVR water were eggs and water samples collected from Experiment 2 while lanes represented by GF Egg and GF water were yolks sac fry and water samples collected from Experiment3. The same samples were amplified with four different annealing temperatures; 54,56,58 and 60°C in a gradient PCR. The rest of the PCR cycling conditions were the same as described in section 2.3.2. The Ill-Jqsa_732_2.R and Ill-JQSA_240_2.F primers were used to amplify parts of *jqsA* gene (Table 5).

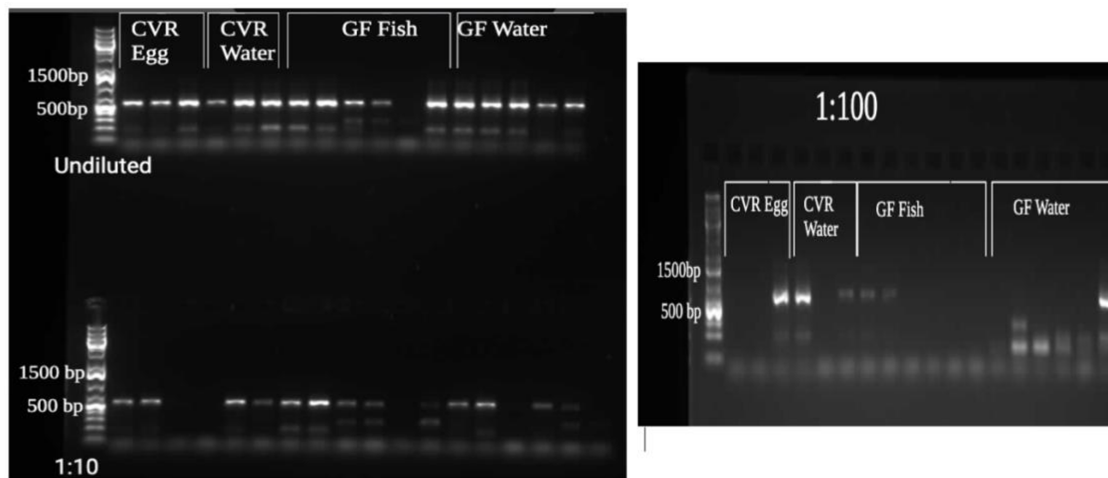

Figure 17: Agarose gel picture showing amplified parts of *jqsA* genes of five *J.sp.* isolates. DNA extraction of samples taken from homogenized Atlantic salmon egg and yolk sac fry, and rearing water system exposed by the five *J.sp.* isolates were used as a template. undiluted 1:10 and 1:100 diluted DNA extracts were used as templates, as indicated in the figure to optimize the PCR products. Samples of CVR fish that had been exposed to the mix of five *J.sp.* isolates were collected from Exp.2, while samples of GF fish that had been exposed to mix of five *J.sp.* isolates were collected from Exp.3. The Ill-Jqsa_732_2.R and Ill-JQSA_240_2.F primers were used to amplify parts of *jqsA* gene.

As addressed earlier, amplifying the *jqsA* fragment from fish samples was challenging due to the primer dimer formation problem. Therefore, we investigated whether reducing the primer concentration (from 300 uM to 150 uM) reduced primer dimer formation. This seemed to efficiently reduce the production of the primer dimers, and specific *jqsA* PCR products were obtained for both fish and water samples (Fig. 18). The final PCR products for all samples were obtained by increasing number of cycles from 36 to 38 (Fig. 18). PCR products from at total of 9 fish samples and 8 from water samples were used to prepare an amplicon library which was later sequenced by Illumina MiSeq technology.

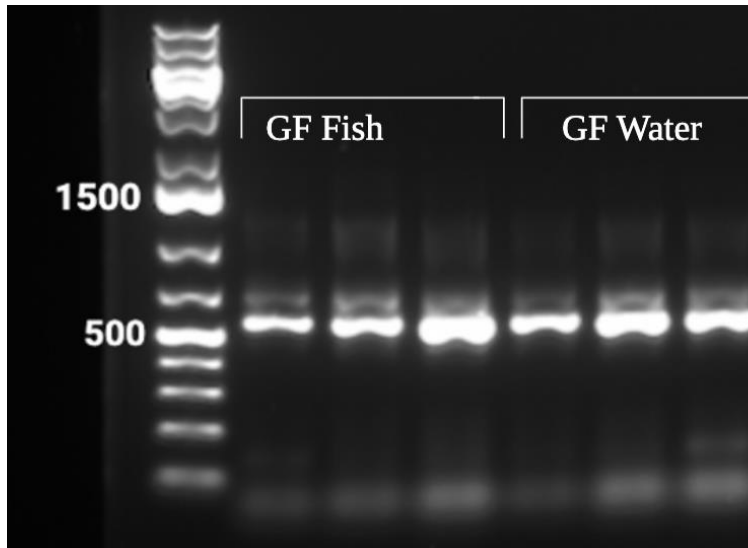

Figure 18: A agarose gel picture showing amplified parts of *jqsA* genes of five *J.sp.* isolates. DNA extracts of samples taken were from homogenized Atlantic salmon yolk sac fry and rearing water exposed by the five *J.sp.* isolates from Experiment 3. The Ill-Jqsa_732_2.R and Ill-JQSA_240_2.F primers were used to amplify parts of *jqsA* gene. Samples were amplified under the following PCR cycling conditions: 58℃ annealing temperature, 38 cycles, and 150 uM primer concentration.

3.3.3.2 Investigating the colonization ability of the five isolates.

The five *Janthinobacterium sp.* strains' ability to colonize the fish and the rearing water and their potential integration during the colonization process when added in a mix was investigated (for details, 2.4.4.1 Experiment 3). The aim was to add 10^5 CFU/ml in the final concertation of each strain based on OD measurements and the McFarland standard equation. In addition to OD measurements, each strain's inoculum dose was also determined by flow cytometry cell count. As explained above, Illumina sequencing of *jqsA* amplicons was performed to determine the relative abundances of the five strains in fish samples and water samples collected from Experiment 3. A process that included OTU clustering at the 99% similarity level is shown in the App. K.2.

The four OTUs with the highest abundance were found to represent PBA,3.109,3.108, and PBB, with 29690, 28275, 15830, and 11472 total reads respectively. The number of reads obtained varied among samples. Three fish samples representing samples from Treatment 2 (exposure after hatching) were discarded due to the low number of reads (from 128 - 242). The number of reads for the remaining fish samples varied from 1514 to 10570, with an average of 2561 per sample (App. K.2). The number of reads for the water samples was higher, with an average of 8671 (App. K.2). Furthermore, there were several OTUs that had lower relative abundance. The most common of these represented Salmo salar and an unknown *Janthinobacterium lividum* sequence and represented respectively 1.3 and 0.9% of total reads respectively. And these OTUs were removed from the OTU table.

The results showed that four of the strains (PBA, PBB, 3.108, and 3.109) were able to colonize the fish (Fig. 19); however, the *J.sp.* strain 3.116 was not observed in any of the

samples. Further, all strains except 3.116 were able to grow in the rearing water system in both treatments.

As addressed earlier, the relative amount added of each of the strains based on flow cytometry data was determined. This showed that 27, 11, 36,9, and 18% of PBA, PBB,3.108,3.109, and3.116 respectively, had been added (Fig. 19). The Illumina sequencing results showed that the relative abundance of 3.109 was high in fish samples, despite the fact that the least amounts of 3.109 was added to the fish flasks. Furthermore, the relative abundance of PBA and PBB was high in water samples (Fig 19). On the other hand, the relative abundance of 3.108 was high in water samples collected in treatment 2. Since no fish samples were successfully amplified from Treatment 2, we further focused on samples collected from Treatment 1 (Fig. 19).

To compare the *Janthinobacterium* communities between samples in water and fish collected in Treatment 1, a PcoA based on Bray-Curtis similarities was performed (Fig. 20). One-way PERMANOVA-test based on Bray-Curtis showed that the difference between fish and water samples is significant (p = 0.026). Interestingly, OTUs representing PBA and PBB, which had been isolated from water, were 1.8 and 4.7 times more abundant in the water samples compared to the fish samples (App. K.3). SIMPER analysis showed that the OTU representing 3.109, having higher relative abundance in the fish samples, contributed most of the Bray-Curtis dissimilarities between the two sample groups (App. K.4).

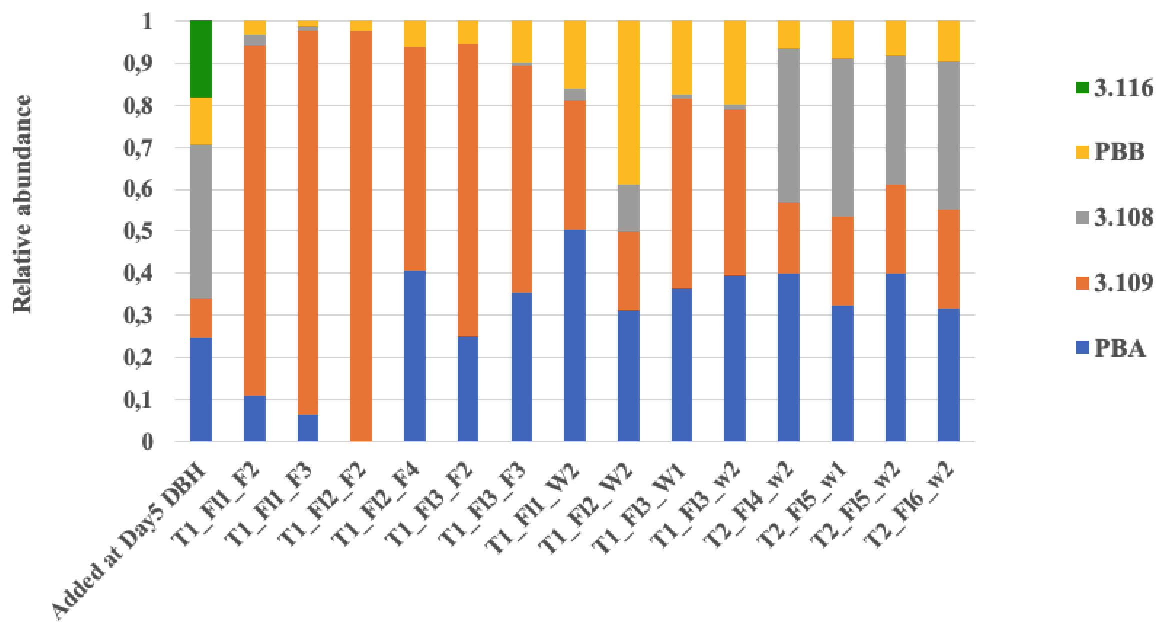

Figure 19: Relative abundance of the ASVs representing the five *Janthinobacterium sp.* isolates in fish and water samples collected in Experiment 3 based on Illumina sequencing of *jqsA* amplicons. Atlantic salmon eggs in Treatment 1(T1) were exposed to a mix of five *J.sp.* isolates prior to hatching, and in Treatment 2 (T2), Atlantic salmon yolk sac fry were exposed after hatching (see Fig. 6). The relative abundances of the five strains added to the fish flasks in treatment 1 at Day5 before hatching (DBH) date was determined by using cell densities as determied for the separate bacterial culture by flow cytometry. Letters T,Fl,F, and W indicate Treatment, flask, fish, and water respectively.

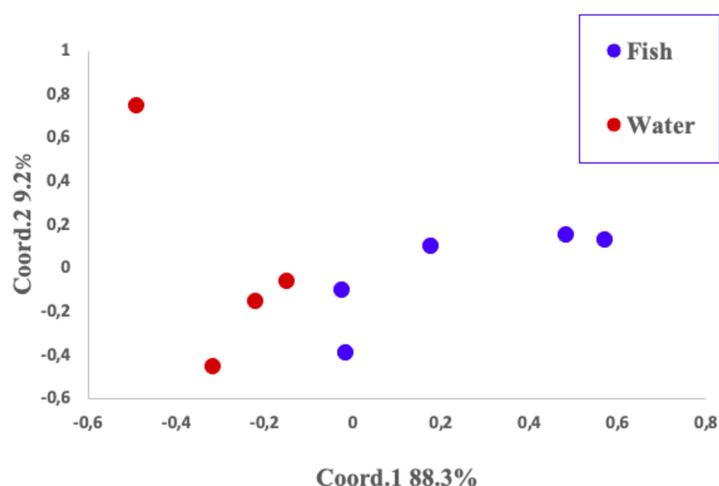

Figure 20: Principal coordinate analysis (PCoA) test based on Bray -Curtis generated with Illumina *jqsA* data for samples from Experiment 3, Treatment 1. PcoA indicated that the water and fish communities differed.

3.3.4 Fish experiment 4

In this experiment, the intention was to investigate the individual *J.sp.* strains' ability to colonize yolk sac fry.

The surface-disinfected salmon eggs were exposed to each of the five strains. Sample collection for CFU analysis was performed at Day 22 dpe, 10 days after hatching (Fig. 7). The amount of bacteria in fish and water samples was examined using CFU analysis (for detail, see section 2.3.4.2 and 2.5.2).

From one of the replicates, a dilution series with reliable bacterial colonies was selected. The average CFU for that dilution serie was then determined. In order to calculate an average CFU from all replicates, we selected dilution series from the rest of the replicates that matched the first replicate. The average CFU and standard deviation between samples were then determined for all water and fish samples.

Furthermore, the CFU results at Day 22 dpe were normalized to the bacterial cell density in the water at the start of the experiments, as determined by flow cytometry for the separate strains prior to the addition to fish flasks. All strains were able to colonize the Atlantic salmon fry (Fig. 21). Among the strains, the variation in colonization density in Atlantic salmon yolk sac fry was low except for 3.109. The 3.109 strain had the highest density in the fish samples. PBA and PBB had the highest density in the water samples, while 3.116 had a very low occurrence in both water and fish samples. This indicates that PBA and PBB (which were originally isolated from water) had the greatest colonization success in water, while 3.109 strain had the best colonization success in fish. On the other hand, the 3.116 strain had very low colonization success in both sample types.

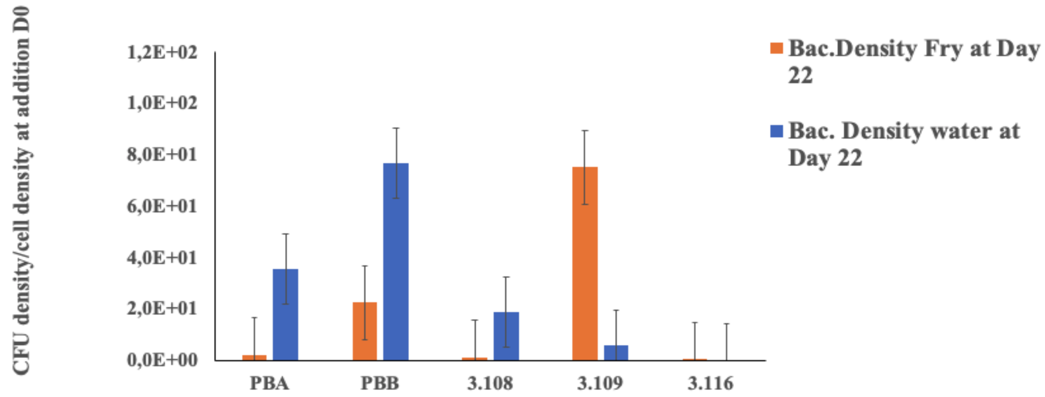

Figure 21: Number of CFU density normalized to the cell density in the water at the start of the experiment (Day 0), as determined by flow cytometry for the separate strains prior to addition to fish flasks. Error bars represent calculated standard errors based on the average CFU for each strain.

4. Discussion

Bacterial characterization refers identifying and describing the specific traits and properties of a particular type of bacteria. It involves various techniques and approaches, such as analyzing the organism's genetic material, observing its colony morphology (size, shape, and structure), testing its biochemical properties, and assessing its behavior in different environments. Characterizing bacteria has several purposes. For one it helps to identify and distinguish bacteria from other types of microorganisms. Furthermore, identifying bacteria's applicable functions, potentially positive effects (on hosts), and their pathogenicity is useful in fields such as medicine and industry to choose microbes as probiotics. Therefore, characterizing bacteria is, essential in understanding and studying microbial metabolic activities, ecological roles, pathogenicity, and its potential purpose in industry.

In this project work, *Janthinobacterium sp*. strains named PBA, PBB, 3.108, 3.109, and 3.116, isolated from Atlantic salmon rearing water and fry skin were studied in different aspects. Sequencing of the 16s rRNA gene of the isolates was conducted to identify the isolates. Furthermore, their growth nature, colony morphology (size, shape, color), biochemical properties, and ability to colonize Atlantic salmon eggs and yolk sac fry were studied.

4.1 Identification of the *Janthinobacterium sp.* isolates.

The 16S rRNA gene is conserved in bacteria, archaea, and some eukaryotes. Some gene regions in this gene are highly conserved, meaning the nucleotide sequences are very similar among different organisms(120). However, there are gene regions named V1 - V9 that are variable. These gene regions, specifically V1 - V3 and V4 - V5, are widely used for identifying and classifying different bacterial species in microbial taxonomy. In microbiological taxonomy and phylogenic studies, the conserved regions needed for designing broad-coverage primers and variable reflecting the diversity of communities are used to identify and classify closely related bacteria (121).

The 16S DNA sequencing was performed in this project to ensure that the strains we worked on were the right ones. Upon alignment with previously sequenced DNA sequences of these strains, the five *J.sp.* strains we worked on were confirmed to be the correct strains. The 16S rRNA gene sequences of these strains are very similar, despite their differences in colony morphology.

4.2 Growth characterization of five *Janthinobacteria sp.* isolates

Phenotypes are observable properties that bacterial strains can exhibit(122). Bacterial strains, including closely related strains, exhibit different phenotypic characteristics. Characterizing various phenotypic traits among strains is therefore essential to differentiate the bacterial strains.

This project aimed to characterize the growth nature of five *Janthinobacterium sp*. strains. To characterize the isolates' phenotypes and growth nature, the five *Janthinobacterium sp.* strains were studied in TSA/TSB with 0.1% Tween-80 medium. The strains' growth nature, including colony morphology and growth conditions, has been studied.

Previously, when the strains grew in LB media without Tween-80 at 120 RPM, all strains showed a clear tendency of growing in aggregates. This has been a challenge in previous work in the group when working with the strains(51). Therefore, we cultivated the strains so that they grow planktonically. Planktonic growth is needed to get reliable cell counts, including in flow cytometry, CFU analyses, and OD measurements quantification methods. All strains grew at room temperature in aerobic conditions with vigorous shaking (300 RPM) in a TSB with 0.1/ Tween-80 medium. Their growth nature was then investigated under a microscope. It was found that all strains grew planktonic. A possible explanation for this could be Tween-80, which reduces cell aggregation(123). Several studies have indicated that the *J.sp* strains normally grow in aggregates in a liquid medium by forming biofilms(124). Biofilm formation in *J.sp*. is believed to be a response to environmental stress(125). Extracellular polymeric substance (EPS) which is the main component of biofilm, makes the bacterial cells grow in aggregates at their stationary phase (95, 96). Biofilm formation in *J.sp* strains has been suggested to help the bacteria to colonize hosts such as fungi and fish skin(92). Vigorous agitation might also have a negative effect on biofilm formation as it causes mechanical forces that disrupt the attachment of bacterial cells to each other as well as to the surfaces. Moreover, high agitation can increase access to oxygen and nutrient. As *J.sp.* is aerobic bacteria, increased oxygen availability may promote planktonic growth rather than biofilms. Additionally, planktonic growth may be favoured by increased nutrient availability over biofilm growth. Generally, as biofilm formation in *J.sp.* is a response to environmental stress, mechanical forces such as high agitation, temperature, nutrient ,and oxygen availability might have their own effects on bacterial growth in aggregates(125).

When the strains were incubated in TSB with Tween-80 without shaking for one week, they showed interesting characteristics. The PBA and PBB strains showed less pigmentation despite they are being known to produce purple pigments. It was especially interesting to notice that PBB produced almost no purple bacterial cells when the strains grew without agitation (Fig.10). According to previous work, when the strains grew in LB medium at 120 RPM without Tween-80 for one week, the bacteria produced purple pigments in high concentrations(51). This could be due to the fact that violacein production in *J.sp.* is regulated by quorum sensing and is produced as a response to environmental stress(97). It is believed that the QS in *J.sp.* regulates the violacein expression through autoinducer genes including the *jqsA* gene (114). Studies done on other bacteria indicated that agitation has a negative effect on violacein production(126, 127). Therefore, it is reasonable to believe the agitation had negative effects on violacein production in these two strains. Furthermore, there is also a possibility that the culture gets less oxygen, which results in less bacterial density. On the other hand, white 3.109 cells grew in the bottom of the tube, while 3.108 and 3.116 had white turbid bacteria throughout the tubes, indicating different growth nature among the strains.

When the strains grew in TSA agar medium, it was found that two of the strains, PBA and PBB showed a clear tendency of growing as a purple pigmented slimy colony while the rest of the strains (3.108, 3.109, and 3.116) grew as a white slimy colony. Of the strains, 3.108 had the most slimy colonies and looked creamy. The PBA and PBB tended to produce more pigmented bacterial colonies as the incubation period increased. This is probably due to the reasons that *J.sp.* produces violacein at stationary phase(97), and slimy colony matrix production is assumed to be due to the presence of EPS (95, 96). There is uncertainty about whether the antifungal effect of *Jantninobacterium* was actually related to its violacein production(88). Our results showed that 3.108 does not produce violacein, despite the strains

having antioomycete properties. This suggests that *Janthinobacterium* strains may be able to have antioomycete activity without producing violacein.

Depending on their colony color, it was possible to differentiate purple pigment and white colony-producing strains. Moreover, colonies from all strains looked circular, flat, and entire. However, when we compare colony size, 3.108 and 3.109 had smaller colonies (slower growth rates) than the rest of the strain's colonies. Furthermore, most of the strains' colonies looked smooth, while PBB's colonies appeared to be irregular/rough.

Despite some limitations, it was possible to differentiat the strains depending on their pigmentation, colony size, and the appearance of their colonies. Further differentiation of the strains, which is not covered by the growth nature study, is required.

4.3 Enzyme activity assessment in the strains

An enzymatic activity test was performed to differentiate the five *J.sp.* strains based on their enzymatic profiles. The API ZYM kit was used to test the enzymatic activity of the strains, including phosphatase, lipase, as well as esterase (128). By analyzing bacterial enzyme activities, we can gain valuable insight into the metabolic abilities and functional potential of the bacteria. The enzyme activity assay is being used for the identification and characterization of bacteria at a functional level, which is not covered by DNA analysis methods(129).
Based on visual observations, it was possible to differentiate the strains as purple pigmented and white phenotypes. Here, the enzyme activity investigation gave us additional knowledge about the strains to further differentiate the strains based on their biochemical activities.

All the strains showed positive enzymatic activity for four enzymes, Alkaline phosphatase, Leucine arlamidase, Acid phosphatase and Naphthol-AS-BI-phosphohydrolase. Except this, there were some significant differences in enzymatic profile among the strains. A principal component analysis showed that PBA and PBB have similar enzymatic profile, while 3.108 and 3.109 have similarities in their enzymatic activities. The strains PBA and PBB have purple phenotypes and were isolated from Atlantic salmon rearing water, whereas the rest have white phenotypes and were originally isolated from salmon fry skin. Therefore, it is reasonable to accept the findings as phenotypically similar and genetically closely related strains can have similar enzymatic profiles. However, this does not mean that the strains do have completely similar enzymatic profiles, as we only tested the presence of 19 enzymes. Even if the strains have closely related genetic makeup and show similar phenotype, they might have variations in their gene sequences, making them to have different biochemical activities(130, 131). The 3.116 showed a different enzymatic profile than the rest of the strains. This strain could be more unique than the rest of the strains in its genetic makeup. The ongoing genome sequencing (based on average nucleotide identity analysis) of these strains indicated that 3.116 was more distinct in terms of genome sequences from the rest of the strains. On the other hand, PBA and PBB were more similar to each other, and 3.109 and 3.108 were more similar to each other (Eirik D. Lorentsen personal communication).

The enzymatic activity test help us differentiate the strains. However, identifying and differentiating strains requires more than just testing 19 enzyme activities. A larger number

of enzymes is suggested to be tested under a variety of conditions. A number of factors can influence the enzyme's activity, including temperature and pH. The biochemical activities of these strains can, therefore, be better understood by conducting several tests under different conditions. The enzymatic assay identification methods could be used as an alternative to identify between *J.sp.* strains as their 16s rRNA gene was not well suited for identification/ differentiation of the strains.

The strains we worked with in this project were isolated from the skin of Atlantic salmon fry and their rearing water showed some interesting characteristics. The strains showed different phenotypic characteristics and enzyme activities despite having closely related 16S rRNA sequences. This could be due to the fact that the strains might have different adaptations according to the environment they inhabit. Phylogenetically closely related microorganisms undergo various adaptations to cope with the environment. This adaptation results in differences in cellular function, metabolism, and biosynthesis among the strains (132, 133). The strains that we worked with might have undergone adaptation, including horizontal gene transfer that resulted in variation in their phenotype and enzymatic activities. The previous study on these strains indicated that the violacein operon of these strains could have been transmitted via horizontal gene transfer(134). As this master's project work is part of a Ph.D. project called "*Janthinobacterium* isolated from salmon skin: genomics, molecular biology, and host-microbe interaction", we hope questions related to genome analysis of the strains as well as its antagonist effects against bacteria and fungus will be answered in the future.

4.4 Fish colonization experiments

In fish, the mucosal surfaces covering their skins, gills, and digestive systems play a key factor in their immunity development(47, 48). The mucosal surface has several layers that trap pathogenic microbes while allowing beneficial microbes to colonize its surface. Mucin is the major component of the mucus covering mucosal surfaces/tissues, and it serves as a barrier against pathogens(50). In spite of this, there are beneficial bacteria, such as *Janthinobacterium sp*, which can use mucin as a carbon source. A previous study of *Janthinobacterium* growth in mucin media indicated that all five of the *Janthinobacterium* strains could grow on mucin as their sole carbon source. This indicates the mucosa of the fish can potentially be colonized by this bacteria, and could potentially have some beneficial effects on the fish (51, 52).

In this master's project, the five *Janthinobacterium sp.* strain's ability to colonize Atlantic salmon eggs and yolks sac fry was investigated. The long yolk sac fry life stage makes Atlantic salmon yolk sac fry ideal for studying host-microbe interactions. When we start external feeding, it is much more challenging to keep the systems germ-fee. But the salmon yolk sac feeds on their yolk sac and do not need external feeding in its entire yolk sac life stage, making it a preferable gnotobiotic model. The ACMS group has recently developed a novel protocol where the salmon fry can be maintained germ-free entire for the yolk sac fry stage (135). By using this protocol, we investigated the colonization ability of the five *J.sp.* isolates on germ-free eggs and yolk sac fry.

PBA and PBB strains were isolated from the same small-scale fish flask we use for gnotobiotic rearing of salmon fry, whereas 3.108, 3.109, and 3.116 were isolated from the skin of Atlantic salmon fry in a commercial Recirculating Aquaculture Systems (RAS). Their colonizing ability when exposed to the salmon yolk sac fry separately and in a mix was examined.

The experiments were conducted in two rounds due to contamination of eggs and yolk sac fry, and also death occurred. For the first round of the fish experiments, we received eggs from Lerøy with 369-degree days, whereas for the second round, from Aquagen with 339-degree days. Eggs from Lerøy were infected, probably by Saprolegnia. Yolk sac fry death occurred in both germ-free and CVR treatment conditions. The disinfection protocol might not have been suited for targeting this kind of oomycetes infection. Therefore, we increased the antibiotic cocktails concentration in the second round.

The eggs and yolks sac fry death could be due to Saprolegniosis, a disease caused by teh oomycete Saprolegnia. The water sources of Norwegian hatcheries normally contain Saprolegnia spores, which means that the eggs are continuously exposed(136). Hence, the Saprolegnia probably came along with the eggs. No further investigation was conducted except for plating the contaminated eggs on agar plates. It was, however, only one egg which died during the second round of the fish experiments. This egg could be a poor egg, as there will always be a few individual deaths due to genetic and developmental factors. Yolk sac stage is the most critical stage in the fish life cycle (137).

4.4.1 Investigating the *Janthinobacterium sp.* strain's ability to colonize Atlantic salmon yolk sac fry by Illumina sequencing.

The Atlantic salmon eggs and yolk sac fry were exposed to a mix of five *J.sp*. isolates in two different treatment groups. In Treatment 1, a mix of the five *J.sp.* was introduced into fish flasks which contained germ-free Atlantic salmon eggs at 5 days prior to hatching, and in Treatment 2, yolk sac fry at 5 days post-hatching. The bacterial density used to expose the eggs and fish was determined based on the McFarland standard. It was intended to add approximately 10^5 CFU/ml of each strain to the fish flakes. To be more precise, the bacteria density added to the fish was measured by flow cytometry cell count. The sample collection was performed at 27 days post-hatching for both treatment groups. Since we were unsuccessful in getting PCR products from the fish samples from Treatment 2, we focused on Treatment 1.

As the strains have little variations in their 16s DNA sequences, we used another marker gene, *jqsA,* which could differentiate better between the strains. The gene codes for the autoinducer synthetase JQSA. Relative abundance of *J.sp.* strains in Atlantic salmon yolk sac fry and its rearing water was determined by Illumina sequencing of the *jqsA* amplicons.

It seemed that most of the strains except 3.116 were able to colonize the yolk sac fry and its rearing water. The 3.109 strain was the most abundant in fish samples, whereas more PBA and PBB were found in water samples. The 3.116 strain was not found in any of the samples. Possible reasons could be that the 3.116 strain was not able to grow in the fish bottles. When bacteria try to colonize the host, they compete for niches and limited resources with other microorganisms. The competition occurs between bacterial populations too (138). The observations from the mono-associations experiments indicated that the 3.116 was the isolate which had the lowest abundances both in the rearing water and the yolk sac fry. It appears that the strain was not affected by interaction with other strains, but was unable to sustain its population in both yolk sac fry and rearing water. Even though the strain was isolated from the skin of salmon fry, it was not successful in colonizing the yolk sac fry in our rearing system. Therefore 3.116 strain may have been isolated from the skin of Atlantic salmon fry, but this does not necessarily mean that it will colonize the fry again.

The strains' host colonization ability can be influenced by several factors. including their genetic makeup and host immune response (139). Additionally, factors such as receptor and adhesion sites and resources are other factors that bacteria compete for during host colonization processes (140). The strains possess different mechanisms for attaching to the host's mucosal surface. This difference can influence their ability to establish and maintain themselves within the host(141). For instance, fimbriae or pili are found on the outer surface of some *Escherichia coli.* strains. The bacteria attach to the host mucosal surface using these structures(142). Different bacterial strains may have different adhesion molecules or surface proteins that facilitate attachment to mucosal surfaces. The difference among the strains in their host colonization ability might have therefore been caused by differences in their adhesion molecules.

Another factor that could affect the ability to colonize the fry could be the formation of biofilms, which provide protection and enhance the colonization of surfaces(93, 97). The *Janthinobacterium species* is a biofilm-forming microorganism, but each strain might have different biofilm-forming abilities, which might account for differences in host colonization abilities (93, 143). The microscopy pictures reveal that some of the isolates were growing with cells attached to each other (see Figure 11). This could maybe be related to the potential for biofilm formation. It is believed that the ability to form or grow in biofilm could be linked to the ability to colonize a mucus surface on the host(124).
Some studies conducted on other bacteria indicated that the host colonizing ability of bacteria was dependent on EPS and biofilm production(144). It is believed that EPS synthesis is important for the bacteria as it provides attachment to the host. This helps the bacteria to grow in aggregates as well as provide optimal environment for the exchange of genetic materials between the cells (144, 145).
Comparing the *J.sp.* community in water and fish samples, it seemed that PBA and PBB were more dominant in water and 3.108 and 3.109 in fish. It is reasonable to believe that the strains that were originally isolated from Atlantic salmon rearing water, were better adapted to grow/live in aquatic environments, while those that were originally isolated from Atlantic fry skin were better at colonizing salmon fry. The strains might have adapted to different environmental conditions that gave them differences in colonization ability. To sum up, the high abundance of four of the *J.sp.* strains in Atlantic salmon showed that the strains were able to colonize the mucosal surface of the fish and that all the strains were able to use mucin as their sole carbon source. The strains' ability to colonize the Atlantic salmon yolk sac fry and its rearing water, indicates these strains could be part of salmon commensal microbiota. The previous study conducted on these strains indicated that *Janthinobacterium sp.* seems to be a part of the commensal microbiota of yolk sac fry(146)

Interestingly, the 3.108 strain, which has an antagonist effect against Saprolegniasis was able to colonize the yolk sac fry, indicating it could be a potential probiotic candidate. As addressed previously, *J.sp.* has a capnophilic behavior, it prefers environments with high CO2 concentrations, including amphibian skin (91, 147). This will favor the bacteria to colonize the fish skin (especially at the yolk sac fry stage) and support the idea that the strain could be a probiotic in salmon fish. However, more studies are needed to evaluate the strain's antifungal properties, host-microbe interactions, and capnophilic behavior.

It would be interesting to investigate the ability of the strains to colonize the yolk sac fry in competition with the commensal microbiota of conventionally reared fry. This was originally an aim for this master project. However, unfortunately, this experiment was hampered by

death in yolks sac fry, and therefore, the number of samples were too few to carry out the analyses. In addition, sample collection from the control group was not performed.

4.4.2 Mono associate bacterial colonization

Among the many methods of quantifying bacterial cells in culture, CFU analysis is one of the most commonly used methods. Quantifying bacterial numbers using CFU has two advantages: it counts only viable bacterial cells, and dilutions allow us to count any number of bacteria(148). In this project, mono-associated methods were used to investigate how the five *J.sp*. strains colonize the yolk sac fry of Atlantic salmon and its rearing water. The inoculum dose of each strain was determined by two bacterial cell quantification methods, namely by McFarland standard and by flow cytometry cell count. Based on McFarland's standard, the aim was to add approximately 10^5 CFU/ml of each strain into fish flasks containing Atlantic salmon eggs. Additionally, a more accurate determination of cell density was made using flow cytometry. The flow cytometry cell count results were further used to normalize the CFU numbers to the amounts of cells originally added to each fish flask. Bacterial host colonization success is determined by how well it establishes and maintains its population within the host. This can be investigated by comparing the initial bacterial density with the density in the yolk sac fry and rearing water samples at the end of the experiment.

The Atlantic salmon eggs were exposed to separate strains at 10 days prior to hatching, and sample collection was performed at 12 days post-hatching. The whole yolk sac fry was homogenized for plating.

The CFU analysis results indicated that the strains had different colonization abilities. For all of the strains', except 3.116, the CFU density was increased in both sample types during the experiment when compared to the CFU density in the water at the start of the exposure.

Comparing bacterial density in yolk sac fry, the strain 3.109 was the most abundant strain in fish samples, indicating 3.109 has better ability to colonize the fish than the rest of the strains. Interestingly, the 3.109 was also the most abundant strain in the fish samples when the strains were added in a mix. Furthermore, the low 3.116 density (below 30 colonies in all plates) indicated that the strain has a low level of colonizing ability in this rearing system. As addressed previously, when mixed with other strains, this strain was not able to colonize water nor fish. This result supports the previously addressed suggestion that 3.116 might have difficulties in maintaining its population in this rearing system.

Comparing the bacterial density among the strains in water samples, the PBA and PBB had the highest abundance. The results were consistent with the findings from the mix experiment (Experiment 3). As stated previously, these strains were isolated from the Atlantic salmon fry rearing water and might have better adaptation in the rearing water environment than other strains. Adaptation to specific host environments is common among bacteria. Different strains may have evolved specific adaptations to exploit and colonize specific host niches. The number of 3.116 colonies per plate was below 30 in all plates, and its density at the end of the experiment was less than inoculum density in both sample types. This indicates that the strain could not be able to maintain its population in rearing water and had difficulties in colonizing both rearing water and yolk sac fry.

Interestingly, the result we found from mono-associate colonization agrees with what we found from the amplicon sequencing: more PBA and PBB in water and more 3.109 in fish

samples. And very little of 3.116 was observed in the CFU analysis in both water and fish samples, but from amplicon sequencing of the samples for which the strains were added together, we did not find 3.116 at all. Generally, the finding in the mono-associate examination agrees with results that we found from the amplicon sequencing, indicating that there were no strong interactions between the five *Janthinobacterium* strains when they were added together.

5 Future work

This master project has provided results that could improve the knowledge about the five *Jantninobacterium sp* strains. The results showed that the bacterium was able to colonize the Atlantic salmon fry, indicating the strains, particularly 3.108 and 3.109, could be part of the fish commensal microbiome. The findings may offer some insight into the idea of using *Janthinobacterium sp.* as a probiotic in Atlantic salmon. Despite this, further research is needed regarding 3.108's antagonist effect on Saprolegnia, and its potential detrimental and beneficial health effects in salmon. We cannot disregard completely that the strains couldn't have detrimental effects, even though we never observed death as a response to exposure to these strains. Considering the findings, the following research topics are suggested for future studies.

- Improve the understanding of the role of the *Janthinobacterial* quorum sensing system on the colonization of the host/yolk sac fry
- Study the capnophilic behaviour of the *Janthinobacterium sp.* and its contribution to Salmon skin colonization
- Study the initial colonization of the five *Janthinobacterium sp* strains on conventionally raised Atlantic salmon eggs and yolk sac fry.
- Study the potential antagonist effect of the strains against Saprolegnia in experimental systems involving salmon eggs and fry.

6 Conclusions

The growth nature of the five *Janthinobacterium sp.*, colony morphology, enzymatic activities, and ability to colonize the germ-free Atlantic salmon eggs and yolk sac fry were studied. Although the 16S rRNA gene sequences were highly similar between the isolates, the colony morphology differed in terms of colony color, size, and appearance between the strains.

The phenotypic characters of PBA and PBB on TSA agar plates were purple pigmented, indicating violacein production, whereas those of 3.108, 3109, and 3.116 are white. The *Janthinobacterium* strains grow in aggregates, but planktonic growth was successful in TSB with 0.1% Tween-80 at 300 RPM.

The test of 19 enzymatic activities for the five *Janthinobactreium sp.* strains revealed that the strains differed in their enzymatic profile. The enzymatic activities of PBA and PBB were similar, as were those of 3.108 and 3.109. On the other hand, the 3.116 strains exhibited a unique combination of enzymatic activities.

The five *Jantnibacterium* strains' initial colonizing ability of germ-free Atlantic salmon yolk sac fry was investigated with the strains added in a mixture and separately. The Amplicon sequencing was used to determine the relative abundance when the isolates were added in a mix, whereas the CFU analysis method was applied when the isolates were added separately.

The colonizing ability when exposing the yolk sac fry to a mix of the five strains was investigated using Illumina amplicon sequencing. This showed that all strains, except, 3.116, were able to colonize yolk sac fry and the rearing water. Moreover, the mono-associate colonizing ability of these strains indicated that the strains were able to colonize yolk sac fry and its rearing water. The PBA and PBB strains, originally isolated from rearing water, were the strains with the best ability to colonize rearing water (found both when isolates were added separately and in a mix). Furthermore, the 3.109 showed better colonizing ability in yolk sac fry than the rest of the stains when added together with other strains as well as when added separately. The density of the 3.116 strain was less than the inoculum density in both sample types. None of the agar plates with 3.116 contained more than 30 CFU, suggesting the strain has a limited ability to colonize both yolk sac fry and their rearing medium.

The *Janthinobacterium sp.* strains appeared to show the same trends when colonizing Atlantic salmon yolk sac fry and their rearing water, whether in a mix or mono-associated. Overall, these results, together with the previously identified antagonistic property of the 3.108 strain against Saprolegnia, make it an interesting candidate for probiotic treatment of Atlantic salmon in early life stages.

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Appendix

A Growth media used to grow *J.sp.* strains.

Tryptic soy broth (TSB)

Table 1: TSB components per liter of deionized water.

Components	**Amounts (g)**
Pancreatic digest of casein (Tryptone)	17
Papaic digest of soybean meal	3
Dipotassium phosphate (K_2 HPO_4	2.5
Glucose	2.5
Sodium chloride	5
MilliQ H_2O	750 ml

And TSA (1%) was prepared by adding 11.25 agar in TSB.

B Components for TAE-Buffer solution

An agarose solution (1.5%) was prepared by dissolving 1g agarose in 100ml 1x TAE buffer. The solution was heat in microwave for 2-3 minutes. The 1x TAE buffer solution was prepared by adding 1960 ml Milli-Q water with 40 ml 50x TAE-buffer. The following table shows how to prepare 50x TAE-buffer.

Table 2: Components used to prepare 50x TAE Buffer.

Component	Amount (g)
Trise base	242 G
Glactic acetic acid	57.1 ml
0.5 M EDTA solution (pH 8.0)	100 ml
Milli-Q H_2O	Up to 1 L

C Components for PBS solution

Table 3: Recipe for 1 L 10xPBS (Phosphate-Buffered Saline)

Compound	**Amount (g)**
Na2HPO4·7H2O	**25.6**
NaCl	**80.0**
KCl	**2.0**
KH2PO4	**2.0**

D API® ZYM enzymatic activity test protocol

- **For microorganisms:**
  Prepare a suspension with a turbidity of 5-6 McFarland in API Suspension Medium (2 mL) (open the ampule as indicated in the paragraph "Warnings and Precautions" of the package insert for this medium), distilled water or an isotonic medium. Pure growth from an agar slant or sediment from a centrifuged broth culture can be used to prepare the suspension. In order to obtain reproducible results, it is important that the micro- organisms to be compared be initially grown on the same isolation medium, the diluter be the same and the suspension be of the same optical density. This technique assays for constitutive enzymes. Inductive enzymes can be detected by adding the corresponding inducer(s) to the culture medium.

INSTRUCTIONS FOR USE Preparation of the strip

- Prepare an incubation box (tray and lid) and distribute about 5 mL of distilled water or demineralized water [or any water without additives or chemicals which may release gases (e.g. Cl_2, CO_2, etc.)] into the honey- combed wells of the tray to create a humid atmosphere.
- Record the sample reference on the elongated flap of the tray. (Do not record the reference on the lid as it may be misplaced during the procedure).
- Remove the strip from its individual packaging. Place the strip in the incubation box.

Inoculation of the strip

- Using a pipette or PSIpette, dispense 65 µL of specimen into each cupule.
- After inoculation, place the plastic lid on the tray and incubate generally for 4 - 4 ½ hours at 37°C (optimum temperature). The time of incubation and temperature may vary depending on the sample to be tested. However, when samples are being compared, all test conditions (time, temperature, growth media, density of suspension) must be the same. The inoculated strip should not be placed in bright light.

READING AND INTERPRETATION Reading the strip

After incubation:

 Add 1 drop of ZYM A reagent and 1 drop of ZYM B reagent (*) to each cupule.
By placing a surface-active agent (ZYM A reagent) in the cupule, solubilization of the ZYM B reagent in the medium is facilitated.
(*) **It is recommended to control** each ampule of ZYM B before using for the first time. To do this, it is recommended to use **the strain ATCC® 27853™** indicated in the Quality Control paragraph in order to eliminate any defective reagents.

- Let the color develop for at least 5 minutes.
- If possible, put the strip under a powerful light source (1000 W bulb) for about 10 seconds with the bulb placed about 10 cm (4") above the cupules. The procedure will eliminate any yellow color which may appear in the cupules due to any excess of Fast Blue BB which has not reacted. After light exposure, negative reactions become colorless. Placing the strip in daylight for a few minutes will produce comparable results.

Recording the reactions

Read the reactions and record the results on the result sheet. A value ranging from 0-5 can be assigned, corresponding to the colors developed: 0 corresponds to a negative reaction, 5 to a reaction of maximum intensity and values 1, 2, 3 or 4 are intermediate reactions depending on the level of intensity (**3, 4 or 5 being considered as positive reactions**).

- The colors remain stable for several hours after the strip has been inoculated with the reagents. After 24 hours, colors may deteriorate, interferring with test interpretation.

READING TABLE

No.	ENZYME ASSAYED FOR	SUBSTRATE	pH	RESULT	
				POSITIVE	NEGATIVE
1	Control			Colorless or color of the sample if it has an intense coloration	
2	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	
3	Esterase (C 4)	2-naphthyl butyrate	6.5	Violet	
4	Esterase Lipase (C 8)	2-naphthyl caprylate	7.5	Violet	
5	Lipase (C 14)	2-naphthyl myristate	"	Violet	
6	Leucine arylamidase	L-leucyl-2-naphthylamide	"	Orange	
7	Valine arylamidase	L-valyl-2-naphthylamide	"	Orange	
8	Cystine arylamidase	L-cystyl-2-naphthylamide	"	Orange	
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange	
10	□-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	Orange	
11	Acid phosphatase	2-naphthyl phosphate	5.4	Violet	
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	"	Blue	
13	□-galactosidase	6-Br-2-naphthyl-□D-galactopyranoside	"	Violet	Colorless or Very pale yellow *
14	ß-galactosidase	2-naphthyl-ßD-galactopyranoside	"	Violet	
15	ß-glucuronidase	Naphthol-AS-BI-ßD-glucuronide	"	Blue	
16	□-glucosidase	2-naphthyl-□D-glucopyranoside	"	Violet	
17	ß-glucosidase	6-Br-2-naphthyl-ßD-glucopyranoside	"	Violet	
18	N-acetyl-ß-glucosaminidase	1-naphthyl-N-acetyl-ßD-glucosaminide	"	Brown	
19	□-mannosidase	6-Br-2-naphthyl-□D-mannopyranoside	"	Violet	
20	□-fucosidase	2-naphthyl-□L-fucopyranoside	"	Violet	

E DNA extracting and purification kits

1 DNeasy® PowerSoil® Kit (Qiagen) protocol

Protocol: Experienced User

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- Shake to mix Solution C4 before use.

Procedure

1. Add up to 0.25 g of soil sample to the PowerBead Tube provided.
2. Add 750 µl of PowerBead Solution to the PowerBead Tube.
3. Add 60 µl of Solution C1 and invert several times or vortex briefly.
4. Bead beating options:
   A. PowerLyzer 24 Homogenizer: Place the PowerBead Tubes into the tube holder for the PowerLyzer 24 Homogenizer. The PowerBead Tubes must be balanced in the tube holder. Run the samples for a time and RPM suitable for your soil type.
   Note: For clay soils, 4,000 RPM for 45 s is the best starting point. For loose, granular and high organic soils, 2,500 RPM for 45 s will provide an optimal result.
   B. Vortex: Secure the PowerBead Tubes horizontally using a Vortex Adapter (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min.
   Note: If you are using a 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Make sure the PowerBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge at 10,000 x *g* for 30 s. Do not exceed 10,000 x *g*.
   Note: Centrifuge for 3 min at 10,000 x *g* for clay soils or if your soil is not completely pelleted after 30 s.
6. Transfer the supernatant to a clean 2 ml Collection Tube (provided).
   Note: Expect 400–500 µl. Supernatant may still contain some soil particles.
7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with the incubation we recommend you retain the step.

8. Centrifuge the tubes for 1 min at 10,000 x *g*. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube (provided).
9. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
   Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with the incubation we recommend you retain the step.
10. Centrifuge the tubes for 1 min at 10,000 x *g*. Avoiding the pellet, transfer up to 750 µl of supernatant into a clean 2 ml Collection Tube (provided).
11. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 s.
12. Load 675 µl of the supernatant onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard the flow-through and add an additional 675 µl of supernatant.
13. Centrifuge at 10,000 x *g* for 1 minute. Load the remaining supernatant onto the MB Spin Column and centrifuge at 10,000 x *g* for 1 min.
    Note: A total of three loads for each sample processed is required.
14. Add 500 µl of Solution C5 and centrifuge for 30 s at 10,000 x *g*.
15. Discard the flow-through. Centrifuge again for 1 min at 10,000 x *g*.
16. Carefully place the MB Spin Column in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the MB Spin Column.
17. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you may use sterile DNA-free PCR-grade water (cat. no. 17000-10) or TE buffer.
18. Centrifuge for 30 s at 10,000 x *g*. Discard the MB Spin Column.
19. The DNA is now ready for downstream applications.
    Note: We recommend storing DNA frozen (–90°C to –15°C) as Solution C6 does not contain EDTA.

2 ZymoBIOMICS™ DNA Miniprep Kit Protocol

1. Add sample to a **ZR BashingBeadTM Lysis Tubes (0.1 & 0.5 mm)**. Add 750 µl **ZymoBIOMICSTM Lysis Solution** to the tube and cap tightly.

   ***Note**: For samples stored and lysed in **DNA/RNA ShieldTM Lysis Tubes**, do not add ZymoBIOMICSTM Lysis Solution and proceed to Step 2*

2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process using optimized beat beating conditions (speed and time) for your device (see Appendix D)[4].
3. Centrifuge the **ZR BashingBeadTM Lysis Tubes (0.1 & 0.5 mm)** in a microcentrifuge at ≥ 10,000 x *g* for 1 minute.

4. Transfer up to 400 µl supernatant to the **Zymo-SpinTM III-F Filter** in a **Collection Tube** and centrifuge at 8,000 x *g* for 1 minute. Discard the Zymo-SpinTM III-F Filter.
5. Add 1,200 µl of **ZymoBIOMICSTM DNA Binding Buffer** to the filtrate in the Collection Tube from Step 4. Mix well.
6. Transfer 800 µl of the mixture from Step 5 to a **Zymo-SpinTM IICR Column** in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.
7. Discard the flow through from the Collection Tube and repeat Step 6.
8. Add 400 µl **ZymoBIOMICSTM DNA Wash Buffer 1** to the Zymo- SpinTM IICR Column in a new Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through.
9. Add 700 µl **ZymoBIOMICSTM DNA Wash Buffer 2** to the Zymo- SpinTM IICR Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through.
10. Add 200 µl **ZymoBIOMICSTM DNA Wash Buffer 2** to the Zymo- SpinTM IICR Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.
11. Transfer the Zymo-SpinTM IICR Column to a clean 1.5 ml microcentrifuge tube and add 100 µl (50 µl minimum) **ZymoBIOMICSTM DNase/RNase Free Water** directly to the column matrix and incubate for 1 minute. Centrifuge at 10,000 x *g* for 1 minute to elute the DNA[5, 6].
12. Place a **Zymo-SpinTM III-HRC Filter** in a new Collection Tube and add 600 µl **ZymoBIOMICSTM HRC Prep Solution**. Centrifuge at 8,000 x *g* for 3 minutes.
13. Transfer the eluted DNA (Step 11) to a prepared Zymo-SpinTM III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 16,000 x *g* for 3 minutes.

    The filtered DNA is now suitable for PCR and other downstream applications.

3 QIAquick PCR Purification Kit (Qiagen) Protocol

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, poly- merases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a

  conventional tabletop microcentrifuge at room temperature.

- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of 7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

10. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

4 QIAquick Gel Extraction kit (Qiagen) protocol

Quick-Start Protocol

QIAquick® Gel Extraction Kit
QIAquick® PCR & Gel Cleanup Kit

The QIAquick Gel Extraction Kit and the QIAquick PCR & Gel Cleanup Kit (cat. nos. 28704, 28706, 28506 and 28115) can be stored at room temperature (15–25°C) for up to 12 months.

Further information

- *QIAquick Spin Handbook*: **www.qiagen.com/HB-1196**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- This protocol is for the purification of up to 10 µg DNA (70 bp to 10 kb).
- The yellow color of Buffer QG indicates a pH ≤7.5. DNA adsorption to the membrane is only efficient at pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge.

Sample to Insight

QIAGEN

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes >800 µl, load and spin/apply vacuum again.
6. If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 µl Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
7. To wash, add 750 µl Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
   Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2–5 min after addition of Buffer PE.
   Centrifuge the QIAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
8. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the

F Amplicon library preparation kits

1 SequalPrep™ Normalization Plate (96) Kit

General Guidelines

- Wear a laboratory coat, disposable gloves, and eye protection when handling reagents and plate.
- Always use proper aseptic techniques when working with DNA and use only sterile, DNase-free tips to prevent DNase contamination.
- If you are using only part of the plate for DNA purification, cover unused wells with the Plate Seal and leave them attached while purifying DNA in the other wells. The plates can be stored at room temperature for up to 6 months.
- The SequalPrep™ Normalization Plates are compatible for use with automated liquid handling workstation; the workstation must be capable of handling and manipulating 96-well plates.
- If you are using automated liquid handling workstations for purification, you may need additional Wash Buffer depending on your type of workstation. See previous page for Wash Buffer ordering information.

Binding Step

1. Transfer the desired volume of PCR product (5–25 µl PCR reaction mix, at least 250 ng amplicon/well) from the PCR plate into the wells of the SequalPrep™ Normalization plate.
2. Add an equivalent volume of SequalPrep™ Normalization Binding Buffer.
For example: To purify 10 µl of PCR product, add 10 µl SequalPrep™ Normalization Binding Buffer.

3. Mix completely by pipetting up and down, or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate.

4. Incubate the plate for 1 hour at room temperature to allow binding of DNA to the plate surface. Mixing is not necessary at this stage.

Note: Incubations longer than 60 minutes do not improve results. However, depending on your workflow you may perform overnight incubation at room temperature for the binding step.

5. **Optional:** If >25 ng DNA/well yield is desired, transfer the amplicon/Binding Buffer mixture from Step 4 to another, fresh well/plate to sequentially bind more DNA. Perform DNA binding at room temperature for 1 hour.

Note: After binding is complete, you can remove the amplicon/Binding Buffer mixture from the well and store at –20oC for up to 30 days to perform additional purifications at a later time.

6. Proceed to **Washing Step**, next page.

Washing Step

1. Aspirate the liquid from wells. Be sure not to scrape the well sides during aspiration.

Note: If you wish to store the amplicon/Binding Buffer mixture for additional purifications at a later time, aspirate the liquid from wells into another plate and store at –20oC for up to 30 days.

2. Add 50 µl SequalPrep™ Normalization Wash Buffer to the wells. Mix by pipetting up and down twice to improve removal of contaminants.

3. Completely aspirate the buffer from wells and discard.

To ensure complete removal of wash buffer and maximize elution efficiency, you may need to invert and tap the plate on paper towels depending on the pipetting technique or instrument used. A small amount of residual Wash Buffer (1–3 µl) is typical and does not affect the subsequent elution or downstream applications.

4. Proceed to **Elution Step**, below.

Elution Step

Review **Elution Options** (previous page).
1. Add 20 µl SequalPrep™ Normalization Elution Buffer to each well of the plate.

Note: Do not use water for elution. If you need to elute in any other buffer, be sure to use a buffer of pH 8.5–9.0. If the pH of the buffer is <8.5, the DNA will not elute efficiently.

2. Mix by pipetting up and down 5 times or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate. Ensure that the buffer contacts the entire plate coating (up to 20 µl level).

3. Incubate at room temperature for 5 minutes.

4. Transfer and pool the purified DNA as desired or store the eluted DNA at 4°C (short-term storage) or –20°C (long-term storage) until further use.

Expected Yield and Concentration

The expected DNA concentration is 1–2 ng/µl when using 20 µl elution volume. The expected DNA yield is ~25 ng/well normalized.

User Guide

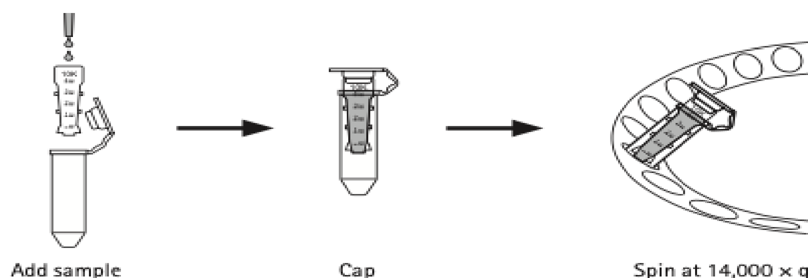
Amicon® Ultra-0.5 Centrifugal Filter Devices

for volumes up to 500 µL

For research use only;
not for use in diagnostic procedures

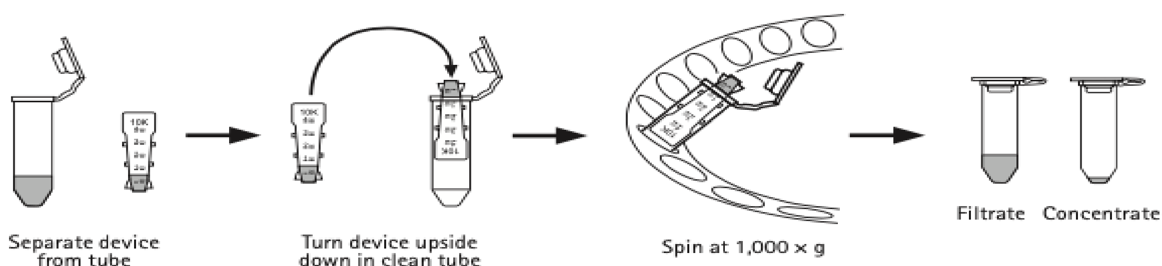
How to Use Amicon® Ultra-0.5 Centrifugal Filter Devices

1. Insert the Amicon® Ultra-0.5 device into one of the provided microcentrifuge tubes.
2. Add up to 500 µL of sample to the Amicon® Ultra filter device and cap it.
3. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.
4. Spin the device at 14,000 × g for approximately 10–30 minutes depending on the NMWL of the device used. Refer to Figure 1 and Table 2 for typical spin times.



5. Remove the assembled device from the centrifuge and separate the Amicon® Ultra filter device from the microcentrifuge tube.
6. To recover the concentrated solute, place the Amicon® Ultra filter device upside down in a clean microcentrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 × g to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.

   NOTE: For optimal recovery, perform the reverse spin immediately.



G Salmon Gnotobiotic media (SGM)

Sol Gómez de la Torre Canny, modified from on US EPA/600/4-90/027F artificial water recipe

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Salt Stocks

All salt stocks are prepared in fish-only 1L glass bottles pre-autoclaved with a stir bar inside. Also, use fish-only graduated cylinders.

$MgSO_4 \cdot 7H_2O$ 100X
Dissolve 12.3 g in 1 l. Autoclave.

KCl 100X
Dissolve 0.4 g in 1 l. Autoclave.

$NaHCO_3$ 100X
Dissolve 9.6 g in 1 l. Filter sterilize.

$CaSO_4 \cdot 2H_2O$ 5X
Dissolve 0.3 g in 1 L.
This salt takes a few hours to go into solution. Usually prepare the solution and leave it ON in stirring. Once dissolved, autoclave

SGM prep

$MgSO_4 \cdot 7H_2O$ 100X	10 ml
KCl 100X	10 ml
$NaHCO_3$ 100X	10 ml
$CaSO_4 \cdot 2H_2O$ 5X	200 ml
Miiq H_2O	770 ml

	1000 ml

Prepare in pre-autoclaved 1 L fish-only glass bottles and use for measuring the water and the $CaSO_4 \cdot 2H_2O$ 5X fish-only graduated cylinders.
Autoclave prepared SGM and cool down to RT before storing in the fish room.

NOTE: Sometimes the salts go out of solution, after autoclaving and because of the low temperature. Just shake the bottle before the water change. Flasks containing fish have a slightly lower pH so the salt will not precipitate after the water change.

H AB-GSM—Salmon Gnotobiotic Media with antibiotic cocktail

Antibiotic cocktail preparation

Antibiotic	Working concentration	Volume of stock to add to 1 L SGM
Rifampicin	10 mg/l	200 µl
Erythromicin	10 mg/l	200 µl
Kanamycin	10 mg/l	200 µl
Ampicillin	100 mg/	1000 µl
Amphotericin B	250 ug/l	1000 µl
Penicillin	150 mg/l	1500 µl
Oxolinic acid	75 mg/l	1800 µl

NOTE: All these operations should be performed at room temperature. Fish room temperature is too cold for one of the antibiotic stocks to remain liquid.

Buffodine solution

NOTE: Buffodine has 1.11% w/w Iodine (~11 g/l). The manufacturer suggests a 1:100 dilution of the buffodine (110 mg/l final concentration). We prepare a 1:200 dilution (0.25 ml Buffodine in 50 ml final volume). **We therefore get a Buffodine solution of 50 mg/l of available iodine.** Sol tested the concentrations, and 50 mg/l should be fine

I Media for sterility checks

- Sabourad Dextrose (Sab Dex): Dissolve 30 g/l, distribute 3 ml / tube, add red cap
- Brain Heart Infusion (BHI): Dissolve 37 g/l, distribute 3 ml / tube, add blue cap
- Nutrient Broth (NB): Dissolve 8 g/l, distribute 3 ml / tube, add green cap
- Glucose Yeast Medium (GY): Dissolve 10 g/l glucose and 2.5 g/l yeast extract, distribute 3 ml / tube, add yellow cap
- Autoclave all tubes with medium afterwards and store in fish room until needed
- Wash and autoclave all tubes after use

J ***Janthinobacterium. sp.*** **isolates and their DNA previously isolated by ACMS group.**

>J._PBA
CATGCAAGTCGAACGGCAGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAA
CGGGTGAGTAATATATCGGAACGTACCCTGGAGTGGGGGATAACGTAGCG
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GTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCA
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GAGCTAATATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTA
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>J._PBB
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TGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTAT
GGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACC
CGCGAGGGGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTC
TGCAACTCGACTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCAT
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CCAC

>J._3.108(MM5)
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GGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACC
CGCGAGGGGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTC
TGCAACTCGACTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCAT
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CCAC

>J._3.109
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CCAC

>J._3.116
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GGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGT
GAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGA
GAGCTAATATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTA
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GGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCA
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GCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAAC
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CCAC

K Results

1 Enzymatic activity test results

Table 4: Enzymatic activity test results of *J.sp.* isolates using API ZYM test kit

No.	**Enzyme assayed for**	**Substrates**	**PH**	**Results** (+/-)				
				PBA	PBB	3.108	3.109	3.116
1	Alkaline phosphatase	2-naphthyl phosphate	8.5	+	+	+	+	+
2	Esterase (C 4)	2-naphthyl butyrate	6.5					
3	Esterase Lipase (C8)	2-naphthyl caprylate	7.5					
4	Lipase (C 14)	2-naphthyl myristate	,,					
5	Leucine arylamidase	L-leucyl-2-naphthylamide	,,	+	+	+	+	+
6	Valine arylamidase	L-valyl-2-naphthylamide	,,			+	+	
7	Cystine arylamidase	L-cystyl-2-naphthylamide	,,	+	+			
8	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5					+
9	α - chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	+				
10	Acid phosphatase	2-naphthyl phosphate	5.4	+	+	+	+	+
11	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	,,	+	+	+	+	+
12	α-galactosidase	6-Br-2-naphthyl α -D-galactopyranoside	,,					
13	ß-galactosidase	2-naphthyl-ßD-galactopyranoside	,,					
14	ß-glucuronidase	Naphthol-AS-BI-ßD-glucuronide	,,					

15	α-glucosidase	2-naphthyl- α D-glucopyranoside	,,					
16	ß-glucosidase	6-Br-2-naphthyl-ßD-glucopyranoside	,,					
17	N-acetyl-ß-glucosaminidase	1-naphthyl-N-acetyl-ßD-glucosaminide	,,					
18	α -mannosidase	6-Br-2-naphthyl- α D-mannopyranoside	,,				+	
19	α-fucosidase	2-naphthyl- α L-fucopyranoside	,,					

2 Illumina sequencing results of *jqsA* amplicons

Table 5: Illumina sequencing of *jqsA* amplicons from fish and rearing water. The samples collected after fish and rearing water exposed to a mix of five *J,sp.* isolates. Only samples with 11 and above total reads per samples were included here.

#OTU ID	193-E1F2	194-E1F3	195-E2F2	196-E3F2	197-E2F4	198-E1W2	199-E2W2	200-E3W1	201-E6F4	202-E5F4	203-E6F3	204-E3W2	205-E4W2	206-E5W2	207-E6W2	208-E3F3	209-E5W1	S137_tru	Sum	Taxonomy
OTU_1	318	117	0	405	709	2929	3313	552	64	41	56	4433	6645	5138	1608	1494	1868		29690	**PBA**
OTU_2	2464	1727	2890	1120	928	1804	1968	679	46	31	53	4374	2808	2704	1203	2276	1200		28275	**3.109**
OTU_3	67	21	0	0	0	160	1172	20	95	75	18	147	6091	3951	1806	37	2170		15830	**3.108**
OTU_4	102	22	64	87	107	943	4117	263	37	18	1	2210	1045	1055	477	411	513		11472	**PBB**
OTU_13	32	35	134	55	48	0	0	0	346	289	111	0	0	0	0	101	0		1151	**Salmo salar**
OTU_48	2	0	0	0	0	21	70	0	2	0	0	24	324	159	76	1	101		780	**J.lividum**
OTU_73	7	2	1	3	3	59	159	22	0	0	0	104	73	53	33	26	46		591	**Salmo**
OTU_33	5	7	17	12	14	0	0	0	78	74	31	0	0	0	0	4	0		242	
OTU_5	0	0	0	0	0	0	108	0	0	0	0	0	0	0	0	0	0		108	
OTU_7	2	1	4	4	4	3	2	1	3	3	2	5	6	5	3	8	4		60	
OTU_71	3	6	2	5	4	0	0	0	23	13	3	0	0	0	0	1	0		60	
OTU_11	4	4	6	3	5	4	4	0	2	1	5	1	0	2	0	2	2		45	
OTU_24	0	0	17	1	19	0	0	0	4	0	3	0	0	0	0	0	0		44	
OTU_8	1	1	4	1	1	3	3	1	3	3	1	1	1	2	2	1	2		31	
OTU_6	0	2	2	16	3	0	0	0	1	1	4	0	0	0	0	2	0		31	
OTU_9	14	0	5	0	0	0	2	0	0	0	0	1	1	1	0	5	0		29	
OTU_10	4	3	1	3	1	2	1	2	1	2	6	0	1	1	0	0	1		29	
OTU_29	2	1	1	1	1	1	0	1	2	2	2	2	0	2	2	0	0		20	
OTU_19	0	0	0	0	0	0	18	0	0	0	0	0	0	0	0	0	0		18	
OTU_31	0	0	1	1	2	2	0	2	0	0	0	3	1	4	1	0	0		17	
OTU_18	1	2	1	0	0	2	2	0	1	1	1	1	0	1	0	1	1		15	
OTU_12	1	0	1	1	0	0	0	0	0	0	1	3	1	2	0	2	3		15	
OTU_45	3	0	0	1	0	0	0	0	4	5	0	0	0	0	0	0	0		13	
OTU_36	1	1	1	2	3	0	1	2	0	0	2	0	0	0	0	0	0		13	
OTU_16	1	0	0	0	0	0	0	1	0	0	0	0	0	10	0	0	0		12	
OTU_15	1	1	2	1	1	0	2	0	1	1	1	0	0	0	0	0	0		11	
OTU_20	1	0	2	0	1	2	3	0	0	1	1	0	0	0	0	0	0		11	
OTU_14	0	0	0	0	0	0	2	0	0	0	0	3	2	1	1	2	0		11	
OTU_23	0	0	0	0	0	0	1	0	0	0	0	1	4	2	3	0	0		11	

3 One-way PERMANOVA based on Bray-Curtis to test

Table 6: One-way PERMAVOVA test based on Bray-Curtis to test if there were significant differenences in the *Janthinobacterium.sp.* communities between fish and water samples:

PERMANOVA	
Permutation N:	9999
Total sum of squares:	0,5969
Within-group sum of squares:	0,2563
F:	9,3
p (same):	0,0261

4 SIMPER test based on Bray-Curtis

Table 7: A SIMPLER test based on Bray-Curtis for identifying OTUs contributing most to the Bray-Curtis dissimilarity between

Taxon	Av. dissim	Contrib. %	Cumulative %	Mean Fish	Mean Water	W/F	F/W
3,109	19,91	48,86	48,86	0,732	0,334	0,456284	2,191617
PBA	9,899	24,3	73,16	0,215	0,394	1,832558	
PBB	9,074	22,27	95,44	0,0492	0,231	4,695122	
3,108	1,859	4,564	100	0,00398	0,0412	10,35176	

5 *Janthinobacterium. sp.* isolates and their sanger sequencing result

>3.108(New)
TTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAG
TCGAACGGCAGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAACGGGTGAG
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GCTAATACCGCATACGATCTAAGGATGAAAGTGGGGGATCGCAAGACCTC
ATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAAGC
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TGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGG
CCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGAGCTAAT
ATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAATCCCCGGG
CTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAATCTGGCAGAGG
GGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAAC
ACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAAC
GATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACGCAGCTAACGC
GTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGA
ATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCA
ACGCGAAAAACCTTACCTACCCTTGACATGGCTGGAATCCTCGAGAGATT
GAGGAGTGCTCGAAAGAGAACCAGTACACAGGTGCTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGG

CTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACCCGCGAGGG
GGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTCTGCAACTC
GACTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGT
GAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGG
GTTTACCAGAAGTAGGTAGCTTAACCGCAAGGA

>J._3.109(New)
GTCGAACGGCAGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAACGGGTGA
GTAATATATCGGAACGTACCCTAGAGTGGGGGATAACGTAGCGAAAGTTA
CGCTAATACCGCATACGATCTAAGGATGAAAGTGGGGGATCGCAAGACCT
CATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAAG
CCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACAC
TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATT
TTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAG
GCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGAGCTAA
TATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTG
GGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAATCCCCGG
GCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAATCTGGCAGAG
GGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAA
CACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAA
CGATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACGCAGCTAACG
CGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG
AATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGC
AACGCGAAAAACCTTACCTACCCTTGACATGGCTGGAATCCCGGAGAGAT
CTGGGAGTGCTCGAAAGAGAACCAGTACACAGGTGCTGCATGGCTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGG
GCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACCCGCGAGG
GGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTCTGCAACT
CGACTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGG
TGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCG
GGTTTACCAGAAGTAGGTAGCTTAACCG

>J._3.116(New)
CATGCAAGTCGAACGGCAGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAA
CGGGTGAGTAATATATCGGAACGTACCCTAGAGTGGGGGATAACGTAGCG
AAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGTGGGGGATCGC
AAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGG
GTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGT
GAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGA
GAGCTAATATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGA

ATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAA
TCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAATCT
GGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGT
GGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCA
TGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCCTAAACGATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACGCA
GCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAAT
TCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGGCTGGAATCCTT
GAGAGATCGAGGAGTGCTCGAAAGAGAACCAGTACACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTAT
GGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACC
CGCGAGGGGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTC
TGCAACTCGACTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCAT
GTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCAT
GGGAGCGGGTTTACCAGAAGTAGGTAGC

>J._PBA(New)
CATGCAAGTCGAACGGCAGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAA
CGGGTGAGTAATATATCGGAACGTACCCTGGAGTGGGGGATAACGTAGCG
AAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGTGGGGGATCGC
AAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGG
GTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGT
GAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGA
GAGCTAATATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAA
TCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAATCT
GGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGT
GGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCA
TGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCCTAAACGATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACGCA
GCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAAT
TCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGGCTGGAATCCTC
GAGAGATTGAGGAGTGCTCGAAAGAGAACCAGTACACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTAT
GGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACC
CGCGAGGGGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTC
TGCAACTCGACTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCAT

GTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCAT
GGGAGCGGGTTTACCAGAAGTAGGTAG

>J._PBB-(New)
CATGCAAGTCGAACGGCAGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAA
CGGGTGAGTAATATATCGGAACGTACCCTAGAGTGGGGGATAACGTAGCG
AAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGTGGGGGATCGC
AAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGG
GTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGT
GAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACGGTGA
GAGCTAATATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAA
TCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAATCT
GGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGT
GGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCA
TGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCCTAAACGATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACGCA
GCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAAT
TCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGGCTGGAATCCCC
GAGAGATTGGGGAGTGCTCGAAAGAGAACCAGTACACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTAT
GGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACC
CGCGAGGGGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTC
TGCAACTCGACTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCAT
GTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCAT
GGGAGCGGGTTTACCAGAAGTAGGTAGCTTAACCGTAAGGAGGGCG

6 *J.sp.* isolate DNA sequences alignment results

3.108 alignment results

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26 Apr 2023                              Alignment Results

Alignment:    Multi-way DNA alignment.
Parameters:   Scoring matrix:  Linear (Mismatch 2, OpenGap 4, ExtGap 1)


   Number of sequences to align:  2
   Total length of aligned sequences with gaps:  1391 bps
   Settings:  Similarity significance value cutoff:  >= 60%


Summary of Percent Matches:
     1:   J._3.108(Old)          1 to   1391   (  1391 bps)    100%
     2:   3.108(New)             1 to   1391   (  1391 bps)    100%


J._3.108(Old)         1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagta
3.108(New)            1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagta

J._3.108(Old)        61 atatatcggaacgtaccctggagtgggggataacgtagcgaaagttacgctaataccgca
3.108(New)           61 atatatcggaacgtaccctggagtgggggataacgtagcgaaagttacgctaataccgca

J._3.108(Old)       121 tacgatctaaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatat
3.108(New)          121 tacgatctaaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatat

J._3.108(Old)       181 ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag
3.108(New)          181 ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag

J._3.108(Old)       241 aggacgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagtg
3.108(New)          241 aggacgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagtg

J._3.108(Old)       301 gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc
3.108(New)          301 gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc

J._3.108(Old)       361 ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa
3.108(New)          361 ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa

J._3.108(Old)       421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta
3.108(New)          421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta

J._3.108(Old)       481 gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc
3.108(New)          481 gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc

J._3.108(Old)       541 tgatgtgaaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatct
3.108(New)          541 tgatgtgaaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatct

J._3.108(Old)       601 ggcagaggggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac
3.108(New)          601 ggcagaggggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac

J._3.108(Old)       661 cgatggcgaaggcagccccctgggtcaagattgacgctcatgcacgaaagcgtggggagc
3.108(New)          661 cgatggcgaaggcagccccctgggtcaagattgacgctcatgcacgaaagcgtggggagc

J._3.108(Old)       721 aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc
3.108(New)          721 aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc

J._3.108(Old)       781 ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca
3.108(New)          781 ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca

J._3.108(Old)       841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat
3.108(New)          841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat

J._3.108(Old)       901 tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatcctcgagagattga
3.108(New)          901 tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatcctcgagagattga
```

```
J._3.108(Old)        961 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt
3.108(New)           961 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt

J._3.108(Old)       1021 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg
3.108(New)          1021 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg

J._3.108(Old)       1081 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca
3.108(New)          1081 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca

J._3.108(Old)       1141 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc
3.108(New)          1141 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc

J._3.108(Old)       1201 cgcgagggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga
3.108(New)          1201 cgcgagggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga

J._3.108(Old)       1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc
3.108(New)          1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc

J._3.108(Old)       1321 gggtcttgtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtagctt
3.108(New)          1321 gggtcttgtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtagctt

J._3.108(Old)       1381 aaccgcaagga
3.108(New)          1381 aaccgcaagga
```

3.109 Alignments

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27 Apr 2023                          Alignment Results

Alignment:   Multi-way DNA alignment.
Parameters:  Scoring matrix:  Linear (Mismatch 2, OpenGap 4, ExtGap 1)


   Number of sequences to align:  2
   Total length of aligned sequences with gaps:  1378 bps
   Settings:  Similarity significance value cutoff:  >= 60%


Summary of Percent Matches:
     1:  J. 3.109(Old)          1 to   1378   (  1378 bps)    100%
     2:  J._3.109(New)          1 to   1378   (  1378 bps)    100%


J._3.109(Old)          1 gtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagtaatatatc
J._3.109(New)          1 gtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagtaatatatc

J._3.109(Old)         61 ggaacgtaccctagagtgggggataacgtagcgaaagttacgctaataccgcatacgatc
J._3.109(New)         61 ggaacgtaccctagagtgggggataacgtagcgaaagttacgctaataccgcatacgatc

J._3.109(Old)        121 taaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatatctgatta
J._3.109(New)        121 taaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatatctgatta

J._3.109(Old)        181 gctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgagaggacga
J._3.109(New)        181 gctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgagaggacga

J._3.109(Old)        241 ccagccacactggaactgagacacggtccagactcctacgggaggcagcagtggggaatt
J._3.109(New)        241 ccagccacactggaactgagacacggtccagactcctacgggaggcagcagtggggaatt

J._3.109(Old)        301 ttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggccttcgggt
J._3.109(New)        301 ttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggccttcgggt

J._3.109(Old)        361 tgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaatgacggt
J._3.109(New)        361 tgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaatgacggt

J._3.109(Old)        421 acctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgtagggtgca
J._3.109(New)        421 acctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgtagggtgca

J._3.109(Old)        481 agcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtctgatgtg
J._3.109(New)        481 agcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtctgatgtg

J._3.109(Old)        541 aaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatctggcagag
J._3.109(New)        541 aaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatctggcagag

J._3.109(Old)        601 gggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacaccgatggc
J._3.109(New)        601 gggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacaccgatggc

J._3.109(Old)        661 gaaggcagccccctgggtcaagattgacgctcatgcacgaaagcgtggggagcaaacagg
J._3.109(New)        661 gaaggcagccccctgggtcaagattgacgctcatgcacgaaagcgtggggagcaaacagg

J._3.109(Old)        721 attagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtcttaattg
J._3.109(New)        721 attagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtcttaattg

J._3.109(Old)        781 acttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgcaagattaa
J._3.109(New)        781 acttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgcaagattaa

J._3.109(Old)        841 aactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaattcgatgc
J._3.109(New)        841 aactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaattcgatgc

J._3.109(Old)        901 aacgcgaaaaaccttacctacccttgacatggctggaatcccggagagatctgggagtgc
J._3.109(New)        901 aacgcgaaaaaccttacctacccttgacatggctggaatcccggagagatctgggagtgc
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J._3.109(Old)        961 tcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgtgagatgt
J._3.109(New)        961 tcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgtgagatgt

J._3.109(Old)       1021 tgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaagggcactcta
J._3.109(New)       1021 tgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaagggcactcta

J._3.109(Old)       1081 atgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctcatggccct
J._3.109(New)       1081 atgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctcatggccct

J._3.109(Old)       1141 tatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacccgcgagg
J._3.109(New)       1141 tatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacccgcgagg

J._3.109(Old)       1201 gggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcgactgcatg
J._3.109(New)       1201 gggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcgactgcatg

J._3.109(Old)       1261 aagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttcccgggtctt
J._3.109(New)       1261 aagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttcccgggtctt

J._3.109(Old)       1321 gtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtagcttaaccg
J._3.109(New)       1321 gtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtagcttaaccg
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3.116 Alignment results

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27 Apr 2023                         Alignment Results

Alignment:   Multi-way DNA alignment.
Parameters:  Scoring matrix:  Linear (Mismatch 2, OpenGap 4, ExtGap 1)


   Number of sequences to align:  2
   Total length of aligned sequences with gaps:  1378 bps
   Settings:  Similarity significance value cutoff:  >= 60%


Summary of Percent Matches:
     1:  J. 3.116(Old)         1 to   1378   (  1378 bps)    100%
     2:  J._3.116(New)         1 to   1378   (  1378 bps)    100%


J._3.116(Old)        1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagta
J._3.116(New)        1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagta

J._3.116(Old)       61 atatatcggaacgtaccctagagtgggggataacgtagcgaaagttacgctaataccgca
J._3.116(New)       61 atatatcggaacgtaccctagagtgggggataacgtagcgaaagttacgctaataccgca

J._3.116(Old)      121 tacgatctaaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatat
J._3.116(New)      121 tacgatctaaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatat

J._3.116(Old)      181 ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag
J._3.116(New)      181 ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag

J._3.116(Old)      241 aggacgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagtg
J._3.116(New)      241 aggacgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagtg

J._3.116(Old)      301 gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc
J._3.116(New)      301 gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc

J._3.116(Old)      361 ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa
J._3.116(New)      361 ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa

J._3.116(Old)      421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta
J._3.116(New)      421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta

J._3.116(Old)      481 gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc
J._3.116(New)      481 gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc

J._3.116(Old)      541 tgatgtgaaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatct
J._3.116(New)      541 tgatgtgaaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatct

J._3.116(Old)      601 ggcagagggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac
J._3.116(New)      601 ggcagagggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac

J._3.116(Old)      661 cgatggcgaaggcagcccctgggtcaagattgacgctcatgcacgaaagcgtggggagc
J._3.116(New)      661 cgatggcgaaggcagcccctgggtcaagattgacgctcatgcacgaaagcgtggggagc

J._3.116(Old)      721 aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc
J._3.116(New)      721 aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc

J._3.116(Old)      781 ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca
J._3.116(New)      781 ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca

J._3.116(Old)      841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat
J._3.116(New)      841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat

J._3.116(Old)      901 tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatccttgagagatcga
J._3.116(New)      901 tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatccttgagagatcga
```

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J._3.116(Old)       961 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt
J._3.116(New)       961 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt

J._3.116(Old)      1021 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg
J._3.116(New)      1021 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg

J._3.116(Old)      1081 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca
J._3.116(New)      1081 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca

J._3.116(Old)      1141 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc
J._3.116(New)      1141 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc

J._3.116(Old)      1201 cgcgagggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga
J._3.116(New)      1201 cgcgagggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga

J._3.116(Old)      1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc
J._3.116(New)      1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc

J._3.116(Old)      1321 gggtcttgtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtagc
J._3.116(New)      1321 gggtcttgtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtagc
```

PBA Alignment Results

```
27 Apr 2023                          Alignment Results

Alignment:   Multi-way DNA alignment.
Parameters:  Scoring matrix:  Linear (Mismatch 2, OpenGap 4, ExtGap 1)


   Number of sequences to align:  2
   Total length of aligned sequences with gaps:  1377 bps
   Settings:  Similarity significance value cutoff:  >= 60%


Summary of Percent Matches:
     1:  J. PBA(Old)           1 to   1377   (  1377 bps)    100%
     2:  J._PBA(New)           1 to   1377   (  1377 bps)    100%


J._PBA(Old)          1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagta
J._PBA(New)          1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagta

J._PBA(Old)         61 atatatcggaacgtaccctggagtgggggataacgtagcgaaagttacgctaataccgca
J._PBA(New)         61 atatatcggaacgtaccctggagtgggggataacgtagcgaaagttacgctaataccgca

J._PBA(Old)        121 tacgatctaaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatat
J._PBA(New)        121 tacgatctaaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatat

J._PBA(Old)        181 ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag
J._PBA(New)        181 ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag

J._PBA(Old)        241 aggacgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagtg
J._PBA(New)        241 aggacgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagtg

J._PBA(Old)        301 gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc
J._PBA(New)        301 gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc

J._PBA(Old)        361 ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa
J._PBA(New)        361 ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa

J._PBA(Old)        421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta
J._PBA(New)        421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta

J._PBA(Old)        481 gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc
J._PBA(New)        481 gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc

J._PBA(Old)        541 tgatgtgaaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatct
J._PBA(New)        541 tgatgtgaaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatct

J._PBA(Old)        601 ggcagagggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac
J._PBA(New)        601 ggcagagggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac

J._PBA(Old)        661 cgatggcgaaggcagccccctgggtcaagattgacgctcatgcacgaaagcgtggggagc
J._PBA(New)        661 cgatggcgaaggcagccccctgggtcaagattgacgctcatgcacgaaagcgtggggagc

J._PBA(Old)        721 aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc
J._PBA(New)        721 aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc

J._PBA(Old)        781 ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca
J._PBA(New)        781 ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca

J._PBA(Old)        841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat
J._PBA(New)        841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat

J._PBA(Old)        901 tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatcctcgagagattga
J._PBA(New)        901 tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatcctcgagagattga
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J._PBA(Old)          961 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt
J._PBA(New)          961 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt

J._PBA(Old)         1021 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg
J._PBA(New)         1021 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg

J._PBA(Old)         1081 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca
J._PBA(New)         1081 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca

J._PBA(Old)         1141 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc
J._PBA(New)         1141 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc

J._PBA(Old)         1201 cgcgagggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga
J._PBA(New)         1201 cgcgagggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga

J._PBA(Old)         1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc
J._PBA(New)         1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc

J._PBA(Old)         1321 gggtcttgtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtag
J._PBA(New)         1321 gggtcttgtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtag
```

PBB **Alignment Results**

```
27 Apr 2023                          Alignment Results

Alignment:   Multi-way DNA alignment.
Parameters:  Scoring matrix:  Linear (Mismatch 2, OpenGap 4, ExtGap 1)


   Number of sequences to align:  2
   Total length of aligned sequences with gaps:  1396 bps
   Settings:  Similarity significance value cutoff:  >= 60%


Summary of Percent Matches:
     1:  J. PBB (Old)          1 to   1396   (  1396 bps)    100%
     2:  J._PBB-(New)          1 to   1396   (  1396 bps)    100%


J._PBB (Old)         1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagta
J._PBB-(New)         1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagta

J._PBB (Old)        61 atatatcggaacgtaccctagagtgggggataacgtagcgaaagttacgctaataccgca
J._PBB-(New)        61 atatatcggaacgtaccctagagtgggggataacgtagcgaaagttacgctaataccgca

J._PBB (Old)       121 tacgatctaaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatat
J._PBB-(New)       121 tacgatctaaggatgaaagtgggggatcgcaagacctcatgctcgtggagcggccgatat

J._PBB (Old)       181 ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag
J._PBB-(New)       181 ctgattagctagttggtagggtaaaagcctaccaaggcatcgatcagtagctggtctgag

J._PBB (Old)       241 aggacgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagtg
J._PBB-(New)       241 aggacgaccagccacactggaactgagacacggtccagactcctacgggaggcagcagtg

J._PBB (Old)       301 gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc
J._PBB-(New)       301 gggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagtgaagaaggcc

J._PBB (Old)       361 ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa
J._PBB-(New)       361 ttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa

J._PBB (Old)       421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta
J._PBB-(New)       421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgta

J._PBB (Old)       481 gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc
J._PBB-(New)       481 gggtgcaagcgttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtc

J._PBB (Old)       541 tgatgtgaaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatct
J._PBB-(New)       541 tgatgtgaaatccccgggctcaacctgggaattgcattggagactgcaaggctagaatct

J._PBB (Old)       601 ggcagagggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac
J._PBB-(New)       601 ggcagagggggtagaattccacgtgtagcagtgaaatgcgtagatatgtggaggaacac

J._PBB (Old)       661 cgatggcgaaggcagccccctgggtcaagattgacgctcatgcacgaaagcgtggggagc
J._PBB-(New)       661 cgatggcgaaggcagccccctgggtcaagattgacgctcatgcacgaaagcgtggggagc

J._PBB (Old)       721 aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc
J._PBB-(New)       721 aaacaggattagataccctggtagtccacgccctaaacgatgtctactagttgtcgggtc

J._PBB (Old)       781 ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca
J._PBB-(New)       781 ttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca

J._PBB (Old)       841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat
J._PBB-(New)       841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaat

J._PBB (Old)       901 tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatccccgagagattgg
J._PBB-(New)       901 tcgatgcaacgcgaaaaaccttacctacccttgacatggctggaatccccgagagattgg
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J._PBB (Old)        961 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt
J._PBB-(New)        961 ggagtgctcgaaagagaaccagtacacaggtgctgcatggctgtcgtcagctcgtgtcgt

J._PBB (Old)       1021 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg
J._PBB-(New)       1021 gagatgttgggttaagtcccgcaacgagcgcaacccttgtcattagttgctacgaaaggg

J._PBB (Old)       1081 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca
J._PBB-(New)       1081 cactctaatgagactgccggtgacaaaccggaggaaggtggggatgacgtcaagtcctca

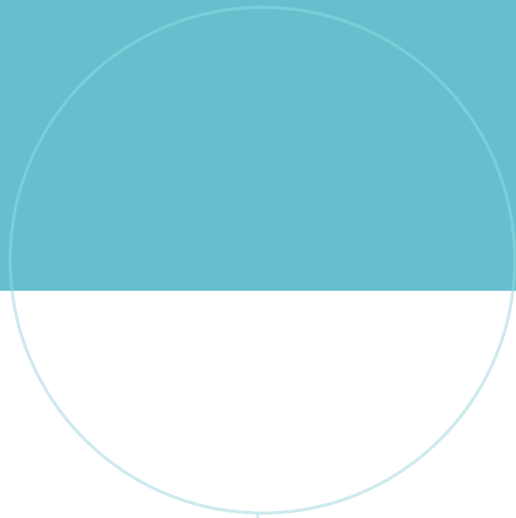
J._PBB (Old)       1141 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc
J._PBB-(New)       1141 tggcccttatgggtagggcttcacacgtcatacaatggtacatacagagcgccgccaacc

J._PBB (Old)       1201 cgcgagggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga
J._PBB-(New)       1201 cgcgagggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga

J._PBB (Old)       1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc
J._PBB-(New)       1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttccc

J._PBB (Old)       1321 gggtcttgtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtagctt
J._PBB-(New)       1321 gggtcttgtacacaccgcccgtcacaccatgggagcgggtttaccagaagtaggtagctt

J._PBB (Old)       1381 aaccgtaaggagggcg
J._PBB-(New)       1381 aaccgtaaggagggcg
```



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Science and Technology