Master's thesis

NTNU
Norwegian University of Science and Technology
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Department of Biotechnology and Food Science

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Janthinobacterium Strains Isolated from Rearing Systems for Atlantic Salmon Fry

Growth Characteristics, Violacein Production and Evolution of the Violacein Operon

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Ingrid Bakke
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Acknowledgements

This master project was carried out in cooperation with the research group Analysis and Control of Microbial Systems (ACMS) at the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU).

First of all, I would like to thank my main supervisor Professor Ingrid Bakke for her guidance and feedback throughout this project. Her valuable experience and knowledge have been highly appreciated, and working with Ingrid has been a true pleasure. I would also like to thank my co-supervisor, PhD-candidate Alexander Willi Fiedler, for the many hours of training in the laboratory. I am beyond grateful for his willingness to share knowledge, and for his support and patience during my master project. To witness my supervisors commitment to the field has been very inspiring.

Further, I would like to thank laboratory engineer Amalie Horn Mathisen for her assistance during experiments. I would also like to thank researcher Eirik Degré Lorentsen for all his advice during the laboratory work, and for always being available for questions. I am also grateful to have been part of such an including research group as ACMS. Thank you for letting me participate in both your academic and social meetings.

I would also like to thank my classmates and friends for sharing these five memorable years at NTNU together. Finally, I want to thank my family and my boyfriend Jonas for all their love and support.

Background Information from Specialization Project

The following master project is a continuation of the work performed during my specialization project (Mølmen, 2020).

Five bacterial strains, referred to as PBA, PBB, MM5, 3.109 and 3.116, were previously isolated from Atlantic salmon fry rearing systems (skin and rearing water). Sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene strongly suggested that these strains belong to the species *Janthinobacterium lividum*. Examination of the *J.* sp. strains growth characteristics showed that several of the strains produced a purple-coloured pigment and a slimy substance, assumed to be violacein and extracellular polymeric substances (EPS) respectively. The colonies of the *J.* sp. strains PBA and PBB were both observed to exhibit a strong purple colour, whereas *J.* sp. MM5 usually produced white colonies, with indications of a slight purple hue. The *J.* sp. strains 3.109 and 3.116 grew in white colonies with no sign of violacein production. Sequencing parts of the violacein operons, only yielded results for the *J.* sp. strains PBA, PBB and MM5, indicating that *J.* sp. 3.109 and 3.116 lack this operon. Despite the five *J.* sp. strains having highly similar 16S rRNA gene sequences, the overall results strongly suggested that they were five different strains of *Janthinobacterium*.

Abstract

Janthinobacterium lividum is commonly isolated from the skin of amphibians and humans. The species is known for producing the purple pigment violacein, exhibiting antifungal, antibacterial, antiviral and antitumoral effects. This wide range of biological activities have made *J. lividum* a promising candidate in medical applications as an antibiotic and as a treatment for cancer. Due to its well known antifungal properties, *J. lividum* might be an interesting probiotic candidate against saprolegniosis, caused by the fungal pathogen *Saprolegnia*. The disease can lead to the death of farmed Atlantic salmon and hence severe economic losses in the aquaculture industry. Today's treatment approaches involve the use of harmful chemicals and are highly debated. Thus, there is a growing need for new sustainable alternatives. However, the mechanisms driving the production of violacein and *J. lividum*'s role as a commensal of Atlantic salmon are not yet fully understood.

In this master project, the five *J.* sp. strains PBA, PBB, MM5, 3.109 and 3.116, previously isolated from rearing systems for Atlantic salmon fry, were studied more closely. This study aimed to sequence the violacein operon of *J.* sp. PBA, PBB and MM5 and conduct phylogenetic analyses to study the evolution of the violacein operon. Growth experiments were performed to provide further understanding of the *J.* sp. strains' growth characteristics, their potential violacein production and antagonistic properties, as well as their ability to colonize the skin and gut of Atlantic salmon yolk sac fry. The sequencing of the violacein operons was successful, providing evidence that *J.* sp. PBA and PBB possessed all five violacein operon genes, VioA-VioE. However, analysis of the amino acid sequences suggested that *J.* sp. MM5 did not have a functional VioE gene product, and that the last half of the VioB gene (3' end) for both *J.* sp. PBA and MM5 was truncated. The phylogenetic analyses indicated that the violacein operon had been subjected to horizontal gene transfer during the evolution of the Proteobacteria. Measuring the maximum absorbance of ethanol extracts of violacein from *J.* sp. PBA, *J.* sp. PBB and the *J. lividum* type strain, resulted in wavelengths of 576 nm, 574 nm and 575 nm, strongly suggesting the presence of violacein. Moreover, all *J.* sp. strains showed indications of growing in cell aggregates and produced copious amounts of slime when cultivated in liquid LB-medium. All strains exhibited both smooth and rugose colony morphology when grown on LA-plates, except for *J.* sp. 3.116, which surface was consistently smooth. Further, the strains were able to grow with mucin and chitin as their sole carbon source, substantiating their mucin and chitin-degrading abilities. Examination of the *J.* sp. strains' antagonistic activity, showed indications of inhibition against *Pedobacter* sp., but not against *Yersinia ruckeri* or *Arthrobacter* sp. Absence of violacein production did not seem to affect the potential antagonistic properties of the *J.* sp. strains against *Pedobacter* sp. Further, all strains were able to colonize the skin of Atlantic salmon yolk sac fry in high numbers. For all *J.* sp. strains, the colonization density of the gut was generally lower than on the skin. The two *J.* sp. strains PBA and 3.109 seemingly lacked the ability to colonize the gut of salmon fry, as well as growing planktonically in the rearing water. Overall, the study support that *Janthinobacterium* might be a commensal of Atlantic salmon skin.

Sammendrag

Janthinobacterium lividum er tidligere isolert fra amfibieskinn og menneskehud. Arten er kjent for å produsere det lilla pigmentet violacein, som har vist seg å ha antifungale, antibakterielle, antivirale og antitumorale egenskaper. Dette brede spekteret av biologiske egenskaper har gjort *J. lividum* til en lovende kandidat i medisinske applikasjoner som antibiotika og som behandling mot kreft. Grunnet *J. lividum*s kjente antifungale egenskaper kan den være en interessant probiotisk kandidat mot saprolegniose forårsaket av sopp-patogenet *Saprolegnia*. Sykdommen kan føre til død blant oppdrettslaks og følgelig store økonomiske tap i havbruksnæringen. Dagens behandlingsmetoder involverer bruk av skadelige kjemikalier og er sterkt omdiskutert. Det er derfor et økende behov for nye bærekraftige alternativer. Foreløpig er mekanismene forbundet med violaceinproduksjon og *J. lividum*s rolle som en kommensal for atlanterhavslaks enda ikke helt forstått.

I dette masterprosjektet ble de fem *Janthinobacterium*-stammene PBA, PBB, MM5, 3.109 og 3.116, tidligere isolert fra oppdrettssystemer for yngel av atlanterhavslaks, studert nærmere. Dette prosjektet hadde som mål å sekvensere violaceinoperonet til PBA, PBB og MM5 og deretter gjennomføre fylogenetiske analyser for å studere evolusjonen av violaceinoperonet. Vekstesperimenter ble utført for å gi en ytterligere forståelse av stammenes vekstegenskaper, deres potensielle violaceinproduksjon og antagonistiske egenskaper, samt deres evne til å kolonisere skinnet og tarmen til plommesekkyngel av atlanterhavslaks. Sekvenseringen av violaceinoperonene var vellykket og viste at PBA og PBB hadde alle fem violaceinoperon-genene, VioA-VioE. Videre analyse av aminosyresekvensene antydet at MM5 ikke hadde et funksjonelt VioE-genprodukt, og at den siste halvdelen av VioB-genet (3' enden) for både PBA og MM5 var trunkert. De fylogenetiske analysene indikerte at violaceinoperonet har gjennomgått horisontal genoverføring i evolusjonen av Proteobacteria. Måling av maksimal absorbans for etanolekstrakt av violacein fra PBA, PBB og *J. lividum* resulterte i bølgelengder på 576 nm, 574 nm og 575 nm, noe som tydelig indikerte at violacein var tilstede. Videre viste alle stammene indikasjoner på å vokse i celleaggregater og produserte store mengder slim i flytende LB-medium. Alle stammene viste både glatt og rynkete kolonimorfologi da de ble dyrket på LA-plater, bortsett fra 3.116 som hele tiden vokste med glatt overflate. Alle stammene var i stand til å vokse med mucin og kitin som eneste karbonkilde, noe som underbygget deres mucin- og kitin-degraderende evner. Undersøkelse av stammenes antagonistiske egenskaper viste indikasjoner på inhibering av *Pedobacter*, men ingen inhibering av *Yersinia ruckeri* eller *Arthrobacter*. Fravær av violaceinproduksjon så ikke ut til å påvirke den potensielle antagonistiske aktiviteten til *Janthinobacterium*-stammene mot *Pedobacter*. Alle stammene koloniserte skinnet til plommesekkyngel av atlanterhavslaks med et høyt antall bakterier. Videre ble det vist at koloniseringstettheten i tarmen generelt var lavere enn på skinnet for alle stammene. De to stammene PBA og 3.109 manglet tilsynelatende evnen til å kolonisere tarmen til lakseyngel, samt evnen til å vokse planktonisk i fiskevannet. Studiet viste at *Janthinobacterium* kan være en kommensal bakterie i skinnet til atlanterhavslaks.

Abbreviations

3.109	*Janthinobacterium* strain
3.116	*Janthinobacterium* strain
ACMS	Analysis and Control of Microbial Systems
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
EPS	Extracellular polymeric substance
Fwd	Forward
GI	Gastrointenstinal
HGT	Horizontal gene transfer
IMG/M	Integrated Microbial Genomes & Microbiomes
J. lividum	*Janthinobacterium lividum*
***J.* sp.**	*Janthinobacterium* species
LA/LB	Luria-Bertani agar/broth
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
MM5	*Janthinobacterium* strain
NCBI	National Center for Biotechnology Information
NTNU	Norwegian University of Science and Technology
ON	Overnight
PBA	*Janthinobacterium* strain
PBB	*Janthinobacterium* strain
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
rDNA	Ribosomal deoxyribonucleic acid
Rev	Reverse
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SGM	Salmon gnotobiotic medium
TSA/TSB	Tryptic soy agar/broth
VioA/B/C/D/E	Violacein operon gene A/B/C/D/E

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1 Introduction

1.1 *Janthinobacterium lividum*

Janthinobacterium lividum (*J. lividum*) is a species of Gram-negative bateria belonging to the phylum Proteobacteria and family of Oxalobacteraceae (Oh et al., 2019; Valdes et al., 2015). It is a rod-shaped, aerobic and heterotrophic bacterial species, commonly isolated from the microbiota of soils and sediments, as well as water originating from rivers, springs and lakes (Pantanella et al., 2007). In addition, several *J. lividum* strains have been isolated from harsh environments, such as Antarctic snow (Baricz et al., 2018) and Himalayan waters (Kumar et al., 2018), demonstrating its versatility and survivability. Moreover, high incidence of *J. lividum* has been reported in the skin microbiota of both amphibians (Brucker et al., 2008) and humans (Grice et al., 2008; Ramsey et al., 2015). Further, *J. lividum* has been reported to exhibit antagonistic properties towards other bacteria and fungi, meaning it inhibits their growth (Munakata et al., 2021). The size of the cells measures approximately 0.8-1.0 x 2.5-3.0 µm, and it is motile by means of a sub-polar to lateral flagellum (O'sullivan et al., 1990). *J. lividum* generally has a temperature range of growth varying between 4 ℃ and 30 ℃, depending on the strain and environmental conditions. With an optimum growth temperature of approximately 25 ℃, *J. lividum* has been proposed to be a psychrotolerant organism (Valdes et al., 2015).

Studies have reported that *J. lividum* is able to produce a strong biofilm in the stationary phase of growth (Pantanella et al., 2007). The produced biofilm contained a significant amount of extracellular polymeric substance (EPS), serving as the main constituent of biofilm and embedding microorganisms within a slimy matrix, providing stability and protection from the external environment. Furthermore, *J. lividum* is one of the main producers of a purple pigment called violacein (Hoshino, 2011; Pantanella et al., 2007). Violacein-producing species have been observed to produce increased amounts of EPS and possess better survival characteristics compared to their non violacein-producing counterparts (Pantanella et al., 2007).

J. lividum is well-known for its antifungal activity, suppressing growth of fungal pathogens on amphibian (Becker et al., 2009) and human (Ramsey et al., 2015) skin. It is believed that these antifungal activities are related to quorum sensing (QS) regulation in the cells as a response to the presence of chitin or chitin degradation products (Cretoiu et al., 2013). QS is an intercellular communication process used to control population density-dependent changes related to cellular behaviour, enabling the bacteria to adjust their gene expression in a density-related manner (Choi et al., 2015). Interestingly, Hack et al. (2016) reported indications of *J. lividum* possessing chitin degrading abilities, encoding chitinases, possibly involved in the fungal growth inhibition.

Fungal diseases are a major threat to amphibian populations, leading to a severe increase in mortality and extinction of several species (Harris et al., 2009). Chytridiomycosis is a lethal amphibian disease caused by the chytrid fungus *Batrachochytrium dendrobatidis*. Studies have shown that the presence of *J. lividum* in the skin microbiota reduced mortality rates in both yellow-legged mountain frog (*Rana muscosa*) and eastern red-backed salamander (*Plethodon cinereus*). In addition, higher concentrations of violacein correlated to decreased mortality rates, indicating the death or inhibition of the pathogen (Brucker et al., 2008). In the future, the survival of such amphibious species could be dependent upon a deeper understanding of the mutualistic relationship between *J. lividum* and the hosts. Additionally, it has been questioned if there in fact is a direct connection between violacein production and the observed anti-fungal properties of *J. lividum* (Haack et al., 2016).

Certain strains of *J. lividum* are known to be capnophilic (Valdes et al., 2015), thriving in presence of high concentrations of carbon dioxide (CO_2). Amphibians have the ability to exchange gases with their environment through their skin. This is made possible by a subdermal capillary network through which carbon dioxide is excreted, leading to an increased concentration of CO_2 in the mucus membrane covering their skin (Tattersall, 2007). A strain of *J. lividum* (MTR) has been shown to be carbon dioxide sensitive. All concentrations of CO_2 above 1% yielded increased bacterial growth. Generally, the CO_2 concentration in amphibian skin is approximately 1.2-2.5%, supporting that amphibian skin is a suitable habitat for capnophilic *J. lividum* strains (Valdes et al., 2015). Moreover, the production of violacein has been postulated to protect amphibian hosts against fungal infections, inhibiting pathogenic growth (Harris et al., 2009). However, the overall beneficial effects provided to the bacteria still remains uncertain (Valdes et al., 2015).

Apart from Amphibia, *J. lividum* has been found to be a member of the fish skin-mucus microbiome. Several studies have reported high abundance of *J. lividum* in the gut and skin microbiota of Atlantic salmon (*Salmo salar)* (Gajardo et al., 2016; Hovda et al., 2007). In addition, previous experiments performed in the ACMS group have also confirmed the presence of *Janthinobacterium* in the gut and skin mucosa of Atlantic salmon yolk sac fry (personal communication, Alexander Willi Fiedler, 2020). However, the effects relating to *Janthinobacterium* in the mucosa of the Atlantic salmon is not fully understood.

As previously mentioned, *J. lividum* is a significant component of the human skin microbiota (Grice et al., 2008). It has been postulated that the observed effects reported in amphibian species might be applicable in human medical technology as treatment against nail and skin fungus. Studies have shown that the fungus *Trichophyton rubrum*, causing the foot infection tinea pedis (athlete's foot), was significantly inhibited in the presence of *J. lividum*. As a result of this, the implementation of *J. lividum* as a probiotic treatment has been proposed to protect against fungal pathogens and skin infections in humans (Ramsey et al., 2015).

1.2 Violacein

Violacein is a natural purple-coloured pigment produced by a variety of Gram-negative bacteria. *J. lividum* is one of the most well-known producers of this compound, explaining the distinct purple-coloured colonies of many *Janthinobacterium* strains (Pantanella et al., 2007). Additionally, the pigment is produced by *Chromobacterium violaceum* (Rettori & Durán, 1998), *Pseudoalteromonas luteoviolacea* (Yada et al., 2008), *Duganella sp.* (Wang et al., 2009), and *Collimonas sp.* (Hakvåg et al., 2009) among others. Besides to these bacterial strains being able to produce violacein, they all belong to the major phylum of Proteobacteria, having variations in class, order and family. Most of the violacein-producing species within the class Betaproteobacteria belong to the two families Chromobacteriaceae and Oxalobacteraceae (Table 1.1).

Table 1.1: Taxonomy for various violacein-producing Proteobacteria. Data is retrieved from The National Center for Biotechnology Information (NCBI)-Taxonomy classifications.

Class	Order	Family	Genus
Betaproteobacteria	Neisseriales	Chromobacteriaceae	*Chromobacterium*
Betaproteobacteria	Neisseriales	Chromobacteriaceae	*Iodobacter*
Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Duganella*
Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Collimonas*
Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Janthinobacterium*
Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Massilia*
Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	*Pseudoalteromonas*
Gammaproteobacteria	Cellvibrionales	Microbulbiferaceae	*Microbulbifer*
Deltaproteobacteria	Myxococcales	Myxococcaceae	*Myxococcus*

Violacein is an indole-derived compound, with the chemical formula $C_{20}H_{13}N_3O_3$ and the molecular weight 343.34 g/mol (Figure 1.1). The biosynthesis of this bisindole is dependent upon a condensation reaction between two molecules of tryptophan and is formed as a secondary metabolite during the metabolism of this amino acid (Asencio et al., 2014; Choi et al., 2015).

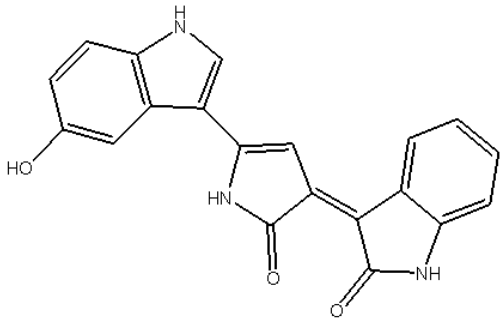


Figure 1.1: Chemical structure of the natural purple-coloured pigment violacein.

Violacein is water insoluble, but easily dissolves in organic solvents such as acetone, methanol and ethanol (Pantanella et al., 2007). The absorbance spectrum of violacein extracts in ethanol is shown to have a maximum absorbance value at 575 nm (Alem et al., 2020; Wang et al., 2009). Additionally, violacein has been reported to show good stability at pH values ranging from 5-9 and temperatures between 25-100 ℃ (Ahmad et al., 2012).

Violacein has been shown to exhibit a number of important biological activities (Pantanella et al., 2007), making it a promising candidate molecule in numerous pharmacological studies. The compound has expressed antiprotozoal (Matz et al., 2004), antiviral (Andrighetti-Fröhner et al., 2003), anticancer (Ferreira et al., 2004), antioxidizing (Konzen et al., 2006), antifungal (Brucker et al., 2008) and antitumoral (Jiang et al., 2015) activities. It has been discussed that the production of violacein is a major part of the defense mechanisms against external stressors in many bacteria (Choi et al., 2015). Through several experiments, it was discovered that many bacterial strains produced violacein as a part of the response to low temperatures and UV radiation (Mojib et al., 2013). Moreover, the antioxidizing properties of violacein have been postulated to provide protection against oxidative stress (Konzen et al., 2006).

Studies have shown that violacein exhibits antibacterial activity towards Gram-negative and Gram-positive bacteria, the latter in particular (Asencio et al., 2014; Choi et al., 2015). From experiments performed in vitro, Cauz et al. (2019) discovered that violacein can directly bind to liposomes and disrupt their structure and permeability. Further, violacein has been shown to permeabilize the cytoplasmic membrane of several Gram-positive bacteria, leading to small ruptures and discontinuities in the membrane. The ruptures and holes in the membrane leads to a leakage of intercellular contents, effectively killing the cell. The bacterial cell wall is unaffected and kept intact during the process. It has been suggested that the reported antifungal activity of violacein is attributed to the same permeabilizing properties (Cauz et al., 2019).

Violacein has been used commercially as a bio-dye for a long time (Xu et al., 2019). In later years, there has been an increased focus on the applicability of violacein in medicine. Violacein has been proposed as a therapeutic agent for cancer treatment, with promising results in leukemia studies. Violacein has expressed cytotoxic activity on transformed cell lines, by induction of apotosis in HL60 leukemic cells, when incubated together with violacein (Ferreira et al., 2004; Melo et al., 2003). However, the cytotoxic effects of vioalcein does not only affect cancer cells, but also attacks healthy human cells. In addition, the poor solubility of violacein in water may also lead to poor biological activity in vivo. Because of this, there has been discussions regarding the potential use of violacein as a cancer therapeutic. Further, production of violacein has been shown to be lethal against the malaria-causing parasite *Plasmodium falciparum* (*P. falciparum).* It has been reported that violacein was therapeutically applicable against malaria infected mice (Lopes et al., 2009), both the wild-type and the drug-resistant strain of *P. falciparum.*

1.3 The Violacein Operon

Bacterial genes are typically organized in operons. An operon is a cluster of genes with functionally related properties and the associated genes are commonly co-expressed in cells' metabolic pathways. An operon is made up of three important DNA components. The first component is a promoter, functioning as a binding site for RNA polymerase, which will initiate transcription. Additionally, the promoter functions as a binding site for regulation of gene expression. Directly following the promoter is the operator, which functions as a repressor binding site. If a repressor is bound to the operator it will inhibit transcription of the functional genes. The final part of the operon is comprised of a set of functional genes (Osbourn & Field, 2009).

J. lividum contains an operon consisting of the five functional genes VioA, VioB, VioC, VioD and VioE, which are all transcribed in the same direction (Figure 1.2). This operon is called the violacein operon and its genes are part of the complex biosynthetic pathway for the production of violacein. The synthesis is initiated when two molecules of L-tryptophan are catalyzed by VioA, functioning as an oxidase, to form two molecules of 2-imino-3-(Indole-3-yl) propionate. Further, the pathway continues with reactions converting these molecules into protodeoxyviolaceinic acid, catalyzed by the two enzymes VioB and VioE. Through the pathway's final reactions, and the catalytic activity of the two enzymes VioC and VioD, violacein is produced. In the absence of VioD in the metabolic pathway of violcein, the byproduct deoxyviolacein is produced instead (Füller et al., 2016).

Figure 1.2: Schematic representation of the five functional genes VioA, VioB, VioC, VioD and VioE in the violacein operon.

Violacein production has been observed to be regulated by quorum sensing. The QS-system in the *Janthinobacterium* sp. strain HH01 is regulated by the autoinducer JAI-1, interacting with receptors in the cells and altering the expression of genes (induce or inhibit). Additionally, the system consists of the three clustered genes *jqsA* (autoinducer synthase gene), *jqsS* (sensor kinase gene) and *jqsR* (response regulator gene) (Haack et al., 2016; Hornung et al., 2013). Hornung et al. (2013) showed that the expression of the violacein operon was dependent on the presence of the *jqsA* gene, by comparing violacein production between *Janthinobacterium* sp. HH01 and a *jqsA* deletion mutant. This resulted in a visible reduction of violacein production when lacking the *jqsA* gene.

Further, phylogenetic analysis based on the violacein operon gene sequences of strains of *Janthinobacterium* (PBA, PBB, MM5, 3.109, 3.116 and *J. lividum* type strain), and other violacein-producing bacterial strains, indicated horizontal gene transfer (HGT) (Lorentsen, 2020; Mølmen, 2020). HGT is the transference of genetic information from one genome to another which is not its offspring (Keeling & Palmer, 2008). However, these analyses were conducted utilizing incomplete data, and further analysis and data acquisition is required to confirm this result.

1.4 Atlantic Salmon (*Salmo salar*)

The Atlantic salmon (*Salmo salar*) is a species of ray-finned fish belonging to the family Salmonidae (Abbate et al., 2020). The species is spread across the northern parts of the Atlantic ocean, and inhabits waters of North America, Europe and north-western Russia (Horreo et al., 2019). It is an anadromous species, meaning it is born in freshwater before migrating to the ocean where it spends the majority of its life, only returning to its river of origin to spawn (Kjærner-Semb et al., 2020).

The life cycle of the Atlantic salmon is complex (Figure 1.3). Normally, it spends the first one to four years in fresh water, before migrating to the sea for one to three winters. As the Atlantic salmon enters the reproduction phase, the adults return to their native river for spawning (Pardo & Hutchings, 2020). The female excavates a nest in the gravel of the riverbed and places her eggs at the bottom, following fertilization by the male. The eggs then incubate for approximately 550-560 degree days before hatching into alevins (yolk sac fry) (Solberg et al., 2014). At this stage the alevins stay in the gravel nest and are completely dependent upon the yolk sac as their primary source of nutrition. When the yolk sac is consumed and encapsulated by the body of the alevin, it emerges from the gravel nest and enters the next phase of development as fry (Dill, 1977). Fry develop into parr which resides in its freshwater territory for 2-4 years (Hansen & Quinn, 1998). After the juvenile stage, the parr must undergo smoltification, enabling it to survive a life in seawater (Heys et al., 2020). The preparatory smolting process involves considerable changes in factors like behavior, physiology and biochemistry (Fjelldal et al., 2018). In addition, the process involves morphological changes leading to a slimmer body and altered skin color to improve camouflaging abilities in the pelagic habitat (Thorstad et al., 2012). As it enters the sea, the post-smolt continues its development and rapid growth until it reaches sexual maturity. When reaching the end of its first winter in the ocean, the Atlantic salmon is considered an adult.

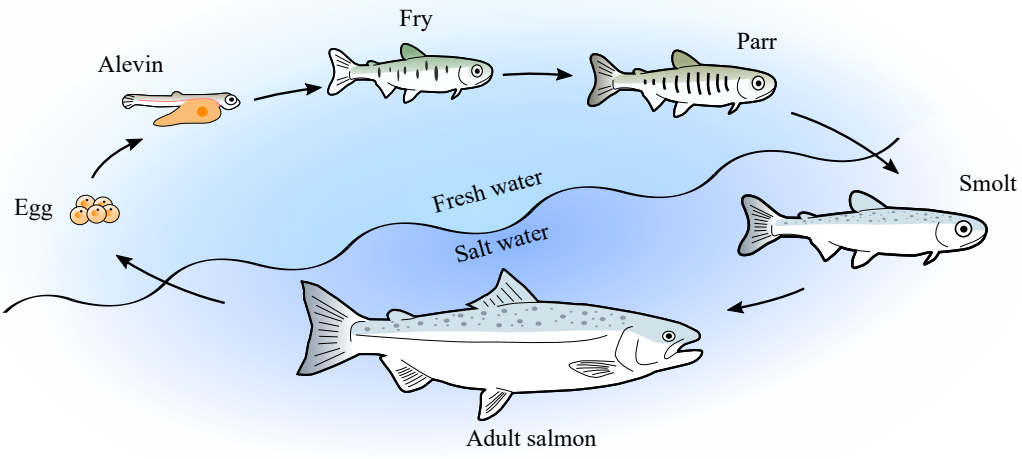


Figure 1.3: The life cycle of the Atlantic salmon.

1.5 Aquaculture of Atlantic Salmon

Aquaculture is the farming and cultivation of aquatic organisms such as fish, molluscs, crustaceans, algae and other aquatic plants (Jobling, 2010), and the fastest growing sector in regards to food production worldwide (Subasinghe et al., 2009). In Norway, the farming of Atlantic salmon at the commercial-scale started around 1970, but was not widely exercised until the beginning of the 1980s. With its long and sheltered coastline, Norway has established itself as the second largest exporter of farmed fish and seafood in the world, with main focus on Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) (Johansen et al., 2019). Innumerable cold, clear fjords and steady sea currents ensure ideal growing and living conditions for the fish.

The Atlantic salmon is, in terms of global economic value, listed as the number one fish species in the aquaculture industry (Adams, 2019). Because of this, and its suitability for farming, the Atlantic salmon is intensively farmed in aquaculture facilities globally. When farming Atlantic salmon, the eggs are incubated and the fish are reared in onshore fresh water facilities until smoltification occurs. Further, the smolts are transferred into sea-based net cages and grown until they reach market size to undergo further processing (Bjørndal & Tusvik, 2019; Verspoor et al., 2007).

Primarily, recirculating aquaculture systems (RAS) have been used for production of Atlantic salmon smolts (Dalsgaard et al., 2013). As the commercial demand for salmon continues to increase, so does the interest towards land-based RAS as a supplementary production method of adult fish. Despite the high costs following building and maintaining RAS, there are several advantages utilizing this production method regarding the environment and sustainability. RAS provide reduced fresh water consumption, and does not lead to pollution of natural water due to nutrient loading (Kristensen et al., 2009). The rearing of fish in RAS is performed under more controlled circumstances as the environment can be monitored and optimized for the most efficient growth (Martins et al., 2010).

Because of several infectious diseases and various pathogens in both the marine and freshwater environment, the fish health is under severe threat. As Atlantic salmon farming is partly performed in open aquatic systems, there is a possibility that disease-causing organisms can be transferred between the farmed salmon and the wild salmon (Verspoor et al., 2007). Due to their high stocking numbers, aquacultured salmon might be exposed to more long-term stress than salmon living in the wild and therefore have a higher chance of being infected (Padra et al., 2014). In 2021, the Norwegian Veterinary Institute reported that 52.1 million Norwegian farmed salmon died during last year's production due to reasons like lethal infectious diseases, parasites and injuries (Norwegian Veterinary Institute, 2020). This high number emphasizes the demand for further research on how to overcome the challenges related to aquacultured salmon.

1.6 The Microbiota and the Positive Effects of Host-microbe Interactions

The microbiota is the complex community of various microorganisms living in an environment. In organisms, the microbiota has been attributed several beneficial functions relating to nutrient uptake and the development of the host's immune system (Valdes et al., 2018). Over the years, the main focus of host-microbe interaction studies has been directed towards pathogens and their associated negative effects on the hosts (Fraune & Bosch, 2010). However, several studies have pointed out the importance of the commensal microbiota of hosts relating to protection against pathogens and infections (Cho & Blaser, 2012; Fraune & Bosch, 2010).

Several studies have investigated the use of probiotics as treatment against pathogens in both humans and animals (Balcazar et al., 2006; Chapman et al., 2012; Cross, 2002). Probiotics are considered as microorganisms that when ingested are intended to provide health benefits to the host (Gilliland, 2003). In aquaculture, the application of probiotic treatments have been proven successful. The bacteria *Thalassobacter utilis* exhibited inhibitory effects against the fish pathogen *Vibiro anguillarum*, increasing the survival rate of crab larvae (*Portunus trituberculatus*) (Nogami & Maeda, 1992). Additionally, bacterial species related to skin and intestinal mucus inhibted growth of the same pathogen in turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*) (Olsson et al., 1992). As the aquaculture industry continues to grow rapidly, the demand for a deeper understanding of the host-microbiome-pathogen interaction in fish is crucial to prevent losses in the industry and to further improve fish welfare.

1.7 The Mucosal Surfaces of Fish

In fish, the main mucosal surfaces consist of the skin, gills and gastrointestinal (GI) tract, representing the first point of contact between microbes and the fish as a host (Padra et al., 2019). The epithelial surfaces of these organs are covered by a mucus layer where the main component is gel-forming mucins. Mucins are epithelial high molecular-weight glycoproteins, which are highly O-glycosylated, with a NH_2-terminal peptide domain, a large central peptide domain and a COOH-terminal peptide domain. Additionally, the fish mucus consists of proteins, lipids and ions, contributing to good growth conditions and enabling microbial attachment. Mucins have shown to be a critical constituent of the mucosal host defense. This is due to the mucus layer serving as a physical barrier, protecting the host against the surroundings (Jin et al., 2015), and ensuring limited adhesion of pathogens (Linden et al., 2008). Due to surface goblet cells continuously producing mucins, the inner mucus layer is always maintained and protected (Linden et al., 2008). The mucosal secretion also ensures that trapped particles are being washed off.

The establishment of the mucosal surfaces of fish and the bacterial community assembly associated with the fish skin and gut mucus have not yet been fully understood. Hence, it is important to continue to focus on the fish's microbiota to alleviate problems related to infections and diseases.

1.8 Saprolegniosis

One of the biggest obstacles in terms of developing sustainable aquaculture is infectious diseases. An example of such a disease is the destructive infection saprolegniosis, caused by the fungal-like *Saprolegina* sp. organism. These infectious organisms are aquatic oomycetes, more commonly known as water molds, and are most seen in salmonoid hatcheries (Hussein & Hatai, 1999). In addition, the disease commonly occurs in wild fish. With the ability of colonizing fish eggs and both mature and juvenile fish, the fungal infection poses a severe threat to the fish health and consequently causing economic losses in the aquaculture industry (Jiang et al., 2013).

Through the intake water, *Saprolegnia* sp. spores easily enter the aquaculture facilities and it is therefore hard to prevent *Saprolegnia* from infecting the fish. The disease has appeared to be more frequent during winter times when the water temperature suddenly drops. This predisposing factor is associated with induction of immunosuppression in fish, which consequently leads to the organisms being more prone to diseases (Bly et al., 1993). In addition, several other factors have been reported to increase the fish's susceptibility of developing the disease. These factors relate to an increased level of stress in the fish due to injuries from rough handling and transportation, overcrowding, changes in water temperature, osmotic shock and water pollution (Noga, 1993; Zaki et al., 2010).

Saprolegnia invades epidermal tissues, and the disease is characterized by symptoms such as grey and white areas of filamentous mycelium growing on the gills and skin. This leads to cellular necrosis and large wounds on the entire surface of the body. As a consequence, the fish may suffer from impaired osmoregulation and haemodilution, as well as respiratory failure, leading to increased mortality (Sarowar et al., 2013). There is a critical demand for finding new functioning control strategies to fight saprolegniosis, since current methods used are either ineffective or pose a serious risk to the environment. Until 2002, saprolegniosis was efficiently treated using the synthetic organic dye malachite green. However, due to findings of toxicological effects directly related to malachite green, the compound was prohibited in several countries (van West, 2006). In addition, treatment using formalin-based products have been commonly used in the later years, but due to potential harmful risk on ecosystems and human health, formalin has also been banned in several countries (Magaraggia et al., 2006). Vaccination is used as a successful treatment for several fish infections, such as furunculosis disease caused by *Aeromonas salmonicida*, but due to severe side effects and following reduction of fish welfare, the treatment is far from ideal (Padra et al., 2014). Currently, there is no vaccine against saprolegniosis and despite several attempts, there has not been found any treatments measuring the same effect as malachite green.

The cell wall of the fungal-like organism *Saprolegnia* serves as a protective barrier and participates in critical biological functions, such as growth, signaling and interaction with the surroundings. It is mainly composed of cellulose, $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 6)$-glucans (Rzeszutek et al., 2019), as well as small amounts of the polysaccharide chitin (Hinkel & Ospina-Giraldo, 2017). As *J. lividum* possess

antifungal properties, possibly chitin degrading activities and the ability of naturally colonizing the skin of yolk sac fry of Atlantic salmon, strains of *Janthinobacterium* could be tested as complete or preventive treatments against saprolegniosis.

1.9 Aims of the Study

The overall aim of this master thesis was to study the properties of five *Janthinobacterium* strains isolated from rearing systems for Atlantic salmon fry, focusing on growth characteristics and the ability of producing the purple pigment violacein. Further, the *Janthinobacterium* strains' ability to colonize the skin and gut of Atlantic salmon yolk sac fry was examined. As a reference, the *J. lividum* type strain was included in all experiments.

The main goals of the study were to

- Obtain complete sequences of the violacein operon for the three *Janthinobacterium* strains PBA, PBB and MM5
- Study the evolution of the ability to produce violacein within Proteobacteria by conducting phylogenetic analyses
- Confirm the presence of violacein produced by the *Janthinobacterium* strains PBA, PBB and the *J. lividum* type strain in liquid culture using spectrophotometry
- Examine the general growth characteristics and colony morphology of all six *Janthinobacterium* strains
- Determine the growth of all six *Janthinobacterium* strains with mucin and chitin as the sole carbon source
- Assess the potential antagonistic properties of the six *Janthinobacterium* strains against other bacterial strains
- Investigate and compare the ability to colonize the skin and gut of Atlantic salmon yolk sac fry for the six *Janthinobacterium* strains

2 Materials and Methods

2.1 Overview of *Janthinobacterium* Strains

Through previous work performed by the research group ACMS, a collection of *Janthinobacterium* strains have been isolated from the skin of Atlantic salmon fry originating from the RAS facility SalMar at Follafoss, and the water from rearing flasks of Atlantic salmon fry originating from AquaGen AS. Additionally, the type strain of the species *Janthinobacterium lividum* (DSM1522) has been used as a reference strain in all experiments. In this master project, experiments and analyses have been performed using the *J.* sp. strains presented in Table 2.1.

Table 2.1: Overview of the *Janthinobacterium* strains worked with in this master project and their origins.

***J.* sp. strain**	**Origin**
PBA	Rearing water of salmon fry (lab scale culture flasks, eggs from; AquaGen AS)
PBB	Rearing water of salmon fry (lab scale culture flasks, eggs from; AquaGen AS)
MM5	Salmon fry skin (commercial RAS; SalMar)
3.109	Salmon fry skin (commercial RAS; SalMar)
3.116	Salmon fry skin (commercial RAS; SalMar)
J. lividum type	DSMZ (German Collection of Microorganisms and Cell Cultures)

2.2 Microscopy Analysis of *J.* sp. Strains

Cells of the six *J.* sp. strains were studied under a Carl Zeiss Axio Imager.Z2 microscope equipped with a camera and 40x magnification to examine their characteristics and growth behaviour. Overnight (ON) cultures of all six *J.* sp. strains were grown in 3 mL Luria-Bertani (LB) medium (Appendix A) in 13 mL glass tubes on an orbital shaker (120 rpm) at 20 ℃ under aerobic conditions. The medium was inoculated by a single colony of the respective *J.* sp. strain from a LA-plate (1.5%) incubated at room temperature for three days. All overnight cultures were carefully mixed using a vortex mixer for 5-10 seconds before the microscopy to homogenize the bacterial suspension.

2.3 Sequencing of the Violacein Operon

Through previous work, the five enzyme coding genes, VioA-VioE, in the *J.* sp. strains PBA, PBB, MM5, 3.109 and 3.116 were attempted amplified and sequenced, following the methods given in the simplified flow scheme (Figure 2.1) (Mølmen, 2020). Sequencing of the violacein operon for the *J.* sp. strains 3.109 and 3.116 did not yield any results and were therefore not examined any further. The parts of the three violacein operons that were missing and attempted sequenced in this master project are presented in Table 2.2.

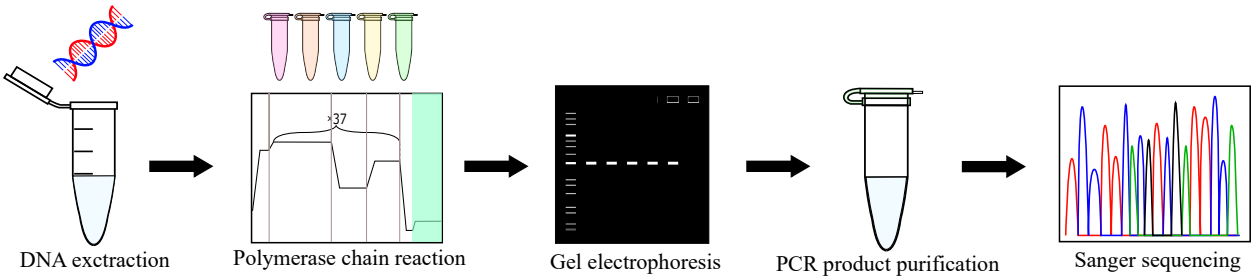


Figure 2.1: Flow scheme showing the techniques utilised to sequence the 16S rRNA gene and the violacein operon of the six *Janthinobacterium* strains (Mølmen, 2020)

Table 2.2: The relevant gene regions that were missing in order to complete the violacein operons of the three *J.* sp. strains PBA, PBB and MM5.

***J.* sp. strain**	**Violacein operon gene**	**Relevant gene region**
PBA	VioB	5' end
PBB	VioC	3' end
MM5	VioC	3' end
MM5	VioD	5' end
MM5	VioE	3' end

2.3.1 Isolation of DNA

To determine the sequences of the violacein operons of the three *J.* sp. strains PBA, PBB and MM5, DNA from liquid cultures was extracted. Overnight cultures of the *J.* sp. strains were prepared and incubated under conditions described in section 2.2. For each strain, a 1.5 mL sample of bacterial culture was centrifuged at 13,000 x *g* for 1 minute to collect the cells. The supernatant was removed and the process was repeated with the remaining 1.5 mL culture. The DNA was extracted using the DNeasy PowerSoil kit (Qiagen) as described by the manufacturers (Appendix E).

2.3.2 Amplification of the Violacein Operon by Polymerase Chain Reaction

To amplify the relevant parts of the violacein operons of the *J.* sp. strains PBA, PBB and MM5 (see Table 2.2), polymerase chain reaction (PCR) was conducted using the extracted DNA from the bacterial strains as templates (diluted 1:50 with PCR-grade water). The primer combinations used for amplification are specified in Table 2.3, and all primers were designed in SnapGene®. Annealing temperatures were varied between 55-62 ℃ by running gradient PCR to investigate if this had an effect on amplification.

For each reaction, a 24 µL of mastermix was prepared (Table 2.4) to which 1 µL of diluted DNA extract was added. The PCRs were run in a T100™ Thermal Cycler (BioRad) with PCR cycling conditions as specified in Table 2.5.

Table 2.3: Primer combinations used for PCR amplification, including their associated target gene regions and expected product length in kilo bases (kb).

Primer combination	**Target gene region**	**Expected product length [kb]**
VioA-1184.F + VioB-2956.R	5' end of VioB	1.8
VioC-5443.F + VioD-7059.R	3' end of VioC and 5' end of VioD	1.6
VioC-5466.F + VioD-7059.R	3' end of VioC and 5' end of VioD	1.6
VioE-6739.F + VioE-7305.R	VioE	0.6
VioE-6739.F + VioE-7376.R	VioE	0.6
VioE-6739.F + VioE-7429.R	VioE	0.7

Table 2.4: Composition of the PCR mastermix used for amplification of the violacein operon of the *Janthinobacterium* strains. Each PCR had a final volume of 25 µL (including the template).

Component	**Supplier**	**Volume 1x [µL]**
PCR-grade H_2O	VWR	16.6
5x Phusion HF Buffer	Thermo Scientific	5.0
dNTP mix (40 mM total)	VWR	0.50
$MgCl_2$ (50 mM)	Thermo Scientific	0.25
Forward primer (10 mM)	Sigma-Aldrich	0.75
Reverse primer (10 mM)	Sigma-Aldrich	0.75
Phusion Hot Start II DNA Polymerase (2 U/µL)	Thermo Scientific	0.15
Total		24.0

Table 2.5: PCR cycling conditions used to amplify DNA sequences of the violacein operon of the *Janthinobacterium* strains. Different annealing temperatures were tested using gradient PCR. Infinity indicates a required user interaction in order for the program to proceed.

Reaction	**Temperature [°C]**	**Time**	
Warm-up	95	∞	
Initial denaturation	98	1 minute	
Denaturation	98	15 seconds	} 37 cycles
Annealing	55/57.7/60.6/62	20 seconds	
Elongation	72	20 seconds	
Final elongation	72	5 minutes	
Cooling	4	1 minute	
Storage	10	∞	

2.3.3 Amplification of the Violacein Operon by Genome Walking

In order to capture the 3' end of the VioE gene of *J.* sp. MM5, the genome walking (GW) method was applied. Genome walking is used for capturing unsequenced genomic regions that are adjacent to already sequenced gene regions, using "walking primers" (reverse primers) in combination with sequence-specific primers (SSP). The PCR-based method requires two consecutive rounds of PCR, using the product from the first round as template in the second round. In the first round of PCR, the forward primer binds to an already known sequence in the target DNA, while the reverse primer, which is based on sequences of restriction enzymes, targets sequences of unknown positions in the template (Kalendar et al., 2019). The annealing temperature in the linear amplification phase is high to ensure stringent binding of the specific forward primer, before decreasing the annealing temperature in the exponential amplification phase which allows a less stringent binding of the reverse primer (Figure 2.2).

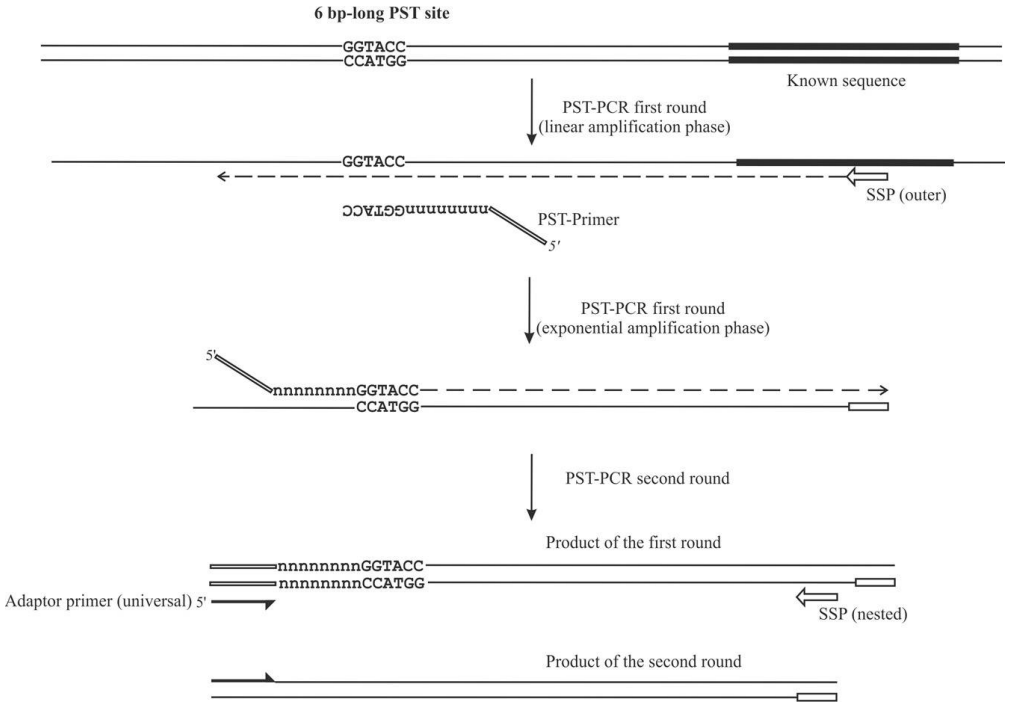


Figure 2.2: Schematic representation of the two consecutive rounds of PCR in the genome walking method. The figure is adapted from Kalendar et al. (2019).

For the first round of PCR in the GW method, a 24 µL of mastermix was prepared for each reaction (Table 2.6) to which 1 µL of diluted DNA extract was added (1:50). Two forward primers and four reverse primers were tested, resulting in a total of eight reactions (Table 2.10). The samples were run in a T100^{TM} Thermal Cycler (BioRad) with PCR cycling conditions as specified in Table 2.7.

Table 2.6: Composition of the mastermix for the first round of PCR with the GW method. Each PCR had a final volume of 25 µL (including the template).

Component	**Supplier**	**Volume 1x [µL]**
PCR-grade H_2O	VWR	19.1
10x DreamTaq Buffer	Thermo Scientific	2.5
dNTP mix (40 mM total)	VWR	0.5
Forward primer (10 µM)	Sigma-Aldrich	0.5
Reverse primer (10 µM)	Sigma-Aldrich	1.3
DreamTaq polymerase (5 U/µL)	Thermo Scientific	0.1
Total		24.0

Table 2.7: PCR cycling conditions used in the first round of PCR to amplify DNA sequences in the violacein operon using the GW approach. Infinity indicates a required user interaction in order for the program to proceed.

Reaction	**Temperature [°C]**	**Time**	
Initial denaturation	95	2 minutes	
Linear amplification	95	15 seconds	18 cycles
	68	10 seconds	
	72	1 minute	
Exponential amplification	95	15 seconds	18 cycles
	52	10 seconds	
	72	1 minute	
Final extraction	72	2 minutes	
Cooling	4	1 minute	
Storage	10	∞	

For the second round of PCR in the GW method, a 23 µL mastermix was prepared for each reaction (Table 2.8) and 2 µL of diluted PCR product from the first round (diluted 1:6 with PCR-grade water) was used as template. One specific forward primer and one specific reverse primer were used for amplification, leading to a total of eight reactions (Table 2.10). The samples were run in a T100^{TM} Thermal Cycler (BioRad) with PCR cycling conditions as specified in Table 2.9.

Table 2.8: Composition of the mastermix for the second round of PCR in the GW method. Each PCR had a final volume of 25 µL (including the template).

Component	**Supplier**	**Volume 1x [µL]**
PCR-grade H_2O	VWR	18.9
10x DreamTaq Buffer	Thermo Scientific	2.5
dNTP mix (40 mM total)	VWR	0.5
Forward primer (10 µM)	Sigma-Aldrich	0.5
Reverse primer (10 µM)	Sigma-Aldrich	0.5
DreamTaq polymerase (5U/µL)	Thermo Scientific	0.1
Total		23.0

Table 2.9: PCR cycling conditions used in the second round of PCR to amplify DNA sequences in the violacein operon using the GW approach. Infinity indicates a required user interaction in order for the program to proceed.

Reaction	**Temperature [°C]**	**Time**	
Initial denaturation	95	2 minutes	
Two-step cycle	95	15 seconds	} 29 cycles
	70	90 seconds	
Final extraction	72	2 minutes	
Cooling	4	1 minute	
Storage	10	∞	

Table 2.10: PCR primers used for amplification in the GW method, including their associated application and sequences. n represents degenerate positions in the primer sequences.

Primer	**Application**	**Sequence (5'-3')**
MM5_GW_Fb	Fwd primer 1st PCR	GCA GAA AGT GGC TTA CGC CAG GCA AGC GG
MM5_GW_Fc	Fwd primer 1st PCR	CGG ATG CCT GGA TAG TCG AGC CAG CGG
GW_AsuII	Rev primer 1st PCR	GTT GCG GCA GGT CCT CAC Cnn nnn nnn nnT TCG AA
GW_NcoI	Rev primer 1st PCR	GTT GCG GCA GGT CCT CAC Cnn nnn nnn nnC CAT GG
GW_AscI	Rev primer 1st PCR	GTT GCG GCA GGT CCT CAC Cnn nnn nnn nnG GCG CGC C
GW_BlpI	Rev primer 1st PCR	GTT GCG GCA GGT CCT CAC Cnn nnn nnn nnG CTN AGC
GW_nested_F	Fwd primer 2nd PCR	GCC TGC TGC GCA TGG TCA CCG GCA AC
GW_nested_R	Rev primer 2nd PCR	GTT GCG GCA GGT CCT CAC C

2.3.4 Gel Electrophoresis and Purification of PCR Products

To investigate the size and quality of the PCR products, gel electrophoresis was performed using 1% w/v agarose gel in Tris-acetate-EDTA (TAE) buffer (Appendix B). The gel was run for 1 hour at 110 V (for details see Appendix I). To determine the size of the PCR products, GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) was used as reference. The gel was examined under UV light using a G:Box HR Geldoc (Syngene).

When only one signal of expected size was visible on the gel, the PCR products were not isolated from the gel before purification and Sanger sequencing (section 2.3.5). The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) as described by the manufacturers (Appendix F). When detecting more than one signal for each product, the entire amount of remaining PCR product (approximately 20 µL) was loaded on a new agarose gel. After the gel had run, the DNA band representing the product of expected size was cut out from the gel under UV light in a G:Box HR Geldoc (Syngene) using a sterile scalpel. Purification of isolated DNA fragments excised from the gel, was performed using the QIAquick® PCR & Gel Cleanup Kit (Qiagen) as described by the manufacturers (Appendix G).

2.3.5 Sanger Sequencing

For each PCR product, one sequence reaction containing a forward primer and one sequence reaction containing a reverse primer was prepared. The sequencing primers used in Sanger sequencing were the ones that were successful in amplifying the gene regions of the violacein operon in the PCRs (Table 2.11). The reactions were prepared by mixing 5 µL purified PCR product with 5 µL sequencing primer (5 mM). The samples were then sent to Eurofins Genomics for Sanger sequencing. The resulting DNA sequences for the three *J.* sp. strains PBA, PBB and MM5 were returned with associated chromatograms, which were used for further evaluation of quality.

Table 2.11: Forward and reverse primers used in Sanger sequencing, with their associated sequences.

***J.* sp. strain**	**Primer**	**Sequence (5'-3')**
PBA	VioA-1184.F	GGA TGG AAG GCA GCC TGA TCA
PBA	VioB-2956.R	TGC CAC ATC AGG CGC GAA TA
PBB	VioC-5443.F	CTG ATT TTC TTC CTG CAG ACG CGC TAC
MM5	VioC-5466.F	CTA CAC GCG CTA CAT GCAC AA
PBB/MM5	VioD-7059.R	GAA ACG GCG TCA CCT CGT CGG
MM5	GW_nested_F	GCC TGC TGC GCA TGG TCA CCG GCA AC
MM5	GW_nested_R	GTT GCG GCA GGT CCT CAC C

2.4 Sequence Alignment and Phylogenetic Analyses

2.4.1 Alignment of Violacein Operon Sequences for *J.* sp. Strains

To compare the violacein operons of the *J.* sp. strains PBA, PBB, MM5 and the *J. lividum* type strain, a DNA sequence alignment was created utilizing the sequences received from Sanger sequencing. Additionally, an amino acid sequence alignment of each strain's individual violacein operon genes was created to further compare the violacein operons. The DNA sequences were assembled and edited using Clone Manager 9.0 (Sci Ed Software). The quality of the different DNA sequences was evaluated by inspection of the chromatograms, using Chromas (Technelysium Pty Ltd). Any regions with poor sequence quality, typically in the 5' and 3' ends of the PCR templates, were removed. The DNA sequences and amino acid sequences of the violacein operons were both aligned using the Global-Ref alignment type with the *J. lividum* type strain as reference.

2.4.2 Screening Bacterial Genomes for Violacein Operon and 16S rRNA Gene Sequences

To construct phylogenetic trees, the sequences of the 16S rRNA gene and the violacein operon of known violacein-producing species of different genera belonging to the phylum Proteobacteria (Table 1.1) were retrieved from Integrated Microbial Genomes and Microbiomes (IMG/M) and NCBI nucleotide database (accession numbers in Appendix K). The violacein operon sequence for the *J. lividum* type strain was used as reference when searching for the violacein operon in the different genomes performing a Basic Local Alignment Search Tool (BLAST) in IMG/M. The sequences of the violacein operons and the 16S rRNA genes of known violacein producers, including the *J.* sp. strains PBA, PBB and MM5, were aligned separately in Molecular Evolutionary Genetics Analysis (MEGA X) using ClustalW with default parameters. The two alignments were trimmed to have a common beginning and end across all strains.

2.4.3 Phylogenetic Analyses

The evolutionary relationship between known violacein-producing strains, including the *J.* sp. strains PBA, PBB and MM5, were examined by conducting phylogenetic analyses. Using the aligned sequences of the violacein operons and the 16S rRNA genes, phylogenetic trees were constructed using MEGA X based on the Maximum Likelihood method. The general principle of this method is generating all possible trees based on the input sequences and calculating the probability of each tree having naturally occurred given an evolutionary model. The Maximum Likelihood trees were constructed using default parameters with the Tamura-Nei model for sequence evolution and 1000-bootstrap replicates. The phylogenetic trees were rooted at the Deltaproteobacteria node (here represented by *Myxococcus stipitatus*).

2.5 Violacein Extraction

To verify the presence of violacein in liquid cultures (LB-medium) of the *J.* sp. strains PBA, PBB and the *J. lividum* type strain, the purple pigment was extracted using ethanol. Findings from previous work showed that strong purple-coloured cell material formed a ring at the air-liquid interface on the vessel wall when a *J.* sp. strain (JF4) was grown in liquid culture (Lyakhovchenko et al., 2021). Therefore, liquid cultures of *J.* sp. strains PBA, PBB and the *J. lividum* type strain were grown under the conditions described in section 2.2, for approximately one week (Figure 2.3A). The liquid was discarded and the ring of purple substance on the inside of the glass tube (Figure 2.3B) was dissolved in approximately 3 mL of 96% ethanol (Figure 2.3C). To clarify the solution, a 1 mL sample from each tube was centrifuged at 18,000 x *g* for 1 minute. The supernatant was decanted and added to a 1 mL quartz kyvette. The maximum absorbance was determined spectrophotometrically using a V-1200 Spectrophotometer (VWR). The absorbance of the ethanol solution containing assumed crude violacein was measured between 300 nm and 800 nm, and the absorbance values were plotted in Python (version 3.7.5).

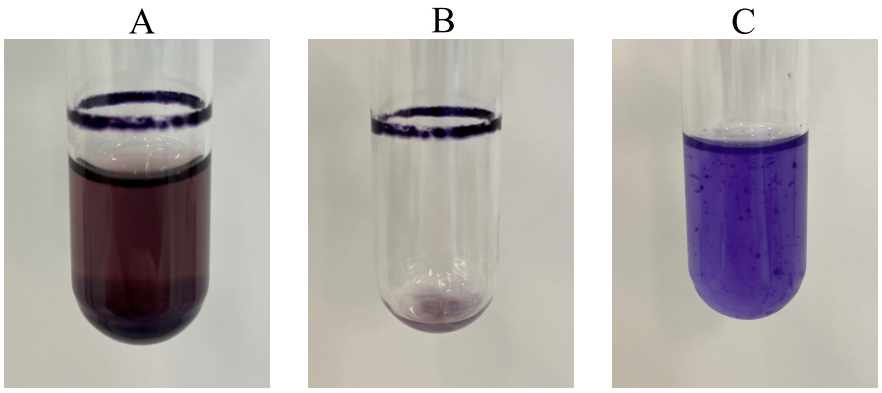


Figure 2.3: Step-by-step procedure for violacein extraction; A) liquid culture of *Janthinobacterium* strain PBA incubated for one week (20 ℃, 120 rpm), B) liquid culture discarded and C) cell material dissolved in ethanol (96%).

2.6 Cultivation of *J.* sp. Strains on Mucin and Chitin Agar Medium

To investigate the six *J.* sp. strains' ability to grow and produce violacein with mucin and chitin as the sole carbon source, a growth experiment was performed. The six *J.* sp. strains were retrieved from cryo stocks stored at -80 ℃ and streaked onto LA-plates (1.5%). The plates were incubated at room temperature for three days. Agar plates containing mucin (section 2.6.1) and chitin (section 2.6.2) were divided into six parts using a marker pen. A single colony from each of the six *J.* sp. strains were streaked onto its respective sector of the plates. The plates were incubated at room temperature for five days and then visually inspected for growth. The experiment was conducted in triplicates and plates containing no additional carbon source were used as control plates to examine the potential growth with agar as the only carbon source.

2.6.1 Preparation of Mucin Agar Medium

Mucin agar medium was prepared using mucin from porcine stomach, M9-broth and agar. The medium was autoclaved at 121 ℃ for 15 minutes. After autoclavation, sterile magnesium sulfate ($MgSO_4$) solution was added using a syringe filter for a final concentration of 2mM (Appendix A).

2.6.2 Preparation of Chitin Agar Medium

Colloidal chitin was prepared by dialysis of chitosan (Appendix J for details). After dialysis, the content of the dialysis bag was freeze dried for 24 hours in liquid N_2 using a rotavapor (Christ Alpha 1-4 LO). The chitin agar medium (0.4% chitin) was prepared by dissolving colloidal chitin in MilliQ water and stirring with a magnet overnight. The chitin solution was added to the agar medium prior to autoclavation at 121 ℃ for 15 minutes. After autoclavation, sterile magnesium sulfate ($MgSO_4$) was added using a syringe filter for a final concentration of 2mM (Appendix A).

2.7 Cross-streaking of *Janthinobacterium* Strains Against Other Bacterial Strains

In order to investigate the potential antagonistic activity of the *J.* sp. strains against other bacteria, a cross-streak experiment on agar plates was conducted (Williston et al., 1947). All six *J.* sp. strains were tested against four bacterial strains on LA-plates (1.5% agar), LA-glycerol plates (1.5% agar, 2% glycerol) and TSA-plates (1.5% agar). The cross-streak experiment included two strains of the fish pathogen *Yersinia Ruckeri* and two bacterial strains that have been isolated from fish skin in an earlier project, *Pedobacter* sp. and *Arthrobacter* sp. For each of the six *J.* sp. strains, a single colony was streaked out in a straight line in the middle of an agar plate. The plates were incubated at room temperature for three days. The strains of *Y. ruckeri*, *Pedobacter* sp. and *Arthrobacter* sp. were streaked out perpendicularly towards the *J.* sp. strains from both sides of the straight line (Figure 2.4). The plates were incubated for three more days and then visually inspected for potential inhibition of bacteria growth. For further examination, the cross-streak experiment was duplicated for all *J.* sp. strains against *Pedobacter* sp. with *Y. ruckeri* as control strains, on LA-glycerol plates.

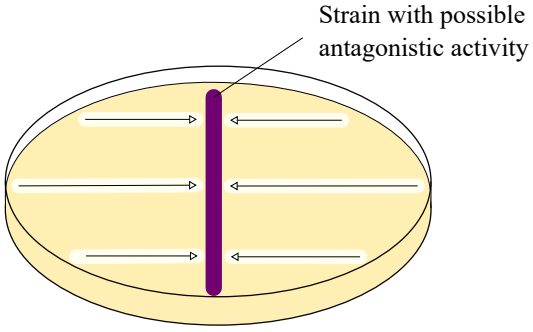


Figure 2.4: Schematic representation of the cross-streaking technique on agar plates. The purple line represents the bacterial strain with potential antagonistic activity. The white lines represent the bacterial strains that were crossed streaked. The arrows represent the direction the bacterial strains were streaked.

2.8 Fish Experiment

2.8.1 Overview of Experimental Design

To investigate the six *J.* sp. strains' ability to colonize the skin and gut of fish, an experiment with Atlantic salmon yolk sac fry was conducted. Additionally, to investigate potential interactions between the strains in the colonization process, the salmon fry were exposed to a mix of all six *J.* sp. strains. For each of the *J.* sp. strains, four germ-free salmon fry were transferred to individual wells in a 6-well plate. The fry were then exposed to the *J.* sp. strains, including the *J.* sp. mix, and incubated for five days. All fry were euthanized and dissected, followed by separate homogenization of the skin and gut. The colonization success of the *J.* sp. strains was examined and compared by CFU analyses of the homogenized skin, gut and rearing water at the end of the experiment. A simplified flow scheme of the experimental design is presented in Figure 2.5.

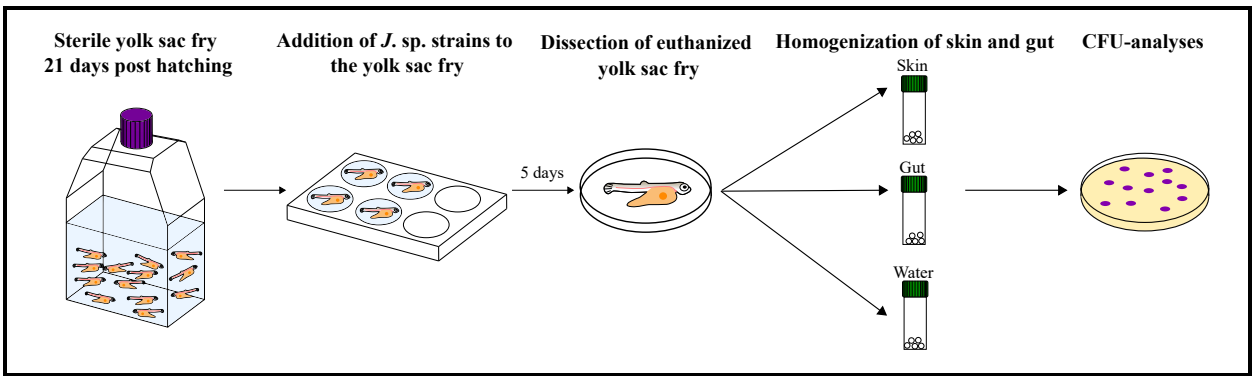


Figure 2.5: Simplified flow scheme showing the experimental design of the bacterial colonization of Atlantic salmon yolk sac fry with the six *J.* sp. strains PBA, PBB, MM5, 3.109, 3.116 and the *J. lividum* type strain. The same set-up was used for all six strains, as well as for the *J.* sp. mix.

2.8.2 Rearing Conditions of the Atlantic Salmon Yolk Sac Fry

The Atlantic salmon eggs used for this experiment were provided by AquaGen AS. The eggs and yolk sac fry were treated and reared under the conditions described in Mallasvik (2019) and performed by Alexander Willi Fiedler and Anders Wilhelm Skovly. The eggs were disinfected and distributed in fish flasks (15-18 fish per flask). The rearing water was changed three times per week to ensure good water quality. A total of two fish flasks (28 fish) was needed to conduct this experiment. The yolk sac fry were hatched germ-free and the experiment was conducted 21 days post hatching (dph).

Prior to the colonization of the yolk sac fry with the *J.* sp. strains (section 2.8.5), approximately two week post hatching, the germ-free status of the fish was examined by a sterility check of the rearing water in the fish flasks. A volume of 100 µL Salmon Gnotobiotic Medium (SGM, Appendix C) from each of the two fish flasks were used to inoculate four different liquid media (nutrient broth, brain heart infusion, sabouraud-2% dextrose broth and glucose yeast extract broth, Appendix D).

Additionally, 100 µL SGM from each fish flask was dispersed on LA-plates (1.5%) using sterile glass beads. The liquid cultures and agar plates were incubated at 6 ℃ and at room temperature for one week and then visually inspected for potential bacterial growth.

2.8.3 Determination of the Relationship Between OD_{600} and CFUs

The relationship between the optical density (OD_{600}) of a bacterial culture and the number of colony forming units (CFU) was determined for each *J.* sp. strain to be able to add approximately the same number of bacterial cells to the rearing water in a simple manner (section 2.8.4). An overnight culture of each *J.* sp. strain was prepared (section 2.2). The OD_{600} of each culture was measured using a spectrophotometer and was adjusted by dilution with LB-medium to obtain approximate values of 0.5, 0.3 and 0.1. For each of these OD_{600} values, a 10-fold dilution series was prepared to obtain a 10^{-5} and 10^{-6} dilution. For each dilution, 100 µL was dispersed on LA-plates (1.5%) using sterile glass beads. The experiment was performed in triplicates for each dilution, *J.* sp. strain and culture with different OD_{600} value, resulting in a total of 108 plates. The plates were incubated for five days at room temperature. The colonies were counted for each plate and the relationship between the OD_{600} and CFUs was determined for each *J.* sp. strain.

2.8.4 Preparation of *Janthinobacterium* Doses for the Fish Experiment

One day prior to the fish experiment, an overnight culture of each *J.* sp. strain was prepared and incubated under the conditions described in section 2.2. Each ON culture were mixed by vortex to dissolve cell aggregates and the OD_{600} was measured for each culture. Based on the ratio between OD_{600} and CFUs (section 2.8.3), dilutions of the six *J.* sp. cultures in SGM were prepared with an intended final concentration of 10^5 CFU/mL. The volumes of bacterial culture added to 50 mL SGM varied between 34 µL and 153 µL. For each of the *J.* sp. strains, their corresponding volumes were added to 300 mL SGM in the *J.* sp. mix solution.

2.8.5 Exposing Atlantic Salmon Yolk Sac Fry to the *Janthinobacterium* Strains

The colonization of Atlantic salmon skin, gut and rearing water by *J.* sp. strains was investigated through bacterial exposure approximately 21 dph. Germ-free Atlantic salmon yolk sac fry were transferred from their fish flasks to 6-well plates (one plate for each *J.* sp. strain and the *J.* sp. mix), with each well containing 8 mL of SGM with 10^5 CFU/mL of the respective *J.* sp. strain. Using a serological pipette, the fry was lifted by carefully aspirating the yolk sac. Each 6-well plate contained four fry in individual wells, which were left in the dark at 6 ℃ for five days.

2.8.6 CFU Analyses for Determination of Colonization Density of Skin and Gut of Atlantic Salmon Yolk Sac Fry

After five days, the yolk sac fry were transferred from the 6-well plates containing SGM with bacteria to new 6-well plates containing sterile SGM with ethyl 3-aminobenzoate methanesulfonate (5.2 g/L) for euthanization of the fry.

The fry were dissected in an empty petri dish under a microscope (Figure 2.6A), using sterile forceps. The yolk sac was carefully removed and the gut was separated from the fry. The gut and the rest of the fish (referred to as skin sample) were transferred to separate sterile precellys tubes (2 mL), containing a 2 mm layer of beads and 300 µL SGM (Figure 2.6B). Additionally, a sample of the rearing water from each well was collected in its own precellys tube. The gut and skin samples were homogenized using a Precellys 24. The skin samples were homogenized in two rounds for 10 seconds at 4,000 x *g* with a 15 second break between the two rounds. The gut samples were homogenized in two rounds for 10 seconds at 2,500 x *g* with a 15 second break between the two rounds.

For the homogenized samples, 1:10 serial dilutions were prepared, and 70 µL was distributed evenly on LA-plates (1.5%) using sterile glass beads. This was performed in triplicates for each fish, sample type (skin, gut and water) and dilution (including the undiluted samples) resulting in 756 plates. All plates were incubated at room temperature for one week before the colonies on each plate were counted. The colonization success of the different *J.* sp. strains in the skin, gut and rearing water of the Atlantic salmon yolk sac fry was then calculated and compared.

Figure 2.6: Picture of A) Atlantic salmon yolk sac fry before dissection and B) salmon fry skin sample in a Precellys tube with SGM and beads before homogenization.

3 Results

3.1 PCR Amplification and Sanger Sequencing of the Violacein Operon

Previous work on the *J.* sp. strains PBA, PBB and MM5 involved sequencing of the five violacein operon genes, VioA-VioE. However, the DNA sequencing did not yield a complete violacein operon for the three *J.* sp. strains (Mølmen, 2020). In this master project, the retrieval of the remaining DNA sequences was performed by PCR amplification, where the size and quality of each PCR product were examined by gel electrophoresis. Further, the PCR products were purified and submitted for Sanger sequencing in order to complete the sequencing of the three violacein operons. The purpose of fully sequencing the violacein operons was to use them in phylogenetic analyses. The relevant gene regions missing in order to complete the three violacein operons were the 5' end of the VioB gene for *J.* sp. PBA and the 3' end of the VioC gene for *J.* sp. PBB. Additionally, the relevant violacein operon genes for *J.* sp. MM5 were the 3' end of the VioC gene, the 5' end of the VioD gene, and the 3' end of the VioE gene (Table 2.2).

In the amplification of the relevant gene regions of the *J.* sp. strains PBA, PBB and MM5, the first three PCR products yielded clear signals of expected product size (Figure 3.1). Moreover, the fourth PCR product associated with the VioE gene of *J.* sp. MM5 yielded a signal of expected product size, however at relatively low intensity (Figure 3.1).

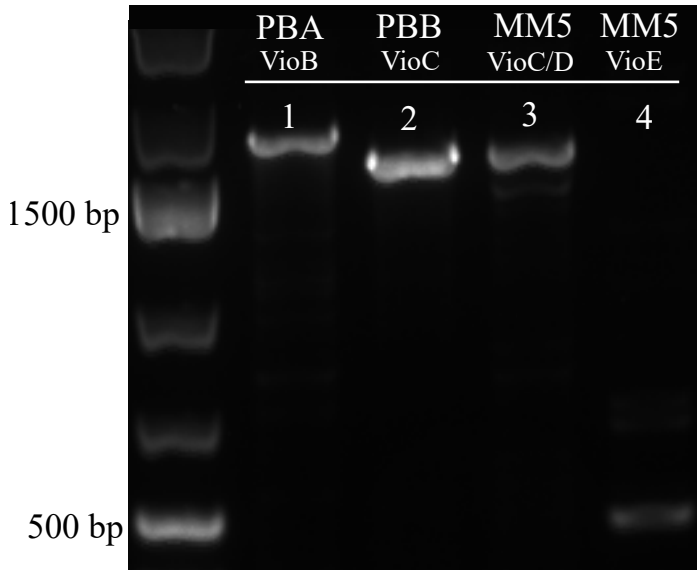


Figure 3.1: Agarose gel showing PCR products for amplification of regions of the violacein operon in the *J.* sp. strains PBA, PBB and MM5. 1) PCR product for *J.* sp. PBA with primer pair VioA-1184.F + VioB-2956.R. 2) PCR product for *J.* sp. PBB with primer pair VioC-5466.F + VioD-7059.R. 3) PCR product for *J.* sp. MM5 with primer pair VioC-5466.F + VioD-7059.R. 4) PCR product for *J.* sp. MM5 with primer pair VioE-6739.F + VioE-7429.R.

Sanger sequencing of the PCR products associated with the 5' end of VioB for the *J.* sp. strain PBA and the 3' end of VioC/5' end of VioD for the *J.* sp. strain MM5, resulted in DNA sequences and chromatograms of good quality. However, Sanger sequencing of the PCR products associated with the 3' end of VioC for *J.* sp. PBB and the VioE gene of *J.* sp. MM5 did not provide sequences and chromatograms of sufficient quality for sequence assembly.

3.2 Amplification of the Violacein Operon by Gradient PCR

In order to retrieve the 3' end of VioC for *J.* sp. PBB and the VioE gene for *J.* sp. MM5, new sets of primers were tested for targeting these regions. Additionally, gradient PCR was run to investigate the effect of different annealing temperatures on the amplification. For *J.* sp. MM5, no specific signals were detected on the gel for any of the primer combinations or annealing temperatures (Figure 3.2). For *J.* sp. PBB, one clear signal of expected size for all the annealing temperatures was obtained. However, one additional non-specific signal of substantially lower product length than expected was also observed for these PCRs (Figure 3.2).

Moreover, Sanger sequencing of the purified gel band of expected size associated with the 3' end of VioC for *J.* sp. PBB, resulted in sequences and chromatograms of good quality.

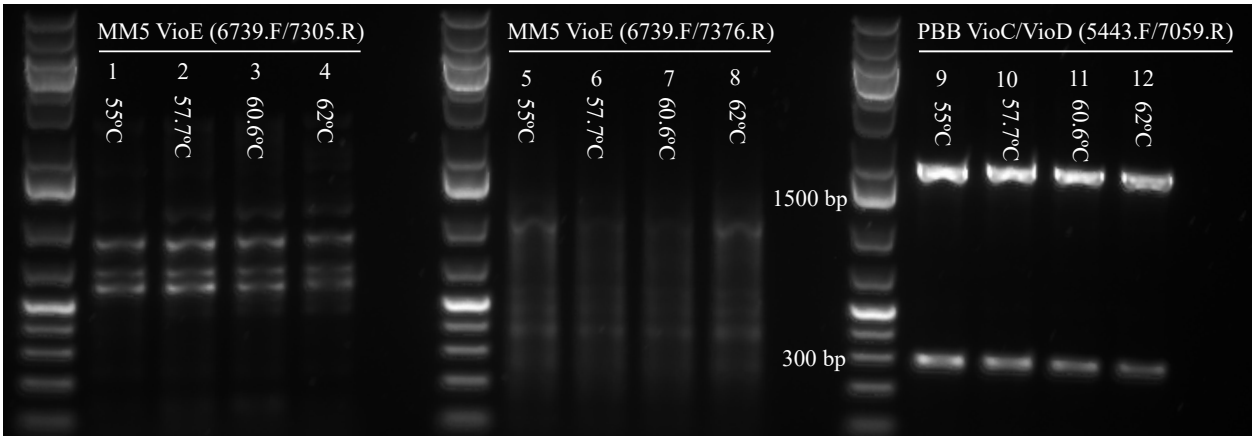


Figure 3.2: Agarose gel showing PCR products for amplification of regions of the violacein operon in *J.* sp. MM5 and PBB. The amplification was performed by gradient PCR with four different annealing temperatures for each primer combination (55 ℃, 57.7 ℃, 60.6 ℃ and 62 ℃). 1-4) PCR products for *J.* sp. MM5 with primer pair VioE-6739.F + VioE-7305.R. 5-8) PCR products for *J.* sp. MM5 with primer pair VioE-6739.F + VioE-7376.R. 9-12) PCR products for *J.* sp. PBB with primer pair VioC-5443.F + VioD-7059.R.

3.3 Amplification of the Violacein Operon by Genome Walking

Several attempts have been made to retrieve the sequence of the VioE gene of the *J.* sp. strain MM5 without success. Therefore, a PCR-based method called genome walking was tested to capture this specific DNA sequence. The genome walking strategy involved two rounds of PCR, using an unspecific primer in the first round. Different combinations of forward and reverse primers were used for amplification of the DNA sequence to increase the probability for success (for details see section 2.3.3). After the second round of PCR, gel electrophoresis was performed to evaluate the quality of the PCR products. The results showed that PCR products amplified with the use of the reverse primer GW_AscI yielded strong signals (Figure 3.3), indicating that the amplification had been successful.

Moreover, Sanger sequencing of the two purified PCR products associated with the 3' end of the VioE gene for *J.* sp. MM5 from the genome walking method resulted in sequences and chromatograms of good quality.

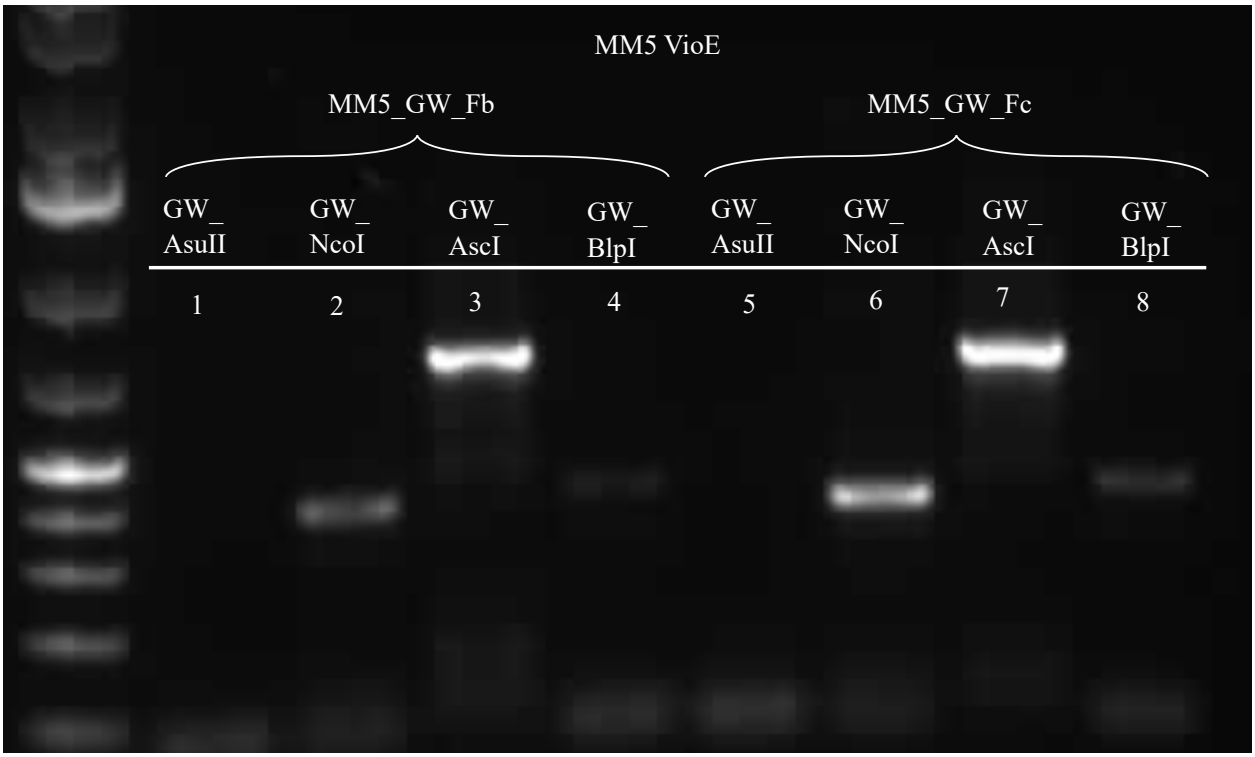


Figure 3.3: Agarose gel showing PCR products for amplification of the VioE gene in the violacein operon of *J.* sp. MM5 using the genome walking method. Unspecific primer pairs used in the first round of PCR: 1) MM5_GW_Fb + GW_AsuII, 2) MM5_GW_Fb + GW_NcoI, 3) MM5_GW_Fb + GW_AscI, 4) MM5_GW_Fb + GW_BlpI 5) MM5_GW_Fc + GW_AsuII 6) MM5_GW_Fc + GW_NcoI, 7) MM5_GW_Fc + GW_AscI, 8) MM5_GW_Fc + GW_BlpI. Specific primer pair used in all PCRs in the second round: GW_nested_F + GW_nested_R.

3.4 Alignment of Violacein Operon Sequences

To compare the violacein operons of the four *J.* sp. strains PBA, PBB, MM5 and the *J. lividum* type strain, a DNA sequence alignment was created. From the alignment, the violacein operons of the *J.* sp. strains PBA, PBB and MM5 were all highly similar to the violacein operon of the *J. lividum* type strain, with match scores of 94%, 91% and 91%, respectively (Appendix M).

Interestingly, examination of the amino acid sequence alignment of the four *J.* sp. strains' individual violacein operon genes, showed discrepancies in the VioB and VioE genes (Appendix N). In the last half of the VioB gene (3' end), the amino acid sequences of both *J.* sp. PBA and MM5 differed from *J.* sp. PBB and the *J. lividum* type strain, as well as from each other. Additionally, several stop codons were detected in the amino acid sequence of the VioB gene for *J.* sp. PBA and MM5, indicating that the amino acid sequences code for non-functional proteins. Moreover, the amino acid sequence of the VioE gene of *J.* sp. MM5 was highly dissimilar compared to the rest of the *J.* sp. strains and included a stop codon close to the 5' end of the sequence, indicating a non-functional gene.

3.5 Phylogenetic Analyses of the 16S rRNA Gene and the Violacein Operon

To study the phylogenetic relationship among the violacein operon sequences for the *J.* sp. strains PBA, PBB and MM5, and previously published violacein operon sequences of violacein-producing strains, phylogenetic analyses were performed based on DNA sequence alignments of the violacein operons. Further, the alignments of the 16S rRNA gene sequences were used to construct phylogenetic trees representing the vertical evolutionary history and the relationship between the strains.

For the Maximum Likelihood trees based on the 16S rRNA gene sequences, the tree topology corresponded to the taxonomy as previously described for these species (Figure 3.4). Interestingly, the branch lengths were generally longer in the tree associated with the violacein operon sequences (Figure 3.5A) than in the tree based on the 16S rRNA gene sequences (Figure 3.4A), indicating a more rapid sequence evolution. The phylogenetic trees showed that the five *J.* sp. strains PBA, PBB, MM5, 3.109 and 3.116 are closely related to the *J. lividum* type strain (Figure 3.4). Moreover, the phylogenetic analyses showed that the resulting trees of the 16S rRNA gene sequences and the violacein operon sequences did not share similar topologies. Comparing the topology of the two trees (Figure 3.4A and Figure 3.5A), there were indications of a closer relationship between *Pseudoalteromonas* and *Chromobacterium* in terms of violacein operon sequences. The same tendency was observed for *Collimonas* and *Duganella*. Moreover, *Massilia* and *Janthinobacterium* seemed more distantly related for the violacein operon sequences than the 16S rRNA gene sequences. Furthermore, the topology of the violacein operon based tree (Figure 3.5B) generally had a lower degree of certainty than the 16S rRNA gene based tree (Figure 3.4B) in terms of bootstrap support values (percentage of bootstrap replicates that yielded the same clustering at each node).

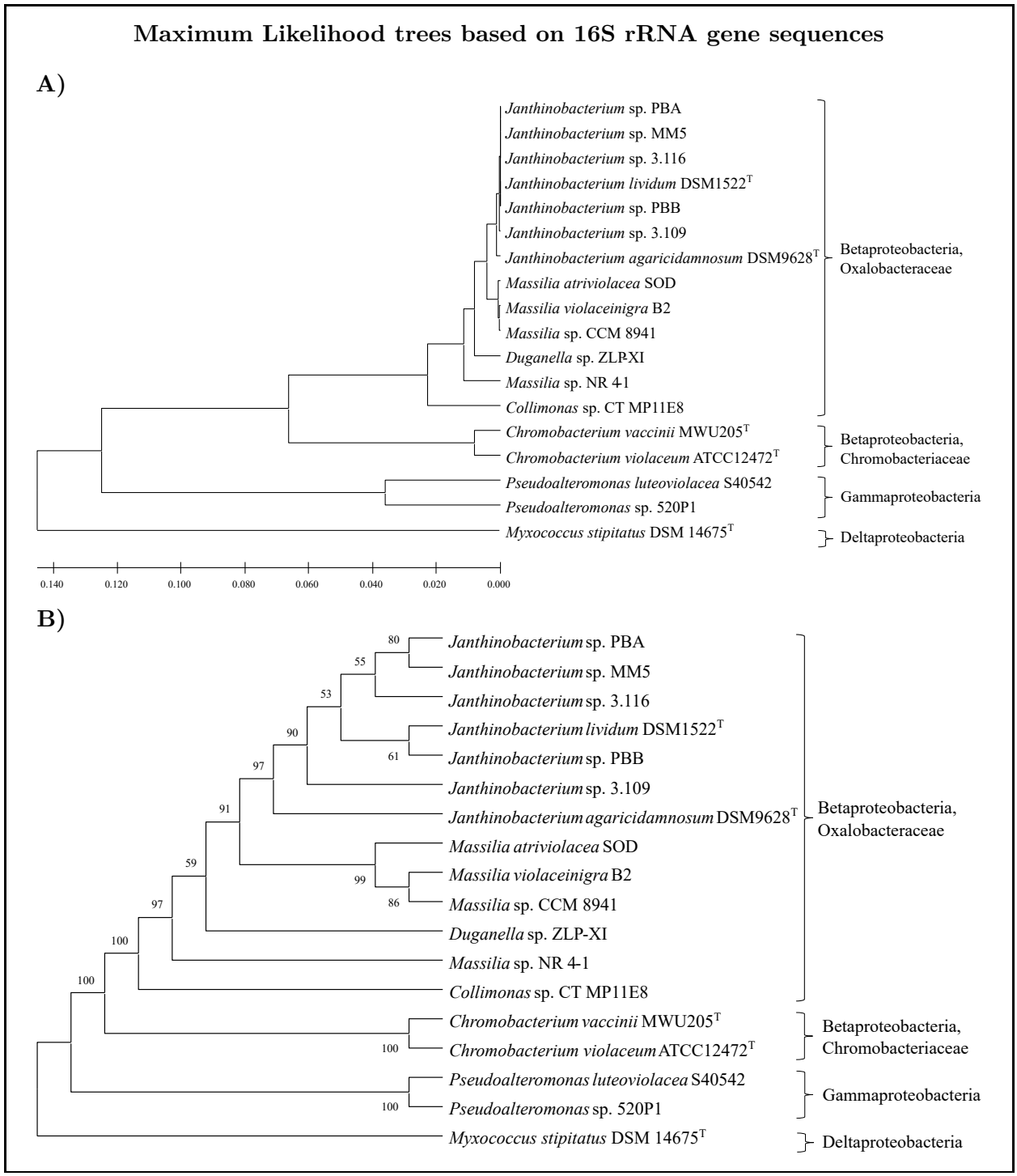


Figure 3.4: Maximum Likelihood trees based on 16S rRNA gene sequences of violacein-producing species. Type strains are indicated by a ^{T}. The analysis was performed in MEGA X and the sequences were aligned using ClustalW with default parameters. The ML analysis was performed with the Tamura-Nei model for sequence evolution and 1000 bootstrap replicates. The phylogenetic trees are rooted at the *Myxococcus stipitatus* node. A) Branch lengths are proportional to the estimated number of nucleotide substitutions per site. B) Tree topology with bootstrap support values at each node.

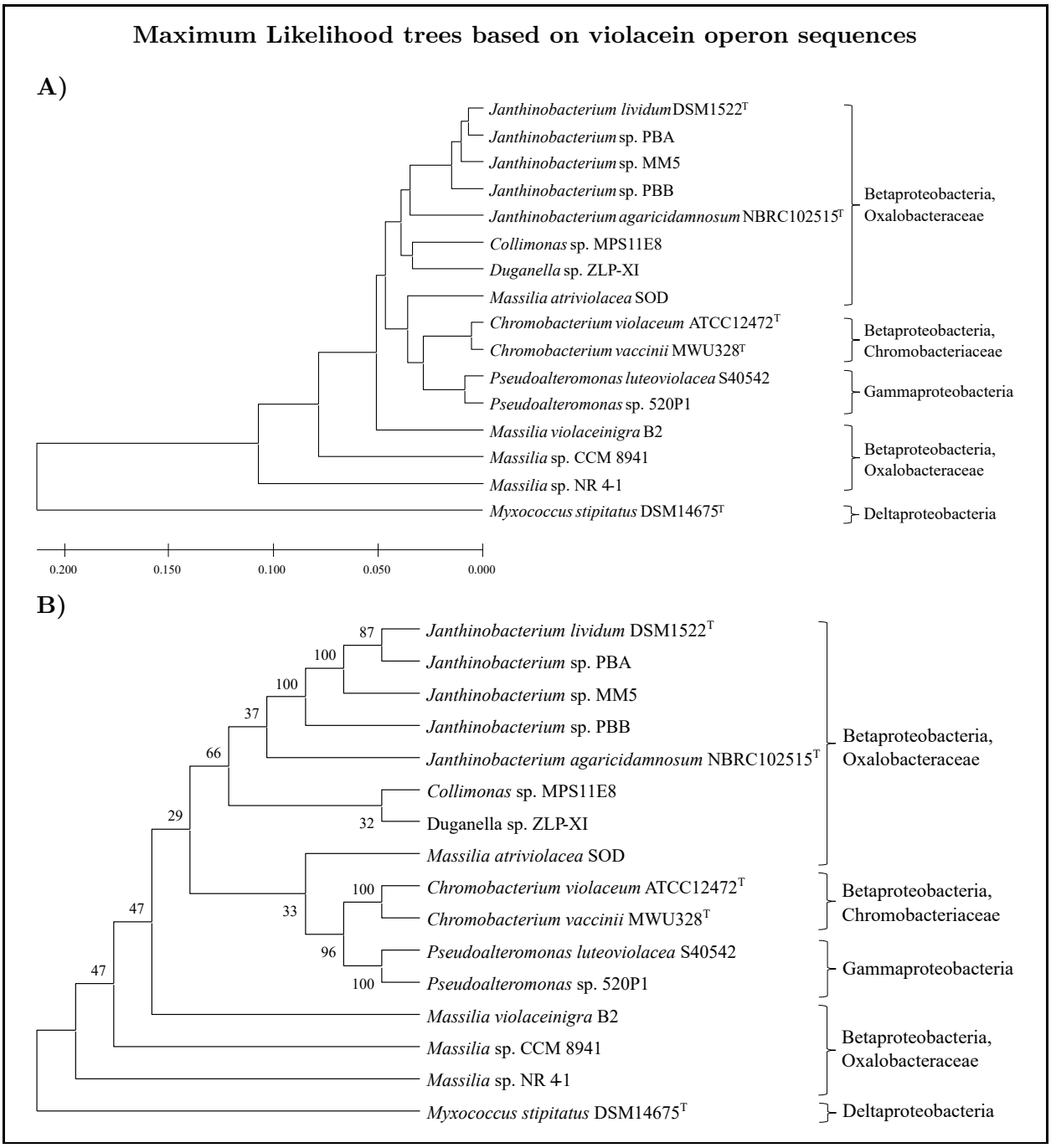


Figure 3.5: Maximum Likelihood trees based on violacein operon sequences of violacein-producing species. Type strains are indicated by a ^{T}. The analysis was performed in MEGA X and the sequences were aligned using ClustalW with default parameters. The ML analysis was performed with the Tamura-Nei model for sequence evolution and 1000 bootstrap replicates. The phylogenetic trees are rooted at the *Myxococcus stipitatus* node. A) Branch lengths are proportional to the estimated number of nucleotide substitutions per site. B) Tree topology with bootstrap support values at each node.

3.6 Extraction of Violacein

Previous studies have shown that several *Janthinobacterium* strains posses the ability to produce the purple-coloured pigment violacein when grown in various liquid and agar media. Among the *J.* sp. strains worked with in this master thesis, the strains PBA, PBB and the *J. lividum* type strain have exhibited purple pigmentation assumed to be violacein when grown on agar plates and in liquid medium. To examine if the violet substance was violacein, the potential violacein was extracted from liquid cultures of the three strains using ethanol, and the absorbance maximums were determined spectrophotometrically. The absorbance spectra showed that the absorption maximum of the purple ethanol extracts was at wavelengths of 576 nm for *J.* sp. PBA, 574 nm for *J.* sp. PBB and 575 nm for the *J. lividum* type strain (Figure 3.6A-C). These absorption maximums corresponded with the expected absorption spectrum for violacein extracted from *C. violaceum* by Rettori and Durán (1998) (Figure 3.6D).

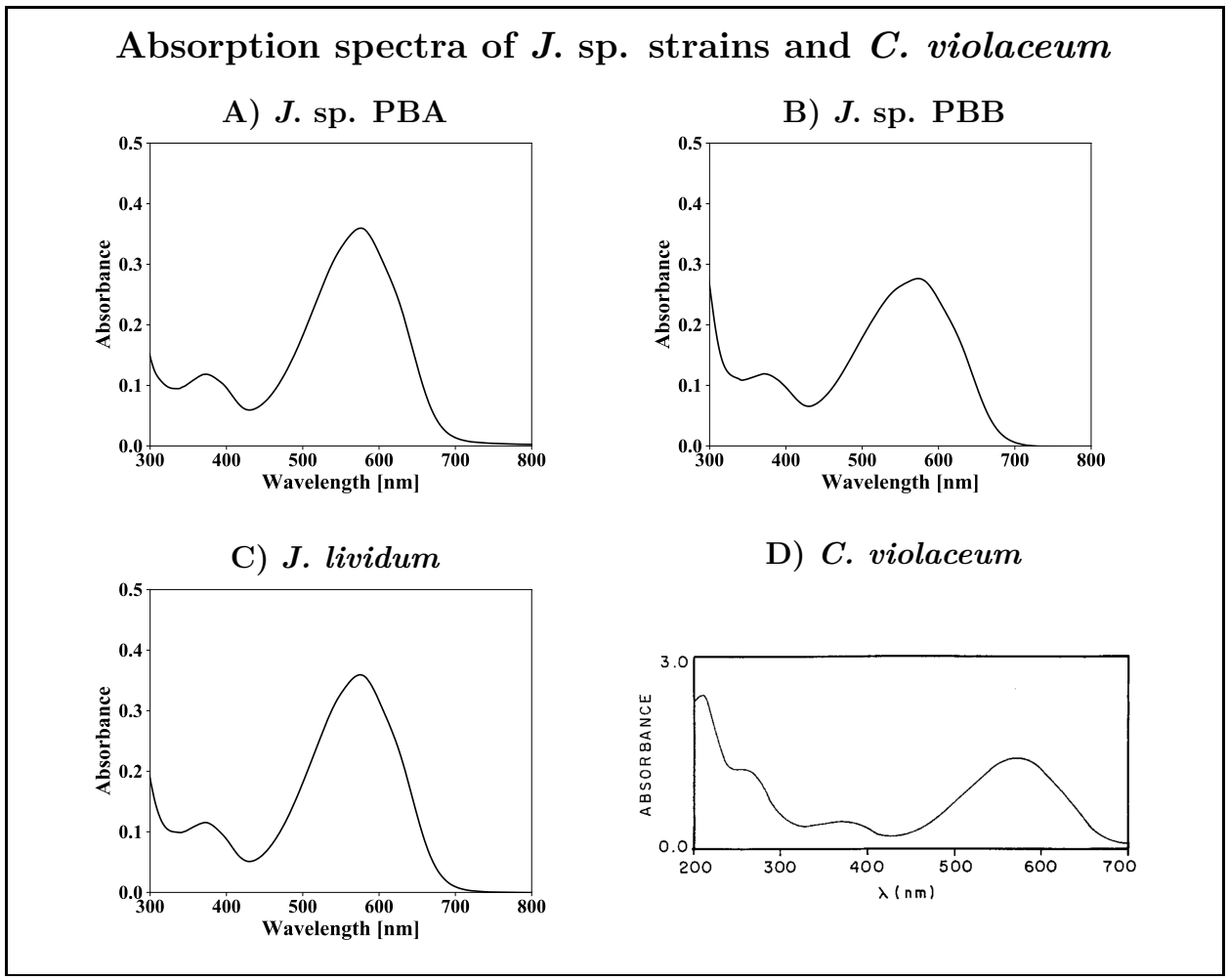


Figure 3.6: Absorption spectra of the ethanol extracts from *J.* sp. strains and *C. violaceum*; A) *J.* sp. PBA, B) *J.* sp. PBB, C) *J. lividum* type strain and D) *C. violaceum* (Figure 3.6D is adapted from Rettori and Durán (1998)).

3.7 Cell Aggregation and Violacein Production

To study the growth of the six *J.* sp. strains in liquid culture, overnight cultures were examined under a microscope. After incubation, all cultures were turbid with a tendency of forming cell clumps. The cultures were vortexed before the microscopy to dissociate clumps of slime. However, both clumps and strings of slime remained undissolved in the liquid medium. The cells of all six *J.* sp. strains were observed to be rod-shaped and motile, and despite the vortexing, the strains showed clear tendencies of growing in aggregates (Figure 3.7). This was especially evident for *J.* sp. MM5, which appeared to have a relatively high cell density compared to the other *J.* sp. strains (Figure 3.7).

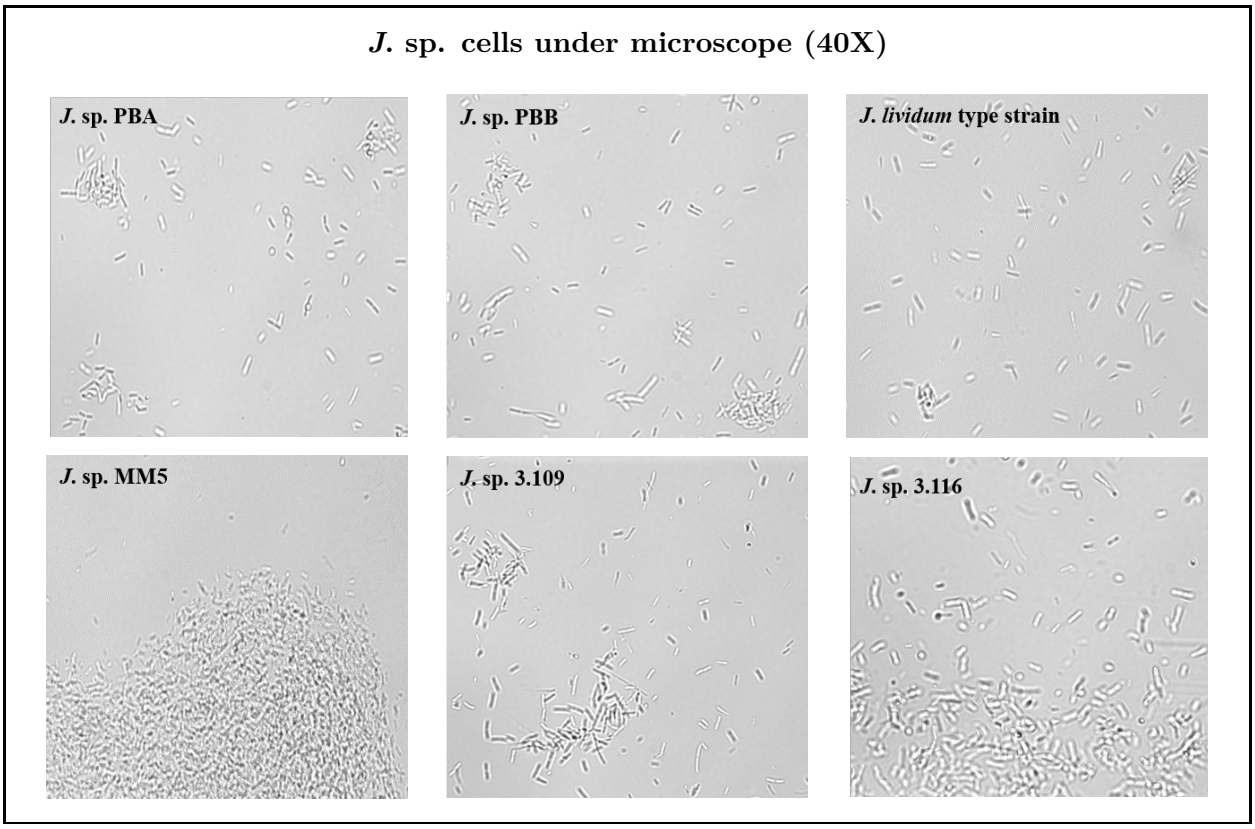


Figure 3.7: Microscope pictures of cells of *J.* sp. strains studied under a Carl Zeiss Axio Imager.Z2 microscope with 40X magnification. The *J.* sp. strains were cultured overnight (20 ℃, 120 rpm) and vortexed before microscopy.

During further incubation of the strains, the turbidity of the cultures increased. After one week of incubation, all cultures had produced a substantial amount of slime, manifesting as clumps and threads in the cultivation liquid, showing clear tendencies of growing in aggregates. The *J.* sp. strain 3.109 appeared particularly slimy and less turbid than the *J.* sp. strains MM5 and 3.116 (Figure 3.8). Moreover, these three strains did not produce violacein during cultivation, unlike the *J.* sp. strains PBA, PBB and the *J. lividum* type strain. Further, the intensity of purple pigmentation for the aforementioned strains increased for each day, making the cell aggregation less visible in the liquid cultures (Figure 3.8). Moreover, a deep-purple ring appeared on the glass wall at the interface between the liquid medium and the airspace in the cultures of the three violacein-producing strains. These rings seemed to stem from an accumulation of violacein, since their purple colour was much stronger than the colour in the medium. These rings were not observed in non-violacein producing cultures (Figure 3.8).

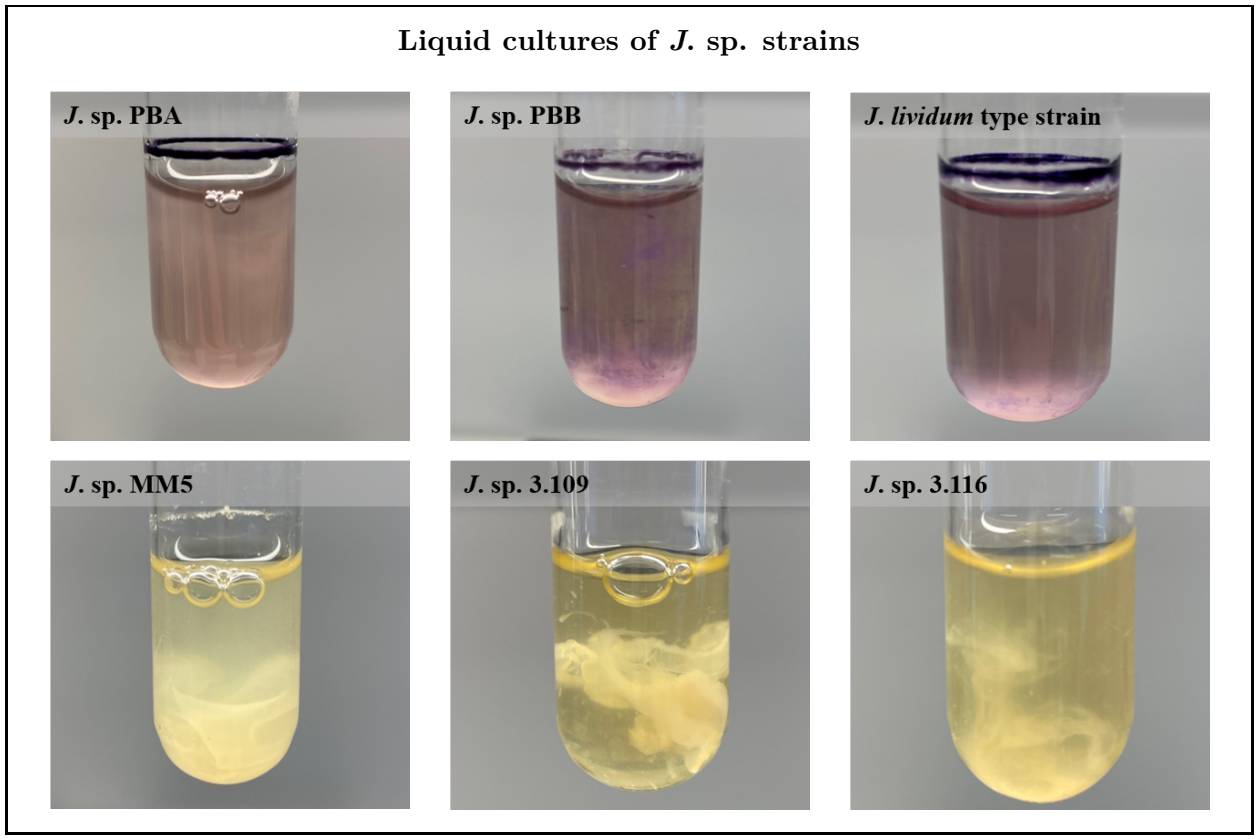


Figure 3.8: *J.* sp. strains grown in LB-medium for one week at 20 ℃ at aerobic conditions and under agitation (120 rpm).

3.8 Colony Morphology of *Janthinobacterium* Strains

During the specialization project in the fall of 2020 (Mølmen, 2020), the *J.* sp. strains were observed to exhibit different phenotypes when grown on agar plates, varying in surface texture (smooth and wrinkled/rugose colonies) and degree of mucus production (Figure 3.9). However, these surface characteristics were only examined for clusters of multiple colonies. Therefore, the phenotypes of single colonies of the six *J.* sp. strains were inspected. The purpose was to investigate whether the six strains could be identified based on colony morphology when grown together on LA-plates. After two weeks of incubation, the colonies of the *J.* sp. strains PBA, PBB and the *J. lividum* type strain had turned deep purple, while the colonies of the *J.* sp. strains MM5, 3.109 and 3.116 were white (Figure 3.10, Table 3.1). All *J.* sp. strains were observed to exhibit both smooth and wrinkled colony morphology on LA-plates, except for *J.* sp. 3.116 which surface consistently appeared smooth (Table 3.1). The colony morphology seemed to be dependent upon the size of the colony, as the degree of wrinkling increased with increasing colony size (Figure 3.10). Consequently, it was not possible to distinguish between the six *J.* sp. strains, other than by the differences in colour.

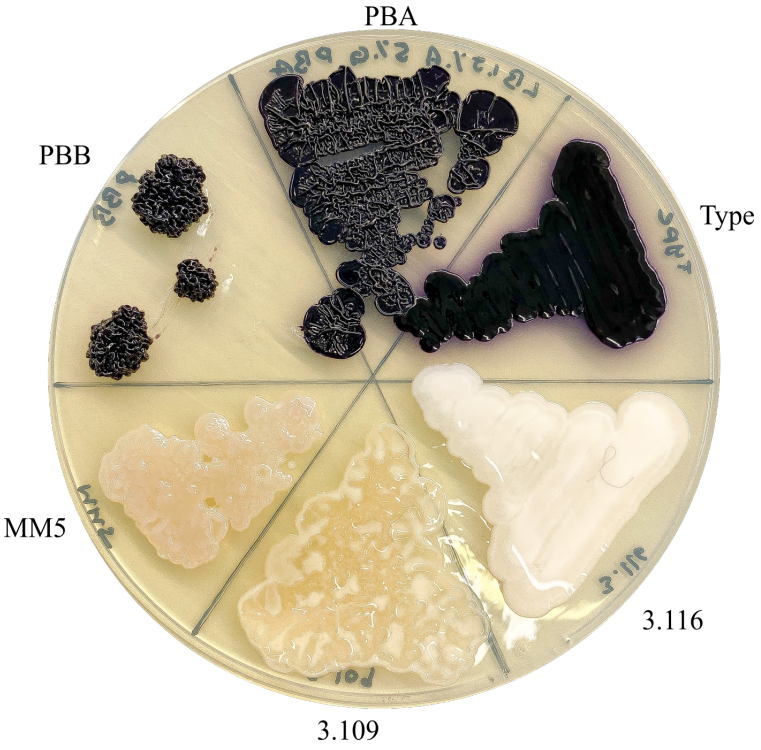


Figure 3.9: Different growth characteristics and phenotypes of the six *J.* sp. strains and their different colony morphologies (smooth and wrinkled/rugose). Picture is adapted from Mølmen (2020).

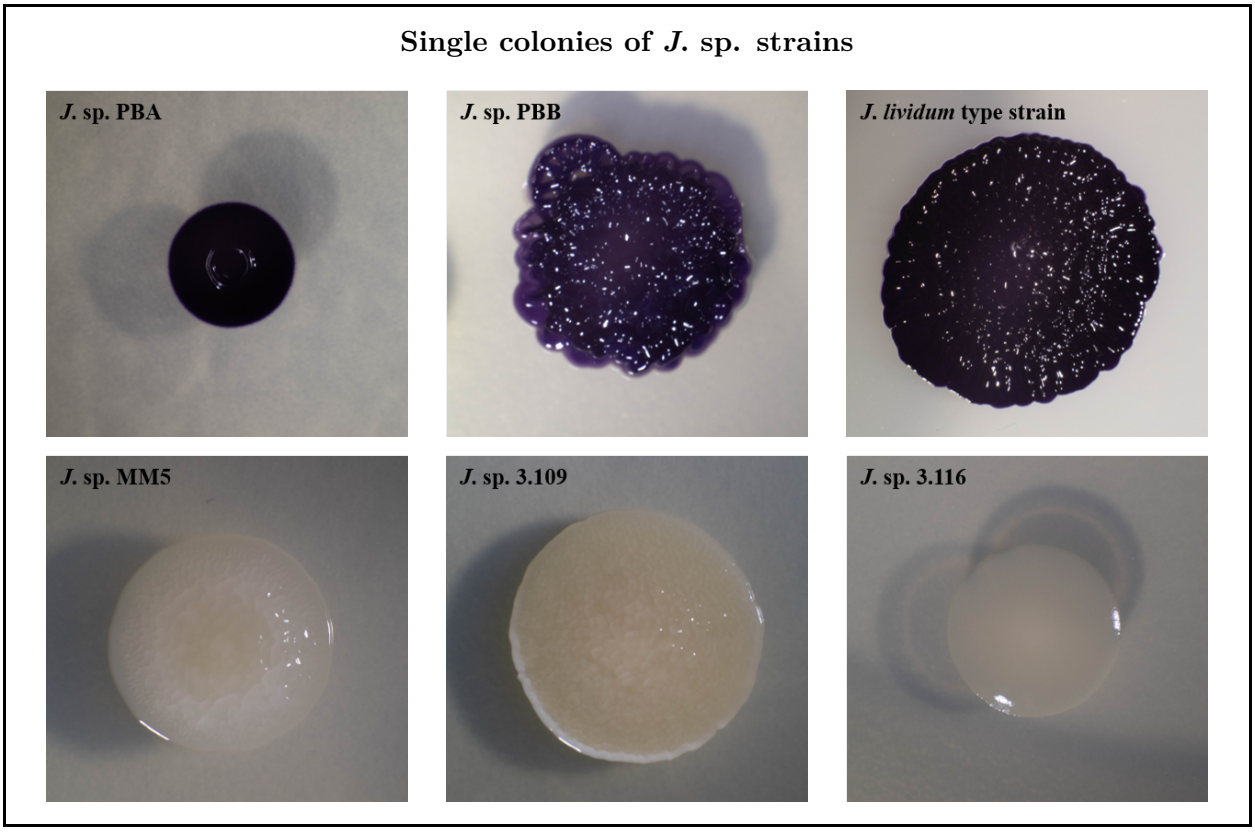


Figure 3.10: Examples of the different phenotypes the six *J.* sp. strains could exhibit after approximately two weeks of incubation at room temperature on LA-plates (1.5%).

Table 3.1: The phenotypes of single colonies of the six *J.* sp. strains grown on LA-plates, characterized by colour (purple or white) and colony morphology (smooth and/or rugose).

***J.* sp. strain**	**Colour of colony**	**Colony Morphology**
PBA	Purple	Smooth and rugose
PBB	Purple	Smooth and rugose
J. lividum type	Purple	Smooth and rugose
MM5	White	Smooth and rugose
3.109	White	Smooth and rugose
3.116	White	Smooth

3.9 Examination of Potential Antagonistic Activity of *Janthinobacterium* Strains

To examine the possible antagonistic properties of the six *J.* sp. strains against other bacteria, a cross-streak experiment was conducted. The agar plates were inoculated with *J.* sp. strains by a single streak in the centre of the agar medium (TSA, LA, LA-glycerol), and then incubated for three days. Further, the strains were challenged against two different strains of the fish pathogen *Y. ruckeri*, one strain of *Pedobacter* sp. and one strain of *Arthrobacter* sp. The antagonistic activity of the individual *J.* sp. strain was determined after three days of incubation and indicated by absence of growth near the field of *J.* sp. growth on the agar plates. The results are summarized in Table 3.2.

None of the six *J.* sp. strains showed any antagonistic activity against the two strains of *Y. ruckeri* on any of the agar media (Table 3.2). The same tendency was observed for the strain of *Arthrobacter* sp., except for indication of inhibition on one TSA-plate. Generally, the *Arthrobacter* sp. strain grew poorly on all LA-plates. On these plates, it was difficult to differentiate inhibition from poor growth (Table 3.2). However, for the *Pedobacter* sp. strain, the bacterial growth was noticeably weaker closer to the *J.* sp. growth line for all of the *J.* sp. strains on several agar plates. Moreover, the growth of *Pedobacter* sp. was generally weak on LA-plates not containing glycerol. The cross-streaking of *J.* sp. strains against *Pedobacter* sp. was repeated on LA-plates containing glycerol to further substantiate the aforementioned results indicating inhibition, using the two strains of *Y. ruckeri* as control. For all six *J.* sp. strains, inhibition zones were observed on both sides of the bacterial growth line, indicating inhibition of *Pedobacter* sp. (Figure 3.11). Moreover, absence of violacein production did not seem to affect the potential antagonistic activity of the *J.* sp. strains against *Pedobacter* sp. (Figure 3.11). Furthermore, on three of the agar plates, there was no growth of the *J.* sp. strain. Therefore, these plates could not be utilized for cross-streaking (Table 3.2).

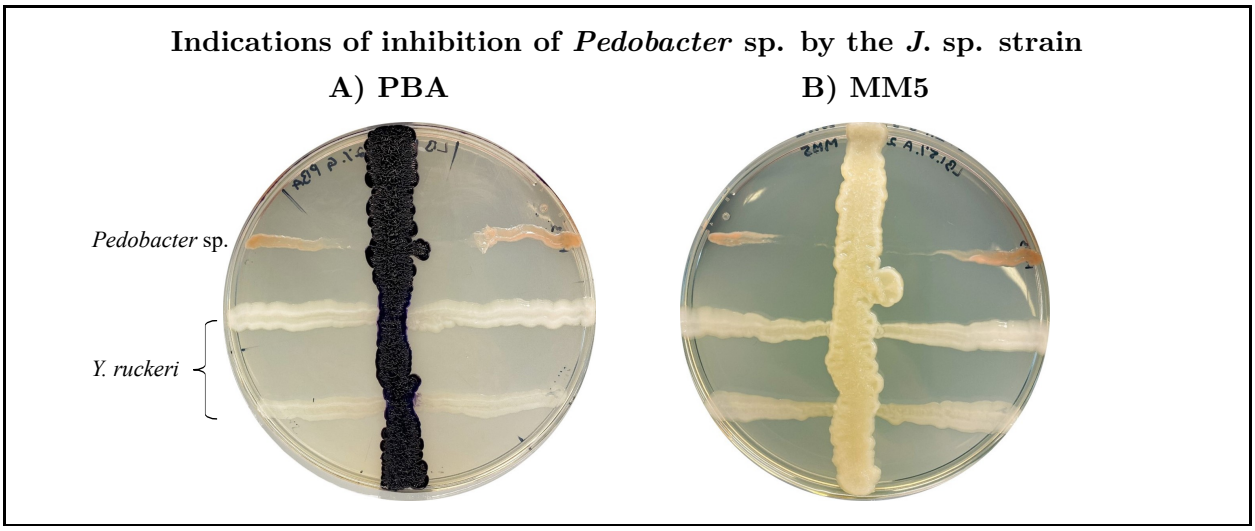


Figure 3.11: Cross-streaking of A) *J.* sp. PBA and B) *J.* sp. MM5 against *Pedobacter* sp. and two *Y. ruckeri* strains on LA-plates (2% glycerol) to examine potential antagonistic activity of the *J.* sp. strains. The results indicated that *Pedobacter* sp. was inhibited by the *J.* sp. strains.

Table 3.2: Result of cross-streak experiment with the six *J.* sp. strains against two strains of *Y. ruckeri*, one strain of *Pedobacter* sp. and one strain of *Arthrobacter* sp. The experiment was conducted on different agar media. Plates were incubated at room temperature for three days before inspection of potential inhibition. The observations were characterized as either inhibition, no inhibition or no growth. Inhibition: lack of growth of the relevant strain in the proximity of the J. sp. growth. No inhibition: no visible inhibition of the relevant strain. No growth: no growth of the relevant strain was observed on the agar medium. Agar is designated A, while glycerol is designated G.

***J.* sp. strain**	**Agar medium**	***Y. ruckeri* 1**	***Y. ruckeri* 2**	***Pedobacter* sp.**	***Arthrobacter* sp.**
PBA	TSA (1.5% A)	No inhibition	No inhibition	Inhibition	No inhibition
	LA (1.5% A)	No inhibition	No inhibition	No growth	No inhibition
	LA (1.5% A, 2% G)	No *J.* sp. growth	No *J.* sp. growth	No *J.* sp. growth	No *J.* sp. growth
PBB	TSA (1.5% A)	No inhibition	No inhibition	Inhibition	Inhibition
	LA (1.5% A)	No inhibition	No inhibition	No growth	No inhibition
	LA (1.5% A, 2% G)	No inhibition	No inhibition	Inhibition	No growth
J. lividum type	TSA (1.5% A)	No inhibition	No inhibition	Inhibition	No inhibition
	LA (1.5% A)	No inhibition	No inhibition	No growth	No inhibition
	LA (1.5% A, 2% G)	No inhibition	No inhibition	Inhibition	No growth
MM5	TSA (1.5% A)	No *J.* sp. growth	No *J.* sp. growth	No *J.* sp. growth	No *J.* sp. growth
	LA (1.5% A)	No *J.* sp. growth	No *J.* sp. growth	No *J.* sp. growth	No *J.* sp. growth
	LA (1.5% A, 2% G)	No inhibition	No inhibition	Inhibition	No growth
3.109	TSA (1.5% A)	No inhibition	No inhibition	Inhibition	No inhibition
	LA (1.5% A)	No inhibition	No inhibition	Inhibition	No inhibition
	LA (1.5% A, 2% G)	No inhibition	No inhibition	Inhibition	No growth
3.116	TSA (1.5% A)	No inhibition	No inhibition	Inhibition	No inhibition
	LA (1.5% A)	No inhibition	No inhibition	No growth	No inhibition
	LA (1.5% A, 2% G)	No inhibition	No inhibition	No inhibition	No growth

3.10 Examination of Growth with Mucin and Chitin as Sole Carbon Source

Mucin is the major component of mucus found on the skin, gills and intestine of Atlantic salmon. Based on this, the five *J.* sp. strains' and the *J. lividum* type strain's ability to grow with mucin as their sole carbon source was examined. The growth experiment showed that all six *J.* sp. strains were able to grow on mucin agar plates (Figure 3.12A).

Furthermore, *Janthinobacterium* strains are known for their chitin-degrading abilities. We therefore examined whether the five *J.* sp. strains and the *J. lividum* type strain were able to grow with chitin as their sole carbon source. From the results, it was clear that all six *J.* sp. strains were able to grow on the chitin agar plates (Figure 3.12B).

To examine if the *J.* sp. strains were able to grow with agar as their sole carbon source, the isolates were cultivated on agar plates without any additional carbon. The agar plates were inspected after five days of incubation and did not display any growth of the six *J.* sp. strains.

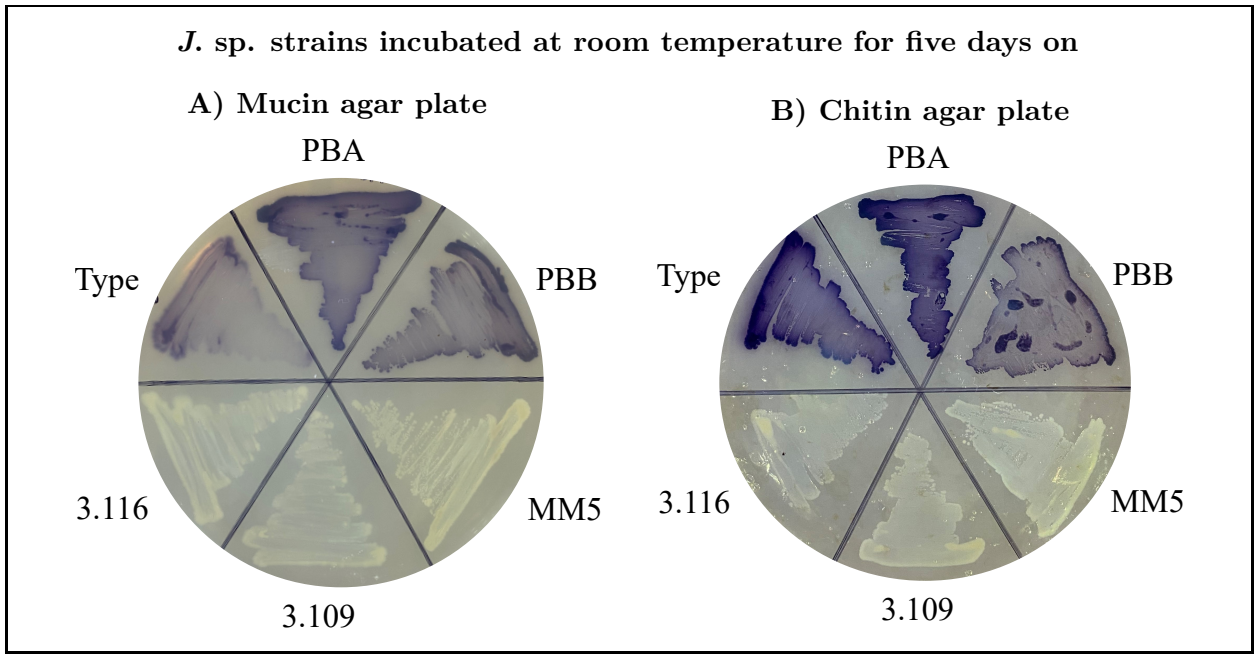


Figure 3.12: Growth of the six *J.* sp. strains on M9-agar plates with A) mucin and B) chitin as the sole carbon source. The plates were incubated at room temperature for five days.

3.11 Colonization of Atlantic Salmon Yolk Sac Fry with *J.* sp. Strains

The six *J.* sp. strains' ability to colonize germ-free Atlantic salmon yolk sac fry skin and gut was investigated. Using 6-well plates, salmon fry were exposed to the *J.* sp. strains and incubated for five days in individual wells (see Figure 2.5 for experimental design). Additionally, the salmon fry were exposed to a mix of all *J.* sp. strains to investigate any interactions between the strains during the colonization process. Through estimation of the relationship between OD_{600} and the number of CFUs, the six strains were added to the rearing water at the beginning of the experiment to obtain an initial concentration of 10^5 CFU/mL. Further, CFU analyses were performed to estimate the colonization success on homogenized skin and gut samples of individual fry. The survival rate of salmon yolk sac fry during the incubation period was high as only one fry exposed to the *J. lividum* type strain died during the experiment. Results regarding individual fry is presented in Appendix H.

Prior to the fish experiment, a sterility check of the rearing water in the fish flasks was conducted (see section 2.8.2 for rearing conditions). The purpose was to ensure germ-free conditions before exposing the salmon fry to the *J.* sp. strains. After one week of incubation, there was no growth in any of the liquid media (nutrient broth, brain heart infusion, sabouraud-2% dextrose broth and glucose yeast extract broth) or on the LA-plates (1.5%) inoculated with the rearing water, indicating that all of the fry were germ-free.

From the CFU analyses, all of the *J.* sp. strains were able to colonize the skin of salmon fry in high numbers (Table 3.3). The results showed the highest colonization density, in terms of CFU/mL of homogenized skin, for *J.* sp. 3.109 and the lowest for *J.* sp. PBA. For all strains, the standard deviation was high, and in the case of *J.* sp. 3.109, higher than the average CFU/mL value (Table 3.3).

The colonization density appeared to be considerably lower for the gut samples than the skin samples (Table 3.3). The results showed the highest colonization density, in terms of CFU/gut, for the *J. lividum* type strain. The lowest colonization density was measured for *J.* sp. strains PBA and 3.109, with an average CFU/gut value of less than one. Similar to the skin samples, there were large variations between individual gut samples (Table 3.3).

Examination of the agar plates associated with the rearing water showed large variations in colonization density between the *J.* sp. strains. For *J.* sp. PBA, no CFUs were detected on any of the agar plates at the end of the experiment. The CFU counts for *J.* sp. 3.109, showed that the number of bacteria in the rearing water had decreased compared to the assumed initial concentration of 10^5 CFU/mL (Table 3.3). Additionally, the colonies were small compared to those of the other *J.* sp. strains. This might indicate that the *J.* sp. strains PBA and 3.109 were unable to grow planktonically in the rearing water. Moreover, the results showed that the colonization density for *J.* sp. 3.116 in the rearing water had increased compared to the assumed initial concentration of 10^5 CFU/mL (Table 3.3).

One of the aims in the fish experiment was to investigate potential interactions between the *J.* sp. strains colonizing the gut and skin of Atlantic salmon yolk sac fry. However, as indicated in section 3.8, it was not possible to consistently differentiate between the strains based on colony morphology. Most of the *J.* sp. colonies were either too small or too similar to observe any morphological differences with certainty, beyond the differences in colour, and were therefore classified as either purple or white (Figure 3.13, Table 3.3). The samples from salmon yolk sac fry exposed to the *J.* sp. mix showed higher CFU values for purple colonies in comparison to the white colonies. This was valid for the samples associated with both skin and gut.

Table 3.3: Results from the colonization of Atlantic salmon yolk sac fry by *J.* sp. strains based on the homogenized skin [CFU/mL], gut [CFU/gut] and rearing water at the end of the experiment. The water samples were classified in terms of growth relative to the assumed initial concentration of 10^5 CFU/mL.

***J.* sp. strain**	**Skin [CFU/mL]**	**Gut [CFU/gut]**	**Rearing water (SGM)**
PBA	$4.29 \cdot 10^3 \pm 1.01 \cdot 10^3$	< 1	Not detectable
PBB	$2.09 \cdot 10^4 \pm 1.52 \cdot 10^4$	179 ± 62	Sustained in numbers
MM5	$2.51 \cdot 10^4 \pm 1.18 \cdot 10^4$	716 ± 444	Sustained in numbers
3.109	$2.17 \cdot 10^5 \pm 2.36 \cdot 10^5$	< 1	Decreased in numbers
3.116	$1.79 \cdot 10^5 \pm 1.13 \cdot 10^5$	189 ± 107	Increased in numbers
Type	$1.53 \cdot 10^5 \pm 1.34 \cdot 10^5$	834 ± 423	Sustained in numbers
Mix purple	$5.43 \cdot 10^4 \pm 3.56 \cdot 10^4$	108 ± 62	Decreased in numbers
Mix white	$2.53 \cdot 10^4 \pm 1.10 \cdot 10^4$	62 ± 10	Decreased in numbers

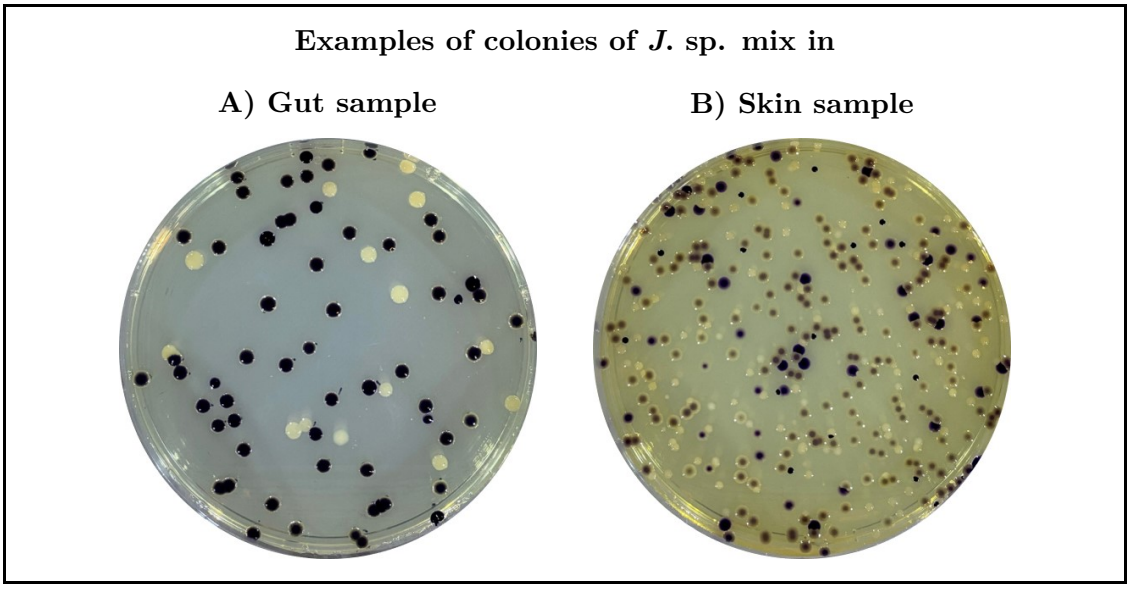


Figure 3.13: LA-plates from CFU analysis of salmon fry A) homogenized gut and B) homogenized skin of Atlantic salmon yolk sac fry after being exposed to a mix of the six *J.* sp. strains. The colonies could only be classified as either purple or white and not as each individual strain.

4 Discussion

In this study, the growth characteristics, violacein production and possible antagonistic activity of five strains of *Janthinobacterium*, previously isolated from rearing systems for Atlantic salmon fry, were examined. Additionally, the *J. lividum* type strain was used as a reference in all experiments and analyses. Furthermore, previous work involving sequencing of the violacein operons for three of the *J.* sp. strains (PBA, PBB and MM5), was completed and the phylogenetic relationship among the violacein operon sequences of different violacein-producing strains was studied. Moreover, the ability of the six *J.* sp. strains to colonize the skin and gut of Atlantic salmon yolk sac fry was investigated through bacterial exposure of these strains in rearing water. The colonization success was determined by performing CFU analyses of homogenized samples of the skin and gut.

4.1 Amplification of the Violacein Operon

The violacein operon encodes for the five enzymes VioA-VioE, which are essential in the violacein biosynthesis (Jiang et al., 2015). In the previous specialization project (Mølmen, 2020), parts of the violacein operon of the three *J.* sp. strains PBA, PBB and MM5 were amplified and sequenced. PCR amplification of the violacein operon of the *J.* sp. strains 3.109 and 3.116 was unsuccessful, indicating that these two strains did not posses the violacein operon. In this master project, the continuation of the amplification and sequencing of the relevant gene regions in the violacein operon of *J.* sp. PBA, PBB and MM5 was successful. Most of the primer combinations used to amplify the relevant regions seemed to work well, yielding products of expected size. However, some signals of unexpected length were detected on the agarose gel. This could be due to other regions in the genome with a similar nucleotide sequence as the intended primer binding sites.

Moreover, several primer combinations and varying annealing temperatures were tested to retrieve the VioE gene for *J.* sp. MM5. The increased annealing temperatures were tested to increase the stringency and specificity of the primer annealing (Malhotra et al., 1998). However, none of the attempts resulted in successful amplification. The primers used in these PCRs were designed from sequence alignments from several reference genomes of *Janthinobacterium* sp. and targeted conserved regions in the violacein operon (Mølmen, 2020). The unsuccessful attempts of amplifying the VioE gene could be explained by a divergent sequence in the primer-binding region, presumably downstream of the VioE gene, reducing the chance for primer binding. Therefore, the genome walking method was used (Kalendar et al., 2019). The method is suitable for identifying DNA sequences adjacent to already sequenced gene regions and filling in gaps in genome sequences (Wang et al., 2013).

As the 3' end of the VioD gene for *J.* sp. MM5 was already sequenced, it was used for primer design in genome walking. One of the tested reverse primers (GW_AscI) appeared to effectively amplify the VioE gene, resulting in strong signals on the agarose gel. Moreover, one additional reverse primer (GW_NcoI) yielded a relatively strong signal on the agarose gel, and could possibly have been effective in capturing the gene region. However, only the PCR products with the strongest visible signals were chosen for Sanger sequencing (PCRs using the reverse primer GW_AscI), which yielded sequences and chromatograms of high quality. Hence, the genome walking method was successful in capturing the VioE gene of *J.* sp. MM5. In genome walking, the PCR product length is unknown as the reverse primers bind to unknown regions in the DNA template. In addition to providing the strongest signals, the PCR products amplified with GW_AscI appeared to be larger than the PCR products amplified with GW_NcoI in terms of base pairs and therefore more likely to include the relevant gene region. Overall, the results indicated that all three *J.* sp. strains possess a complete violacein operon, with all of the five violacein operon genes. Therefore, all of the three strains should theoretically be able to produce violacein.

Interestingly, the *J.* sp. strain MM5 has never been observed to produce violacein in liquid culture. However, the strain has exhibited light pink/purple growth when grown on glycerol agar plates (Mølmen, 2020) and EPS-sucrose agar medium (Lorentsen, 2020). Translating the violacein operon DNA sequences to amino acid sequences (aa-sequences), and aligning the aa-sequences of each violacein operon gene, showed that the aa-sequences of the two *J.* sp. strains PBA and MM5 differed from both *J.* sp. PBB and the *J. lividum* type strain in the VioB gene. Additionally, the aa-sequence of the VioB genes of *J.* sp. PBA and MM5 differed from each other. This is due to nucleotide insertions or deletions in the DNA sequences, leading to highly dissimilar aa-sequences compared to those of *J.* sp. PBB and the *J. lividum* type strain. For both strains, especially for *J.* sp. MM5, there were several stop codons in the aa-sequences of VioB, indicating truncated VioB genes. For both strains, the first stop codon appeared approximately 500 aa into the VioB aa-sequence (approximately 1000 aa in total), indicating that the last half of the of the VioB gene (3' end) would not be expressed. Consequently, the VioB genes of *J.* sp. PBA and MM5 might encode for different proteins than those of *J.* sp. PBB and the *J. lividum* type strain. However, *J.* sp. PBA has been found to produce violacein regardless of these findings. August et al. (2000) conducted a study on the violacein biosynthetic pathway in *C. violaceum* and reported that any disruptions of either the VioA or the VioB gene would lead to the inability of producing violacein intermediates and hence violacein. Another study investigating the expression of the violacein gene cluster, using *Escherichia coli* K12 as a model organism, proposed that in the case of white colonies, a mutation in either the VioA or the VioB gene had occurred (Philip et al., 2009). This is not in line with our findings for *J.* sp. PBA, and may suggest that the function of the VioB gene in the violacein biosynthesis is not well understood. Another possibility is that the functional part of the protein coding gene is preserved in the first half of the aa-sequence (5' end).

Further investigation of the violacein operon aa-sequences showed that the VioE gene of *J.* sp. MM5 was completely different from the other *J.* sp. strains. In addition, the first stop codon was located very close to the start codon in the VioE gene. Based on this, it reasonable to assume that *J.* sp. MM5 is unable to produce a functional gene product of VioE. This enzyme is responsible for a [1,2]-shift of an indole ring to form the violacein precursor protodeoxyviolaceinic acid. In absence of VioE, the compound chromopyrrolic acid is formed instead (Balibar & Walsh, 2006). This is not an intermediate in the violacein biosynthesis, making VioE essential for the production of violacein (Shinoda et al., 2007). This might explain *J.* sp. MM5's inability to produce violacein to the same degree as *J.* sp. PBA, PBB and the *J. lividum* type strain.

4.2 Phylogenetic Analyses

Phylogenetic analyses of the 16S rRNA gene sequences and the violacein operon sequences from different violacein-producing species, were performed by construction of Maximum Likelihood trees. The analyses included the fully sequenced violacein operons and the previously sequenced 16S rRNA genes (Mølmen, 2020) of the *J.* sp. strains worked with in this master project. The violacein-producing species belonged to the major phylum Proteobacteria, representing the class of either Betaproteobacteria, Gammaproteobacteria or Deltaproteobacteria. All of the phylogenetic trees generated were rooted at the Deltaproteobacteria which is the oldest class of the three mentioned Proteobacteria (Letunic & Bork, 2019), represented by *Myxococcus stipitatus* in these analyses. The 16S rRNA gene can be used to investigate the relationship between bacteria at different taxonomic levels (Johnson et al., 2019). Additionally, the 16S rRNA gene is less likely to have undergone horizontal gene transfer (HGT) between species (Kitahara & Miyazaki, 2013), and is therefore commonly used to describe the vertical evolution (Rajendhran & Gunasekaran, 2011).

Comparison of the 16S rRNA and violacein operon Maximum Likelihood-trees revealed that they did not share the same topology, indicating that the violacein operon sequences of *Pseudoalteromonas* and *Chromobacterium* were more closely related than the 16S rRNA gene sequences. Additionally, the same applied for *Collimonas* and *Duganella*, while *Janthinobacterium* and *Massilia* seemed more distantly related in the violacein operon tree than in the 16S rRNA gene tree. However, the bootstrap support values in the violacein operon tree were generally lower than in the 16S rRNA gene tree, indicating that the proposed branching structure is more uncertain in the violacein operon tree. These observations suggest the occurrence of HGT events of the violacein operon during the evolution of Proteobacteria. If the violacein operon sequences have been subjected to HGT, it is no longer certain that *Myxococcus stipitatus* was the first species of the ones analyzed to develop the violacein operon. As previously mentioned, all of the violacein-producing strains found when screening bacterial genomes for the violacein operon were part of the phylum Proteobacteria, with the majority belonging to the class Betaproteobacteria. This might indicate that the ability to produce violacein first occurred in the class Betaproteobacteria and was later transferred to

Deltaproteobacteria and Gammaproteobacteria by HGT. However it is also possible that the ability to produce violacein occurred early in the evolution of Proteobacteria, and was later lost for most taxa, but is still preserved mainly in the class Betaproteobacteria. A previous study reported indications of HGT of the violacein operon between species of *Janthinobacterium*, *Duganella* and *Collimonas* (Hakvåg et al., 2009). Further, the two *Janthinobacterium* strains ERGS5:01 and AU11, have been suggested to have acquired specific enzyme coding genes related to cold-adaptation by HGT (Kumar et al., 2018; Martinez-Rosales et al., 2015). The findings from these studies, support the idea that horizontal gene transfer has occurred in the evolution of the violacein operon among species in the Proteobacteria.

4.3 Extraction of Violacein

Numerous strains belonging to the genus *Janthinobacterium* possess the ability to produce the purple pigment violacein. Of the six *J.* sp. strains worked with in this master project, the three strains *J.* sp. PBA, PBB and the *J. lividum* type strain have been shown to produce a purple coloured pigment, assumed to be violacein, when cultivated in liquid LB-medium and when grown on different agar plates. To further support this assumption, we extracted the presumed violacein in order to confirm its presence.

Several studies have reported methods of extracting violacein from violacein-producing genera like *Duganella* (Wang et al., 2009), *Chromobacterium* (Rettori & Durán, 1998) and *Janthinobacterium* (Lyakhovchenko et al., 2021) using ethanol as solvent with great success. Moreover, violacein is known to be an intracellular pigment (Choi et al., 2015; Poddar et al., 2021), meaning the violacein pigment is contained within the cell. As this was an attempt to qualitatively verify the production of violacein by different *J.* sp. strains, increasing the yield of the purple pigment was not prioritized. If this had been the aim, a more efficient method would be to spin down and collect the cells before cell lysis and extraction of pigment with ethanol, as described by Mendes et al. (2001). Instead, the *J.* sp. cultures were discarded and the deep purple cell material on the inside of the glass tube wall was dissolved in ethanol. A spectrophotometric study of the ethanol extracts of the purple pigment from the three *J.* sp. strains showed that the maximum absorbance value of the solutions was detected at wavelengths of 576, 574 and 575 nm, which is in agreement with the absorption maximum of crude violacein at 575 nm (Alem et al., 2020). Based on the data obtained, and by comparison of the UV-VIS spectrum of ethanol extracts from liquid cultures of the violacein-producing *C. violaceum* (Rettori & Durán, 1998), it is reasonable to assume that the pigment is violacein. Thus, the three *J.* sp. strains PBA, PBB and the *J. lividum* type strain belong to the violacein-producing group.

4.4 Antagonistic Properties of *Janthinobacterium* Strains

Janthinobacterium is known for exhibiting antagonistic activity against bacteria and fungi (Munakata et al., 2021). Recently, *J.* sp. have been reported to display antagonistic properties against soilborne fungal pathogens such as *Rhizoctonia solani* and *Pythium ultimum* (Yin et al., 2021). Additionally, strains of *J. lividum* and *J. agaricidamnosum* have been shown to exhibit strong inhibitory effects against the fungal plant pathogen *Fusarium graminearum* (Munakata et al., 2021), making the species an interesting candidate as a biocontrol in the agricultural industry.

From the cross-streak experiment, the *Y. ruckeri* strains did not show any signs of inhibition by the *J.* sp. strains. The same tendency was observed for the *Arthrobacter* sp. strain, with one exception on the TSA-plate with the *J.* sp. strain PBB, possibly indicating inhibition. However, *Arthrobacter* sp. was observed to grow poorly on LA-plates, making it difficult to determine the difference between actual inhibition or inadequate growth conditions. It would be interesting to examine the inhibitory effects of *J.* sp. more closely, as *Arthrobacter* is a genus of Gram-positive bacteria. Violacein is known to exhibit strong inhibition against Gram-positive bacteria, permeabilizing and killing the cells (Cauz et al., 2019). Unfortunately, as this experiment can be time consuming in regards to the incubation time for the bacterial strains involved, the antagonistic properties of the six *J.* sp. strains against *Arthrobacter* sp. were not examined any further.

It is, however, noteworthy that the strain belonging to the *Pedobacter* sp. showed indications of being inhibited by all of the *J.* sp. strains on different agar media. *Pedobacter* sp. is a bacterium with Gram-negative cells belonging to the class of Sphingobacteria (Steyn et al., 1998). The bacterium is often reported to be isolated from soil (Dahal & Kim, 2016; Yoon et al., 2007), which is interesting, considering the previously mentioned findings of antagonistic activity of *J.* sp. against other soil bacteria. However, the *Pedobacter* sp. strain used in this experiment was isolated from the skin of Atlantic salmon yolk sac fry during previous work in the ACMS group. Further, the *Pedobacter* sp. strain also seemed to grow poorly on LA-plates, which raises the question if the poor growth towards the center of the agar plate that was classified as inhibition, is in fact just insufficient growth. This experiment will have to be repeated with more replicates and control strains to verify the possible antagonistic effects of *J.* sp. strains against *Pedobacter* sp.

Moreover, there were no signs indicating that absence of violacein production had any effect on the potential antagonistic activities of *J.* sp. against *Pedobacter* sp. This is also in line with what was reported by Hack et al. (2016), finding indications that violacein was most probably not the main reason for inhibitory effects against the fungal plant pathogen *Fusarium graminearum*. As previously mentioned, violacein is an intracellular pigment and this result was therefore not unexpected. To investigate if violacein affects the antagonistic activity associated with *Janthinobacterium*, the pigment could have been extracted and integrated in the agar medium. However, this was not investigated considering that the main goal was to examine the potential antagonistic properties

of the *J.* sp. strains in general. Furthermore, bacterial species, such as *Pseudomonas fluorescens*, have been reported to inhibit growth of the pathogenic oomycyte *Saprolegnia* (Bly et al., 1997; Carbajal-González et al., 2011), which is the causative agent of the devastating disease saprolegniosis affecting fresh water fish. There might be a possibility that *Janthinobacterium* is capable of the same, and it would be interesting to investigate the antagonistic activity of the *Janthinobacterium* strains against *Saprolegnia*.

Antifungal properties have been associated with the production of violacein for a long time. However, a recent study has been questioning this hypothesis, attributing the antifungal properties of *Janthinobacterium* to its ability to degrade chitin (Haack et al., 2016). Interestingly, the six *J.* sp. strains used in this study were able to grow with chitin as their sole carbon source. Chitin is one of the most common polymers in nature and a component in fungal cell walls, including the cell wall of *Saprolegnia*. This supports the possibility that *Janthinobacterium* can be used as a preventative treatment of the fish disease. However, the content of chitin reported to be present in the cell wall of this fungal-like pathogen is low (Guerriero et al., 2010). Strains of *Janthinobacterium* sp. have previously appeared to utilize chitin as a carbon and nutrient source (Hornung et al., 2013) and therefore been characterized as chitinolytic bacteria (Xiao et al., 2005). The genomes of these chitin-degrading bacteria were discovered to possess chitinase genes, supporting the reported degrading abilities. Since the *J.* sp. strains were able to grow on chitin plates, it would be interesting to sequence regions of their genomes to confirm the presence of chitin-degrading genes. Interestingly, a study performed on *Collimonas* sp., a relative of *Janthinobacterium* in the class of Betaproteobacteria, provided evidence that *Collimonas fungivorans* Ter331 expressed inhibitory effects against *Saprolegnia paracitica* (Song et al., 2015). The strain of *C. fungivorans* was reported to lack violacein encoding genes. These results indicate that it might not be violacein activity affecting the inhibition of the fungal-like oomycyte *Saprolegnia paracitica*.

4.5 Colony Morphology and Violacein Production

Independent from the fact that some of our strains produced violacein, we have previously observed that they grow in colonies exhibiting different morphologies on agar plates (Mølmen 2020). We therefore investigated this interesting behaviour and found indications that all of the *J.* sp. strains were able to grow in both smooth and wrinkled colonies, except for 3.116 which surface was consistently smooth. The findings made it clear that the six *J.* sp. strains could not be identified based on colony morphology when grown together on agar dishes. The only consistent differentiating factor was the colour of the colonies. These observations lead to difficulties in investigating potential interactions between the strains in the salmon fry colonization process (further discussed in section 4.6).

The wrinkled colony morphology (rugose) have been associated with production of EPS (Yildiz & Schoolnik, 1999). This is in line with our results, assuming the substantial amounts of slime produced by the *J.* sp. strains is EPS. Moreover, the results indicated that increasing size of the single colonies lead to increased expression of a rugose phenotype. Several studies have reported findings of the aquatic bacterium *Vibrio cholerae* being able to shift from a smooth phenotype to a rugose phenotype (Beyhan & Yildiz, 2007; Rashid et al., 2003). This adaptive regulation was suggested to have an increased effect on the bacteria's persistence, and hence survivability, in nature (Ali et al., 2002). This could mean that environmental effects such as temperature (Rahman et al., 2014) or nutrient depletion (Wai et al., 1999) could be the reason for the changing phenotypes observed in the *J.* sp. strains.

Further, the *J.* sp. strains PBA, PBB and the *J. lividum* type strain have been growing in purple-coloured colonies and produced a purple pigment when cultivated in liquid LB-medium. *J.* sp. strains 3.109 and 3.116 grew in white colonies on agar plates and showed no signs of violacein production in liquid culture. These observations, and the unsuccessful sequencing of their potential violacein operon (Lorentsen, 2020; Mølmen, 2020), suggest that these two *J.* sp. strains might lack the violacein operon. Interestingly, previous findings have provided evidence that *J. lividum* ERGS5:01 did not contain the violacein operon in its genome (Kumar et al., 2018), which might substantiate this theory. Similarly to our *J.* sp. strains, the aforementioned *J. lividum* strain was also isolated from an aquatic environment, more precisely Himalayan glacial water. However, there could be a possibility that the violacein operon of the two *J.* sp. strains 3.109 and 3.116 is organized in a different manner compared to the other strains. Therefore, we cannot completely exclude the possibility that these two *J.* sp. strains are able to produce violacein under certain cultivation conditions.

4.6 Fish Experiment

Mucus of skin, gills and intestine of fish contains mucins, protecting the epithelial surfaces. Previously, it has been a common belief that degradation of mucin could be detrimental for human gut health (Norin et al., 1985), since it has been postulated that mucin-degrading probiotics could alter the mucosal barrier of the intestine (Ruas-Madiedo et al., 2008). However, it is now well known that many important commensal bacteria, associated with the gut of mammalian species, use the mucin as nutrient source and substrate for growth (Derrien et al., 2010). Our results showed that all *J.* sp. strains were able to utilize mucin as their sole carbon source for growth. Bacteria that are able to degrade mucin, such as *Janthinobacterium*, may have an advantage in terms of colonizing the mucosal surfaces of a host and in turn provide health benefits to the host.

One of the aims of this master project was to examine the six *J.* sp. strains' ability to colonize the gut and skin of Atlantic salmon yolk sac fry. As the salmon has a long fry stage, it is a suitable model for studying the host-microbe interaction in fish over time. The bacteria colonizing the

larvae presumably originates from the surrounding water (Vadstein et al., 2018) and possibly from the egg or from the mother (Hansen & Olafsen, 1999). The exposure of salmon yolk sac fry was performed using 6-well plates, with each fry separated in its own well. One of the fish exposed to *J. lividum* type strain died during the incubation time, due to unknown reasons, but most likely due to handling stress when it was transferred into the 6-well plate. However, because of the experimental set-up, the probability of polluting the water or exposing the other fish to any health risk was low. The close to 100% survival rate of *J.* sp. exposed salmon fry, indicates that these strains were not detrimental for the fry.

Most of the *J.* sp. strains were able to colonize both the skin and gut in high numbers. However, comparison of the colonization density between the skin samples and the gut samples proved difficult. For the gut samples, the average CFU/gut was calculated by neglecting the volume of the gut. Unfortunately, due to the fact that the volume of the skin (the rest of the fry) was not measured prior to homogenization, it was not possible to calculate the average CFU/skin. Therefore, the colonization density of the skin was determined in CFU/mL of homogenized skin. A possible solution could be to estimate the average volume of a salmon fry. However, this could affect the CFU/skin value if the volume of the fry deviated from the average. In this case, a large number of biological replicates would be necessary to provide a reliable result.

From the results, it was observed that *J.* sp. PBA and 3.109 did not colonize the gut of the yolk sac fry after five days of bacterial exposure. For a newly-hatched salmon larvae, the colonization of the gastrointestinal tract is expected to occur shortly after the mouth opens for the first time, at least within seven dph (Sahlmann et al., 2015). In this project, the bacterial exposure of the salmon fry was conducted approximately 21 dph, when the yolk sac fry were far past this stage in development. Consequently, these results were quite unexpected as it is reasonable to believe that the gut provides a nutrient-rich environment for colonization. In absolute number, the *J.* sp. strains were generally more prevalent on the skin of the salmon fry than the gut. This is in accordance with the findings from previous work on *J.* sp. in the ACMS group (Mallasvik, 2019). A possible explanation for this might be that the skin is more readily available for colonization of the *J.* sp. strains than the gut. Additionally, the skin has a substantially larger surface area than the gut and can therefore be inhabited by more bacteria, potentially impacting the results. Further, there is a possibility that the comparatively higher colonization success on the skin is because of a lower tolerance for less aerobic conditions such as in the gut. However, the conditions in the gut may be assumed to be relatively aerobic prior to exogenous feeding as it is close to the surface of the skin. Due to the fact that most studies have focused on bacterial colonization of adult fish (Lescak & Milligan-Myhre, 2017; Padra et al., 2014), the knowledge of the early colonization of skin and gut in fish larvae is limited, and therefore an interesting field of study.

When inoculating the rearing water of the yolk sac fry, the intention was to achieve an initial concentration of 10^5 CFU/mL for each *J.* sp. strain and the *J.* sp. mix. In the preparation of the *J.* sp. bacterial doses, the relationship between OD_{600} and CFUs was established. The CFU method is one of the most widely used approaches for determining the absolute number of bacterial cells in a culture (Hazan et al., 2012). The bacterial cultures were vortexed before the OD_{600} measurement to dissolve potential aggregates and ensure a homogenized suspension. However, from the CFU analyses, the abundance of bacteria was much lower than expected, often lower than 30 CFUs per plate, leading to unreliable results. This might be due to the fact that *J. lividum* commonly forms biofilm (Pantanella et al., 2007; Valdes et al., 2015), which is a complex community of microorganisms embedded in an extracellular matrix. The biofilm matrix primarily consists of water and extracellular polymeric substances (EPS). *Janthinobacterium* are among the bacteria reported to produce biofilm consisting of substantial amounts of EPS (Petrushin et al., 2020). Moreover, cells of biofilm-producing bacteria commonly grow in aggregates (Di Martino, 2018). This was indicated through microscopy of liquid cultures of the six *J.* sp. strains, observing clear tendencies of cell aggregation. Further, the cells of the *J.* sp. strains in liquid culture had a tendency of growing in slime aggregates, even when incubated overnight. The copious amount of slime produced by the cultures was assumed to be EPS. It is reasonable to believe that the cell aggregation and EPS formation have lead to difficulties related to the CFU analysis and consequently provided inaccurate results. There is a possibility that such cell aggregates were aspirated when pipetting and in turn disturbed the measurements, leading to an incorrect OD value. Therefore, different amounts of bacteria were most likely added to the SGM when exposing the salmon yolk sac fry to the *J.* sp. strains. The strains' ability to colonize skin and gut of salmon fry is therefore not easily comparable as the initial conditions could have been different for each strain. To avoid this problem, the liquid cultures could have been incubated in larger containers, such as an Erlenmeyer flask, and mixed more intensively to avoid cell aggregation (Dangcong et al., 1999). Kim et al. (2017) reported reduced cell aggregation when agitating the liquid cultures at 300 rpm, as opposed to 120 rpm used when incubating the *J.* sp. strains in this master project.

From the CFU counts of the water samples it was evident that the number of CFUs in the rearing water at the end of the experiment was highly variable. Therefore, the water samples were classified as either increased, decreased or sustained in numbers in comparison to the intended initial concentration of 10^5 CFU/mL. Surprisingly, the results showed that for *J.* sp. PBA, no CFUs were detectable in the rearing water at the end of the experiment. Further, *J.* sp. 3.109 showed a severely decreased CFU/mL value compared to the initial concentration in the rearing water. This could be explained by the aforementioned tendency of growing in cell aggregates and biofilm, indicating that these two *J.* sp. strains might not have a free-living, planktonic life phase. It is known that bacteria may posses the ability to transition between being planktonic cells and biofilm-forming cells (O'Toole et al., 2000). This transition often occurs as a response to changes in nutrient availability. Therefore, it is possible that the salmon yolk sac fry provided enough nutrients to the bacteria

to avoid the transition to the planktonic mode of growth. Additionally, the colonies were smaller compared to the other *J.* sp. colonies. It can be questioned if this due to some biological factors affecting the cells when they are added to and incubated in the rearing water, leading to less viable bacteria. Moreover, the concentration of *J.* sp. 3.116 in the rearing water had increased at the end of the experiment. This supports our assumption that different amounts of bacteria were added at the beginning of the experiment.

As previously addressed, *J. lividum* is a common commensal of both amphibian (Becker et al., 2009) and human (Ramsey et al., 2015) skin. The fact that the strains worked with in this project were isolated from salmon fry and salmon rearing systems, and that they are able to grow on mucin, indicates that *Janthinobacterium* might be a potential commensal of Atlantic salmon. This assumption was further supported during this master project by the diverse presence of *Janthinobacterium* on the skin of Atlantic salmon yolk sac fry. Interestingly, *Janthinobacterium* have been shown to thrive on a wide variety of skin surfaces, on hosts inhabiting both land and water, demonstrating its versatility. Moreover, the *J.* sp. strains were able to utilize chitin as their sole carbon source. This has further strengthen our suspicion that *J. lividum* could be a candidate for probiotic treatment to protect fish against *Saprolegnia* infection.

5 Future Work

This master project has provided results supporting that *Janthinobacterium* could be a potential commensal of Atlantic salmon skin. This is interesting considering the many biological activities associated with *J. lividum* and violacein production. However, more knowledge regarding its presence and potential physiological benefits in Atlantic salmon should be established in future studies. Based on the findings from this study, the following research topics are suggested for future studies

- Investigate the *J.* sp. strains' potential antagonistic activity against the fungal pathogen *Saprolegnia*
- Examine the potential presence of chitin-degrading genes within the genomes of the six *J.* sp. strains
- Study the expression of the violacein operon genes in the *J.* sp. strains under different cultivation conditions, through qPCR
- Provide more knowledge regarding the function of each violacein operon gene product, especially the VioB gene product
- Systematically investigate the factors affecting the changes in colony morphology of the different *J.* sp. strains
- Detect genes involved in the quorum sensing system, previously identified in *Janthinobacterium*, and investigate how QS affects violacein and EPS production

6 Conclusion

Sequencing of the relevant gene regions missing in order to complete the violacein operons of the three *J.* sp. strains PBA, PBB and MM5 was successful. The two *J.* sp. strains PBA and PBB possessed the five violacein operon genes VioA-VioE. However, based on amino acid sequences, *J.* sp. MM5 showed indications of being unable to produce a functional VioE gene product. Moreover, the analysis showed that the last half of the VioB gene (3' end) for both *J.* sp. PBA and MM5 was truncated. Further, phylogenetic analyses showed indications of a closer relationship between the violacein operon sequences of *Pseudoalteromonas* and *Chromobacterium* than between the 16S rRNA gene sequences. The same tendency was observed for *Collimonas* and *Duganella*. Moreover, *Massilia* and *Janthinobacterium* seemed more distantly related for the violacein operon sequences than 16S rRNA gene sequences. These findings support that the violacein operon might have been subjected to horizontal gene transfer during the evolution of Proteobacteria.

The spectrophotometric study of the assumed crude violacein, extracted from the three *J.* sp. strains PBA, PBB and the *J. lividum* type strain using ethanol, showed maximum absorbance values at 576 nm, 574 nm and 575 nm, respectively. This strongly suggested that the purple pigment observed both in liquid medium and on agar medium was violacein.

From microscopy analysis, the *J.* sp. strains showed tendencies of growing in cell aggregates. This was substantiated by visual inspection of liquid cultures revealing copious amounts of slime assumed to be EPS. Further, all *J.* sp. strains were able to exhibit both smooth and rugose colonies when grown on LA-plates, except for *J.* sp. 3.116 which surface was consistently smooth.

Moreover, all six *J.* sp. strains were able to grow with mucin and chitin as their sole carbon source, confirming mucin and chitin-degrading abilities within *Janthinobacterium*.

Examination of the *J.* sp. strains' antagonistic behaviour, indicated inhibitory effects against the *Pedobacter* sp. for all of the six strains. However, the lack of growth near the *J.* sp. strains could also be due to poor growth in general. None of the six strains showed any consistent antagonistic activity against *Y. rukceri* or *Arthrobacter* sp. Interestingly, absence of violacein production did not seem to affect the potential antagonistic properties of the *J.* sp. strains against *Pedobacter* sp.

Investigation of the *J.* sp. strains' ability to colonize the skin and gut of Atlantic salmon yolk sac fry showed that all six strains were able to colonize the skin in high numbers. Moreover, the colonization density was generally lower in the gut than on the skin. The two *J.* sp. strains PBA and 3.109 seemingly lacked the ability of colonizing the gut of salmon fry, and showed indications of being unable to grow planktonically in the rearing water. It was not possible to investigate the interaction between the six *J.* sp. strains in the colonization process, as the strains could not be visually differentiated. Overall, the study support that *Janthinobacterium* might be a commensal of Atlantic salmon skin, and a potential probiotic candidate for treatment of *Saprolegnia* infection.

Bibliography

Abbate, F., Guerrera, M. C., Levanti, M., Laurà, R., Germanà, G. P., Montalbano, G., Cavallaro, M., & Germanà, A. (2020). Morphology of the Atlantic salmon (Salmo salar) tongue. *Journal of Veterinary Medicine Series C: Anatomia Histologia Embryologia*, *49*(6), 686–694.

Adams, A. (2019). Progress, challenges and opportunities in fish vaccine development. *Fish and Shellfish Immunology*, *90*, 210–214.

Ahmad, W. A., Yusof, N. Z., Nordin, N., Zakaria, Z. A., & Rezali, M. F. (2012). Production and characterization of violacein by locally isolated chromobacterium violaceum grown in agricultural wastes. *Applied Biochemistry and Biotechnology*, *167*(5), 1220–1234.

Alem, D., Marizcurrena, J. J., Saravia, V., Davyt, D., Martinez-Lopez, W., & Castro-Sowinski, S. (2020). Production and antiproliferative effect of violacein, a purple pigment produced by an Antarctic bacterial isolate. *World Journal of Microbiology and Biotechnology*, *36*(8).

Ali, A., Rashid, M. H., & Karaolis, D. K. R. (2002). High-Frequency Rugose Exopolysaccharide Production by Vibrio cholerae. *Applied and Environmental Microbiology*, *68*(11), 5773–5778.

Andrighetti-Fröhner, C. R., Antonio, R. V., Creczynski-Pasa, T. B., Barardi, C. R., & Simões, C. M. (2003). Cytotoxicity and Potential Antiviral Evaluation of Violacein Produced by Chromobacterium violaceum. *Memorias do Instituto Oswaldo Cruz*, *98*(6), 843–848.

Asencio, G., Lavin, P., Alegría, K., Domínguez, M., Bello, H., González-Rocha, G., & González-Aravena, M. (2014). Antibacterial activity of the Antarctic bacterium Janthinobacteriumsp. SMN 33.6 against 2 multi-resistant Gram-negative bacteria. *Electronic Journal of Biotechnology*, *17*(1), 1–5.

August, P., Grossman, T., Minor, C., Draper, M., Macneil, I., Pemberton, J., Call, K., Holt, D., & Osburne, M. (2000). Sequence analysis and functional characterization of the violacein biosynthetic pathway from chromobacterium violaceum. *Journal of molecular microbiology and biotechnology*, *2*, 513–9.

Balcazar, J., Blas, I., Ruizzarzuela, I., Cunningham, D., Vendrell, D., & Muzquiz, J. (2006). The role of probiotics in aquaculture. *Veterinary Microbiology*, *114*(3-4), 173–186.

Balibar, C. J., & Walsh, C. T. (2006). In vitro biosynthesis of violacein from L-tryptophan by the enzymes VioA-E from Chromobacterium violaceum. *Biochemistry*, *45*(51), 15444–15457.

Baricz, A., Teban, A., Chiriac, C. M., Szekeres, E., Farkas, A., Nica, M., Dascălu, A., Oprișan, C., Lavin, P., & Coman, C. (2018). Investigating the potential use of an Antarctic variant of Janthinobacterium lividum for tackling antimicrobial resistance in a One Health approach. *Scientific Reports*, *8*(1), 1–12.

Becker, M. H., Brucker, R. M., Schwantes, C. R., Harris, R. N., & Minbiole, K. P. (2009). The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. *Applied and Environmental Microbiology*, *75*(21), 6635–6638.

Beyhan, S., & Yildiz, F. H. (2007). Smooth to rugose phase variation in Vibrio cholerae can be mediated by a single nucleotide change that targets c-di-GMP signalling pathway. *Molecular Microbiology*, *63*(4), 995–1007.

Bjørndal, T., & Tusvik, A. (2019). Economic analysis of land based farming of salmon. *Aquaculture Economics & Management*, *23*(4), 449–475.

Bly, J. E., Quiniou, S. M., Lawson, L. A., & Clem, L. W. (1997). Inhibition of Saprolegnia pathogenic for fish by Pseudomonas fluorescens. *Journal of Fish Diseases*, *20*(1), 35–40.

Bly, J. E., Lawson, L. A., Szalai, A. J., & Clem, L. W. (1993). Environmental factors affecting outbreaks of winter saprolegniosis in channel catfish, ictalurus punctatus (rafinesque). *Journal of Fish Diseases*, *16*(6), 541–549.

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., & Minbiole, K. P. (2008). Amphibian chemical defense: Antifungal metabolites of the microsymbiont Janthinobacterium lividum on the salamander Plethodon cinereus. *Journal of Chemical Ecology*, *34*(11), 1422–1429.

Carbajal-González, M. T., Fregeneda-Grandes, J. M., Suárez-Ramos, S., Rodríguez Cadenas, F., & Aller-Gancedo, J. M. (2011). Bacterial skin flora variation and in vitro inhibitory activity against Saprolegnia parasitica in brown and rainbow trout. *Diseases of Aquatic Organisms*, *96*(2), 125–135.

Cauz, A. C., Carretero, G. P., Saraiva, G. K., Park, P., Mortara, L., Cuccovia, I. M., Brocchi, M., & Gueiros-Filho, F. J. (2019). Violacein targets the cytoplasmic membrane of bacteria. *ACS Infectious Diseases*, *5*(4), 539–549.

Cauz, A. C., Carretero, G. P., Saraiva, G. K., Park, P., Mortara, L., Cuccovia, I. M., Brocchi, M., & Gueiros-Filho, F. J. (2019). Violacein targets the cytoplasmic membrane of bacteria. *ACS Infectious Diseases*, *5*(4), 539–549.

Chapman, C. M., Gibson, G. R., & Rowland, I. (2012). In vitro evaluation of single- and multi-strain probiotics: Inter-species inhibition between probiotic strains, and inhibition of pathogens. *Anaerobe*, *18*(4), 405–413.

Cho, I., & Blaser, M. J. (2012). The human microbiome: At the interface of health and disease. *Nature Reviews Genetics*, *13*(4), 260–270.

Choi, S. Y., Yoon, K. H., Lee, J. I., & Mitchell, R. J. (2015). Violacein: Properties and production of a versatile bacterial pigment. *BioMed Research International*, *2015*.

Cretoiu, M. S., Korthals, G. W., Visser, J. H., & Van Elsas, J. D. (2013). Chitin amendment increases soil suppressiveness toward plant pathogens and modulates the actinobacterial and oxalobacteraceal communities in an experimental agricultural field. *Applied and Environmental Microbiology*, *79*(17), 5291–5301.

Cross, M. L. (2002). Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. *FEMS Immunology & Medical Microbiology*, *34*(4), 245–253.

Dahal, R. H., & Kim, J. (2016). Pedobacter humicola sp. nov., a member of the genus Pedobacter isolated from soil. *International Journal of Systematic and Evolutionary Microbiology*, *66*(6), 2205–2211.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., & Pedersen, P. B. (2013). Farming different species in ras in nordic countries: Current status and future perspectives [Workshop on Recirculating Aquaculture Systems]. *Aquacultural Engineering*, *53*, 2–13.

Dangcong, P., Bernet, N., Delgenes, J. P., & Moletta, R. (1999). Aerobic granular sludge - A case report. *Water Research*, *33*(3), 890–893.

Derrien, M., van Passel, M. W., van de Bovenkamp, J. H., Schipper, R. G., de Vos, W. M., & Dekker, J. (2010). Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut Microbes*, *1*(4), 254–268.

Di Martino, P. (2018). Extracellular polymeric substances, a key element in understanding biofilm phenotype. *AIMS Microbiology*, *4*(2), 274–288.

Dill, P. A. (1977). Development of behaviour in alevins of atlantic salmon, Salmo salar, and rainbow trout, S. gairdneri. *Animal Behaviour*, *25*(PART 1), 116–121.

Ferreira, C. V., Bos, C. L., Versteeg, H. H., Justo, G. Z., Durán, N., & Peppelenbosch, M. P. (2004). Molecular mechanism of violacein-mediated human leukemia cell death. *Blood*, *104*(5), 1459–1464.

Fjelldal, P. G., Schulz, R., Nilsen, T. O., Andersson, E., Norberg, B., & Hansen, T. J. (2018). Sexual maturation and smoltification in domesticated Atlantic salmon (*Salmo salar* L.) - is there a developmental conflict? *Physiological Reports*, *6*(17), e13809.

Fraune, S., & Bosch, T. C. (2010). Why bacteria matter in animal development and evolution. *BioEssays*, *32*(7), 571–580.

Füller, J. J., Röpke, R., Krausze, J., Rennhack, K. E., Daniel, N. P., Blankenfeldt, W., Schulz, S., Jahn, D., & Moser, J. (2016). Biosynthesis of violacein, structure and function of L-tryptophan oxidase VioA from chromobacterium violaceum. *Journal of Biological Chemistry*, *291*(38), 20068–20084.

Gajardo, K., Rodiles, A., Kortner, T. M., Krogdahl, Å., Bakke, A. M., Merrifield, D. L., & Sørum, H. (2016). A high-resolution map of the gut microbiota in Atlantic salmon (Salmo salar): A basis for comparative gut microbial research. *Scientific Reports*, *6*(August), 1–10.

Gilliland, S. (2003). PROBIOTICS. *Encyclopedia of food sciences and nutrition* (pp. 4792–4798). Elsevier.

Grice, E. A., Kong, H. H., Renaud, G., Young, A. C., Bouffard, G. G., Blakesley, R. W., Wolfsberg, T. G., Turner, M. L., & Segre, J. A. (2008). A diversity profile of the human skin microbiota. *Genome Research*, *18*(7), 1043–1050.

Guerriero, G., Avino, M., Zhou, Q., Fugelstad, J., Clergeot, P. H., & Bulone, V. (2010). Chitin synthases from saprolegnia are involved in tip growth and represent a potential target for anti-oomycete drugs. *PLoS Pathogens*, *6*(8), 81–82.

Haack, F. S., Poehlein, A., Kröger, C., Voigt, C. A., Piepenbring, M., Bode, H. B., Daniel, R., Schäfer, W., & Streit, W. R. (2016). Molecular keys to the Janthinobacterium and Duganella spp. Interaction with the plant pathogen Fusarium graminearum. *Frontiers in Microbiology*, *7*(OCT).

Hakvåg, S., Fjærvik, E., Klinkenberg, G., Borgos, S. E. F., Josefsen, K. D., Ellingsen, T. E., & Zotchev, S. B. (2009). Violacein-producing Collimonas sp. from the sea surface microlayer of costal waters in Trøndelag, Norway. *Marine Drugs*, *7*(4), 576–588.

Hansen, L. P., & Quinn, T. P. (1998). The marine phase of the atlantic salmon (salmo salar) life cycle, with comparisons to pacific salmon. *Canadian Journal of Fisheries and Aquatic Sciences*, *55*(S1), 104–118.

Hansen, G. H., & Olafsen, J. A. (1999). Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology*, *38*(1), 1–26.

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., Lam, B. A., Woodhams, D. C., Briggs, C. J., Vredenburg, V. T., & Minbiole, K. P. C. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *The ISME Journal*, *3*(7), 818–824.

Hazan, R., Que, Y. A., Maura, D., & Rahme, L. G. (2012). A method for high throughput determination of viable bacteria cell counts in 96-well plates. *BMC Microbiology*, *12*(1), 1–7.

Heys, C., Cheaib, B., Busetti, A., Kazlauskaite, R., Maier, L., Sloan, W. T., Ijaz, U. Z., Kaufmann, J., McGinnity, P., & Llewellyn, M. S. (2020). Neutral processes dominate microbial community assembly in Atlantic Salmon, Salmo salar. *Applied and Environmental Microbiology*, *86*(8).

Hinkel, L., & Ospina-Giraldo, M. D. (2017). Structural characterization of a putative chitin synthase gene in Phytophthora spp. and analysis of its transcriptional activity during pathogenesis on potato and soybean plants. *Current Genetics*, *63*(5), 909–921.

Hornung, C., Poehlein, A., Haack, F. S., Schmidt, M., Dierking, K., Pohlen, A., Schulenburg, H., Blokesch, M., Plener, L., Jung, K., Bonge, A., Krohn-Molt, I., Utpatel, C., Timmermann, G., Spieck, E., Pommerening-Röser, A., Bode, E., Bode, H. B., Daniel, R., ... Streit, W. R. (2013). The Janthinobacterium sp. HH01 Genome Encodes a Homologue of the V. cholerae CqsA and L. pneumophila LqsA Autoinducer Synthases. *PLoS ONE*, *8*(2), 55045.

Horreo, J. L., Griffiths, A. M., Machado-Schiaffino, G., Stevens, J. R., & Garcia-Vazquez, E. (2019). Northern areas as refugia for temperate species under current climate warming: Atlantic salmon (Salmo salar L.) as a model in northern Europe. *Journal of Fish Biology*, *95*(1), 304–310.

Hoshino, T. (2011). Violacein and related tryptophan metabolites produced by Chromobacterium violaceum: Biosynthetic mechanism and pathway for construction of violacein core. *Applied Microbiology and Biotechnology*, *91*(6), 1463–1475.

Hovda, M. B., Lunestad, B. T., Fontanillas, R., & Rosnes, J. T. (2007). Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (Salmo salar L.) *Aquaculture*, *272*(1-4), 581–588.

Hussein, M. M. A., & Hatai, K. (1999). Saprolegnia salmonis sp. nov. isolated from sockeye salmon,Onchrhynchus nerka. *Mycoscience*, *40*(5), 387.

Jiang, Y., Sigmund, F., Reber, J., Deán-Ben, X. L., Glasl, S., Kneipp, M., Estrada, H., Razansky, D., Ntziachristos, V., & Westmeyer, G. G. (2015). Violacein as a genetically-controlled, enzymatically amplified and photobleaching-resistant chromophore for optoacoustic bacterial imaging. *Scientific Reports*, *5*(December 2014), 1–9.

Jiang, R. H. Y., de Bruijn, I., Haas, B. J., Belmonte, R., Löbach, L., Christie, J., van den Ackerveken, G., Bottin, A., Bulone, V., Díaz-Moreno, S. M., Dumas, B., Fan, L., Gaulin, E., Govers, F., Grenville-Briggs, L. J., Horner, N. R., Levin, J. Z., Mammella, M., Meijer, H. J. G., ... van West, P. (2013). Distinctive Expansion of Potential Virulence Genes in the Genome of the Oomycete Fish Pathogen Saprolegnia parasitica (J. M. McDowell, Ed.). *PLoS Genetics*, *9*(6), e1003272.

Jin, C., Padra, J. T., Sundell, K., Sundh, H., Karlsson, N. G., & Lindén, S. K. (2015). Atlantic Salmon Carries a Range of Novel O-Glycan Structures Differentially Localized on Skin and Intestinal Mucins. *Journal of Proteome Research*, *14*(8), 3239–3251.

Jobling, M. (2010). Fish culture: Achievements and challenges. *Finfish aquaculture diversification* (pp. 3–32). CABI Publishing.

Johansen, U., Bull-Berg, H., Vik, L. H., Stokka, A. M., Richardsen, R., & Winther, U. (2019). The Norwegian seafood industry – Importance for the national economy. *Marine Policy*, *110*, 103561.

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*, *10*(1), 1–11.

Kalendar, R., Shustov, A. V., Seppänen, M. M., Schulman, A. H., & Stoddard, F. L. (2019). Palindromic sequence-targeted (PST) PCR: a rapid and efficient method for high-throughput gene characterization and genome walking. *Scientific Reports*, *9*(1), 1–11.

Keeling, P. J., & Palmer, J. D. (2008). Horizontal gene transfer in eukaryotic evolution. *Nature Reviews Genetics*, *9*(8), 605–618.

Kim, M., Kim, B. C., Choi, Y., & Nam, K. (2017). Minimizing mixing intensity to improve the performance of rice straw anaerobic digestion via enhanced development of microbe-substrate aggregates. *Bioresource Technology*, *245*, 590–597.

Kitahara, K., & Miyazaki, K. (2013). Revisiting bacterial phylogeny. *Mobile Genetic Elements*, *3*(1), e24210.

Kjærner-Semb, E., Edvardsen, R. B., Ayllon, F., Vogelsang, P., Furmanek, T., Rubin, C. J., Veselov, A. E., Nilsen, T. O., McCormick, S. D., Primmer, C. R., & Wargelius, A. (2020). Comparison of anadromous and landlocked Atlantic salmon genomes reveals signatures of parallel and relaxed selection across the Northern Hemisphere. *Evolutionary Applications*, *14*(2).

Konzen, M., De Marco, D., Cordova, C. A., Vieira, T. O., Antônio, R. V., & Creczynski-Pasa, T. B. (2006). Antioxidant properties of violacein: Possible relation on its biological function. *Bioorganic and Medicinal Chemistry*, *14*(24), 8307–8313.

Kristensen, T., Åtland, Å., Rosten, T., Urke, H. A., & Rosseland, B. O. (2009). Important influent-water quality parameters at freshwater production sites in two salmon producing countries. *Aquacultural Engineering*, *41*(2), 53–59.

Kumar, R., Acharya, V., Singh, D., & Kumar, S. (2018). Strategies for high-altitude adaptation revealed from high-quality draft genome of non-violacein producing Janthinobacterium lividum ERGS5:01. *Standards in Genomic Sciences*, *13*(1), 11.

Lescak, E. A., & Milligan-Myhre, K. C. (2017). Teleosts as model organisms to understand host-microbe interactions. *Journal of Bacteriology*, *199*(15), 868–884.

Letunic, I., & Bork, P. (2019). Interactive Tree of Life (iTOL) v4: Recent updates and new developments. *Nucleic Acids Research*, *47*(W1).

Linden, S. K., Sutton, P., Karlsson, N. G., Korolik, V., & McGuckin, M. A. (2008). Mucins in the mucosal barrier to infection. *Mucosal Immunology*, *1*(3), 183–197.

Lopes, S. C. P., Blanco, Y. C., Justo, G. Z., Nogueira, P. A., Rodrigues, F. L. S., Goelnitz, U., Wunderlich, G., Facchini, G., Brocchi, M., Duran, N., & Costa, F. T. M. (2009). Violacein extracted from Chromobacterium violaceum inhibits Plasmodium growth in vitro and in vivo (2009/03/09). *Antimicrobial agents and chemotherapy*, *53*(5), 2149–2152.

Lorentsen, E. D. (2020). *Characterization of two Janthinobacterium isolates originating from salmon fry* (Master's thesis). Norwegian University of Science and Technology.

Lyakhovchenko, N. S., Abashina, T. N., Polivtseva, V. N., Senchenkov, V. Y., Pribylov, D. A., Chepurina, A. A., Nikishin, I. A., Avakova, A. A., Goyanov, M. A., Gubina, E. D., Churikova, D. A., Sirotin, A. A., Suzina, N. E., & Solyanikova, I. P. (2021). A blue-purple pigment-producing bacterium isolated from the vezelka river in the city of belgorod. *Microorganisms*, *9*(1), 1–19.

Magaraggia, M., Faccenda, F., Gandolfi, A., & Jori, G. (2006). Treatment of microbiologically polluted aquaculture waters by a novel photochemical technique of potentially low environmental impact. *Journal of Environmental Monitoring*, *8*(9), 923–931.

Malhotra, K., Foltz, L., Mahoney, W. C., & Schueler, P. A. (1998). Interaction and effect of annealing temperature on primers used in differential display RT-PCR. *Nucleic Acids Research*, *26*(3), 854–856.

Mallasvik, H. (2019). *Initial bacterial colonization of Atlantic salmon (Salmo salar) yolk sac fry using a gnotobiotic model system* (Master's thesis). Norwegian University of Science and Technology.

Martinez-Rosales, C., Marizcurrena, J. J., Iriarte, A., Fullana, N., Musto, H., & Castro-Sowinski, S. (2015). Characterizing proteases in an Antarctic Janthinobacterium sp. isolate: Evidence of a protease horizontal gene transfer event. *Advances in Polar Science*, *1*(1), 012.

Martins, C., Eding, E., Verdegem, M., Heinsbroek, L., Schneider, O., Blancheton, J., D'Orbcastel, E. R., & Verreth, J. (2010). New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering*, *43*(3), 83–93.

Matz, C., Deines, P., Boenigk, J., Arndt, H., Eberl, L., Kjelleberg, S., & Jürgens, K. (2004). Impact of Violacein-Producing Bacteria on Survival and Feeding of Bacterivorous Nanoflagellates. *Applied and Environmental Microbiology*, *70*(3), 1593–1599.

Melo, P. S., Justo, G. Z., De Azevedo, M. B., Durán, N., & Haun, M. (2003). Violacein and its β-cyclodextrin complexes induce apoptosis and differentiation in HL60 cells. *Toxicology*, *186*(3), 217–225.

Mendes, A. S., De Carvalho, J. E., Duarte, M. C., Durán, N., & Bruns, R. E. (2001). Factorial design and response surface optimization of crude violacein for Chromobacterium violaceum production. *Biotechnology Letters*, *23*(23), 1963–1969.

Mojib, N., Farhoomand, A., Andersen, D. T., & Bej, A. K. (2013). UV and cold tolerance of a pigment-producing Antarctic Janthinobacterium sp. Ant5-2. *Extremophiles*, *17*(3), 367–378.

Mølmen, N. P. (2020). Janthinobacterium Strains Isolated from Salmon Skin: Cultivation Conditions for Violacein Production and DNA Sequencing of the 16S rRNA Gene and the Violacein Operon (Unpublished specialization project).

Munakata, Y., Gavira, C., Genestier, J., Bourgaud, F., Hehn, A., & Slezack-Deschaumes, S. (2021). Composition and functional comparison of vetiver root endophytic microbiota originating from different geographic locations that show antagonistic activity towards Fusarium graminearum. *Microbiological Research*, *243*, 126650.

Noga, E. J. (1993). Water mold infections of freshwater fish: Recent advances. *Annual Review of Fish Diseases*, *3*(100), 291–304.

Nogami, K., & Maeda, M. (1992). Bacteria as biocontrol agents for rearing larvae of the crab Portunus trituberculatus. *Canadian Journal of Fisheries and Aquatic Sciences*, *49*(11), 2373–2376.

Norin, K. E., Gustafsson, B. E., Lindbland, B. S., & Midtvedt, T. (1985). The Establishment of Some Microflora Associated Biochemical Characteristics in Feces from Children during the First Years of Life. *Acta Pædiatrica*, *74*(2), 207–212.

Norwegian Veterinary Institute. (2020). *Fiskehelserapporten 2020* (Report, Norwegian Veterinary Institute, Oslo, Norway).

Oh, W. T., Giri, S. S., Yun, S., Kim, H. J., Kim, S. G., Kim, S. W., Kang, J. W., Han, S. J., Kwon, J., Jun, J. W., & Park, S. C. (2019). Janthinobacterium lividum as an emerging pathogenic bacterium affecting rainbow trout (Oncorhynchus mykiss) fisheries in Korea. *Pathogens*, *8*(3).

Olsson, J. C., Westerdahl, A., Conway, P. L., & Kjelleberg, S. (1992). Intestinal colonization potential of turbot (Scophthalmus maximus)- and dab (Limanda limanda)-associated bacteria with inhibitory effects against Vibrio anguillarum. *Applied and Environmental Microbiology*, *58*(2), 551–556.

Osbourn, A. E., & Field, B. (2009). Operons. *Cellular and Molecular Life Sciences*, *66*(23), 3755–3775.

O'sullivan, J., McCullough, J., Johnson, J. H., Bonner, D. P., Clark, J. C., Dean, L., & Trejo, W. H. (1990). Janthinocins A, B and C, novel peptide lactone antibiotics produced by janthinobacterium lividum: I. taxonomy, fermentation, isolation, physico-chemical and biological characterization. *The Journal of Antibiotics*, *43*(8), 913–919.

O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). Biofilm Formation as Microbial Development. *Annual Review of Microbiology*, *54*(1), 49–79.

Padra, J. T., Sundh, H., Jin, C., Karlsson, N. G., Sundell, K., & Lindén, S. K. (2014). Aeromonas salmonicida binds differentially to mucins isolated from skin and intestinal regions of Atlantic salmon in an N-acetylneuraminic acid-dependent manner. *Infection and Immunity*, *82*(12), 5235–5245.

Padra, J. T., Murugan, A. V. M., Sundell, K., Sundh, H., Benktander, J., & Lindé Nid, S. K. (2019). Fish pathogen binding to mucins from Atlantic salmon and Arctic char differs in avidity and specificity and is modulated by fluid velocity. *PLoS ONE*, *14*(5), e0215583.

Pantanella, F., Berlutti, F., Passariello, C., Sarli, S., Morea, C., & Schippa, S. (2007). Violacein and biofilm production in Janthinobacterium lividum. *Journal of Applied Microbiology*, *102*(4), 992–999.

Pardo, S. A., & Hutchings, J. A. (2020). Estimating marine survival of Atlantic salmon using an inverse matrix approach. *PLoS ONE*, *15*(5).

Petrushin, I., Belikov, S., & Chernogor, L. (2020). Cooperative interaction of janthinobacterium sp. Slb01 and flavobacterium sp. slb02 in the diseased sponge lubomirskia baicalensis. *International Journal of Molecular Sciences*, *21*(21), 1–13.

Philip, D. S., Sarovich, D. S., & Pemberton, J. M. (2009). Complete sequence and analysis of the stability functions of pPSX, a vector that allows stable cloning and expression of Streptomycete genes in Escherichia coli K12. *Plasmid*, *62*(1), 39–43.

Poddar, K., Padhan, B., Sarkar, D., & Sarkar, A. (2021). Purification and optimization of pink pigment produced by newly isolated bacterial strain Enterobacter sp. PWN1. *SN Applied Sciences*, *3*(1).

Rahman, M., Jubair, M., Alam, M. T., Weppelmann, T. A., & Azarian, T. (2014). High-Frequency Rugose Exopolysaccharide Production by Vibrio cholerae Strains Isolated in Haiti. *PLoS ONE*, *9*(11), 112853.

Rajendhran, J., & Gunasekaran, P. (2011). Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond. *Microbiological Research*, *166*(2), 99–110.

Ramsey, J. P., Mercurio, A., Holland, J. A., Harris, R. N., & Minbiole, K. P. (2015). The cutaneous bacterium Janthinobacterium lividum inhibits the growth of Trichophyton rubrum in vitro. *International Journal of Dermatology*, *54*(2), 156–159.

Rashid, M. H., Rajanna, C., Ali, A., & Karaolis, D. K. (2003). Identification of genes involved in the switch between the smooth and rugose phenotypes of Vibrio cholerae. *FEMS Microbiology Letters*, *227*(1), 113–119.

Rettori, D., & Durán, N. (1998). Production, extraction and purification of violacein: An antibiotic pigment produced by Chromobacterium violaceum. *World Journal of Microbiology and Biotechnology*, *14*(5), 685–688.

Ruas-Madiedo, P., Gueimonde, M., Fernández-García, M., De Los Reyes-Gavilán, C. G., & Margolles, A. (2008). Mucin degradation by Bifidobacterium strains isolated from the human intestinal microbiota. *Applied and Environmental Microbiology*, *74*(6), 1936–1940.

Rzeszutek, E., Díaz-Moreno, S. M., & Bulone, V. (2019). Identification and Characterization of the Chitin Synthase Genes From the Fish Pathogen Saprolegnia parasitica. *Frontiers in Microbiology*, *10*.

Sahlmann, C., Gu, J., Kortner, T. M., Lein, I., Krogdahl, Å., & Bakke, A. M. (2015). Ontogeny of the digestive system of Atlantic salmon (Salmo salar L.) and effects of soybean meal from start-feeding. *PLoS ONE*, *10*(4).

Sarowar, M. N., van den Berg, A. H., McLaggan, D., Young, M. R., & Van West, P. (2013). Saprolegnia strains isolated from river insects and amphipods are broad spectrum pathogens. *Fungal Biology*, *117*(11-12), 752–763.

Shinoda, K., Hasegawa, T., Sato, H., Shinozaki, M., Kuramoto, H., Takamiya, Y., Sato, T., Nikaidou, N., Watanabe, T., & Hoshino, T. (2007). Biosynthesis of violacein: A genuine intermediate, protoviolaceinic acid, produced by VioABDE, and insight into VioC function. *Chemical Communications*, (40), 4140–4142.

Solberg, M. F., Fjelldal, P. G., Nilsen, F., & Glover, K. A. (2014). Hatching time and alevin growth prior to the onset of exogenous feeding in farmed, wild and hybrid Norwegian Atlantic salmon. *PLoS ONE*, *9*(12).

Song, C., Schmidt, R., de Jager, V., Krzyzanowska, D., Jongedijk, E., Cankar, K., Beekwilder, J., van Veen, A., de Boer, W., van Veen, J. A., & Garbeva, P. (2015). Exploring the genomic traits of fungus-feeding bacterial genus Collimonas. *BMC Genomics*, *16*(1).

Steyn, P. L., Segers, P., Vancanneyt, M., Sandra, P., Kersters, K., & Joubert, J. J. (1998). Classification of heparinolytic bacteria into a new genus, Pedobacter, comprising four species:

Pedobacter heparinus comb. nov., Pedobacter piscium comb. nov., Pedobacter africanus sp. nov. and Pedobacter saltans sp. nov. proposal of the family Sphingobac. *International Journal of Systematic Bacteriology*, *48*(1), 165–177.

Subasinghe, R., Soto, D., & Jia, J. (2009). Global aquaculture and its role in sustainable development. *Reviews in Aquaculture*, *1*(1), 2–9.

Tattersall, G. J. (2007). Skin breathing in amphibians. In W. C. Aird (Ed.), *Endothelial biomedicine* (pp. 85–91). Cambridge University Press.

Thorstad, E. B., Whoriskey, F., Uglem, I., Moore, A., Rikardsen, A. H., & Finstad, B. (2012). A critical life stage of the Atlantic salmon Salmo salar: behaviour and survival during the smolt and initial post-smolt migration. *Journal of Fish Biology*, *81*(2), 500–542.

Vadstein, O., Attramadal, K. J. K., Bakke, I., Forberg, T., Olsen, Y., Verdegem, M., Giatsis, C., Skjermo, J., Aasen, I. M., Gatesoupe, F.-J., Dierckens, K., Sorgeloos, P., & Bossier, P. (2018). Managing the microbial community of marine fish larvae: A holistic perspective for larviculture. *Frontiers in Microbiology*, *9*, 1820.

Valdes, A. M., Walter, J., Segal, E., & Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *BMJ (Online)*, *361*, 36–44.

Valdes, N., Soto, P., Cottet, L., Alarcon, P., Gonzalez, A., Castillo, A., Corsini, G., & Tello, M. (2015). Draft genome sequence of Janthinobacterium lividum strain MTR reveals its mechanism of capnophilic behavior. *Standards in Genomic Sciences*, *10*(1), 1–10.

van West, P. (2006). Saprolegnia parasitica, an oomycete pathogen with a fishy appetite: new challenges for an old problem. *Mycologist*, *20*(3), 99–104.

Verspoor, E., Stradmeyer, L., & Nielsen, J. (2007). *The Atlantic Salmon: Genetics, Conservation and Management*.

Wai, S. N., Mizunoe, Y., & Yoshida, S.-i. (1999). How Vibrio cholerae survive during starvation. *FEMS Microbiology Letters*, *180*(2), 123–131.

Wang, H., Yao, T., Cai, M., Xiao, X., Ding, X., & Xia, L. (2013). A genome walking strategy for the identification of nucleotide sequences adjacent to known regions. *Biotechnology Letters*, *35*(2), 279–284.

Wang, H., Jiang, P., Lu, Y., Ruan, Z., Jiang, R., Xing, X. H., Lou, K., & Wei, D. (2009). Optimization of culture conditions for violacein production by a new strain of Duganella sp. B2. *Biochemical Engineering Journal*, *44*(2-3), 119–124.

Williston, E. H., Zia-Walrath, P., & Youmans, G. P. (1947). Plate Methods for Testing Antibiotic Activity of Actinomycetes against Virulent Human Type Tubercle Bacilli. *Journal of bacteriology*, *54*(5), 563–568.

Xiao, X., Yin, X., Lin, J., Sun, L., You, Z., Wang, P., & Wang, F. (2005). Chitinase genes in lake sediments of Ardley Island, Antarctica. *Applied and Environmental Microbiology*, *71*(12), 7904–7909.

Xu, X., Tian, L., Zhang, S., Jiang, L., Zhang, Z., & Huang, H. (2019). Complete genome sequence of Janthinobacterium sp. B9-8, a violacein-producing bacterium isolated from low-temperature sewage. *Microbial Pathogenesis*, *128*, 178–183.

Yada, S., Wang, Y., Zou, Y., Nagasaki, K., Hosokawa, K., Osaka, I., Arakawa, R., & Enomoto, K. (2008). Isolation and characterization of two groups of novel marine bacteria producing violacein. *Marine Biotechnology*, *10*(2), 128–132.

Yildiz, F. H., & Schoolnik, G. K. (1999). Vibrio cholerae O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(7), 4028–4033.

Yin, C., Casa Vargas, J. M., Schlatter, D. C., Hagerty, C. H., Hulbert, S. H., & Paulitz, T. C. (2021). Rhizosphere community selection reveals bacteria associated with reduced root disease. *Microbiome*, *9*(1), 1–18.

Yoon, J. H., Kang, S. J., & Oh, T. K. (2007). Pedobacter terrae sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology*, *57*(11), 2462–2466.

Zaki, M. S., Fawzi, O. M., Omar, S., & Taha, N. (2010). Pathological and Biochemical Studies in Tilapia Zilli Infected with Saprolegnia parasitica and Treated with Potassium Permanganate. *J. Agric. & Environ. Sci*, *6*(9), 395–398.

A Recipes for Growth Media

Luria-Bertani (LB) Agar

Table A.1: Components used in preparations of 1 L LB media.

Component	Amount
Yeast Extract	5 g
Tryptone	10 g
Sodium Chloride	5 g
Agar[1]	15 g
MilliQ H_2O	Up to 1 L

Tryptic Soy Agar (TSA)

Table A.2: Components used in preparations of 1 L TSB media.

Component	Amount
TSB powder	30 g
Agar	15 g
MilliQ H_2O	Up to 1 L

Mucin Agar

Table A.3: Components used in preparations of 500 mL mucin agar media.

Component	Amount
M9 broth	5.25 g
Mucin	2 g
Agar	7.5 g
MilliQ H_2O	Up to 500 mL

Agar Base for Chitin Agar Medium

Table A.4: Components used in preparations of 250 mL chitin agar base.

Component	Amount
M9 broth	5.25 g
Agar	7.5 g
MilliQ H_2O	Up to 250 mL

[1]For preparation of Luria-Bertani broth; agar is omitted.

B Recipe for TAE Buffer

When performing gel electrophoresis, an agarose solution was prepared by dissolving agarose in 1x TAE buffer. Table C.1 shows have to make 1 L of 50x TAE buffer. From this, it is possible to prepare 1x TAE buffer by diluting 20 mL 50x TAE-buffer in 980 mL Milli-Q water.

Table B.1: Components used in preparations of 1 L 50x TAE buffer.

Component	**Amount**
Tris base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA solution (pH 8.0)	100 mL
MilliQ H_2O	Up to 1 L

C Recipe for Salmon Gnotobiotic Media (SGM)

Table C.1: Components used in preparations of 1 L salmon gnotobiotic media.

Component	**Stock concentration [g/L]**	**Amount [mL]**
100x $MgSO_4 \cdot 7H_2O$	12.3	10
100x KCl	0.4	10
100x $NaHCO_3$	9.6	10
5x $CaSO_4 \cdot 2H_2O$	0.3	200
MilliQ H_2O	-	770

D Media for Sterility Testing of Rearing Water in Fish Flasks

To ensure germ-free conditions in the rearing water of the fish flasks prior to exposing the salmon yolk sac fry to *Janthinobacterium* strains, a sterility check was performed. The sterility check was performed using four different liquid medium, as described below. A volume of 100 µL from each fish flask were cultured in 13 mL pre-autoclaved (121 ℃, 60 min) glass tubes containing 5 mL liquid medium. The samples were incubated at 6 ℃ and at room temperature for one week.

Nutrient Broth

Table D.1: Components used in preparations of 1 L nutrient broth.

Component	Amount
Nutrient broth	8 g
MilliQ H_2O	Up to 1 L

Brain Heart Infusion

Table D.2: Components used in preparations of 1 L brain heart infusion.

Component	Amount
Brain heart infusion	37 g
MilliQ H_2O	Up to 1 L

Saboraud-2% Dextrose Broth

Table D.3: Components used in preparations of 1 L saboraud-2% dextrose broth.

Component	Amount
Saboraud-2% dextrose	30 g
MilliQ H_2O	Up to 1 L

Glucose Yeast Extract Broth

Table D.4: Components used in preparations of 1 L glucose yeast extract broth.

Component	Amount
Glucose	10 g
Yeast extract	2.5 g
MilliQ H_2O	Up to 1 L

E 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.
- 2 ml Collection Tubes are provided.

Procedure

1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex to mix.
2. Add 60 µl of Solution C1 and invert several times or vortex briefly.
   Note: Solution C1 may be added to the PowerBead tube before adding soil sample
3. Secure PowerBead Tubes horizontally using a Vortex Adapter for 24 (1.5–2.0 ml) tubes (cat. no. 13000-V1-24).
4. Vortex at maximum speed for 10 min.
   Note: If using the 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Centrifuge tubes at 10,000 x *g* for 30 s.
6. Transfer the supernatant to a clean 2 ml Collection Tube.
   Note: Expect between 400–500 µl of supernatant. 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 DNeasy PowerSoil extractions with this incubation we recommend you retain the step.
8. Centrifuge the tubes for 1 min at 10,000 x *g*.
9. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube.
10. 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 this incubation we recommend you retain the step.

11. Centrifuge the tubes for 1 min at 10,000 x *g*.
12. Avoiding the pellet, transfer up to 750 µl of supernatant to a clean 2 ml Collection Tube.
13. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.
14. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard flow-through.
15. Repeat step 14 twice, until all of the sample has been processed.
16. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x *g*.
17. Discard the flow-through. Centrifuge again for 1 min at 10,000 x *g*.
18. Carefully place the MB Spin Column into a clean 2 ml Collection Tube. Avoid splashing any Solution C5 onto the column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-free PCR-grade water for this step (cat. no. 17000-10).
20. Centrifuge at room temperature for 30 s at 10,000 x *g*. Discard the MB Spin Column. The DNA is now ready for downstream applications.
    Note: We recommend storing DNA frozen (–20°C to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA, see the Troubleshooting Guide.

F QIAquick PCR Purification Kit (Qiagen) Protocol

QIAquick PCR Purification Kit Protocol

using a microcentrifuge

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, polymerases, 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.

G QIAquick Gel Extraction Kit (Qiagen) Protocol

July 2018

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

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

H Fish Experiment Results

Table H.1: Results from the colonization of Atlantic salmon yolk sac fry by *J.* sp. strains based on the homogenized skin [CFU/mL], gut [CFU/gut] and rearing water at the end of the experiment. The water samples were classified in terms of growth relative to the assumed initial concentration of 10^5 CFU/mL.

***J.* sp. strain**	**Fish**	**Skin [CFU/mL]**	**Gut [CFU/gut]**	**Rearing water (SGM)**
PBA	1	Above countable	< 1	Not detectable
	2	$3.57 \cdot 10^3 \pm 0$	< 1	Not detectable
	3	$3.57 \cdot 10^3 \pm 0.50 \cdot 10^3$	< 1	Not detectable
	4	$5.71 \cdot 10^3 \pm 0$	< 1	Not detectable
PBB	1	$3.45 \cdot 10^4 \pm 0.90 \cdot 10^4$	< 1	Sustained in numbers
	2	Above countable	199 ± 33	Sustained in numbers
	3	Above countable	160 ± 77	Sustained in numbers
	4	$7.29 \cdot 10^3 \pm 3.51 \cdot 10^3$	< 1	Sustained in numbers
MM5	1	Above countable	431 ± 233	Sustained in numbers
	2	$2.19 \cdot 10^4 \pm 0.69 \cdot 10^4$	776 ± 227	Sustained in numbers
	3	$4.54 \cdot 10^4 \pm 0$	1180 ± 245	Decreased in numbers
	4	$1.83 \cdot 10^4 \pm 0.60 \cdot 10^4$	60 ± 0	No growth
3.109	1	$2.57 \cdot 10^4 \pm 0.29 \cdot 10^4$	< 1	Sustained in numbers
	2	$3.45 \cdot 10^5 \pm 2.29 \cdot 10^5$	< 1	No growth
	3	< 1	< 1	No growth
	4	< 1	< 1	Decreased in numbers
3.116	1	< 1	239 ± 5	Increased in numbers
	2	Above countable	< 1	Increased in numbers
	3	$9.43 \cdot 10^4 \pm 1.71 \cdot 10^4$	69 ± 13	Increased in numbers
	4	$2.63 \cdot 10^5 \pm 1.06 \cdot 10^5$	220 ± 132	Increased in numbers
Type	1	$4.81 \cdot 10^4 \pm 1.21 \cdot 10^4$	1250 ± 181	Sustained in numbers
	2	$3.41 \cdot 10^5 \pm 0.15 \cdot 10^5$	940 ± 183	Sustained in numbers
	3	$7.00 \cdot 10^4 \pm 1.11 \cdot 10^4$	311 ± 118	Sustained in numbers
	4	Dead	Dead	Dead
Mix purple	1	$1.71 \cdot 10^4 \pm 0.14 \cdot 10^4$	71 ± 6	Decreased in numbers
	2	$4.38 \cdot 10^4 \pm 3.07 \cdot 10^4$	177 ± 65	Decreased in numbers
	3	$8.95 \cdot 10^4 \pm 1.30 \cdot 10^4$	118 ± 19	Decreased in numbers
	4	< 1	59 ± 13	Decreased in numbers
Mix white	1	$1.71 \cdot 10^4 \pm 0.14 \cdot 10^4$	69 ± 0	Decreased in numbers
	2	$3.00 \cdot 10^4 \pm 0$	60 ± 0	Decreased in numbers
	3	$2.95 \cdot 10^4 \pm 1.46 \cdot 10^4$	73 ± 0	Decreased in numbers
	4	$2.43 \cdot 10^4 \pm 0$	47 ± 0	Decreased in numbers

I Gel Electrophoresis

An agarose solution (1% w/v) is prepared by dissolving agarose in Tris-acetate-EDTA (TAE) buffer (Appendix B). The solution is heated in a microwave until boiling. GelRed (Biotum) is added to an erlenmeyer flask containing agarose solution at 55-60 ℃. For 100 mL agarose solution, 5 µL GelRed is added. The mix is poured gently into a gel chamber with a gel comb. After 15 minutes, the gel has solidified and the gel comb can be removed. A volume of 4 µL PCR product is mixed with 1 µL of 6X Loading Dye (Thermo Scientific) and added to a gel well. The gel is run at 110 V, 220 mA and 20 W for 1-2 hours.

J Dialysis of Chitosan

To prepare colloidal chitin, 3 g of chitosan was dissolved to 1% w/v in acetic acid (0.33 M). A stock solution of 1L NaCl (2M) was made. The dialysis bag was transferred to a bucket containing 7L NaCl (50mM). The bucket was left at room temperature for at least four hours (up to 15 hours when incubated overnight), before transferring the dialysis bag to a new bucket. After five shifts against NaCl (50mM), the solution was dialysed against ion free water (five more shifts).

K Accession Numbers

Table K.1: Species and strains used in phylogenetic analyses with accompanying NCBI accession numbers.

Species	**Strain**	**NCBI Accession Number**
Janthinobacterium lividum	DSM1522	LRHW01000061
Janthinobacterium agaricidamnosum	DSM9628	NZ_HG322949
Massilia atriviolacea	SOD	RXLQ01000002
Massilia violaceinigra	B2	NZ_CP024608
Massilia sp.	CCM 8941	NZ_CP065053
Duganella sp.	ZLP-XI	KJ131413
Massilia sp.	NR 4-1	NZ_CP012201
Collimonas sp.	CT MP11E8	GQ160908
Collimonas sp.	MPS11E8	FJ965838
Chromobacterium vaccinii	MWU205	NZ_JZJL00000000
Chromobacterium violaceum	ATCC12472	AE016825
Pseudoalteromonas luteoviolacea	S40542	NZ_CP015413
Pseudoalteromonas sp.	520P1	NZ_BBZB00000000
Myxococcus stipitatus	DSM14675	CP004025

L 16S rRNA Gene Sequence Alignment

04 Nov 2020 **Alignment Results**

Alignment: Assembled DNA alignment against reference molecule
Parameters: Method: FastScan - Max Qual

Reference molecule: DSM1522 (Type), Region 1 to 1405
Number of sequences to align: 6
Total length of aligned sequences with gaps: 1405 bps

Sequence	Start	End	Match	NonMatch	%Match
DSM1522 (Type)	1	1405			
PBA consensus	1	1404	1401	4	99
PBB consensus	1	1404	1403	2	99
MM5 consensus	1	1404	1401	4	99
3.109 consensus	1	1404	1401	4	99
3.116 consensus	1	1404	1400	5	99

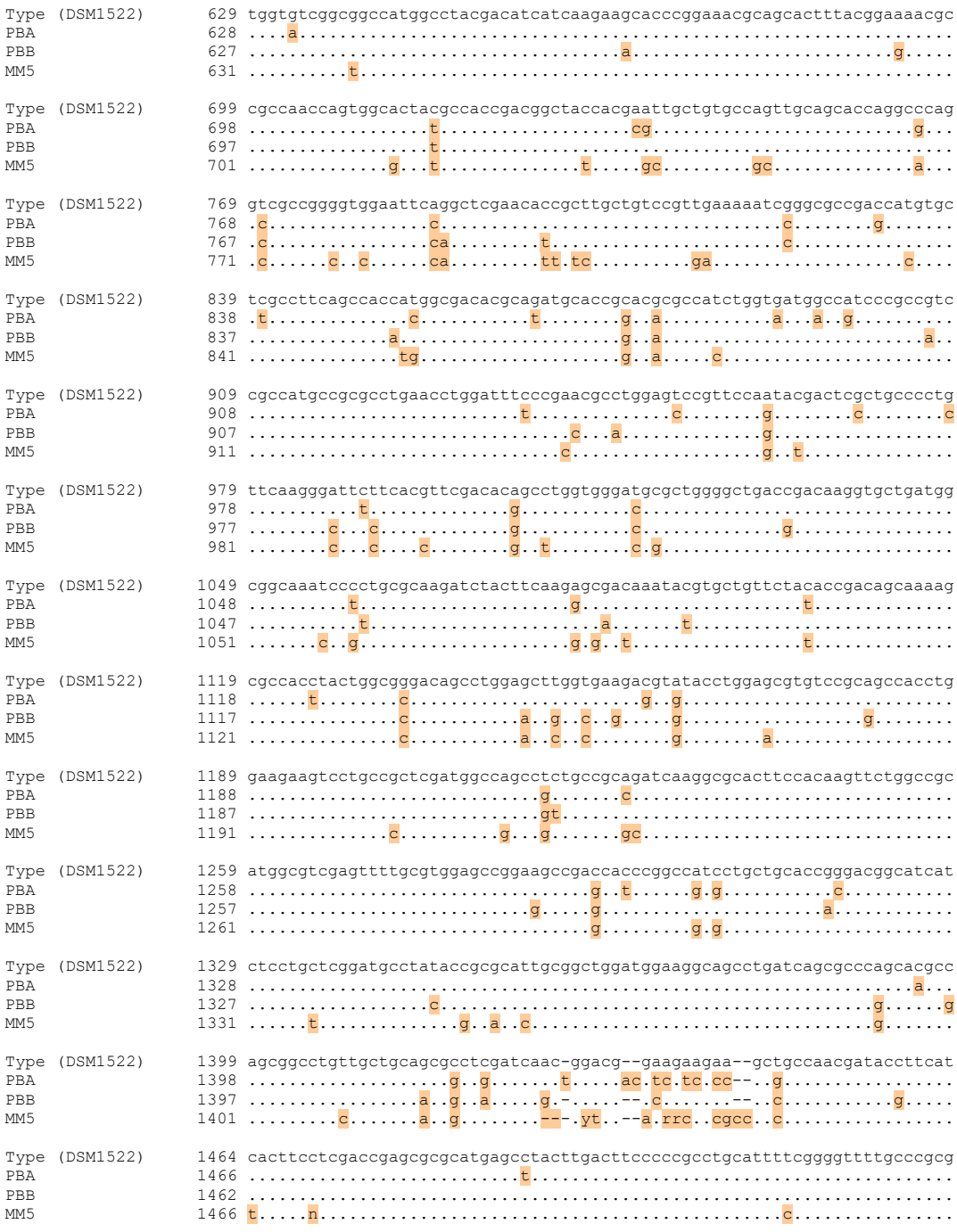
```
DSM1522 (Type)        1 catgcaagtcgaacggcagcacggagcttgctctggtggcgagtggcgaacgggtgagtaatatatcgga
PBA consensus         1 ............................................................................
PBB consensus         1 ............................................................................
MM5 consensus         1 ............................................................................
3.109 consensus       1 ............................................................................
3.116 consensus       1 ............................................................................

DSM1522 (Type)       71 acgtaccctagagtgggggataacgtagcgaaagttacgctaataccgcatacgatctaaggatgaaagt
PBA consensus        71 .........g..................................................................
PBB consensus        71 ............................................................................
MM5 consensus        71 .........g..................................................................
3.109 consensus      71 ............................................................................
3.116 consensus      71 ............................................................................

DSM1522 (Type)      141 gggggatcgcaagacctcatgctcgtggagcggccgatatctgattagctagttggtagggtaaaagcct
PBA consensus       141 ............................................................................
PBB consensus       141 ............................................................................
MM5 consensus       141 ............................................................................
3.109 consensus     141 ............................................................................
3.116 consensus     141 ............................................................................

DSM1522 (Type)      211 accaaggcatcgatcagtagctggtctgagaggacgaccagccacactggaactgagacacggtccagac
PBA consensus       211 ............................................................................
PBB consensus       211 ............................................................................
MM5 consensus       211 ............................................................................
3.109 consensus     211 ............................................................................
3.116 consensus     211 ............................................................................

DSM1522 (Type)      281 tcctacgggaggcagcagtggggaattttggacaatgggcgaaagcctgatccagcaatgccgcgtgagt
PBA consensus       281 ............................................................................
PBB consensus       281 ............................................................................
MM5 consensus       281 ............................................................................
3.109 consensus     281 ............................................................................
3.116 consensus     281 ............................................................................

DSM1522 (Type)      351 gaagaaggccttcgggttgtaaagctcttttgtcagggaagaaacggtgagagctaatatctcttgctaa
PBA consensus       351 ............................................................................
PBB consensus       351 ............................................................................
```

```
MM5 consensus       351 ....................................................................
3.109 consensus     351 ....................................................................
3.116 consensus     351 ....................................................................

DSM1522 (Type)      421 tgacggtacctgaagaataagcaccggctaactacgtgccagcagccgcggtaatacgtagggtgcaagc
PBA consensus       421 ....................................................................
PBB consensus       421 ....................................................................
MM5 consensus       421 ....................................................................
3.109 consensus     421 ....................................................................
3.116 consensus     421 ....................................................................

DSM1522 (Type)      491 gttaatcggaattactgggcgtaaagcgtgcgcaggcggttttgtaagtctgatgtgaaatccccgggct
PBA consensus       491 ....................................................................
PBB consensus       491 ....................................................................
MM5 consensus       491 ....................................................................
3.109 consensus     491 ....................................................................
3.116 consensus     491 ....................................................................

DSM1522 (Type)      561 caacctgggaattgcattggagactgcaaggctagaatctggcagagggggtagaattccacgtgtagc
PBA consensus       561 ....................................................................
PBB consensus       561 ....................................................................
MM5 consensus       561 ....................................................................
3.109 consensus     561 ....................................................................
3.116 consensus     561 ....................................................................

DSM1522 (Type)      631 agtgaaatgcgtagatatgtggaggaacaccgatggcgaaggcagccccctgggtcaagattgacgctca
PBA consensus       631 ....................................................................
PBB consensus       631 ....................................................................
MM5 consensus       631 ....................................................................
3.109 consensus     631 ....................................................................
3.116 consensus     631 ....................................................................

DSM1522 (Type)      701 tgcacgaaagcgtggggagcaaacaggattagataccctggtagtccacgccctaaacgatgtctactag
PBA consensus       701 ....................................................................
PBB consensus       701 ....................................................................
MM5 consensus       701 ....................................................................
3.109 consensus     701 ....................................................................
3.116 consensus     701 ....................................................................

DSM1522 (Type)      771 ttgtcgggtcttaattgacttggtaacgcagctaacgcgtgaagtagaccgcctggggagtacggtcgca
PBA consensus       771 ....................................................................
PBB consensus       771 ....................................................................
MM5 consensus       771 ....................................................................
3.109 consensus     771 ....................................................................
3.116 consensus     771 ....................................................................

DSM1522 (Type)      841 agattaaaactcaaaggaattgacggggacccgcacaagcggtggatgatgtggattaattcgatgcaac
PBA consensus       841 ....................................................................
PBB consensus       841 ....................................................................
MM5 consensus       841 ....................................................................
3.109 consensus     841 ....................................................................
3.116 consensus     841 ....................................................................

DSM1522 (Type)      911 gcgaaaaaccttacctacccttgacatggctggaatccccgagagattggggagtgctcgaaagagaacc
PBA consensus       911 ....................................t.........r.....................
PBB consensus       911 ....................................t.........r.....................
MM5 consensus       911 ....................................y.........r.....................
3.109 consensus     911 ....................................g.......ct.....................
3.116 consensus     911 ....................................tt......c.a.....................

DSM1522 (Type)      981 agtacacaggtgctgcatggctgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcg
```

```
PBA consensus       981 ....................................................................
PBB consensus       981 ....................................................................
MM5 consensus       981 ....................................................................
3.109 consensus     981 ....................................................................
3.116 consensus     981 ....................................................................

DSM1522 (Type)     1051 caacccttgtcattagttgctacgaaagggcactctaatgagactgccggtgacaaaccggaggaaggtg
PBA consensus      1051 ....................................................................
PBB consensus      1051 ....................................................................
MM5 consensus      1051 ....................................................................
3.109 consensus    1051 ....................................................................
3.116 consensus    1051 ....................................................................

DSM1522 (Type)     1121 gggatgacgtcaagtcctcatggcccttatgggtagggcttcacacgtcatacaatggtacatacagagc
PBA consensus      1121 ....................................................................
PBB consensus      1121 ....................................................................
MM5 consensus      1121 ....................................................................
3.109 consensus    1121 ....................................................................
3.116 consensus    1121 ....................................................................

DSM1522 (Type)     1191 gccgccaacccgcgaggggagctaatcgcagaaagtgtatcgtagtccggattgtagtctgcaactcga
PBA consensus      1191 ....................................................................
PBB consensus      1191 ....................................................................
MM5 consensus      1191 ....................................................................
3.109 consensus    1191 ....................................................................
3.116 consensus    1191 ....................................................................

DSM1522 (Type)     1261 ctgcatgaagttggaatcgctagtaatcgcggatcagcatgtcgcggtgaatacgttcccgggtcttgta
PBA consensus      1261 ....................................................................
PBB consensus      1261 ....................................................................
MM5 consensus      1261 ....................................................................
3.109 consensus    1261 ....................................................................
3.116 consensus    1261 ....................................................................

DSM1522 (Type)     1331 cacaccgcccgtcacaccatgggagcgggttttaccagaagtaggtagcttaaccgcaaggagggcgctt
PBA consensus      1331 ............................-...........................................
PBB consensus      1331 ............................-..........................t.................
MM5 consensus      1331 ............................-...........................................
3.109 consensus    1331 ............................-...........................................
3.116 consensus    1331 ............................-...........................................

DSM1522 (Type)     1401 accac
PBA consensus      1400 .....
PBB consensus      1400 .....
MM5 consensus      1400 .....
3.109 consensus    1400 .....
3.116 consensus    1400 .....
```

M Violacein Operon Sequence Alignment

```
04 May 2021                Alignment Results

Alignment:   Global DNA alignment against reference molecule
Parameters:  Scoring matrix:  Linear (Mismatch 2, OpenGap 4, ExtGap 1)

Reference molecule:  Type (DSM1522), Region 1 to 7670
Number of sequences to align:  4
Total length of aligned sequences with gaps:  7689 bps


Sequence               Start     End      Match    NonMatch %Match

Type (DSM1522)             1    7670

PBA                        1    7671       7255     420        94

PBB                        1    7664       7044     630        91

MM5                        1    7670       7033     650        91


Type (DSM1522)         1 aagaatcgaccgtaccagacacaacgccagt-tctcgccagg-cttttcactccccagccgataacgctc
PBA                    1 ...........a.t.................-.t........-..c....t.t.................
PBB                    1 ...........a.tt....a...........-..........-.gc....t..............a.c.
MM5                    1 ...t.......a.t......c..........g..........t..c....t...................

Type (DSM1522)        69 gccaagtgcggtaacaccctgcgttccccctcccttgcctacacgtgctggggaaacttttgctagcgat
PBA                   69 ..g.............g.a...-...........c............c.....................
PBB                   69 ...c........g....g.a............t.............c......a..........c...a..
MM5                   71 .......a....g...tg.a....c..t..c....c...c.gcg....a.....g..............

Type (DSM1522)       139 tttgcggctttttgattaatgaacgttaaaaaggaatttcgtatgagcacgtattctgacatttgcatcg
PBA                  138 .....a..............c.....................................t..........
PBB                  139 .........-...................-..g...........t.........t..............
MM5                  141 .....a...........c.....................................a..t..........

Type (DSM1522)       209 ttggcgccggcataggaggcttgacttgcgccaacaacctgatcgacgccgccgccggcaggaacctgcg
PBA                  208 .g...........c......c.............tc.a...................a........g.....
PBB                  207 ..........t..c.....................c.....................a.............
MM5                  211 ....t.........c..c...c....c.........c.........c.tc.......g.aa.......g.....

Type (DSM1522)       279 catccgcgtattcgacctgaatgccaccgtaggcggccgcatccagtcgcggaaaatagatggcgaggaa
PBA                  278 ......................c.........c.....................a......a.c..........
PBB                  277 ......g.....................g..c.....................t..g..c.......a.....
MM5                  281 ...................c...c..g.t..c...................ac...................

Type (DSM1522)       349 atcgccgaactcggcgccgcccgctactcgccgcagctgcatccgcatttccagcaactcatgcagggca
PBA                  348 ..t.........................................a...............g..........
PBB                  347 ..............................................c.........g..............
MM5                  351 .....................................a.........a................g..........

Type (DSM1522)       419 gcggcttgccgcatgcggtctacccgttcaccgaggtcatctcccacgatagcgtgctggaagagctgaa
PBA                  418 ....ac..........c.............................g.......................
PBB                  417 ..................................................................a.....
MM5                  421 .....c.................................gg.t...................c..........

Type (DSM1522)       489 ggcaacgctggatgagctgagcccgatgctgaaaatgcatccgaacgactccttcctcgagttcgtcagc
PBA                  488 .........c....gc.....................ga.........................c....t.....
PBB                  487 .........t.........................gac.......................t..t.........
MM5                  491 ...c.........c.......a.............gcc.........c....t...................

Type (DSM1522)       559 cattacctgggcgccgccaaggccagccacatcatcaaggcgaccggctatgacgccctgctgctgccga
PBA                  558 ....................ga....c...g.............................t.............
PBB                  557 ............................a.....................................................
MM5                  561 ..................a......g.c.................c.................t.............
```


Type (DSM1522) 629 tggtgtcggcggccatggcctacgacatcatcaagaagcacccggaaacgcagcactttacggaaaacgc
PBA 628 ....a.....
PBB 627 .....a.....g.....
MM5 631 .....t.....
Type (DSM1522) 699 cgccaaccagtggcactacgccaccgacggctaccacgaattgctgtgccagttgcagcaccaggcccag
PBA 698 .....t.....cg.....g...
PBB 697 .....t.....
MM5 701 .....g...t.....t.....gc.....gc.....a...
Type (DSM1522) 769 gtcgccggggtggaattcaggctcgaacaccgcttgctgtccgttgaaaaatcgggcgccgaccatgtgc
PBA 768 .c.....c.....c.....g.....
PBB 767 .c.....ca.....t.....c.....
MM5 771 .c.....c..c.....ca.....tt.tc.....ga.....c....
Type (DSM1522) 839 tcgccttcagccaccatggcgacacgcagatgcaccgcacgcgccatctggtgatggccatcccgccgtc
PBA 838 .t.....c.....t.....g..a.....a...a..g.....
PBB 837 .....a.....g..a.....a..
MM5 841 .....tg.....g..a.....c.....
Type (DSM1522) 909 cgccatgccgcgcctgaacctggatttcccgaacgcctggagtccgttccaatacgactcgctgcccctg
PBA 908 .....t.....c.....g.....c.....c
PBB 907 .....c...a.....g.....
MM5 911 .....c.....g..t.....
Type (DSM1522) 979 ttcaagggattcttcacgttcgacacagcctggtgggatgcgctggggctgaccgacaaggtgctgatgg
PBA 978 .....t.....g.....c.....
PBB 977 .....c...c.....g.....c.....g.....
MM5 981 .....c...c....c.....g..t.....c.g.....
Type (DSM1522) 1049 cggcaaatcccctgcgcaagatctacttcaagagcgacaaatacgtgctgttctacaccgacagcaaaag
PBA 1048 .....t.....g.....t.....
PBB 1047 .....t.....a.....t.....
MM5 1051 .....c..g.....g.g..t.....t.....
Type (DSM1522) 1119 cgccacctactggcgggacagcctggagcttggtgaagacgtatacctggagcgtgtccgcagccacctg
PBA 1118 .....t.....c.....g..g.....
PBB 1117 .....c.....a..g..c..g.....g.....g.....
MM5 1121 .....c.....a..c..c.....g.....a.....
Type (DSM1522) 1189 gaagaagtcctgccgctcgatggccagcctctgccgcagatcaaggcgcacttccacaagttctggccgc
PBA 1188 .....g.....c.....
PBB 1187 .....gt.....
MM5 1191 .....c.....g...g.....gc.....
Type (DSM1522) 1259 atggcgtcgagttttgcgtggagccggaagccgaccacccggccatcctgctgcaccgggacggcatcat
PBA 1258 .....g..t.....g.g.....c.....
PBB 1257 .....g.....g.....a.....
MM5 1261 .....g.....g.g.....
Type (DSM1522) 1329 ctcctgctcggatgcctataccgcgcattgcggctggatggaaggcagcctgatcagcgcccagcacgcc
PBA 1328 .....a...
PBB 1327 .....c.....g.....g
MM5 1331 .....t.....g..a..c.....g.....
Type (DSM1522) 1399 agcggcctgttgctgcagcgcctcgatcaac-ggacg--gaagaagaa--gctgccaacgataccttcat
PBA 1398 .....g..g.....t.....ac.tc.tc.cc--..g.....
PBB 1397 .....a..g..a.....g.-.....--.c.....--..c.....g.....
MM5 1401 .....c.....a..g.....---..yt..--a.rrc..cgcc..c.....
Type (DSM1522) 1464 cacttcctcgaccgagcgcgcatgagcctacttgacttcccccgcctgcattttcggggttttgcccgcg
PBA 1466 .....t.....
PBB 1462 .....
MM5 1466 t.....n.....c.....

```
Type (DSM1522)     1534 ccaatgtgccgacggggaatcgcaatacgcacggcaacatcgatatcgccacgaatgcggtatcgatggc
PBA                1536 ......................t.............................................
PBB                1532 ............c.....................................................
MM5                1536 ..........................................................c.........a

Type (DSM1522)     1604 gggcgaggctgtcgacctgagccggccgccagccgaattccatgcgcacctgaaacagctcgccccccgc
PBA                1606 ......a.................a...a..g..............................t.........
PBB                1602 ......a..g.....................................................t.........
MM5                1606 ....a.t.............a.....................a.............t.........

Type (DSM1522)     1674 ttcaacgcacagggcaagcccgatccggacggcatcttcagccaggcgacaggctataatttttgcgggga
PBA                1676 ..................................g.........t....g.....c.c......g......
PBB                1672 .....t..ggc.................a.............t....g.g.....c.....c.....c.
MM5                1676 .....t..cg.a.....................................g.......c......g....c.

Type (DSM1522)     1744 acaaccatttctcgtgggaaaacgcgcggatcacgggcgtccagttgcgtgatggcgaggtcgataccca
PBA                1746 ....t.....................................t.......c....c..g.............g..
PBB                1742 ....t.....................................................c.........a.......g..
MM5                1746 .t..t.............g.........................c.tg.c.c.............c..g..

Type (DSM1522)     1814 ggacgcgctggtgggcgccaagctgggcctgtggggccactacaacgagtacctgcgcacgacgttcaac
PBA                1816 a.....................g......a.............................................
PBB                1812 ...t..a.................................a.........t.....t.................
MM5                1816 ...t..a.............g.............................a............c.........

Type (DSM1522)     1884 cgcgcaaggtggatcgacaacaacccggcgcagcccgacaccacgctgatctacgcgggccagttcacct
PBA                1886 .....c.........................a..g.....................c..t.........c
PBB                1882 .....c...................t..c..c.....g.....g.............................
MM5                1886 .....c.....................c..cgc.........................c.............

Type (DSM1522)     1954 tgagcgacaagctggccacgcccaatacgcccacgctgttcacggccgacatcgcgcaggcgcactcggt
PBA                1956 ............................c.........ct.....................................
PBB                1952 ....t....g.................c.....a........t..c.............................
MM5                1956 ............................c.........ct.....................................

Type (DSM1522)     2024 gcgctggctcggcagcggccacatcacggaacgcagcgggcatttcctggacgaggaattcggccgctcc
PBA                2026 ......................t.........................t..............t.........g
PBB                2022 ...................g..t.............................c..........a...........
MM5                2026 ......................t....t.....................................g..

Type (DSM1522)     2094 aggctgttccagttttccgtggccaagcaggacccgcatttcctgttcaatccggacctgccgctgccgg
PBA                2096 c.....................................................g.....................
PBB                2092 ..................................t.........t.........g.....................
MM5                2096 ..................g.....................................g.....................

Type (DSM1522)     2164 ccagtatgcatgccttgcagcaagccctggccgacgacgaggtgctgggcctgacggtgcaatactgcct
PBA                2166 ....c.................................t.....c.....................g.........
PBB                2162 ....c....tc.......g...........a.........................c.....g..tgc...
MM5                2166 ....c.....g.......g...c...................c.....................g..tgc.t.

Type (DSM1522)     2234 gttcaatatgtcgacgccgcaaaaacccgattcgcccgtgttctacgacctggccggcagcatcggcctg
PBA                2236 ......................t.......g..c.....................................
PBB                2232 c.....................................................................
MM5                2236 ......................tc.....g..c.....................................

Type (DSM1522)     2304 tggcggcgcggcgagctggccacctatccggccggccgcctgctgcagccgcgccagggcagcctggggc
PBA                2306 ..........a.......................a.........................c...........
PBB                2302 ............................c.....................................................
MM5                2306 ...........................................................cag.........

Type (DSM1522)     2374 cggtgctggtgaaagtgcatgcggaccgcgtctcgttcaacatgccgaccgccatccccttcaccacgcg
PBA                2376 ............c.....................g.................g.....................
PBB                2372 ..............g.....................................................t.............
MM5                2376 ............c.......c.........g...g.....................g.tg.............
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Type (DSM1522) 2444 cgacgcgggcgccgtctcggaacagcatcccacgcatgccttgggcggcaagcaggcgctgggcgacctg
PBA 2446 .....c.....gc.....c.....g...
PBB 2442 ....a.....gc.....g...c.....t...
MM5 2446 ..g..a.....t.....ac.....c.....
Type (DSM1522) 2514 ctgctgcatgacggcgccggcaccgttctggcgcggattcccgagcagctgtaccgcgactactggcgcc
PBA 2516 .....gg.....t.g.....c..a.....c.....c.....
PBB 2512 .....a.....t.g.....c..c.....a.c.....c.....
MM5 2516 .....c.....a.....t.g.....c..a.....a.c.....c.....
Type (DSM1522) 2584 atcacggcgtcttcgacgtgccgctgcagcacgctggcgcggcgccaggctcgctcagcctgggcagcgc
PBA 2586 .....t.....t.....ct..---..g..c.....
PBB 2582 .....t.....c..c..g..t.....a.....
MM5 2586 .....t.....c..c..g..t.....a.....
Type (DSM1522) 2654 gcaggcgcagtgggacgaagccgactgggtgctgcaatcggacagcaaccagctgtacctggaagcgccg
PBA 2653 t.....g.....g.....
PBB 2652 .....a.....g.....t.....c
MM5 2656 .....a.....g.....t.....c
Type (DSM1522) 2724 aaccggaacaagcacgagcaatttccgcagaccatcaccgtgcaaagccgctttcgcggcgagctggcgg
PBA 2723 ....at..a.....c.....
PBB 2722 ...g.a..a.....g.....g....tgc.....c.....a...
MM5 2726 ...g.a..a.....g.....g....tgc.....c.....a...
Type (DSM1522) 2794 cgcccccgtccttggcggagg---cggaagacggcgtgctgctggccgtggagcagcaaccgtcgccgct
PBA 2793 .....c....ac.a.---.....c.....c.....g.....
PBB 2792 ....t...g..c.....c..aag.c..g..t....c.....cgc.....g.....
MM5 2796 ....t...g..c.....c..aag.c..g..t....c.....cgc.....g.....
Type (DSM1522) 2861 cgggcacggctacacgacgctgacgctgacggggcgcaagccgggcgcgacccgcatcgtgctgggcaca
PBA 2860 .....t.....g.....c.....a...c.....t.....
PBB 2862 .t...tt.....c.....c...cg...t.....g.....g..c
MM5 2866 .t...tt.....c.....c...cg...t.....g.....g..c
Type (DSM1522) 2931 ggcaaggcaaagcaatacctcggcgtgcgcgtgctgcccgacgactgggacctcgacgacgtgccggccg
PBA 2930 ...c...at..a.....a.....a.....
PBB 2932 .....gc.c...g.....c.....g..g..a.....g.
MM5 2936 .....gc.c...g.....c.....g..g..a.....g.
Type (DSM1522) 3001 aacaggtcgactacgccttcctctaccggcatgtgatgagctactac-gagctc-gtgtatcccttcatg
PBA 3000 .....t.....t.....-sa.....
PBB 3002 .....t.....-.....t.....
MM5 3006 .....t.....-.....-.....
Type (DSM1522) 3069 tcggacaaggtcttcagcctggcggaccagtgcaagtgcgaaacgtattcgcgcctgatgtggcagatgt
PBA 3069 .....
PBB 3071 .....-.....a.....
MM5 3074 .....-.....a.....
Type (DSM1522) 3139 gcgatccgcagaaccgcgacaagagctactacatgcccagcacccgcgaactgtcgctgccaaagtcgcg
PBA 3139 .....a..a.....a.....
PBB 3140 .....t.....
MM5 3143 .....gc.....
Type (DSM1522) 3209 cctgttcctgaaatacctgacgcaggtcgaggcggcagccgcggccaaggcggcggcaccggaaccggcc
PBA 3209 .....c.....tg.....g....g
PBB 3210 .....------.....c....tg....cg..a..g
MM5 3213 .....c.....c.....a.g.....g.....tg.....g.....
Type (DSM1522) 3279 gcgccgcatgccatcggcggcaaggcggagttgatcgacgagctgaaaaaagccatcgatctggaactgt
PBA 3279 .t.....c..t.....a.....a.c..ac.....g.....g.....c.....
PBB 3274 .t.....c.t.....t.....c.....t.....g.....c.....g....
MM5 3283 tt.....c..t.....a.....c.g.c.....g..a.....g.....c.....

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Type (DSM1522)     3349 cgctgatgctgcaatacctgtatgccgcgtattcgattcccaattatgcgcaggggggcggcgctggtgca
PBA                3349 ..........................................................aa..........
PBB                3344 ....c.....c..g.............c.....c.........c.............aa..........
MM5                3353 ......................c.....................c.........................

Type (DSM1522)     3419 gtccggccgttggctgccggccgagctggagctggcctgcggcgccgaagaccggcgccgcaacagcggc
PBA                3419 .g.......c.....................................................................
PBB                3414 ......t..c....aa.....................t.....t.........................t
MM5                3423 .g.......c........t..........c.............a..................g.......t

Type (DSM1522)     3489 acgcgcggcgcgctgctggaaatcgcccatgaagaaatgattcactacttattggtgaacaatgtattga
PBA                3489 ........................t..g.............................a...........gc...
PBB                3484 ..a.....................................................................gc...
MM5                3493 ..........t..............g.....................t........c.........gc...

Type (DSM1522)     3559 tggcgcttggcgaaccgttttacagcggtaccccgctgctgggccagcaggcgcgccagcgtttcggcct
PBA                3559 ........g.....g.................g.....................................
PBB                3554 ....................c.........c.......g.....................g.............
MM5                3563 ........g......gg.............c..a..................................t.....

Type (DSM1522)     3629 ggacacggaatttgcgttcgaaccattttccgaacacgtgctggcccgcttcgtgcgttttgaatggccc
PBA                3629 ...t..................g.....................................c..c.........
PBB                3624 ......................g............g.....................g..c.........
MM5                3633 ......................g.........................g..............c.........

Type (DSM1522)     3699 gactacattcccacgccgggcaaatccatcgccaccttctatatcgcgatccgccaggccctggccgagc
PBA                3699 ............................g..t.....................................g..
PBB                3694 ............................g..t.................t..............g.........
MM5                3703 ......c.................g..g..t..................a.....................t.

Type (DSM1522)     3769 tgcccggcctgttcgaaagcggcggcggcaagcgcggcggcgagcaccacctgttcctgaaagaactgac
PBA                3769 .................t.....................t.....a.....tt.....................
PBB                3764 ........a........g.............................a.....tt.....................
MM5                3773 ..............t..c.........t..................a.....tt.....................

Type (DSM1522)     3839 caaccgcgcctatcccggctaccagctggaagtatccgaccgcgacagcgcgttgttcgccatcgatttc
PBA                3839 ....................................g.....................c...............
PBB                3834 ..........................................g.................c..............c...
MM5                3843 ............c.........................................c..............c...

Type (DSM1522)     3909 gtcacggaacagggcgaaggcgtggccgtcgattcgccgcatttcgcctcctcgcacttccagcggctgc
PBA                3909 ..................................................g.....................
PBB                3904 .....c........g.....................................t.....................
MM5                3913 ..................................................g.g.....t.........c....

Type (DSM1522)     3979 gcgccatcgccggcaggttttcggcctgcgacaagccgttcgaaccggcgctgccggcgctgaagaatcc
PBA                3979 ..a..g..........aa.....a.....................g.....cg.....................
PBB                3974 ................a........t..c.....................................................
MM5                3983 ..ag.g....a....a..acg.c........g...a.........g.....................................

Type (DSM1522)     4049 cgtgctggaagcgcgcgcggactgcagcgtggtgaccgatcagaaggcgcgcgcgctgatgcagctgtat
PBA                4049 ............a................c.................c.............................
PBB                4044 ......a.....................c.................a.............................c
MM5                4053 ............a..............c..............c.g.................................

Type (DSM1522)     4119 cagggctgctatgaactgaccttcctgatgatggcgcaccattttgcgcagcagccgctgggcagcctgc
PBA                4119 ..a.........c.....................................c.............................
PBB                4114 ..a.........c.....................................................................
MM5                4123 ..a.........c.........................a.........c.............................

Type (DSM1522)     4189 gccgctcgcgcctgatgaacgcgtccatcgacatcatgacaggcctgttgcgccccctgtcggcggccct
PBA                4189 ........................c.....................c.............t.............c.....
PBB                4184 ....................t.....................c.............t..g.................
MM5                4193 ....t.....................................g.............t.........a..c.....
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Type (DSM1522)     4259 gatgaacatgccgtccggcctgcctggccgccatgctggaccgcccgtgcccgagccggtcagcagccgg
PBA                4259 ..................g....c..........g..g..........a.c...c............
PBB                4254 ......................c........a..a...........a........c............
MM5                4263 ............a..g...g..g.c..........g..g..............................

Type (DSM1522)     4329 gtcagcagcgactacagcctgggctgcgacatgctggcgcagagatgcctggcgctggcgcagtacgcgc
PBA                4329 ..............................................a.........................
PBB                4324 ..............................................a.ag......a.............c.
MM5                4333 ..................a.....................a.a.........a.................

Type (DSM1522)     4399 gcagcctggagagcgatgccatcggcatggcgccgatagaaatgttggagtttttttaatcagcaacttac
PBA                4399 .......t.............................................a..................
PBB                4394 ..........a.......t.....................................................
MM5                4403 ........t.............................................t..tccgc.a..t.

Type (DSM1522)     4469 cgatttatctcggggaaagatgtcaagagaggcttgaaatgcataaaatcattatcgtcggcggaggcct
PBA                4469 .....................................................................
PBB                4464 ....................................c....................................
MM5                4473 ...-.......-..........-.....-.....-..........-.....c..t..t.g....-......

Type (DSM1522)     4539 ggcaggcagcctcagcgccatttatctggcgcaacgggggcacgatgtccacgttgtcgaaaagcgcggc
PBA                4539 ......t.................................a.............................
PBB                4534 ......t.................................a.............c.......a......
MM5                4536 ............g.t.....-...t..........c..a.............a....g..a......

Type (DSM1522)     4609 gatccgctgctggagaatgccgcaaacgccgaccccgtcaactcgcgcgccatcggcgtgagcatgacgg
PBA                4609 ............a..c..g.............g.....................................
PBB                4604 ..........a.a.agcgt....c.........t..g.........................c.c......c.
MM5                4605 ..............c.c......c.........t..g.....t.............................

Type (DSM1522)     4679 tacgcggcatcaaggccgtcctggccgccggcatcagcaagcaggagctcgaccagtgcggcgaacccat
PBA                4679 .g.....................ga..g.................a..............t........
PBB                4674 .g......g.......g..g.....g.............cg.............g.t...........g...
MM5                4675 .g................g.....a..g.a.........a.........t....t..............

Type (DSM1522)     4749 cgtcggcatggcattcagcgtgggcggccggcaccggatacgcgagctgaccccgctcgaaggcctgttc
PBA                4749 ............c.....................................a.....t...............
PBB                4744 .c.g........g..ttc...c...c..acatt.aa.g.g.....a...c...a..g.......t....t
MM5                4745 ............c..tt.................a.................................c...

Type (DSM1522)     4819 cccctgtcgctggaccgcaccgccttccagcgcctgctgaaccggcatgccgccctgcacgaggtgaagt
PBA                4819 ...........c.....................................................aa.....a.
PBB                4814 ..g............a.gt....a............t.....acct.c......ag......a.....a.
MM5                4815 ....................g.................................t.aa.....cc.....a.

Type (DSM1522)     4889 attactttgagcataaatgcctggatgtcgacctggaaagaaagatcgtgctgatccagggcccggacgg
PBA                4889 .....................................................................
PBB                4884 .c.....c.....................................ag........g.................
MM5                4885 ...........................................................g.a....a.........

Type (DSM1522)     4959 cgccttgcagaagctgcatggcgacctggtcattggcgccgacggcgcccactctgccgtgcggcgcgcc
PBA                4959 ..t.......c.a.............................................g.............
PBB                4954 ....c.....c.c.................a....c..t.............t..c........t......
MM5                4955 ....g.....c.....................................g.....g.............

Type (DSM1522)     5029 atgcaaagcggcatgcgccgtttcgagttcaggcaaagttacttccgccacggctacaagacgctggtgt
PBA                5029 ..............................c......................................
PBB                5024 ..................g..c..t..a....a......c.t.........t...........c..c....
MM5                5025 ............g.............a...........c..............................

Type (DSM1522)     5099 tgccgaacgcggcggatctgggtttcaggaaggatttgctgtacttcttcggcatggattccaagggcct
PBA                5099 .....................................................................
PBB                5094 .......t....a..gc..............a...........t.............cg..........a
MM5                5095 .......t.....c..c.........c.....................t.......................
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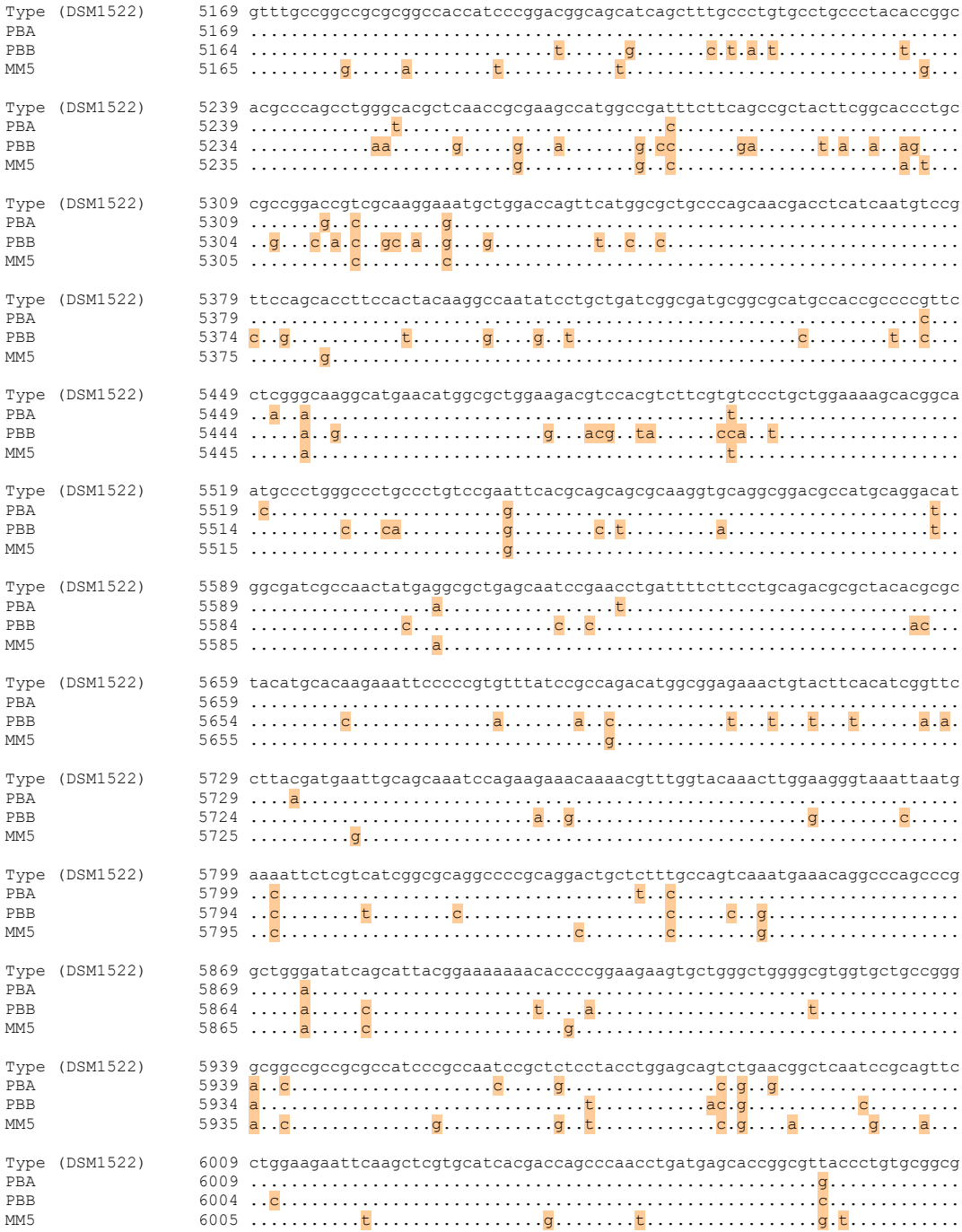
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PBA 5169
PBB 5164
MM5 5165
Type (DSM1522) 5239 acgcccagcctgggcacgctcaaccgcgaagccatggccgatttcttcagccgctacttcggcaccctgc
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PBB 5234
MM5 5235
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PBB 5374
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Type (DSM1522) 5449 ctcgggcaaggcatgaacatggcgctggaagacgtccacgtcttcgtgtccctgctggaaaagcacggca
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Type (DSM1522) 5519 atgccctgggccctgccctgtccgaattcacgcagcagcgcaaggtgcaggcggacgccatgcaggacat
PBA 5519
PBB 5514
MM5 5515
Type (DSM1522) 5589 ggcgatcgccaactatgaggcgctgagcaatccgaacctgattttcttcctgcagacgcgctacacgcgc
PBA 5589
PBB 5584
MM5 5585
Type (DSM1522) 5659 tacatgcacaagaaattcccccgtgtttatccgccagacatggcggagaaactgtacttcacatcggttc
PBA 5659
PBB 5654
MM5 5655
Type (DSM1522) 5729 cttacgatgaattgcagcaaatccagaagaaacaaaacgtttggtacaaacttggaagggtaaattaatg
PBA 5729
PBB 5724
MM5 5725
Type (DSM1522) 5799 aaaattctcgtcatcggcgcaggccccgcaggactgctctttgccagtcaaatgaaacaggcccagcccg
PBA 5799
PBB 5794
MM5 5795
Type (DSM1522) 5869 gctgggatatcagcattacggaaaaaaacaccccggaagaagtgctgggctggggcgtggtgctgccggg
PBA 5869
PBB 5864
MM5 5865
Type (DSM1522) 5939 gcggccgccgcgccatcccgccaatccgctctcctacctggagcagtctgaacggctcaatccgcagttc
PBA 5939
PBB 5934
MM5 5935
Type (DSM1522) 6009 ctggaagaattcaagctcgtgcatcacgaccagcccaacctgatgagcaccggcgttaccctgtgcggcg
PBA 6009
PBB 6004
MM5 6005

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Type (DSM1522)     6079 tcggacgccaggccctggtgcaggcactgcgcgccaagtgcgtggcggccggcatcgccatccgttacga
PBA                6079 ............t..........c.............................a.g...a.......
PBB                6074 .......g..................g.....................a.........a.g.....c..t..
MM5                6075 ....................a.....ct..................a.............a......c.....

Type (DSM1522)     6149 aacgccgccggcggacaaggcgcagctggaagccgagtacgacctggtggtggtatcgaatggcgtcaat
PBA                6149 .........a........a..............gc.a..t..............c..............
PBB                6144 ...a..........c..g.....................................c..............
MM5                6145 ..........ca.....................gc.a..................gg....c.........

Type (DSM1522)     6219 tacaaatcgctggagttgccgccagcactggcgccacacatcgatttcggccgcaacaaatacatctggt
PBA                6219 ..............c.........g..g...a....g.....................................
PBB                6214 ..............t...........g.........g.....................................
MM5                6215 ..............c.........g..g.........g...g.................................

Type (DSM1522)     6289 acggcaccacccagctgttcgaccagatgaacctggtgttccgcagcaatgagcacggcatattcatcgg
PBA                6289 ............................................................t.........
PBB                6284 ..........................................................c..a.....t.........
MM5                6285 ............................................................t.........

Type (DSM1522)     6359 ccatgcctacaaatactcggacacgatgagcacctttatcgtcgaatgcagcgaagagacgtacgccagg
PBA                6359 .........t.......................................g............a............
PBB                6354 .........t.............................................t.....a............
MM5                6355 .........t.........................g...........g.........................

Type (DSM1522)     6429 gccgggctggaggcgctgtccgagcgcgatgccgccgcgtacatcgccaaaacgttcaaggccgaactcg
PBA                6429 ............a............a.....c.....................c.........a..........
PBB                6424 ..gca.............t...........c...........t............c...g..........g.
MM5                6425 ............a......c..........c.........actg......gc.........a..........

Type (DSM1522)     6499 gtgagcatggactgcagagccaaccgggccagggctggcgcaacttcatgaccctcagccacgaccaggc
PBA                6499 .c......c..................a.............................................
PBB                6494 .......c..g....tc.....................................................g...
MM5                6495 .c..a..c.tg.....................................................g.....g...

Type (DSM1522)     6569 ctgcgacggcaagttcgtcctgctcggcgatgcgctgcaatcggggcatttttccatcggccatggcacc
PBA                6569 ..........................................................c..................
PBB                6564 ..............................................................................
MM5                6565 .a.....................................g.....a.........................

Type (DSM1522)     6639 accatggcggtggtggtcgccctgctgttggtcaaaatcctcaataccgaagacggcaaggccgccgcac
PBA                6639 ........c........ca.........c........g.....................ct....c.......g..c.
PBB                6634 ..................cg..a......c..........c....g..........c..a.gc......t..c.
MM5                6635 ..................cg.........c........g.c................c.aat.c...t.....c.

Type (DSM1522)     6709 tggacagtttcaatgcgcgtgccgtgcccctggtgcaattgttcaaggagcacgccaacaacagccgcct
PBA                6709 ........c.........c..c.....................c.......................g.........
PBB                6704 ........c......c..c......a......t.........c.......................g.........
MM5                6705 ........c.........c..c..............a..................c..........gc.........

Type (DSM1522)     6779 gtggtttgaaagcgtgggcgaacgtatcgaactgagcaatgaagagctgaccgccagcttcgacgcccgc
PBA                6779 ................c..............................................................
PBB                6774 ....................c.g........g............c.....................................
MM5                6775 ................................g.....................................................

Type (DSM1522)     6849 cgcaaggacttgccgtcgctacaagaagcgctgatggccagcctcggctacgcgctgggccgctaaggga
PBA                6849 .........a............g..g.....................t.......................-....
PBB                6844 ..............................................................................
MM5                6845 ........................g.....................................................

Type (DSM1522)     6919 gataccatgccgacacacgtctccccgccgctgctgc-cgatgcaatggagcagcgcctatgtttc-cta
PBA                6919 ..c.....................................-.............................-...
PBB                6913 .ccg.......a..c...c..g..............-.............t.....t...a.a..-...
MM5                6914 ..cg.......ag.cgc.c..a..............a..c.....................a.c..a...
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Type (DSM1522)     6987 ctggacgccgatgcaggcggatgaccaggtcacctccggctattgctggttcgactatgcgcgcaatatc
PBA                6987 ..............................g................................c...
PBB                6981 ................aa..c...........g................................c...
MM5                6984 ......a.........a...c........a..g................................c...

Type (DSM1522)     7057 tgccgcatcgacggcctgttcaacccctggtcggaaaaggaacatggacacctgctgtggatgtcggaaa
PBA                7057 ......................t........a.............c.......................
PBB                7051 ..........t.....................................................
MM5                7054 ..........t.....................................t.................

Type (DSM1522)     7127 tcggcgacgccaggcgcgaacaaagccgcaagcagaaagtggcctacgcaaggcaagcggaggcggctgg
PBA                7127 ................t.........................t.....c.....g...c....aa....
PBB                7121 ................t.........................a...a..........c..a..c.....
MM5                7124 ..................g..c.....................t.....c..........c.c.aa....

Type (DSM1522)     7197 cgagcagctgcagggcacggcgctggccgatgaggtgaccccgttccatgagctgttcctgccgcaggcg
PBA                7197 ............................c........g...........c.............a...
PBB                7191 ...a........c.........................g...........c.............a...
MM5                7194 ...a.........................c.....a..g.........c...................

Type (DSM1522)     7267 gtgctgctggacggcggtgcccgtcacgacggccgccacaccgtgctgggccgggaggcggacgcctggg
PBA                7267 ......g.c......a.................t.....g............a................
PBB                7261 a.c.....t.......cg..g........t.....................a................
MM5                7264 ......g.c.......c.....................g.....................t......a

Type (DSM1522)     7337 tagtcgagcgggcgggcaagccgccatcggtcttttacctggaggccggtggcaaccgcctgctgcgcat
PBA                7337 ...........a.....................................................t.......
PBB                7331 ....................g.ct..........c.............a.....................
MM5                7334 .........ca.................t.....................................

Type (DSM1522)     7407 ggtcaccggcaatgacccgcagcacctgtcggtacgcgactttcccaacctgtttgtcagcgacattccg
PBA                7407 ............c.....................................t......g...g...........
PBB                7401 ............c.....................................................g...........
MM5                7404 ............ctt.a...g......t..c.................t....c....g....t......

Type (DSM1522)     7477 gacagcgtctttacgtcttgcaacacctgacc-gggccgtggcgcgcgcaagggccgcatgcggc---cg
PBA                7477 ........................c.......a...a............ccc..............---a.
PBB                7471 ........t............g..........-a.ca........t...ccc..t..........---.a
MM5                7474 ........g...g........gc.......g.-at.gt...........-......a........gcg..

Type (DSM1522)     7543 gagcgcgcgccacgcctgg-caagtgtttttgttattactctcgaaggatattgatatgccgctggttgt
PBA                7544 t..................-.gg............g.....................c.................
PBB                7537 .g.................-..........a..ct.....................c.......t.......
MM5                7542 .g..a...c..c.c..cc.a.c.................................c.................

Type (DSM1522)     7612 ttacattttggggttaacgatcttttcgttaacgacatccgaattcatggtggcgggca
PBA                7613 ..............g..................c..c.......................
PBB                7606 a......c.......g........c.....g.....................a.......
MM5                7612 a......c.......g..................c..c.......................
```

N Amino Acid Sequence Alignment of Violacein Operon Sequences

Alignment of amino acid sequences of VioA

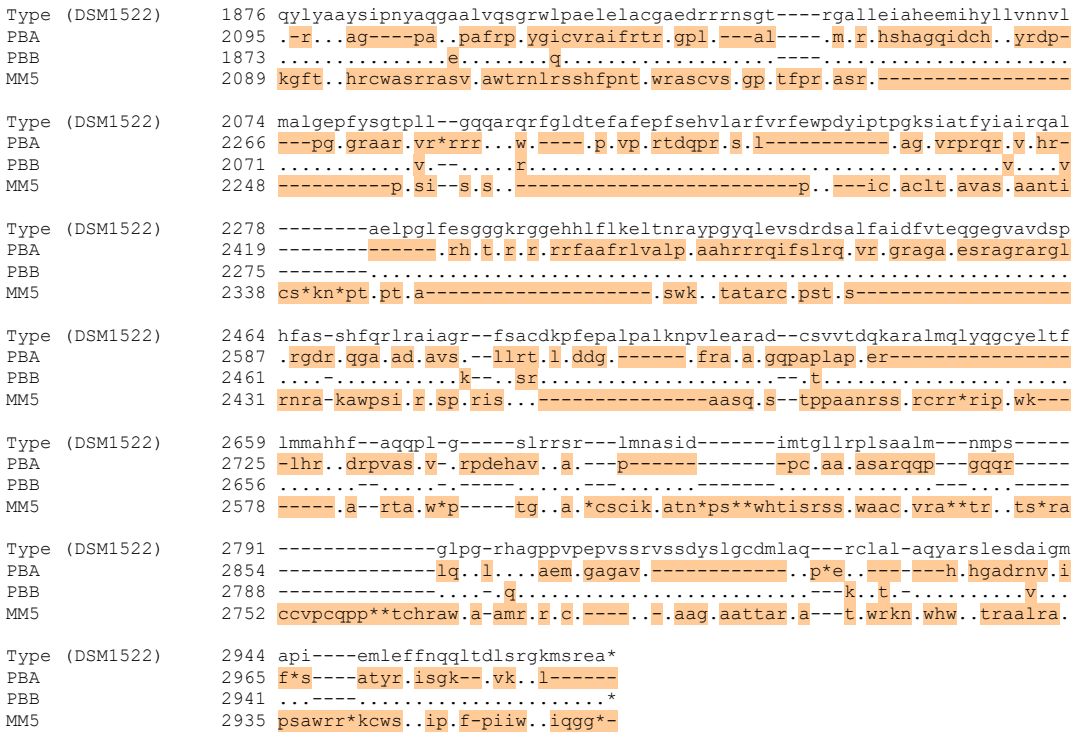
Sequence	Start	End	Match	NonMatch	%Match
Type (DSM1522)	1	1307			
PBA	1	1311	406	31	92
PBB	1	1308	418	18	95
MM5	1	1307	397	38	91

```
Type (DSM1522)        1 mstysdicivgagiggltcannlidaaagrnlrirvfdlnatvggriqsrkidgeeiaelgaaryspqlh
PBA                   1 ......................q......s.k......................n..................
PBB                   1 ......................h......s.......................k..................
MM5                   1 ......................h..hp.gn.k..........pa.......h......................

Type (DSM1522)      211 phfqqlmqgsglphavypftevishdsvleelkatldelspmlkmhpndsflefvshylgaakashiika
PBA                 211 ..........................r............g......e.......a.f........r.tq....
PBB                 211 ..................................................d.......d...........n.....
MM5                 211 ......................v......a.........n....a...h.............d..t.....

Type (DSM1522)      421 tgydalllpmvsaamaydiikkhpetqhftenaanqwhyatdgyhellcqlqhqaqvagvefrlehrlls
PBA                 421 ..............................................a...........a..............
PBB                 421 ..................................................................a.....q.......
MM5                 421 ....................................r............r.......a.....q...c...

Type (DSM1522)      631 veksgadhvlafshhgdtqmhrtrhlvmaippsamprlnldfpnawspfqydslplfkgfftfdtawwda
PBA                 631 ......e...........h.........tv.....................................
PBB                 631 ..............n.............................t.................l.........
MM5                 631 .k............l.............................h.................l........g

Type (DSM1522)      841 lgltdkvlmaanplrkiyfksdkyvlfytdsksatywrdslelgedvylervrshleevlpldgqplpqi
PBA                 841 ......................g.........................e.......................p.
PBB                 841 ......................n.................................g.................
MM5                 841 ......................g.............................................r...r.

Type (DSM1522)     1051 kahfhkfwphgvefcvepeadhpaillhrdgiiscsdaytahcgwmegslisaqhasglllqrldqrtee
PBA                1051 ......................e...v.............................q............lddv
PBB                1051 ......................e........q.........................r..............a.
MM5                1051 ......................e...v.............................r............?e?s

Type (DSM1522)     1261 -eaandtfitsstera-
PBA                1261 va..............*
PBB                1261 -..............*
MM5                1261 -a........?.....-
```

Alignment of amino acid sequences of VioB

Sequence	Start	End	Match	NonMatch	%Match
Type (DSM1522)	1	3021			
PBA	1	3019	590	507	53
PBB	1	3018	942	66	93
MM5	1	3018	564	566	49

```
Type (DSM1522)        1 mslldfprlhfrgfaranvptgnrnthgnidiatnavsmageavdlsrppaefhahlkqlaprfnaqgkp
PBA                   1 .............................................q......................
PBB                   1 ..................................................................a...
MM5                   1 ....................................l..e.n.........................e...

Type (DSM1522)      211 dpdgifsqatgynfcgnnhfswenaritgvqlrdgevdtqdalvgaklglwghyneylrttfnrarwidn
PBA                 211 ....v..l.a.h..g...................e.............r.....................
PBB                 211 ..e....l.a.....................i.....................................
MM5                 211 .........a....g.................ga.............r.....................

Type (DSM1522)      421 npaqpdttliyagqftlsdklatpntptlftadiaqahsvrwlgsghitersghfldeefgrsrlfqfsv
PBA                 421 ..........................................................................
PBB                 421 ....................r.....................................i.........
MM5                 421 ...a.....................................m..............a.......

Type (DSM1522)      631 akqdphflfnpdlplpasmhalqqaladdevlgltvqyclfnmstpqkpdspvfydlagsiglwrrgela
PBA                 631 ..........a...................d.................l.....................d...
PBB                 631 ..........a.........l..r...d.............a.............................
MM5                 631 ..........a.........q..rh.....d.........a.......l.....................

Type (DSM1522)      841 typagrllqprqgslgpvlvkvhadrvsfnmptaipfttrdagavseqhpthalggkqalgdlllhdgag
PBA                 841 ............a.......q......a..............a.....................e...r....
PBB                 841 ..........................................t.................g.........d..
MM5                 841 ............q.......q......a......va....ge.........................t.

Type (DSM1522)     1051 tvlaripeqlyrdywrhhgvfdvplqhagaapgslslgsaqaqwdeadwvlqsdsnqlyleapnrnkheq
PBA                1051 .l......p....h...........l....s-.......................g.........hk....
PBB                1051 .l......p....h.....................................................gk...e
MM5                1051 .l......p....h.....................................................gk...e

Type (DSM1522)     1261 fpqtitvqsrfrgelaappsla-eaedgvllaveqqpsplghgyttltltgrkpgatrivlgtgkakqyl
PBA                1258 .....................-q....a....q............a......q......l....qd....
PBB                1261 .....ml.........t...a..a.....a....r.......wl..........r.....v.......gt...
MM5                1261 .....ml.........t...a..a.....a....r.......wl..........r.....v.......gt...

Type (DSM1522)     1468 gvrvlpddwdlddvpaeqvdyaflyrhvmsyy------------elvypfmsdk----------------
PBA                1465 ................................*a?vslhvgqglqpgg.vqvrn----------------
PBB                1471 .........h.ee...................------------..cvslhvgq----------------
MM5                1471 .........h.ee...................------------.........rssawrtsanakrira*

Type (DSM1522)     1594 --------vfs--ladqckcetysrlmw---------------qmcd----p------------------
PBA                1627 --------..apdv..vrsa.prkk.lhaqhprtvaakiapvpeip.-----.grggsrgqgggagsggaa
PBB                1597 ---------...--.....................---------------....-----.------------------
MM5                1645 cgrcairatat--r.tt.papanc.cqs---------------ra.ssnt*.------------------

Type (DSM1522)     1663 ----qnr-----dk---------------syymps--------------------trelslpksrlflky
PBA                1801 chrq.d.tdrgae.----------------ghrpgt--------------------vadaai.vc.v.dsq
PBB                1666 ----...-----..----------------.....---------------------.............
MM5                1750 ----rs.-----rrprpgrrcrkrpcrls.aar.g*srn*krpstwncr*ccntc.prirf.tm.rgrrw

Type (DSM1522)     1741 ltqveaaaaaka--aa------------pepaaphaiggkaeli---del---kkaidlelslml-----
PBA                1906 .caggs.g.grp--l.agragaglrrrr.a.qqr..rraagnca*rn.s.liseqca.gagrav.-----
PBB                1744 .....--.....--.v------------.a..v..v..c.....---...---.............-----
MM5                1933 crpaagclpswrwp..------------.ktg.at.vraaccwk---srm---..*ftiyw.t.c*wrwa
```


Type (DSM1522) 1876 qylyaaysipnyaqgaalvqsgrwlpaelelacgaedrrrnsgt----rgalleiaheemihyllvnnvl
PBA 2095 .-r...ag----pa..pafrp.ygicvraifrtr.gpl.---al----.m.r.hshagqidch..yrdp-
PBB 1873 .....e.....q.....----.....
MM5 2089 kgft..hrcwasrrasv.awtrnlrsshfpnt.wrascvs.gp.tfpr.asr.----------
Type (DSM1522) 2074 malgepfysgtpll--gqqargrfgldtefafepfsehvlarfvrfewpdyiptpgksiatfyiairqal
PBA 2266 ---pg.graar.vr*rrr...w.----.p.vp.rtdqpr.s.l----------.ag.vrprqr.v.hr-
PBB 2071 .....v.--.....r.....v....v
MM5 2248 ----------p.si--s.s..----------p..---ic.aclt.avas.aanti
Type (DSM1522) 2278 --------aelpglfesgggkrggehhlflkeltnraypgyqlevsdrdsalfaidfvteqgegvavdsp
PBA 2419 --------------.rh.t.r.r.rrfaafrlvalp.aahrrrqifslrq.vr.graga.esragrargl
PBB 2275 --------.....
MM5 2338 cs*kn*pt.pt.a----------.swk..tatarc.pst.s----------
Type (DSM1522) 2464 hfas-shfqrlraiagr--fsacdkpfepalpalknpvlearad--csvvtdqkaralmqlyqgcyeltf
PBA 2587 .rgdr.qga.ad.avs.--llrt.l.ddg.------.fra.a.gqpaplap.er----------
PBB 2461 ....-.....k--..sr.....--.t.....
MM5 2431 rnra-kawpsi.r.sp.ris...----------aasq.s--tppaanrss.rcrr*rip.wk---
Type (DSM1522) 2659 lmmahhf--aqqpl-g-----slrrsr---lmnasid-------imtgllrplsaalm---nmps-----
PBA 2725 -lhr..drpvas.v-.rpdehav..a.---p------------pc.aa.asarqqp---gqqr-----
PBB 2656 .....--.....-.-----.....---.....-------.....---....-----
MM5 2578 -----.a--rta.w*p-----tg..a.*cscik.atn*ps**whtisrss.waac.vra**tr..ts*ra
Type (DSM1522) 2791 --------------glpg-rhagppvpepvssrvssdyslgcdmlaq---rclal-aqyarslesdaigm
PBA 2854 --------------lq..l....aem.gagav.----------..p*e..---k..t.-.....v...
PBB 2788 --------------....-.q.....
MM5 2752 ccvpcqpp**tchraw.a-amr.r.c.----..-.aag.aattar.a---t.wrkn.whw..traalra.
Type (DSM1522) 2944 api----emleffnqqltdlsrgkmsrea*
PBA 2965 f*s----atyr.isgk--.vk..l------
PBB 2941 ..-----.....*
MM5 2935 psawrr*kcws..ip.f-piiw..iqgg*-

Alignment of amino acid sequences of VioC

Sequence	Start	End	Match	NonMatch	%Match
Type (DSM1522)	1	1290			
PBA	1	1290	424	6	98
PBB	1	1290	364	66	84
MM5	1	1287	399	32	92

```
Type (DSM1522)        1 mhkiiivggglagslsai-ylaqrghdvhvvekrgdpllenaanadpvnsraigvsmtvrgikavlaagi
PBA                   1 ................-.............................................g...
PBB                   1 ................-.................qkas.............t.....v.........
MM5                   1 ...sf--swarp.rqprch...........i.........dt...........................d.

Type (DSM1522)      208 skqeldqcgepivgmafsvggrhrireltpleglfplsldrtafqrllnrhaalhevkyyfehkcldvdl
PBA                 208 ..................................................k..................
PBB                 208 .r...el...r.l........rtfkv...pqr..........s.......ty..r..............
MM5                 205 ......l..........c.....................................vk.a..............

Type (DSM1522)      418 erkivliqgpdgalqklhgdlvigadgahsavrramqsgmrrfefrqsyfrhgyktlvlpnaadlgfrkd
PBA                 418 ............v..q.....................................................
PBB                 418 ...v..v.........h.....i.........................k..f.............eg......
MM5                 415 ......v.d....v.q.........................v..............................

Type (DSM1522)      628 llyffgmdskglfagraatipdgsisfalclpytgtpslgtlnreamadffsryfgtlppdrrkemldqf
PBA                 628 ...........................................................e.........
PBB                 628 .......a...q.............v..vi....s.....n.....t..a..d..ydk..aas.q..v...
MM5                 625 ..........................................................n.......d.....

Type (DSM1522)      838 malpsndlinvrsstfhykanilligdaahatapflgqgmnmaledvhvfvsllekhgnalgpalseftq
PBA                 838 ................................................................................
PBB                 838 i...................g.v.........................t.i.at.........a.t.....h
MM5                 835 ..............a.............................................................

Type (DSM1522)     1048 qrkvqadamqdmaianyealsnpnlifflqtrytrymhkkfprvyppdmaeklyftsvpydelqqiqkkq
PBA                1048 ..........................................................*.............
PBB                1048 l................................n....q....i......d..f...i.............
MM5                1045 ................................................................................

Type (DSM1522)     1258 nvwyklgrvn*
PBA                1258 ..........*
PBB                1258 ..........*
MM5                1255 ..........*
```

Alignment of amino acid sequences of VioD

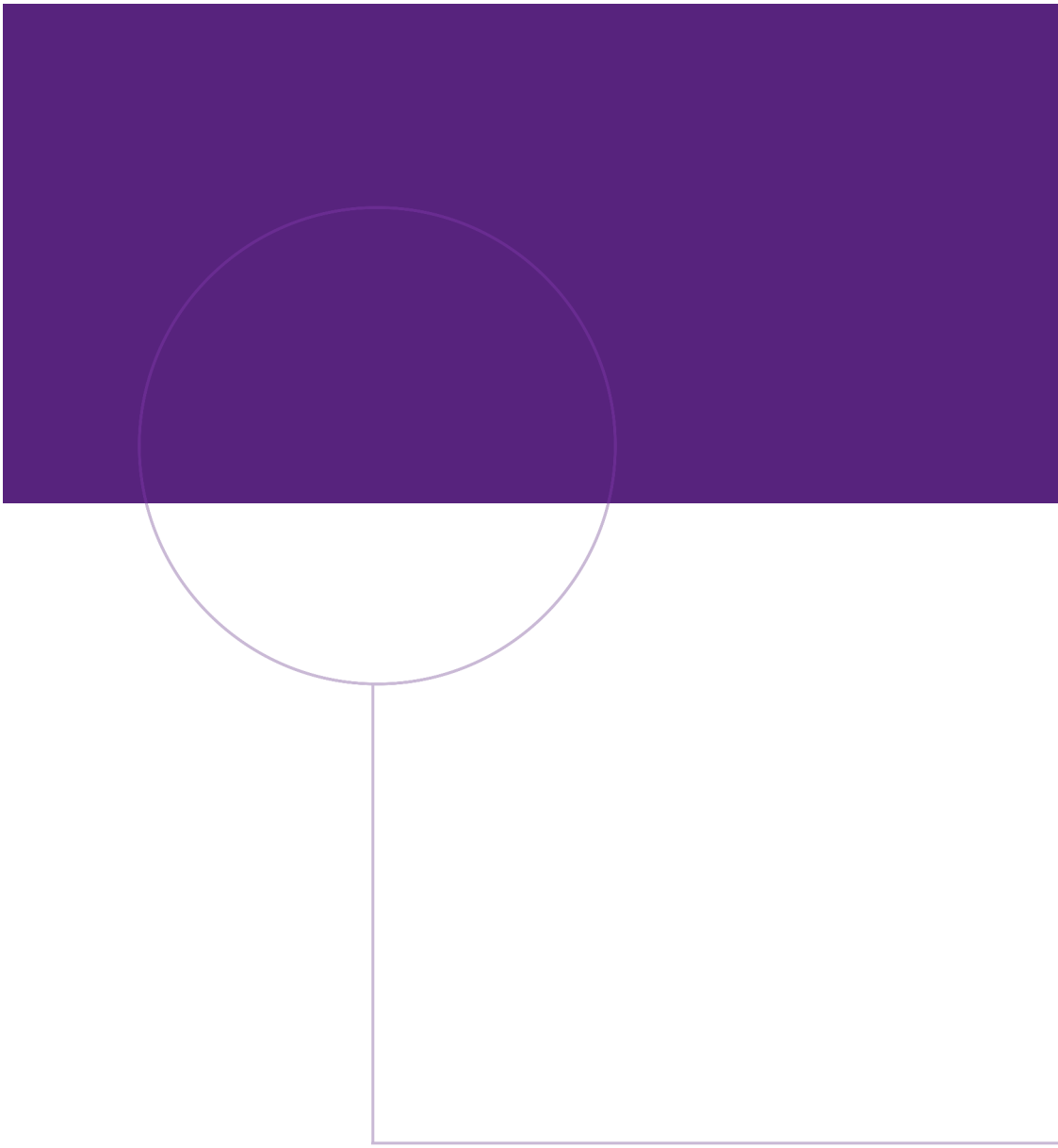
Sequence	Start	End	Match	NonMatch	%Match
Type (DSM1522)	1	1119			
PBA	1	1119	359	14	96
PBB	1	1119	348	25	93
MM5	1	1119	343	30	91

```
Type (DSM1522)        1 mkilvigagpagllfasqmkqaqpgwdisitekntpeevlgwgvvlpgrpprhpanplsyleqserlnpq
PBA                   1 .n.....................n.....................................p......
PBB                   1 .n.....................n.r.....q.............................p......
MM5                   1 .n.....................n.r.................................p.q..a.

Type (DSM1522)      211 fleefklvhhdqpnlmstgvtlcgvgrqalvqalrakcvaagiairyetppadkaqleaeydlvvvsngv
PBA                 211 ..........................................t.s.............q..........
PBB                 211 ..........................................t........ar.....................
MM5                 211 ..........e.........i.....................d.......q.......q......a...

Type (DSM1522)      421 nykslelppalaphidfgrnkyiwygttqlfdqmnlvfrsnehgifighaykysdtmstfivecseetya
PBA                 421 .....d.....t.....................................................
PBB                 421 .....d.....................................dq.....................
MM5                 421 .....d.........v.....................................................

Type (DSM1522)      631 raglealserdaaayiaktfkaelgehglqsqpgqgwrnfmtlshdqacdgkfvllgdalqsghfsighg
PBA                 631 ..................n.....................................................
PBB                 631 ..q..................e.........l.................r.....................
MM5                 631 .......p......tv.s..........v..................r.r.s.....................

Type (DSM1522)      841 ttmavvvalllvkilntedgkaaaldsfnaravplvqlfkehannsrlwfesvgerielsneeltasfda
PBA                 841 ......a...........a.t.....................s.........................
PBB                 841 ......a.......t.d..ada.....................s.........q..g...a........
MM5                 841 ......a......t....ant..............m....d...a.........................

Type (DSM1522)     1051 rrkdlpslqealmaslgyalgr*
PBA                1051 ...e..................*
PBB                1051 .....................*
MM5                1051 .....................*
```

Alignment of amino acid sequences of VioE

Sequence	Start	End	Match	NonMatch	%Match
Type (DSM1522)	1	582			
PBA	1	582	183	11	94
PBB	1	582	180	14	92
MM5	1	584	37	192	16

```
Type (DSM1522)        1 mpthvsppllpmqwssa-yvsyw---------------tpmqaddqvtsgycwfdyarni-----cr---
PBA                   1 .................-.....---------------.....................-----..---
PBB                   1 ....la...........-.i...---------------....e.................-----..---
MM5                   1 ..aalt....haameqrl.lttghrcrrttr*rpaiagst.r.tsaasmacstpgrk..mdtccg..ksa

Type (DSM1522)      139 idglfnpwsek--ehghllwmseigdarreqsr-kqkvayarqaeaageqlqgtaladevtpfhelflpq
PBA                 139 ...........--.....................-..........q.t.................d.....
PBB                 139 ...........--.....................-......t..........h.............d.....
MM5                 211 tp.astaa.r.wltp.krrqlanscr...wpt.*rrsmrcscrrrcwsta.pv.ta.tacwagrr-----

Type (DSM1522)      340 avlldg-garhdgrhtv-----lgreadaw--vveragkppsvfyleaggnrllrmvtgndpqhlsvrdf
PBA                 340 ...v..-s.....l.s.-----.........--....t.................................
PBB                 340 .i....-a.........-----..q.....--.......as.............................
MM5                 406 ---mp.*ssqras..lsftwrpvatacc..spats.gtf.yat.-----------------.ic..a-i.

Type (DSM1522)      526 pnlfvsdipdsvftscnt*
PBA                 526 ...l.g...........p*
PBB                 526 .....g.........d.*
MM5                 553 rtaclrla.p---------
```



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