Introductory studies on bacterial agents infecting cleaner fish

Philosophiae Doctor (PhD) Thesis

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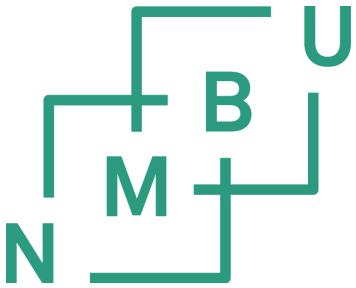

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“I'd take the awe of understanding over the awe of ignorance any day.”

- Douglas Adams (author, 1952-2001)

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Oslo, October 2015
Snorre Gulla

List of abbreviations

16S rDNA	16S (Svedberg) ribosomal ribonucleic acid genes
16S rRNA	16S (Svedberg) ribosomal ribonucleic acid
A^-	*Aeromonas salmonicida* lacking the Additional layer protein
A^+	*Aeromonas salmonicida* possessing the Additional layer protein
AGD	Amoebic gill disease
A-layer	Additional layer protein (*A. salmonicida*: virulence array protein)
atpA	α-subunit of bacterial adenosine triphosphate synthase (gene)
BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
CBB	Coomassie Brilliant Blue agar
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
ftsZ	Cell division protein (gene)
in vitro	Within an artificial environment
in vivo	Within a living organism
L.	Linnaeus
LPS	Lipopolysaccharide
ML	Maximum Likelihood
MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
NVI	The Norwegian Veterinary Institute
PCR	Polymerase chain reaction
pyrH	Uridylate kinase (gene)
qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
rpoA	Ribonucleic acid polymerase α-subunit (gene)
rpoD	Ribonucleic acid polymerase σ-factor (gene)
sensu lato	In the wide/broad sense
sensu stricto	In the narrow/strict sense
S-layer	Surface layer
sp.	Species (singular)
spp.	Species (plural)
subsp.	Subspecies (singular)
vapA	Virulence array protein (gene)
VBNC	Viable but non-culturable
vibriostatic agent O/129	2,4-diamino-6,7-diisopropyl-pteridine phosphate
WGS	Whole Genome Sequencing

List of papers

Paper I:

***Aeromonas salmonicida* infection levels in pre- and post-stocked cleaner fish assessed by culture and an amended qPCR assay**

Authors: Snorre Gulla, Samuel Duodu, Arve Nilsen, Inge Fossen, Duncan J. Colquhoun

Published: Journal of Fish Diseases, 2015 (*Epub ahead of print; DOI: 10.1111/jfd.12420*)

Paper II:

***vapA* (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes**

Authors: Snorre Gulla, Vera Lund, Anja B. Kristoffersen, Henning Sørum, Duncan J. Colquhoun

Published: Journal of Fish Diseases, 2015 (*Epub ahead of print; DOI: 10.1111/jfd.12367*)

Paper III:

Phylogenetic analysis and serotyping of *Vibrio splendidus*-related bacteria isolated from salmon farm cleaner fish

Authors: Snorre Gulla, Henning Sørum, Øyvind Vågnes, Duncan J. Colquhoun

Published: Diseases of Aquatic Organisms, 2015, 117(2):121-131

Summary

Cleaner fish (wrasse and lumpsucker) are used extensively in Norwegian salmon farms for biocontrol of sea louse infestation, but cleaner fish health and welfare has only recently become a focus of attention. Bacterial diseases represent one of the main threats to the sustained use of cleaner fish, and atypical *Aeromonas salmonicida* and *Vibrio splendidus*-related strains remain the most commonly isolated bacterial agents in this regard. While *A. salmonicida* is an established fish pathogen, the relevance of *V. splendidus* for fish disease is less clear. Development of vaccines against bacterial pathogens of cleaner fish may be needed, but more knowledge is required regarding strains associated with these fish.

In the present study, which focused mainly on wrasse, we examined bacterial infection levels in cleaner fish before and after salmon cage stocking, by culture and *A. salmonicida* real-time quantitative PCR (qPCR). Cleaner fish isolates of *A. salmonicida* and *V. splendidus* were characterised by virulence gene (*vapA*) typing, and Multilocus Sequence Analysis (MLSA) in addition to serotyping, respectively. In both cases, isolates of distinct spatiotemporal- and biological origin were also included for comparison.

Our results showed that systemic bacterial infections in Norwegian cleaner fish are largely contracted after salmon farm stocking. In the particular case of *A. salmonicida*, we found (by qPCR) infection levels of <4% before stocking, and 68% in diseased cleaner fish post stocking in salmon farms. Sequence analysis of the *vapA* (A-layer) gene further revealed that 99% of the examined *A. salmonicida* cleaner fish isolates belonged to two distinct subtypes (A-layer types V and VI). In contrast, *V. splendidus*-related cleaner fish isolates showed extensive genetic microdiversity, as well as antigenic dissimilarity. Such strain diversity was also often observed amongst isolates from individual mortality episodes.

Overall, our findings suggest that vaccination of cleaner fish against bacterial pathogens prior to salmon farm stocking may be useful, as infection primarily occurs post capture. Vaccine strain selection for the established fish pathogen *A. salmonicida* seems relatively straightforward, considering the overwhelming dominance of two subtypes in Norwegian cleaner fish. Whether vaccination against *V. splendidus* would help is unclear however, and a lack of strain clonality, even within clinical cases, suggests that fish-to-fish transmission is not the main route by which these agents spread. Perhaps more likely, *V. splendidus* infection in cleaner fish reflects opportunistic invasion of weakened hosts by environmental and/or commensal bacteria.

Sammendrag (Summary in Norwegian)

Rensefisk (leppefisk og rognkjeks) brukes hyppig i norsk lakseoppdrett for biologisk kontroll av lakselus, men hensynet til rensefiskens helse og velferd har inntil relativt nylig i stor grad blitt oversett. Bakterielle sykdommer utgjør i dag kanskje den største utfordringen med tanke på bærekraftig bruk av rensefisk i Norge, og de hyppigst isolerte agensene i så måte er stammer av atypisk *Aeromonas salmonicida* og *Vibrio splendidus*-beslektede bakterier. Mens *A. salmonicida* er en velkjent fiskepatogen, er situasjonen mer uklar for *V. splendidus*. Utvikling av vaksiner til rensefisk mot bakterielle sykdommer kan være nødvendig, men det trengs mer kunnskap om stammer som infiserer disse fiskene.

Med fokus på leppefisk utførte vi, ved hjelp av bakteriekultur og *A. salmonicida* sanntids kvantitativ PCR (qPCR), en screening av bakterielle infeksjonsnivåer i rensefisk før og etter merdsetting. Isolater av *A. salmonicida* og *V. splendidus* fra rensefisk ble karakterisert med henholdsvis virulensgen (*vapA*) typing, og Multilokus sekvensanalyse (MLSA) samt serotyping. I begge tilfeller ble en rekke isolater fra andre opphav (tid, sted, biologisk) også inkludert for sammenligning.

Resultatene viser at norsk rensefisk i all hovedsak blir infisert med bakterielle agens etter utplassering i laksemerd. Med tanke på *A. salmonicida* spesielt fant vi (ved qPCR) <4% positive prøver før merdsetting, og 68% positive prøver fra syk rensefisk i laksemerd. Sekvensanalyse av *vapA* (A-lags) genet viste videre at 99% av de undersøkte *A. salmonicida* isolatene fra rensefisk tilhørte to spesifikke subtyper (A-lagstyper V og VI) av bakterien. Dette stod i kontrast til *V. splendidus*-beslektede isolater fra rensefisk, som viste stor grad av genetisk mikrodiversitet, så vel som antigeniske forskjeller. Slike stammeforskjeller ble også observert blant isolater fra samme sykdomsutbrudd.

I sum antyder funnene våre at vaksinering av rensefisk mot bakterielle patogener før utsett i laksemerd kan være nyttig, da infeksjon hovedsakelig inntrer senere. Når det gjelder den kjente fiskepatogenen *A. salmonicida* fremstår seleksjon av vaksinestammer som relativt enkelt, ettersom to subtyper er nesten utelukkende dominerende blant norsk rensefisk. Det er imidlertid mer usikkert om vaksinering mot *V. splendidus* vil hjelpe, og den store diversiteten, selv innad i kliniske utbrudd, indikerer at direkte smitte fra fisk til fisk antagelig ikke er den viktigste spredningsruten for disse bakteriene. *V. splendidus* infeksjon i rensefisk kan vel så sannsynlig reflektere opportunistisk invasjon av svekkede verter med miljøbakterier og/eller kommensaler.

1. Introduction

1.1. Norwegian salmon farming – a brief disease history

Since its modest beginning in the late 1960s (review: Liu et al. 2011), farming of Atlantic salmon, *Salmo salar* (L.), has now grown to become one of Norway's most profitable export industries, with over one million tonnes of salmon being sold annually in recent years (Norwegian Directorate of Fisheries 2015). Development of the industry has not been problem free, however, with infectious diseases representing one of the main constraints.

During the 1980s and early 1990s the main health concerns were related to bacterial diseases, including furunculosis (*Aeromonas salmonicida*), coldwater vibriosis (*Vibrio salmonicida*[1]), and vibriosis (*Vibrio anguillarum*) (Austin & Austin 2007). Following the introduction of commercial oil-adjuvanted injection vaccines in the fall of 1992 however, many bacterial diseases have in effect been eradicated from salmon farming (Press & Lillehaug 1995, Markestad & Grave 1997, Sommerset et al. 2005). This is well illustrated by the dramatic decline in antibiotic consumption in Norwegian aquaculture, despite the steep and continuing increase in production (Figure 1). Multivalent vaccination regimes are today an integral part of farmed salmon production in Norway.

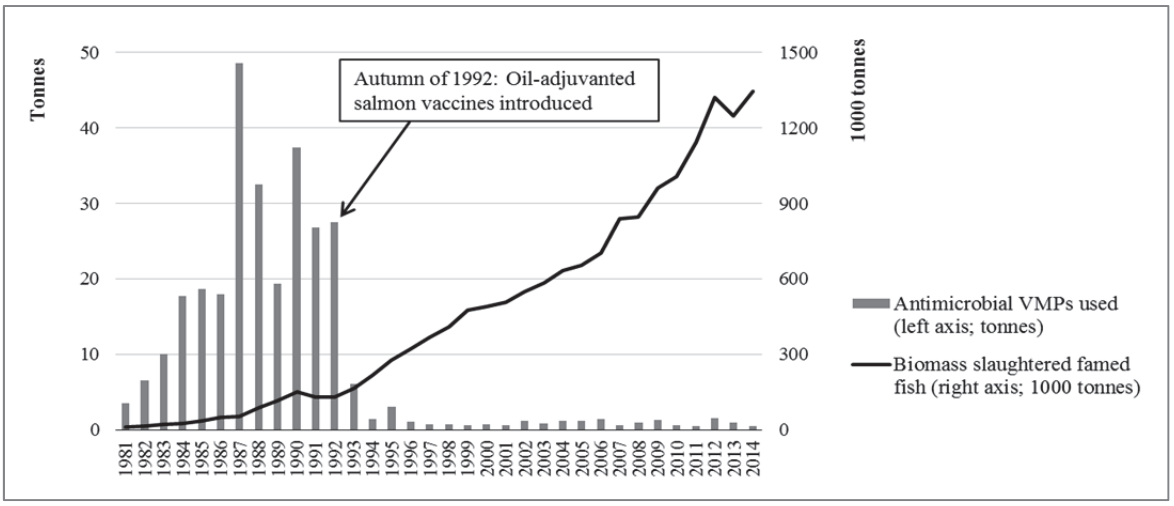

Figure 1: Total production of farmed fish (mainly Atlantic salmon) versus total sales, in tonnes of active substance, of antimicrobial veterinary medicinal products (VMPs) in Norwegian aquaculture in the period 1981-2014. Modified after Figure 5 in NORM/NORM-VET (2015), with statistics provided by Kari Grave.

[1] Members of the genus *Aliivibrio* will be referred to here as *Vibrio* spp. This in order to avoid possible confusion following abbreviation of *Aliivibrio salmonicida* and *Aeromonas salmonicida*.

In recent years, viral agents have dominated diagnoses made by the Norwegian Veterinary Institute (NVI) (Johansen 2013, Hjeltnes 2014, Bornø & Lie Linaker 2015). The most important viral diseases in this regard are pancreas disease (PD), infectious salmon anaemia (ISA), cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation (HSMI), and infectious pancreatic necrosis (IPN). Amoebic gill disease (AGD), caused by the parasite *Paramoeba perurans*, has also recently emerged as an important salmon disease in Norway (Bornø & Lie Linaker 2015). While these infectious agents are undoubtedly important in terms of disease-related losses in Norwegian salmon farming today, the parasite *Lepeophtheirus salmonis* (Krøyer) represents, perhaps, an even greater problem.

1.2. *Lepeophtheirus salmonis* – the salmon louse

1.2.1. The parasite and its impact

L. salmonis is a marine copepod ectoparasite with a circumpolar distribution in the Northern hemisphere (Torrissen et al. 2013). It is a natural parasite of salmonids, and attaches to, and feeds off the skin, mucus and blood of its hosts. Heavy lice burdens can result in significant epidermal- and dermal erosions, which may ultimately kill the fish e.g. due to osmoregulatory failure or secondary infections (Grimnes & Jakobsen 1996, Bowers et al. 2000). In modern aquaculture however, the situation is more commonly characterised by low level infection of an extremely large number of hosts, which may not necessarily be directly detrimental to the health and welfare of individual farmed fish.

The main perceived problem today lies in the potential for spread of lice from the ever increasing population of farmed salmon to wild anadromous salmonids (Costello 2009). Johansen et al. (2011) estimated the standing stock of farmed salmon in Norway to outnumber wild Atlantic salmon returning from sea migration by a factor of 250-700. In fjords with intensive salmon farming, very high lice numbers are also regularly reported in sea trout, *Salmo trutta trutta* (L.), which typically remain in coastal inshore waters during their marine residence (Thorstad et al. 2015).

In order to reduce infection levels in farms and minimise infection pressures towards wild salmonids, compulsory lice counts are required in Norwegian salmon farms every one or two weeks, depending on water temperature. On identification of >0.5 adult female lice per fish, the affected facility must by law initiate corrective measures (Lovdata.no 2012). These measures may be either pharmaceutical or non-pharmaceutical, and may in some serious cases involve stamping out and prolonged fallowing.

1.2.2. Salmon lice control measures

Pharmaceutical treatment and drug resistance

Traditionally, pharmacological agents have been the main tool for combatting salmon lice infections in Norwegian aquaculture. These can be administered as bath treatments (e.g. pyrethroids, organophosphates and hydrogen peroxide) or orally through the feed (e.g. avermectins and chitin synthesis inhibitors). While pyrethroids, organophosphates and avermectins all act by interfering with neuronal signal pathways in the salmon louse, chitin synthesis inhibitors prevent formation of the parasite's exoskeleton, and hydrogen peroxide (H_2O_2), being a strong oxidizer, disrupts cell membranes (Torrissen et al. 2013). No new pharmacological delousing agents have been commercially launched since the introduction of emamectin benzoate (an avermectin) in 1999 (Stone et al. 2000, Torrissen et al. 2013). Figure 2 shows annual use of the main pharmacological delousing agents in Norwegian aquaculture in recent years.

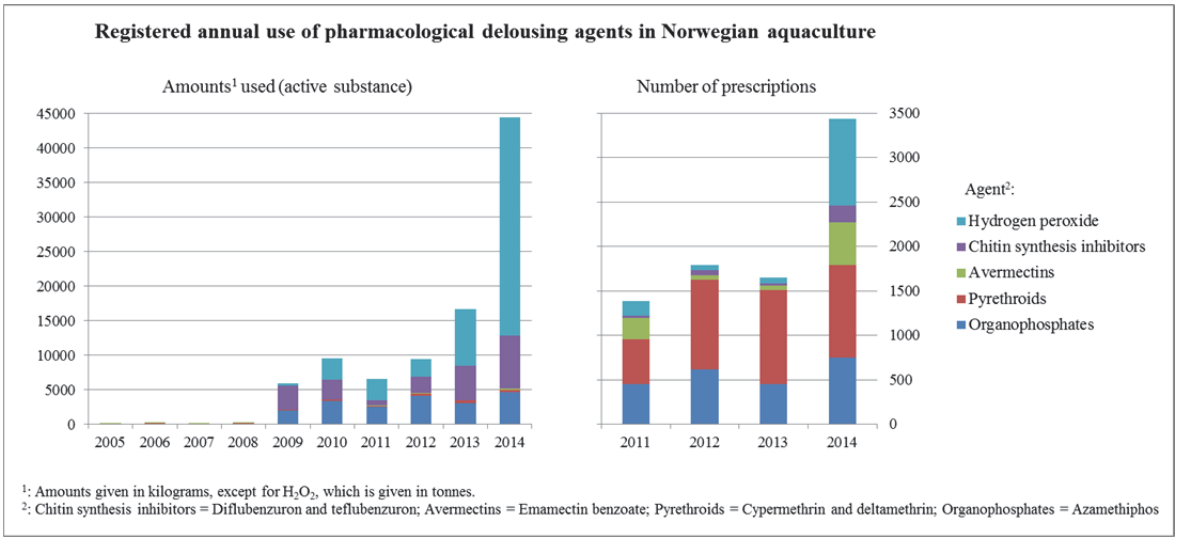

Figure 2: Registered annual use of pharmacological delousing agents in Norway (aquaculture only) in recent years. Stacked bar charts show (on left) the amounts (kilograms; tonnes for H_2O_2) of active substance used (Norwegian Institute of Public Health 2015) and (on right) the number of prescriptions (Grøntvedt, Jansen, et al. 2015). It should be noted that H_2O_2 is also used for AGD treatment.

The dramatic increase in use of pharmaceuticals for delousing from 2008 onwards, has resulted mainly from an increasing prevalence of drug-resistance in Norwegian salmon louse populations. Currently, examples of resistance and/or reduced sensitivity to almost all available drug groups have been reported (Denholm et al. 2002, Sevatdal et al. 2005, Espedal et al. 2013, Aaen et al. 2015, Helgesen et al. 2015). While the 'Surveillance programme for resistance to chemotherapeutants in salmon lice' (Grøntvedt, Jansen, et al.

2015) reported geographical variation with regard to type of resistance, reduced sensitivity to one or more therapeutic agent(s) is generally widespread along the Norwegian coast. In order to halt, and ideally reverse, this trend, implementation of non-pharmaceutical delousing measures is sorely needed.

Non-pharmaceutical alternatives

Various non-pharmaceutical approaches for combatting salmon lice have been tested and/or are under development (Torrissen et al. 2013). These involve e.g. use of cleaner fish (see below), immunological interference through the feed (EWOS 2011), cage design (snorkels, skirts, closures; Norwegian Research Council 2014), physical/mechanical removal with water jets (Nilsen et al. 2010), thermal removal using warm water (Grøntvedt, Nerbøvik, et al. 2015), laser removal (Optics.org 2014), and selective salmon breeding for louse resistance (Jones et al. 2002). None of these strategies alone have yet produced satisfactory results however, and effective vaccination against salmon lice remains a distant goal.

1.3. Cleaner fish used for salmon delousing

1.3.1. History of use and relevant species

Cleaning symbioses between some European wrasse (Labridae) species (the cleaners) and ectoparasite-infested fish of other species (the clients) have long been documented in both wild and aquaria-held fish (Potts 1973, Hilldén 1983). The ability of wrasse to cleanse lice-infested salmon was first described in the late 1980s (Bjordal 1988), and several full-scale trials were attempted with relative success (Bjordal 1992, Deady et al. 1995, Kvenseth 1996, Treasurer 1996). Nevertheless, it was not until the drug resistance problems accelerated that the demand for cleaner fish increased in earnest in Norway (Figure 3).

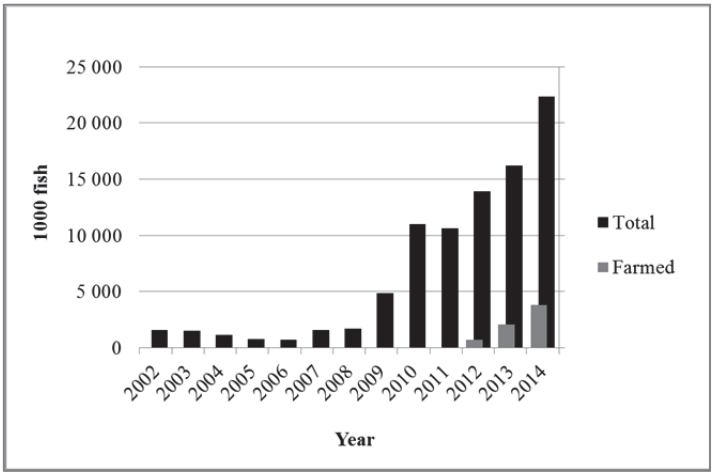

Figure 3: Registered use of cleaner fish (total and farmed) in Norwegian salmon farms in the period 2002-2014 (Norwegian Directorate of Fisheries 2015). Numbers stated in thousand individuals.

As of week 43 in 2015, 63% of Norwegian salmon farms reported active use of cleaner fish for combatting salmon louse infestation (Lusedata.no 2015). Stocking proportions may range from 2-10 cleaner fish per 100 salmon (Lusedata.no 2014a, b, Nilsen et al. 2014), depending on species used, water temperature, and louse burden.

Wrasse

Wrasse species currently dominate in terms of numbers of cleaner fish used, and the majority are wild-caught. Of the wrasse species indigenous to Norwegian coastal waters, three are most sought after for cleaning purposes, i.e. the ballan-, *Labrus bergylta* (Ascanius), goldsinny-, *Ctenolabrus rupestris* (L.), and corkwing-, *Symphodus melops* (L.), wrasses (Figure 4). Rock cook, *Centrolabrus exoletus* (L.), and cuckoo wrasse, *Labrus mixtus* (L.), are used to a lesser extent.

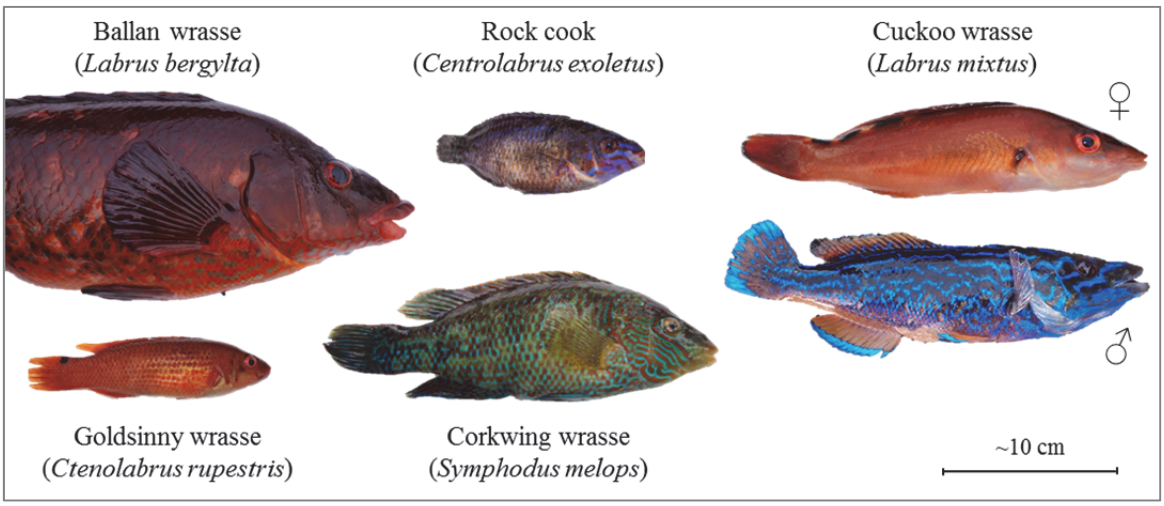

Figure 4: Norwegian wrasse species (adult specimens) caught for use as cleaner fish in salmon farms. Photos: Snorre Gulla.

They all represent benthic, inshore fish species, dwelling e.g. in kelp forests during the warmer seasons (Sayer & Reader 1996, Espeland et al. 2010, Skiftesvik, Durif, et al. 2014). Much is still unknown about their ecology, but at water temperatures roughly below 5-7°C, several species have been shown to enter a state of reduced physiological activity (torpor), accompanied by movement to deeper waters (Espeland et al. 2010, Lusedata.no 2014a). Norwegian wrasse species do not migrate over long distances however, and initially conspecific populations may, as a result of long term geographical separation, have evolved into distinct subspecies/races (Sundt & Jørstad 1993, 1998, Espeland et al. 2010).

Lumpsucker

In recent years, the lumpsucker, *Cyclopterus lumpus* (L.), (Figure 5) has also been introduced with success as a cleaner fish species in Norway (Imsland et al. 2014). Only relatively young (small) specimens are used for this purpose. While lumpsuckers and wrasses are not particularly close relatives (belonging to the orders Scorpaeniformes and Perciformes, respectively), both appear able to fulfil the cleaner fish role. The main perceived advantage of the lumpsucker compared to the wrasses, is its maintained lice-eating activity at lower water temperatures (Imsland et al. 2014), which extends its geographical- and seasonal applicability in Norwegian salmon farms. Lumpsuckers used for cleaning purposes in Norway are exclusively of farmed origin.

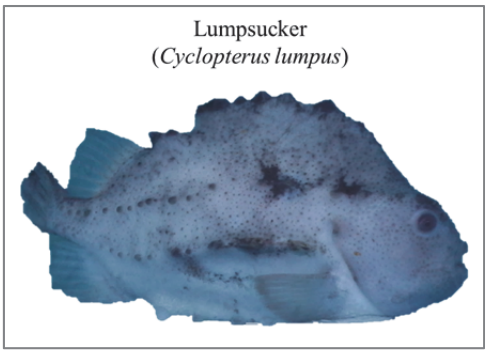

Figure 5: Adult female lumpsucker. Photo: Snorre Gulla.

1.3.2. Supply channels and associated risks

Wild-caught wrasse currently constitute the majority of cleaner fish used in Norway, with an estimated 18 million[2] being harvested in 2014 (Norwegian Directorate of Fisheries 2015). The capture season is restricted to the summer months, with legally regulated regional differences. The supply chain usually involves capture in creels or fyke nets, storage for up to one week in makeshift holding-pens, and transport to- and stocking in salmon farms following on-site health checks/certification. While some batches, especially in Western Norway, are transported only short distances or delivered directly after capture, batches from Southern Norway and South-western Sweden are often transported long distances by truck to Central Norway for stocking (Skiftesvik, Blom, et al. 2014). Detailed guides for capture, storage, transport and delivery/receipt of cleaner fish have been developed (Lusedata.no 2014c, d, e), and are publicly available online (www.lusedata.no).

The effects of intensive harvesting of autochthonous and largely uncharted wild wrasse populations have not been thoroughly investigated (Skiftesvik, Durif, et al. 2014).

[2] Estimate based on ~22 mill. total minus ~4 mill. farmed, the latter of which may be an underestimate.

Furthermore, while transmission of infectious diseases to salmon has not (to my knowledge) been unambiguously linked to introduction of cleaner fish, the potential for this cannot be discounted (Treasurer 2012). Although wild-caught wrasse continue to dominate, farming of both ballan wrasse and lumpsucker has been initiated to meet the growing demand. Increased production of farmed cleaner fish will facilitate improved infection control and a less season-dependent delivery, while also relieving the exploitation pressure imposed upon wild wrasse populations (Treasurer 2012). Production is increasing (Figure 3) and in 2014, 3.8 million farmed cleaner fish were reportedly sold to Norwegian salmon farms (Norwegian Directorate of Fisheries 2015). This is almost certainly an underestimate however (personal communication with D. Knappskog, Vaxxinova). Lumpsucker account for approximately 90% of current farmed cleaner fish production in Norway (Norwegian Directorate of Fisheries 2015).

1.3.3. Best practice care – health and welfare considerations

The importance of good health and welfare of cleaner fish in captivity has only relatively recently become recognised. Species-specific (wrasse and lumpsucker) best practice guidelines for care and handling of cleaner fish in salmon farms are now available online at www.lusedata.no (Lusedata.no 2014a, b, f). Topics covered include the need for species-adapted refuges (e.g. mimicking kelp forests; Figure 6), stocking proportions, fish-/mesh size considerations, health monitoring, implementation of supplemental feeding regimes, and fouling removal.

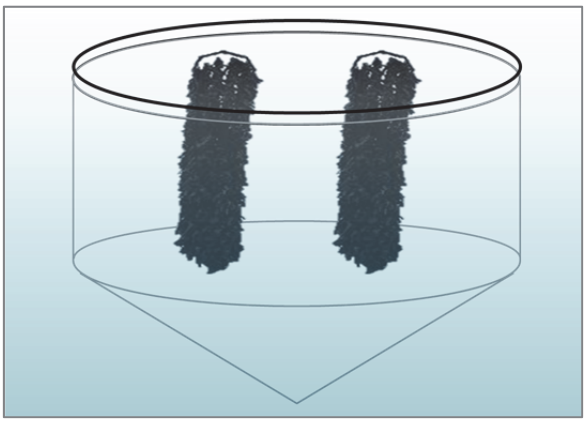

Figure 6: Illustration of a salmon cage with synthetic 'kelp forest' refuges installed to improve cleaner fish welfare. Illustration: Snorre Gulla.

While efforts are made to facilitate good health and welfare, intensive use of cleaner fish is a relatively new practice in Norwegian salmon farming, and much optimisation work remains. The supply chain for wild wrasse undoubtedly involves substantial physical- and mental stress, and stocking together with large predators in the alien salmon cage

environment must represent a stressful transition for all cleaner fish species. Variations in spawning time, during which the fish may be highly territorial and vulnerable to stress (Espeland et al. 2010, Skiftesvik, Durif, et al. 2014), are not considered for the various wrasse species on definition of the common capture season. Post stocking mortalities related to sexual maturation, particularly early in the capture season, have repeatedly been reported (Nilsen et al. 2014). Moreover, although guidelines for wrasse overwintering in salmon cages exist, the present equipment and measures implemented to facilitate hibernation (e.g. lowering of refuges) may be suboptimal, as detailed knowledge of their naturally preferred winter habitat(s) remain lacking. It is very likely that all of these factors combined contribute to a stressed and possibly immunocompromised cleaner fish with increased susceptibility to infectious disease.

As will be discussed in more detail below, it seems clear that very few cleaner fish, irrespective of species, survive through a full salmon culture cycle. A certain proportion will, however, remain at harvest, as cleaner fish stocks are continually replenished during spring/summer. The fate of surviving specimens is poorly documented, although guidelines encourage euthanasia and destruction, or alternatively re-use within the same farm (Lusedata.no 2014a, b). Release of surviving, locally caught, wrasse back into the wild is also suggested, but this is in fact illegal according to statutory regulations for aquaculture operations (Lovdata.no 2008a), which forbids release of live fish from such facilities.

1.4. The current health situation of cleaner fish

1.4.1. Mortality levels and associated causes

While ballan wrasse in the wild may reach almost 30 years of age (Dipper et al. 1977), farmed and wild-caught cleaner fish in salmon farms have considerably shorter life expectancies. A comprehensive investigation of cleaner fish mortality in Norwegian salmon farms during 2013 registered 33% cumulative mortality within six months of stocking (Nilsen et al. 2014). The authors also pointed out that this was almost certainly an underestimate, and the actual proportion was predicted to be almost twice as high.

The study of Nilsen et al. (2014) involved eighteen salmon farms and followed the fate of almost one million cleaner fish (wild-caught wrasse and farmed lumpsucker) post cage stocking. Registered mortality levels were lowest in ballan wrasse (18%) and highest in lumpsucker (48%). On-site categorisation of mortalities was conducted, but due to rapid post mortem autolysis the exact cause of death could often not be established. Over half of

the dead fish could not be unambiguously placed in any of the predefined categories based on visual inspection. For those that could be categorised, putative causes of death were dominated by (in descending order) ulcers/fin rot, bacterial infections, mechanical injury and sexual maturation. Notably, these categories are not (biologically) mutually exclusive.

Individual episodes of high cumulative mortality could often be attributed to acute outbreaks of bacterial disease, and diagnostic investigations (bacteriology, histopathology etc.) demonstrated that bacterial infections were much more prevalent than suggested by initial gross categorisation. The lumpsucker seemed more prone than wrasse to acute bacterial disease, and lumpsucker mortality levels associated with such outbreaks could rapidly approach 100%. The significance of bacterial pathogens with regards to cleaner fish losses is supported by statistics from NVI diagnostic services in recent years (Johansen 2013, Hjeltnes 2014, Bornø & Lie Linaker 2015).

1.4.2. Bacterial infections – common agents and current knowledge

The registered numbers of diagnosed clinical cases at NVI (1997-2015), with identification of the most common cleaner fish-associated bacterial agents, are listed in Table 1.

Table 1: Number of NVI cleaner fish cases (1997-2015) with registered identification of the most common bacterial agents (in descending order).

Bacterial genus, species and/or subtype	**Cleaner fish host and number of cases**		
	Wrasse	Lumpsucker	All
Vibrio splendidus/*V. splendidus*-related	131	103	234
Atypical *Aeromonas salmonicida*	120	48	168
Aliivibrio (*V. logei*/*V. wodanis*/*V. fischeri*/*V. salmonicida*)	42	114	156
Tenacibaculum spp.	27	55	82
Vibrio anguillarum	40	41	81
Pasteurella sp.	0	54	54
Moritella viscosa	12	14	26
Vibrio tapetis	17	1	18
Vibrio ordalii	0	15	15
Pseudomonas anguilliseptica	0	9	9

Members of the genus *Aliivibrio* are commonly found, but their eventual role as cleaner fish pathogens remains largely unresolved. *V. anguillarum* is pathogenic to cleaner fish species (Rønneseth et al. 2014, Vågnes, Gulla, et al. 2014), and while isolates recovered from

wrasse most commonly belong to serotype O2a/O2aII, lumpsucker isolates belong almost exclusively to serotype O1. *Tenacibaculum* spp. are regularly recovered from skin- and fin injuries, and *Moritella viscosa,* the main aetiological agent of winter ulcer in Atlantic salmon, is occasionally isolated from cleaner fish. Their relevance for disease in these fish is unclear however. *V. tapetis* is mainly isolated from wrasse, while *V. ordalii, Pseudomonas anguilliseptica* and a recently described *Pasteurella* sp. (a close relative of the salmon pathogen *Pasteurella skyensis*; Alarcón et al. 2015), appear to be exclusively associated with lumpsucker.

Nevertheless, and in congruence with Nilsen et al. (2014), atypical *A. salmonicida* and *V. splendidus*-related strains remain the most commonly recovered bacterial agents from diseased cleaner fish in Norway. In-depth knowledge on the distribution and population structure of cleaner fish-associated strains of these bacteria was however lacking at the time of initiation of the present project.

1.5. *Aeromonas salmonicida*

1.5.1. Background

Aeromonas salmonicida was first described as 'Bacillus der Forellenseuche' (Eng.: 'Bacillus of trout disease') following isolation from diseased brown trout, *Salmo trutta* (L.), (Emmerich & Weibel 1894). The bacterium was subsequently re-named *Bacterium salmonicida* (Lehmann & Neumann 1896), and has since undergone a series of taxonomic reorganisations (Austin & Austin 2007). It currently resides in the genus *Aeromonas*, within the family Aeromonadaceae (Griffin et al. 1953). Disease in salmonids associated with *A. salmonicida* subsp. *salmonicida* is known as 'furunculosis', so termed due to the muscular boils/furuncles often observed (Austin & Austin 2007). The disease had a huge impact on salmon farming in many countries until effective vaccines were introduced in the early 1990s (Midtlyng 1996). Although rarely observed in farmed salmon today, furunculosis remains a notifiable fish disease in Norway (Lovdata.no 2008b)

A. salmonicida was long considered to represent a highly homogeneous species compared to the other members of the genus, until an increasing number of isolates deviating from the original description started to appear (reviewed by Austin & Austin 2007). The species currently comprises five validly described and published subspecies (Euzéby 1997, Parte 2014), i.e. subsp. *salmonicida* (Lehmann & Neumann 1896, Schubert 1967), subsp. *achromogenes* (Smith 1963, Schubert 1967), subsp. *masoucida* (Kimura 1969a, b), subsp.

smithia (Austin et al. 1989), and subsp. *pectinolytica* (Pavan et al. 2000). As isolates that cannot be ascribed to any of the five subspecies are frequently identified, many laboratories are satisfied with separation of subsp. *salmonicida* and non-subsp. *salmonicida* isolates, commonly referred to as 'typical' and 'atypical' *A. salmonicida*, respectively (Wiklund & Dalsgaard 1998).

Due to its pathogenicity towards commercially important salmonids, it took several decades before the infectious potential of *A. salmonicida* against non-salmonid fish became acknowledged. Over the years however, the number of fish species proven susceptible to this bacterium has increased steadily (Wiklund & Dalsgaard 1998). Infection has been detected in both freshwater and marine fishes, and in all six temperate continents[3]. While typical isolates are often related to disease in salmonids (furunculosis), atypical isolates are most commonly recovered from non-salmonid fish (disease often termed atypical furunculosis).

A. salmonicida is perhaps the most well-studied fish-pathogenic bacterium (Austin & Austin 2007), although, due to the commercial importance of salmonids, most studies have focused on typical strains. Following expanding aquaculture production of non-salmonid fish however, particularly marine species, atypical strains have received increased attention. Yet, despite extensive investigation, much remains uncertain regarding the epizootiology of *A. salmonicida*. Two major factors hampering investigations have been the lack of adequate tools for sensitive detection and typing of the bacterium.

1.5.2. Susceptible fish species and the disease

In addition to the salmonids (Salmonidae), fish species in which *A. salmonicida* has been detected include members of the families Cyprinidae (carps and minnows), Gadidae (cods), Scophthalmidae (turbots), Pleuronectidae (righteye flounders), Anarhichadidae (wolffishes), Percidae (perches), Serranidae (sea basses), Clupeidae (herrings), Anoplopomatidae (sablefishes), Petromyzontidae (lampreys), Sciaenidae (drums and croakers), Anguillidae (freshwater eels), Hexagrammidae (greenlings), Sebastidae (rockfishes), Engraulidae (anchovies), Soleidae (soles), Ammodytidae (sand lances), Lotidae (cuskfishes), Esocidae (pikes), various catfish families, and, evidently, Labridae (wrasses) and Cyclopteridae (lumpfishes) (e.g. Hellberg et al. 1996, Dalsgaard et al. 1998, Wiklund & Dalsgaard 1998, Austin & Austin 2007, Kim et al. 2013). This is not to say that *A. salmonicida* infection has

[3] Asia, Africa, North America, South America, Europe, and Australia

been linked to clinical disease in all these fishes, and some may function merely as covertly infected carriers (Gustafson et al. 1992).

Disease caused by *A. salmonicida* in non-salmonids may take on a range of different manifestations, with described/named conditions including carp erythrodermatitis (Fijan 1972), goldfish ulcer disease (Dror et al. 2006), head ulcer disease (Hidaka et al. 1983), and (most commonly) atypical furunculosis (Wiklund & Dalsgaard 1998). Experimental challenge trials have established certain strains of atypical *A. salmonicida* as pathogens of ballan wrasse and lumpsucker through confirmation of Koch's postulates (Rønneseth et al. 2014, Vågnes, Biering, et al. 2014). Atypical furunculosis in these fish may e.g. be characterised externally by lethargy and increased skin pigmentation (Laidler et al. 1999, Nilsen et al. 2014, Rønneseth et al. 2014, Vågnes, Biering, et al. 2014). Autopsy often reveals haemorrhagic and/or granulomatous internal organs, with microscopy showing bacterial microcolonies in internal organs (Figure 7).

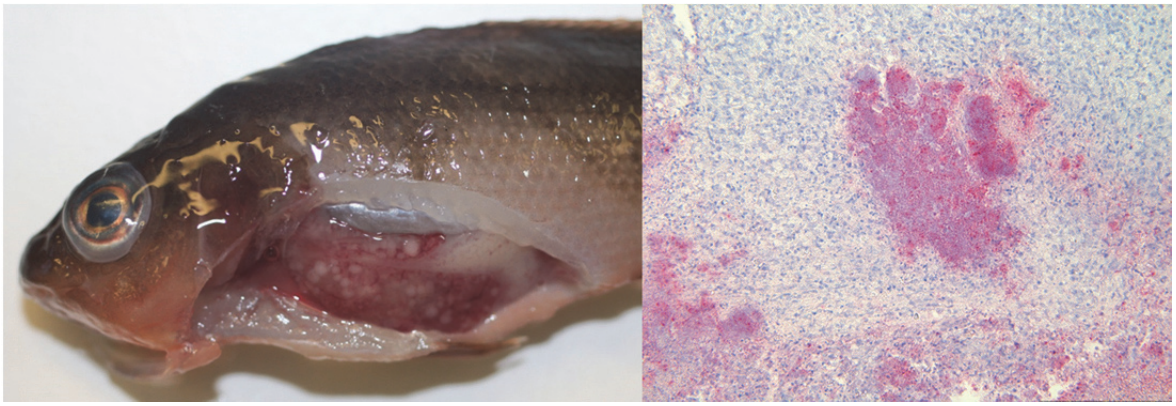

Figure 7: On left: Ballan wrasse with hepatic granulomas due to atypical *A. salmonicida* infection. On right: Bacterial microcolony in ballan wrasse tissue section, immunohistochemistry-stained with polyclonal *A. salmonicida* antiserum. Photos: Hilde Welde (left) and Kristin Almås (right).

1.5.3. The bacterium

Phenotypic traits

A. salmonicida is a Gram-negative, non-motile, facultatively anaerobic, oxidase- and catalase positive, coccobacillus, which does not grow at 37°C (Austin & Austin 2007). Lack of motility has been regarded as an important diagnostic trait distinguishing it from other aeromonads. Separation of typical from atypical isolates is largely based on phenotypic testing (outlined in Figure 8). Both typical and atypical isolates have been described with phenotypic traits conflicting with established criteria. Examples include motile isolates

(McIntosh & Austin 1991, Austin 1993), typical isolates lacking pigment production (Wiklund et al. 1993, Koppang et al. 2000), and catalase- (Kaku et al. 1999) and oxidase-negative isolates (Wiklund & Bylund 1993, Pedersen et al. 1994). The low resolution (i.e. typical/atypical), makes phenotypic investigation inappropriate for inferring diversity amongst atypical *A. salmonicida* strains.

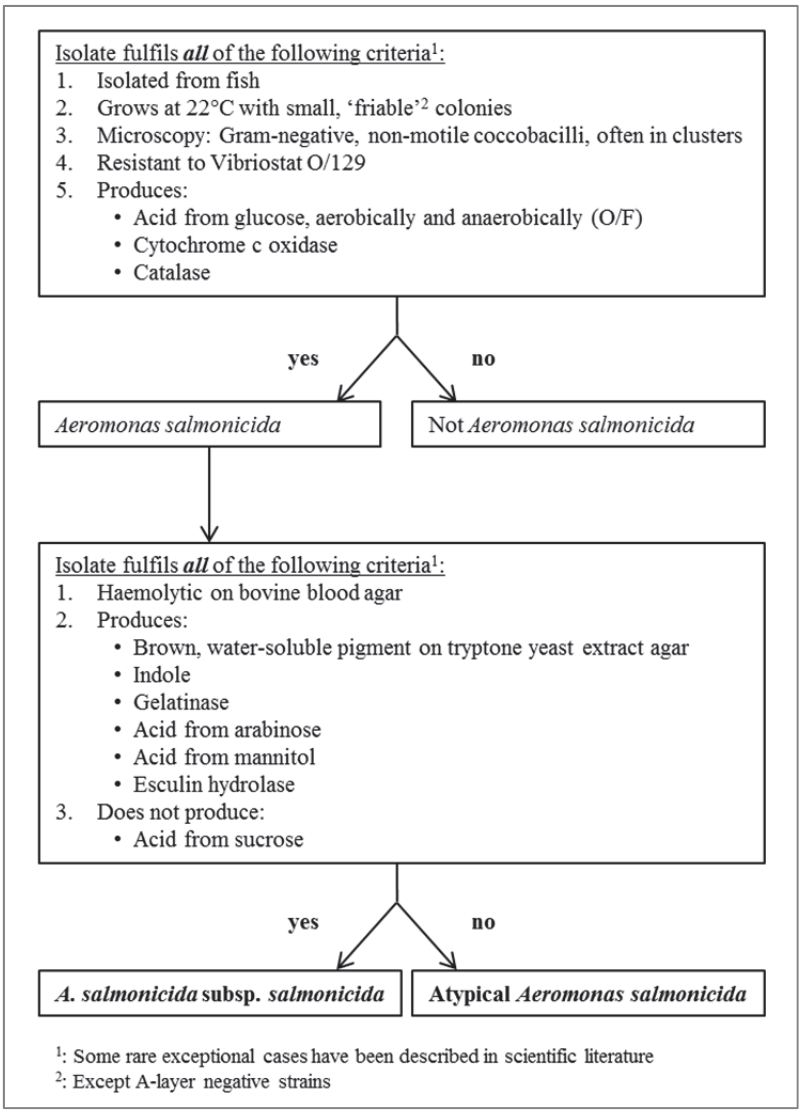

Figure 8: Outline of the phenotypic method (NVI; in-house, accredited) by which bacterial isolates are routinely diagnosed as *A. salmonicida*, and further distinguished as 'typical' or 'atypical'.

Taxonomy

While retention of the non-motile *A. salmonicida* in the otherwise motile *Aeromonas* genus is well supported by genetic data (e.g. Martinez-Murcia et al. 2011, Roger et al. 2012), its intra-species relationships remain largely unresolved. As previously mentioned, it is generally accepted that 'typical *A. salmonicida*' refers only to the subspecies *salmonicida*,

while 'atypical *A. salmonicida*' refers to all other strains (including the other four described subspecies). It is perhaps unsurprising then, that typical isolates form a highly homogeneous group, both genetically and phenotypically, whereas atypical isolates are much more diverse (e.g. Dalsgaard et al. 1994, Miyata et al. 1995, Austin et al. 1998, Umelo & Trust 1998, O'hIci et al. 2000). From a taxonomic viewpoint it is possible that the increasingly wide range of atypical strains represent, in reality, an undefined number of (as yet) undescribed subspecies (Austin et al. 1998).

1.5.4. Virulence factors

A wide range of bacterial products from *A. salmonicida* have been proposed as virulence factors underlying pathogenesis. These include cell-associated factors, such as the A-layer (Additional layer) protein (see below), iron-regulated outer membrane proteins (IROMPs; Hirst & Ellis 1994), a type III secretion system (Dacanay et al. 2006), a type IV pilin (Masada et al. 2002), capsular polysaccharides (Merino et al. 1996, 1997), a conserved porin (Lutwyche et al. 1995), and lipopolysaccharides (LPS; Chart et al. 1984). Additionally, a diverse array of extracellular products, including haemolysins, proteases, leucocidins etc. have been described (e.g. Fuller et al. 1977, Rockey et al. 1988, Gudmundsdottir 1996, Arnesen & Eggset 1999). The isolated and individual contribution to pathogenesis conveyed by each of these components is hard to assess however, and studies have occasionally provided contradictory evidence (Austin & Austin 2007). Quorum sensing further presumably plays a role in terms of regulating expression of virulence genes (Swift et al. 1997, Natrah et al. 2012).

It is worth considering in this regard the diverse nature of *A. salmonicida*, which may, conceivably, also be reflected in its virulence factors, e.g. through varying rates of production, mechanisms of action and/or host-specific cell interactions. Studies on *A. salmonicida* subsp. *salmonicida* pathogenesis cannot, therefore, necessarily be extrapolated to the species as a whole. The presence of one particular proteinaceous structure (i.e. the A-layer) has however, in light of accumulating evidence, been proposed as a principal determinant of *A. salmonicida* virulence (Austin & Austin 2007).

The A-layer protein

The A-layer protein, first reported by Udey & Fryer (1978), is today probably the best studied cell-associated structure of presumed importance for *A. salmonicida* pathogenesis. It is a highly stable, paracrystalline, outer membrane structure (S-layer, or Surface layer), with

a molecular mass of approximately 50 kDa (Kay et al. 1981, Chu et al. 1991). *A. salmonicida* auto-agglutination in culture has been directly linked to the protein's hydrophobic nature (Evenberg & Lugtenberg 1982, Evenberg et al. 1982). The A-layer is anchored to the LPS (Belland & Trust 1985) and exposed on the bacterial surface, thus serving as part of the cell-environment interface and making it well positioned for a direct role in the host-pathogen interplay (Noonan & Trust 1997).

Phipps et al. (1983) described the A-layer as a protein barrier essential for *A. salmonicida* virulence. Subsequent studies have supported the A-layer's role as a virulence factor, and several mechanisms of action have been proposed. It is believed to protect the bacterium from serum complement-activated killing (Munn et al. 1982) and protease degradation (Kay & Trust 1991). Its hydrophobicity is thought to promote association with phagocytes (Trust et al. 1983), and A-layer mediated resistance to superoxide anions may permit intracellular macrophage survival (Daly et al. 1996). The A-layer can further bind small ferric molecules (Kay et al. 1985, Kay & Trust 1991, Hirst et al. 1994), which possibly facilitates survival in the iron-deprived *in vivo* environment.

Following serial laboratory passage, especially at elevated temperatures (≥25°C), *A. salmonicida* cells may lose expression of a functional A-layer (Ishiguro et al. 1981). Such A-layer negative (A^-) strains display grey- or pale blue colonies when grown on Coomassie Brilliant Blue (CBB) agar (Cipriano & Bertolini 1988), while A-layer positive (A^+) strains grow with deep blue colonies (Figure 9). A^- strains are usually avirulent (Ishiguro et al. 1981, Garduño et al. 2000), which supports the hypothesis that the A-layer has a pivotal role for *A. salmonicida* virulence. Some rare exceptions exist however, as both avirulent A^+ strains (Olivier 1990) and, perhaps more interestingly, virulent A^- strains (Ellis et al. 1988) have been reported.

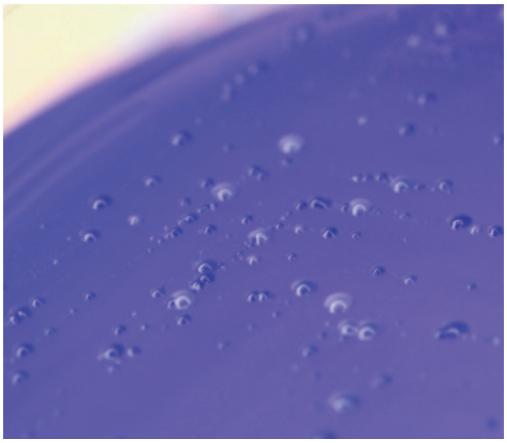

Figure 9: *A. salmonicida* grown on CBB agar displaying both deep blue (A^+) and grey/pale blue (A^-) colonies. Photo: Snorre Gulla.

The vapA (A-layer) gene

The *A. salmonicida* A-layer protein is encoded by the virulence array protein gene (*vapA*) (Chu et al. 1991), for which no homologous gene has to my knowledge been found in any other bacterial species. Variation in *vapA* sequence has been identified amongst strains, as represented by distinct clusters ('A-layer types') in similarity dendrograms (Lund, Espelid, et al. 2003, Lund & Mikkelsen 2004, Han et al. 2011, Kim et al. 2011).

1.5.5. Vaccination

While farmed salmon are efficiently vaccinated against subsp. *salmonicida*, atypical strains represent a serious hazard to farming of marine fish species in temperate areas. In addition to the cleaner fish species, susceptible marine fish species in Norway include Atlantic cod, *Gadus morhua* (L.), Atlantic halibut, *Hippoglossus hippoglossus* (L.), spotted wolffish, *Anarhichas minor* (Ólafsson), and turbot, *Scophthalmus maximus* (L.), (e.g. Ingilæ et al. 2000, Magnadóttir et al. 2002, Björnsdóttir et al. 2005). Commercial furunculosis vaccines developed for salmon have essentially proven ineffective in terms of protecting non-salmonid fish against atypical furunculosis (Gudmundsdóttir & Björnsdóttir 2007). Experimental vaccines against atypical furunculosis have been tested, but inconclusive (and occasionally contradictory) results suggest that antigenic strain differences, as well as host response variations, are of importance (Gudmundsdóttir & Björnsdóttir 2007).

Relevance of the A-layer for vaccination

The highly homogeneous nature of subsp. *salmonicida* makes vaccine development against typical *A. salmonicida*, in terms of strain selection, relatively straightforward. In contrast, the heterogeneous nature of atypical strains renders development of a generic atypical furunculosis vaccine difficult and perhaps unlikely. The phenotypic variability amongst atypical isolates is also likely to be reflected in variability of antigenic structures. Indeed, the most variable area of the *vapA* gene was identified in a region believed to encode an antigenic- and surface exposed proportion of the A-layer protein (Doig et al. 1993, Lund & Mikkelsen 2004). In some studies, superior vaccine protection was achieved when strains with identical A-layers were used for both vaccination and challenge (Lund, Espelid, et al. 2003, Arnesen et al. 2010). The recurrent observation of A^- strains being incapable of inducing a strong protective immune response following vaccination further supports the A-layer's role as an essential component of *A. salmonicida* vaccines (Lund, Arnesen, et al. 2003, 2008, Lund, Mikkelsen, et al. 2008, Lund et al. 2009, Arnesen et al. 2010).

1.5.6. Epizootiology

While various motile aeromonads such as *A. hydrophila* and *A. caviae* are abundant in aquatic environments (e.g. Schubert 1967, Araujo et al. 1991), *A. salmonicida* has, with the exception of the mesophilic subspecies *pectinolytica* (Pavan et al. 2000), almost exclusively been cultured directly from infected fish hosts. Experimental survival studies have nevertheless confirmed *A. salmonicida*'s ability to persist for some time in free-living form, and perhaps also for prolonged periods in a viable but non-culturable (VBNC) state (Allen-Austin et al. 1984, Effendi & Austin 1995). The extent to which such 'survival-modes' occur naturally, and whether or not these cells retain (or are able to revert to) full infectivity towards their target hosts, remains unclear however. Covertly infected fish specimens, which may or may not develop disease, are currently believed to represent *A. salmonicida*'s main reservoir and route of dissemination (Austin & Austin 2007).

Nevertheless, our understanding of this pathogen's interactions with its hosts and environment remains incomplete. In culture, *A. salmonicida* often fares poorly in competition with faster growing opportunists and less fastidious environmental microbes (Wiklund & Dalsgaard 1998, Austin & Austin 2007). Traditional culture methodology is therefore not well suited for detection of low bacterial densities, as would be expected in covert carriers and environmental samples. The increased availability and sophistication of molecular detection tools, such as polymerase chain reaction (PCR), has proven useful in this regard. This will be discussed in detail later.

1.6. *Vibrio splendidus*

1.6.1. Background

The type strain of *Vibrio splendidus* was isolated from an unspecified marine fish species in the North Sea, and was initially classified as *Photobacter splendidum* by Beijerinck (1900). Following a comprehensive description by Reichelt et al. (1976), the bacterium was relocated to the genus *Beneckea*. This genus was later abolished and incorporated into the genus *Vibrio*, in the family Vibrionaceae (Baumann et al. 1980).

Early phenotypic and taxonomic studies divided *V. splendidus* into two conspecific biotypes (Reichelt et al. 1976, Baumann et al. 1980), but an extreme degree of heterogeneity (both genetic and phenotypic) has since been revealed within this *sensu lato* species. Although an almost confluent phylogenetic population structure has complicated attempts at further taxonomic delineation, novel species descriptions have been proposed for a range of more

or less clearly defined strains. *V. splendidus*-related species (including *V. splendidus*) are collectively referred to by some authors as the Splendidus-clade (Sawabe et al. 2007, 2013).

V. splendidus-related bacteria are ubiquitous in temperate marine environments, and for many years these organisms were thought to be non-pathogenic (Le Roux & Austin 2006). While an increasing number of reports linking them to disease in various marine mollusc- and fish species have sown doubts in this regard, their actual role as pathogens, opportunists and/or commensals in relation to marine animals remains a matter of debate (Le Roux & Austin 2006).

1.6.2. Relevance for fish disease

Isolation of *V. splendidus*-related bacteria in association with disease outbreaks has been reported from several marine fish species, including turbot (e.g. Lupiani et al. 1989, Angulo et al. 1994), European seabass, *Dicentrarchus labrax* (L.), (Myhr et al. 1991), Atlantic cod (Santos et al. 1996), gilt-head sea bream, *Sparus aurata* (L.), (Balebona et al. 1998), and wrasses (Jensen et al. 2003). Losses of cultured marine invertebrates (primarily molluscs) have also been repeatedly linked to such infections (e.g. Nicolas et al. 1996, Faury et al. 2004, Le Roux et al. 2005).

Infection trials involving *V. splendidus* and wrasse species have produced contradictory results. An isolate recovered from corkwing wrasse displaying anorexia, reduced swimming behaviour and skin ulcers (Jensen et al. 2003), induced mortalities in adult corkwing- but not goldsinny wrasse, following bath challenge (Bergh & Samuelsen 2006). A recent bath infection trial on farmed ballan wrasse fry, utilising six strains, did not suggest any of the strains to be pathogenic however (S. Mortensen, Norwegian Institute of Marine Research, unpublished data). Data interpretation was, in both trials, complicated by non-specified mortalities in the control groups. Poor reproducibility has been a common observation following *V. splendidus* challenge trials on other marine animals, indicating that factors related to the host (e.g. immune status) and/or environment may be of importance (Le Roux & Austin 2006).

1.6.3. The bacterium

Phenotypic traits

V. splendidus-related bacteria are Gram-negative, motile, facultatively anaerobic, oxidase- and catalase positive, rods, which are normally sensitive to vibriostatic agent O/129 (Austin

& Austin 2007). Isolates classified at NVI as *V. splendidus* are generally associated with the phenotypic profile outlined in Table 2. The biochemical tools used in today's routine diagnostics are, however, too indiscriminate for reliable differentiation of the diverse *V. splendidus*-related (Splendidus-clade) strains and species (Austin et al. 1997, Thompson et al. 2004, Le Roux et al. 2004, Le Roux & Austin 2006, Austin & Austin 2007). Their phenotypic identification to the species-level should therefore be considered tentative, pending further (e.g. molecular) examination.

Table 2: The phenotypic profile commonly associated with fish-related *V. splendidus* recovered at NVI. Plus-/minus-signs indicate the reaction observed for ≥90% of examined isolates unless otherwise stated in parentheses.

Miscellaneous		**Production of**	
Culture conditions:		Cytochrome c oxidase	+
Grows well on 5% bovine blood agar with 2% NaCl within two days when incubated at 15°C and 22°C. Slower growth with 0.5% NaCl.		Alginase	+
		Gelatinase	+
Morphology:		Indole	+
1-4 mm grey/beige, opaque, usually β-haemolytic, colonies, often with production of green diffusible pigment and a strong characteristic smell.		Acid from arabinose	-
		Acid from cellobiose	+
Microscopy:		Acid from lactose	-
Motile rods, often curved or pleomorphic. Gram-negative following Gram-staining.		Acid from mannitol	+
Aerobic/anaerobic glucose fermentation (O/F test)	+/+	Acid from mannose	+
Vibriostat O/129 sensitivity	Sensitive*	Acid from sucrose	- (~75%)
Arginine, lysine and/or ornithine metabolism (ALO)	-/-/- (~85%)	Acid from trehalose	+

* Vibriostat O/129 sensitive *V. splendidus* often display small inhibitory zones and/or colonies appearing within the zones.

Taxonomy

Advances in molecular biology have, since the initial comprehensive description of *V. splendidus* (Reichelt et al. 1976), allowed for taxonomic studies of much higher resolution. This has, as mentioned, led to the description of a number of species closely related to *V. splendidus*. Those commonly considered members of the Splendidus-clade now include *V. cyclitrophicus* (Hedlund & Staley 2001), *V. lentus* (Macián et al. 2001), *V. kanaloae*, *V. pomeroyi*, *V. chagasii* (Thompson, Thompson, Li, et al. 2003), *V. tasmaniensis* (Thompson, Thompson, & Swings 2003), *V. crassostreae* (Faury et al. 2004), *V. gigantis* (Le Roux et al.

2005), *V. gallaecicus* (Beaz-Hidalgo et al. 2009), *V. celticus* (Beaz-Hidalgo et al. 2010), *V. atlanticus*, *V. artabrorum* (Diéguez et al. 2011), and *V. toranzoniae* (Lasa et al. 2013). Sawabe et al. (2007, 2013) also included the more distantly related *V. pelagius* (Baumann et al. 1971, 1980) and *V. fortis* (Thompson, Thompson, Hoste, et al. 2003) in the clade.

Novel Splendidus-clade species are, as we see, frequently published, and it can be expected that this trend will continue. One study (Thompson, Pacocha, et al. 2005) estimated the co-existence of over one thousand distinct *V. splendidus*-related genotypes in coastal seawater samples gathered from a restricted geographical area, each occurring at very low concentrations (<1 cell ml^{-1} on average). This illustrates the extreme genetic diversity existing amongst these environmental bacteria. Isolates occupying 'intermediate' taxonomic positions, and potentially representing undescribed *V. splendidus*-related species, are frequently identified (Le Roux & Austin 2006).

1.6.4. Virulence factors

The virulence of certain *V. splendidus*-related strains, especially towards various mollusc species (e.g. Waechter et al. 2002, Gay, Renault, et al. 2004), has been demonstrated through infection trials, but phenotypic and/or genetic markers enabling separation of pathogenic and non-pathogenic strains are lacking (Le Roux & Austin 2006). Again following the advances in molecular biology however, such as the sequencing of the full genome of *V. splendidus*-related strain LGP 32 (Le Roux et al. 2009), several putative virulence genes have, in recent years, been reported. The products of such genes include a metalloprotease (Vsm) toxic to oysters (Le Roux et al. 2007, Binesse et al. 2008), an outer membrane protein (OmpU) mediating resistance to antimicrobial peptides and facilitating invasion of oyster immune cells (Duperthuy et al. 2010, 2011), and a haemolysin (vibrioaerolysin) possibly causing damage to the intestinal tract of fish larvae (Macpherson et al. 2012). The relevance of pathogenesis studies in larval fish for disease in adult fish is questionable however. It has also been suggested that synergistic mechanisms between distinct *V. splendidus*-related strains may be important for development of disease in molluscs (Gay, Renault, et al. 2004, Gay, Berthe, et al. 2004), but no such studies on fish have been reported.

One recurring observation from *V. splendidus* experimental infection of marine animals has been a lack of reproducibility, and the fact that bath (as opposed to injection) challenges have often resulted in no mortalities at all (Le Roux & Austin 2006). Factors pertaining to

the host and/or environment are therefore also presumably of importance, both for entrance into the host and initiation of pathogenesis. Together with their complex population structure and abundance in marine environments, this apparent status of (at least some) *V. splendidus*-related strains/species as opportunistic pathogens complicates execution of controlled and verifiable *in vivo* studies.

1.6.5. Vaccination

One study does report on experimental (oral, immersion and injection) vaccination of red drum, *Sciaenops ocellatus* (L.), against *V. splendidus*, using formalin-killed cells (Ai-min et al. 2010). From the English version of the abstract it can (presumably) be inferred that vaccination by injection resulted in some degree of protection.

1.6.6. Ecology

Members of the Splendidus-clade occur ubiquitously in temperate marine environments worldwide (e.g. Nealson et al. 1993, Sobecky et al. 1998, Urakawa et al. 1999, Thompson, Pacocha, et al. 2005). In a thorough investigation of the ecology of *V. splendidus*-related bacteria in a brackish environment in the Northern Adriatic Sea, a natural association with various coastal matrices (including zooplankton, sediment, benthic macro-crustaceans, seaweeds and molluscs) was identified (Vezzulli et al. 2015). Year-round sampling indicated water temperature and salinity to be of marginal importance with regards to concentrations of *V. splendidus*-related bacteria in the respective matrices. *In vitro* examination of the oyster-pathogenic strain LGP 32 further revealed an ability for chitin attachment and biofilm formation, and demonstrated its capability for entering a VBNC state (Vezzulli et al. 2015). In addition to environmental reservoirs, *V. splendidus*-related strains may also be abundant in the gut flora of several marine fish larvae, including wrasse, during rearing (Verner-Jeffreys et al. 2003, Thomson et al. 2005, Birkbeck & Treasurer 2014).

1.7. Bacterial diagnostics

1.7.1. Isolation and identification methods

Culturing and biochemical tests

Bacteriological samples of marine origin received at NVI from clinical field cases usually consist of primary and/or secondary blood agar cultures (typically 5% bovine blood with 2% and 0.5% NaCl). Whole fish are also occasionally submitted. For systemic infections,

sampling from head kidney (optionally spleen) is encouraged, and moribund fish should ideally be sampled in addition to (fresh) mortalities. Primary cultures are incubated for one week at 15°C (2% NaCl) or 22°C (0.5% NaCl). Secondary cultures from numerically dominating (or otherwise conspicuous) colonies are usually incubated for 2-3 days prior to further testing. With the notable exceptions of *Renibacterium salmoninarum*, *Francisella noatunensis* and *Tenacibaculum* spp., all of which require specialised media, this method should result in visually detectable growth of nearly all (known) pathogenic marine bacteria of importance for Norwegian aquaculture.

Following morphological description of colonies/strains isolated in pure culture, further examination and evaluation consists of microscopy (Gram-stains and phase contrast microscopy), aerobic/anaerobic production of acid from glucose (O/F test), cytochrome c oxidase production, sensitivity to vibriostatic agent O/129, and, depending on the above presentation, a range of further biochemical parameters. Application of specialised culturing media (e.g. CBB; Figure 9), incubation at different temperatures/atmospheric conditions, and antimicrobial resistance testing, may also be required. Isolates recovered and identified from NVI fish diagnostic cases are routinely cryopreserved at -80°C.

Assuming high numbers of viable cells on (uncontaminated) primary culture, both *A. salmonicida* and *V. splendidus*-related bacteria are considered to be readily identified using culture-based techniques (Table 2 and Figure 8).

16S rDNA sequencing

Due to the indispensable function of 16S rRNA in prokaryote protein synthesis, the ubiquitous and slowly evolving 16S rRNA genes (16S rDNA) are commonly used as universal sequencing targets for reconstruction of prokaryotic phylogenies (reviewed by Clarridge 2004). During bacteriological investigations at NVI, the technique is frequently applied in cases where culture-based methods produce inconclusive results, e.g. due to strains with unusual biochemical profiles, or following the initial emergence of previously undescribed agents. In this regard, 16S rDNA sequencing should however be considered as a supplement to phenotypic description, as its taxonomic discriminatory power commonly does not allow for definite strain identification at the sub-genus level. This is particularly applicable to members of the *Aeromonas* genus (Küpfer et al. 2006) and the Splendidus-clade (Le Roux et al. 2004). For the latter group, the usefulness of this multi-copy gene is

further hampered by the fact that intra-cell allele heterogeneity may surpass that observed between distinct strains of the bacterium (Le Roux et al. 2004, Jensen et al. 2009).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS can be used for fast, accurate and cheap identification of microbes based on their protein content, and may soon become the standard method by which bacterial isolates are identified in diagnostic laboratories (Seng et al. 2009). Identification is achieved through a process involving laser-induced vaporisation and ionisation of peptide molecules in the matrix-suspended sample (MALDI), followed by measurement of the time required for charged (primarily ribosomal) peptides to pass over a length of vacuum (TOF). The resulting (MS) profile can then be compared to databases containing profiles of described bacterial species, thus dictating that these must be known beforehand. The technique has been applied to *A. salmonicida* (e.g. Benagli et al. 2012) and some Splendidus-clade species (e.g. Beaz-Hidalgo et al. 2010, Diéguez et al. 2011).

1.7.2. Non culture-based detection methods

PCR and real-time quantitative PCR (qPCR)

Culture-based methods are often unable to detect covert bacterial infections in healthy carrier individuals (e.g. as discussed for *A. salmonicida*). PCR offers a more sensitive alternative for specific detection from samples (e.g. tissues) containing low concentrations of the target agent, a prerequisite being the preceding identification of an appropriate genetic target locus and good primer/probe design.

The technique is based on thermal cycles of repeated heating and cooling, with enzymatic- and primer-assisted replication of a target genetic locus (or loci) for each cycle (Saiki et al. 1988). Nucleotide sequences thus generated will serve as templates in following cycles, leading to exponential amplification of the target. Subsequent sample treatment may e.g. involve gel electrophoresis for visualisation and size determination of DNA products and/or sequencing (16S rDNA etc.). While PCR theoretically allows for amplification and detection of only a single initial copy of the target locus, few diagnostic protocols display (in practice) sensitivities this high.

As a continuation of the concept, qPCR employs an optical detection system (Higuchi et al. 1992) whereby signal strength increases together with increasing amounts of the target

DNA (probe-based assays). Quantitative detection can thus be performed alongside the amplification process, which, for bacteriological diagnostic purposes, ultimately allows for relative estimation of infection loads in investigated samples.

Several attempts have been made at developing PCR assays for detection of the occasionally fastidious bacterium *A. salmonicida* (e.g. Hiney et al. 1992, Gustafson et al. 1992, O'Brien et al. 1994, Miyata et al. 1996, Byers, Cipriano, et al. 2002, Byers, Gudkovs, et al. 2002, Balcázar et al. 2007, Beaz-Hidalgo et al. 2008, Rattanachaikunsopon & Phumkhachorn 2012, Keeling et al. 2013). Once again however, the variable nature of this species must be considered, as high protocol specificity (i.e. detection of only *A. salmonicida*) may come at the expense of reduced sensitivity (i.e. failure to detect all *A. salmonicida* strains and/or low cell numbers). An optimal PCR target locus for specific and sensitive detection of *A. salmonicida* would therefore be a completely conserved single copy gene (or gene-region) exclusive to, but occurring ubiquitously within, all members of the species. To my knowledge, none of the previously published PCR protocols had at the start of the present project been tested on *A. salmonicida* isolated from cleaner fish.

Loop-mediated isothermal amplification (LAMP)

LAMP represents an affordable and sensitive alternative to PCR-based detection of genetic targets, without the requirement for a thermal cycler (Notomi et al. 2000). The method has been applied to several fish pathogens (Biswas & Sakai 2014), including *A. salmonicida* (Kulkarni et al. 2009).

Immunohistochemistry (IHC)

IHC is a well-known method utilised for detection of microbial antigens in fixed tissue sections (e.g. Figure 7). This is achieved using specific antibodies (see Serotyping) which are, directly or indirectly, visualised following antigen binding. The method is commonly used to detect *A. salmonicia* in fish tissues (e.g. Bergh et al. 1997, Lødemel et al. 2001, Vågnes, Biering, et al. 2014), and has also been employed for *V. splendidus* (Macpherson et al. 2012).

1.8. Bacterial populations and population genetics

Bacterial populations are groups of conspecific bacteria existing in a defined time and space/habitat. Different strains or subspecies within a population possess some degree of genetic distinctiveness, and may also vary with regards to their phenotype (metabolic capabilities, serotype etc.). Through comparative characterisation of large numbers of

isolates using molecular typing tools, it is possible to infer the existence (or eventual non-existence) and nature of strain diversity within bacterial populations, i.e. the genetic population structure. For pathogenic bacteria, such knowledge may provide an insight into their ecology (e.g. geographical distributions and history of dissemination) and epizootiology (e.g. virulence factors, host specificities and transmission routes). This information is also vital when examining the biological mechanisms underlying development of antimicrobial resistance.

Recent advances in molecular biology, especially sequencing technology, have revealed the often highly complex population structures of bacterial pathogens (Feil & Spratt 2001), with pan-genomes ranging from highly conserved to extremely diverse (Spratt & Maiden 1999). Increasingly sophisticated computer analyses and software (eBURST, ClustalX, PhyML, BioNumerics etc.) have made possible discernment of detailed evolutionary patterns within such populations. The relative contribution of genome-altering incidents (point mutation vs. recombination) to diversification, may for instance differ greatly between bacterial species (e.g. Suerbaum et al. 1998, Feil et al. 1999, 2003).

As far as *A. salmonicida* is concerned, prior to introduction of successful vaccination programmes in salmonid aquaculture, investigated isolates primarily belonged to the highly homogeneous (e.g. O'hIci et al. 2000) subspecies *salmonicida*. Studies on such populations thus invariably identified genetic stability. Molecular investigations involving large numbers of atypical strains are relatively scarce, but one study revealed correlations between host fish species and (temporally stable) genetic clusters (Lund et al. 2002). Another study showed that plasmid profiles could provide useful information during epidemiological investigations (Sørum et al. 2000). Sequencing of the entire genome of subsp. *salmonicida* strain A449 revealed a large number of mobile genetic elements compared to an *A. hydrophila* strain (Reith et al. 2008). This indicates that recombination may have played a significant role in the diversification of *A. salmonicida*.

Genetic studies on co-existing *V. splendidus*-related bacteria have, as mentioned, revealed a high degree of diversity (e.g. Thompson, Pacocha, et al. 2005). Following examination of gene sequences from sympatric isolates, Hunt et al. (2008) was able to link phylogenetic clusters to their environmental origins (i.e. zoo- or phytoplankton). This supports a previous notion that the vague taxonomic boundaries often observed between *V. splendidus*-related strains and species could in reality reflect fine-scale ecological specialisation to a range of

marine microniches (Le Roux & Austin 2006). In terms of evolutionary driving forces, authors have argued both for (Shapiro et al. 2012) and against (Sawabe et al. 2009) recombination acting as a major contributor in the Splendidus-clade. Again, at the start of the present project, few (if any) *V. splendidus*-related isolates infecting cleaner fish had been thoroughly and systematically investigated.

1.9. Bacterial typing

Phenotypic methods commonly used in diagnostic laboratories are typically well suited for efficient and inexpensive identification of bacteria at the genus-/species level. These techniques often fall short however, when it comes to unambiguous differentiation of closely related bacterial strains or subspecies. Methodologies of higher resolution are usually required for such purposes.

1.9.1. Serotyping

Serotyping remains widely used for characterisation of pathogenic bacteria, exploiting the fact that closely related strains often differ in surface-exposed antigenic determinants. Antigenic epitopes are e.g. found in LPS O-antigens, flagellar H-antigens, capsular K-antigens, pili, and S-layers. Polyclonal antisera (e.g. from rabbits), raised against bacterial antigens, allow identification of antigenic variability between isolates. In diagnostic laboratories, slide agglutination, a simple procedure whereby antisera and bacterial suspensions are mixed and checked for an agglutination reaction, is commonly used. O-antigen-based serotyping has been extensively used for characterisation of antigenic diversity within various *Vibrio* species, such as *V. cholerae* (Qadri et al. 1994) and *V. anguillarum* (Sørensen & Larsen 1986).

1.9.2. Molecular typing – available tools and methods

The genomic era has opened up a new world of possibilities for study of bacterial population genetics. Molecular typing techniques allow for extremely detailed strain description, and may enable distinction of isolates varying in only one single base pair.

The molecular toolkit available to scientists is ever growing. Relatively recent advances include 16S rDNA sequencing (e.g. Weisburg et al. 1991), virulence gene sequencing (e.g. Beall et al. 1996), Multilocus Sequence Typing/Analysis (MLST; Maiden et al. 1998/MLSA; e.g. Thompson, Gomez-Gil, et al. 2007), Internal Transcribed Spacer (ITS) sequencing (Gurtler & Stanisich 1996), plasmid profiling (e.g. Sørum et al. 2000), Pulsed-Field Gel Electrophoresis (PFGE; Schwartz & Cantor 1984), Random Amplified

Polymorphic DNA (RAPD; Welsh & McClelland 1990), Restriction Fragment Length Polymorphism (RFLP; e.g. Otal et al. 1991), Amplified Fragment Length Polymorphism (AFLP; Vos et al. 1995), Variable Number Tandem Repeat (VNTR)/Multilocus VNTR Analysis (MLVA) (reviewed by Lindstedt 2005), Single Nucleotide Polymorphism (SNP) analysis (e.g. Filliol et al. 2006), and, most recently, Whole Genome Sequencing (WGS; e.g. Snitkin et al. 2012).

Although a wide range of molecular tools and methods are available today, no single approach exists which is optimal for all forms of bacterial typing studies. The various methods differ greatly e.g. in terms of cost-, labour- and time consumption involved, ease of use, sample requirements, reproducibility, and opportunities for inter-lab comparison of results (Li et al. 2009). Their potential area of application (examples outlined in Figure 10) may be limited by low discriminatory power, which is further complicated by the fact that different bacterial species often vary in degree of intra-species genetic polymorphism.

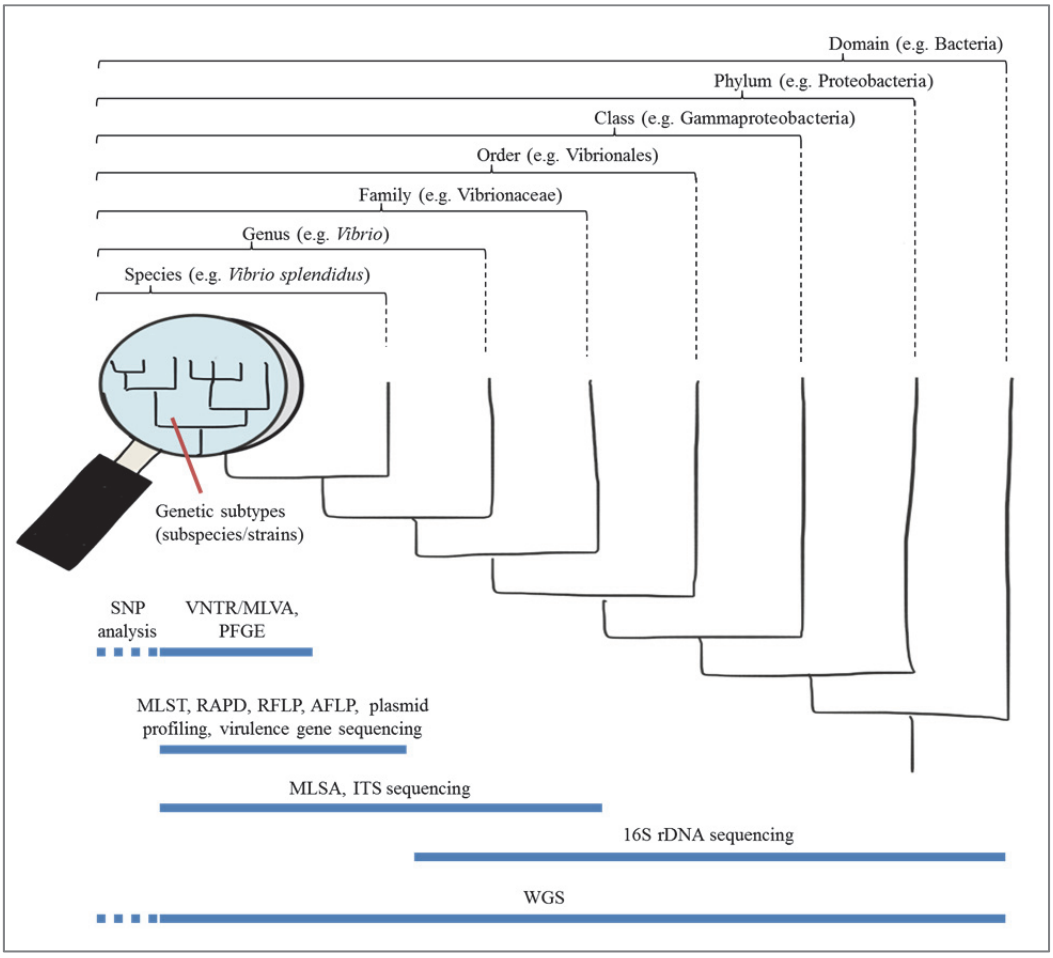

Figure 10: Some molecular techniques commonly used for bacterial typing. Blue lines give gross estimates of their typical discriminatory power relative to different taxonomic levels, although this may vary considerably according to the particular bacteria under investigation. Illustration: Snorre Gulla.

Generally, sequence-based methods (e.g. MLSA and WGS), as opposed to non-sequence based methods (e.g. AFLP and PFGE), have the inherent advantage of being easily portable between laboratories via online databases.

1.9.3. Molecular typing methods relevant to the present study

Virulence gene typing

Sequence typing of virulence genes encoding antigenic surface structures has been employed for surveillance and epidemiological studies of several pathogenic bacteria. Examples include the *emn* and *flaB* genes of, respectively, group A streptococci (*Streptococcus pyogenes*) (Beall et al. 1996, Steer et al. 2009) and *Campylobacter* spp. (Mellmann et al. 2004). Due to the exposed nature of their encoded products to the immune defences of the host, such genes are often under positive selection (Jiggins et al. 2002, Fitzpatrick et al. 2005), and genetic diversity often reflects, therefore, antigenic diversity. For example, in the case of *S. pyogenes*, global *emn* type distribution patterns have indicated that continent-specific vaccines may be necessary (Steer et al. 2009).

The rapid evolutionary rate resulting from positive selection means that typing of virulence genes may provide relatively high resolution at the subspecies level (i.e. enable distinction of very closely related strains). For the same reason however, the relationships revealed by these genes may not reflect true patterns of phylogenetic descent. If such inferences are to be made from virulence gene sequence data, it should ideally be coupled with other methods, such as housekeeping gene analysis (Li et al. 2009, Sabat et al. 2013).

Sequence analysis of the virulence gene *vapA* in *A. salmonicida* has, as mentioned, previously been used for investigating strain differences in this bacterium (Lund, Espelid, et al. 2003, Lund & Mikkelsen 2004, Han et al. 2011, Kim et al. 2011). Upon initiation of the present study however, relatively few isolates, and from a limited number of hosts (none from cleaner fish), had been reported examined by *vapA* sequencing.

Housekeeping genes, MLST and MLSA

Sequencing of so-called 'housekeeping genes' (Butte et al. 2001) may provide sufficient resolution for phylogenetic delineation at the sub-genus level (Glaeser & Kämpfer 2015). Housekeeping genes are constitutively expressed and encode products essential for normal cell function. Under the assumption that housekeeping genes (as opposed to virulence genes) are under stabilising selection, they are hypothesised to evolve at a relatively slow

and constant rate. Selection of candidate loci will depend on the taxon/taxa in question, but selected genes should be single-copy and ubiquitous within the investigated population(s). A low (ideally absent) recombination rate within the genes is also recommended. In order to reduce the influence of eventual bias in any particular locus (e.g. due to recombination or varying degrees of resolution), multiple housekeeping genes are usually combined in phylogenetic studies.

MLST was first described by Maiden et al. (1998). It involves sequencing of internal fragments (~450-500 bp) of (usually) seven housekeeping genes (Li et al. 2009). Unique sequences for each locus are, in the order of discovery, assigned an allele number or type. Each investigated isolate will thus, according to the seven allele types, receive a numerical allele profile which represents its sequence type, and is used for downstream analysis. While MLST is highly efficient for epidemiological studies of bacterial populations, it is unsuitable for phylogenetic investigations as the degree of variability is ignored (Glaeser & Kämpfer 2015).

This information is conserved when using the same sequence input for MLSA (first coined by Gevers et al. 2005). MLSA permits deeper phylogenetic inference, and also allows inclusion of more distantly related taxa. As such, while MLST is often applied to strains belonging to well-defined species (Figure 10), MLSA is better suited for investigations involving taxa with less established species boundaries (e.g. *V. splendidus* and the Splendidus-clade). The number of genes used for MLSA varies greatly, but the method usually employs 4-5 loci (Glaeser & Kämpfer 2015). As a general note, the number of genetic loci used, and the length of the fragments analysed, will always result from a compromise between discriminatory power required and practical considerations (costs and labour involved etc.).

Phylogenetic investigations of vibrios have often employed MLSA, and a range of different housekeeping genes have been utilised, including *atpA*, *rpoA*, *rpoD*, *pyrH*, *ftsZ*, *recA*, *topA*, *mreB*, *gyrB*, *gapA* and *rctB* (e.g. Thompson, Gevers, et al. 2005, Urbanczyk et al. 2007, Pascual et al. 2010, Lasa et al. 2013, Sawabe et al. 2013).

1.10. Knowledge gaps

In relation to the preceding introduction on the subject of bacterial infections in Norwegian cleaner fish, a brief synopsis of some of the major perceived knowledge gaps on initiation of the present PhD project will be given here.

Bacterial agents, including atypical *A. salmonicida* and *V. splendidus*-related strains, were (and are) frequently recovered from dead or moribund cleaner fish used for salmon delousing. Little was however known about the occurrence of these (and other) bacteria in cleaner fish species prior to stocking in salmon farms. Such information will be relevant with regard to the eventual implementation of vaccination regimes, as this (as a preventive measure) would presumably have the greatest effect if infection occurs primarily after transfer to salmon cages. In the case of *A. salmonicida*, traditional culturing techniques are insufficient for detection of low concentrations of viable cells, and molecular approaches would presumably be needed in a screening survey investigating *A. salmonicida* infection levels in fish.

Knowledge regarding the population structures of *A. salmonicida* and *V. splendidus*-related strains associated with cleaner fish mortality was also lacking. We did not know if one or a few strains/subtypes dominated in diseased fish, or if the recovered isolates comprised an array of distinct genetic variants. Such information will be important in order to facilitate qualified selection of representative vaccine strains. It could also provide us with a better understanding of the epizootiology underlying eventual disease outbreaks caused by these bacteria.

A lack of tools enabling detailed subtyping of *A. salmonicida* has complicated previous studies on this species. Additionally, relatively few atypical strains (and to my knowledge none from cleaner fish) had, much due to a historical focus on typical strains, been thoroughly examined. Robust subtype clusters verified through characterisation of large numbers of associated isolates had therefore, with the exception of subsp. *salmonicida*, not been established.

In contrast to the confirmed cleaner fish pathogen *A. salmonicida*, uncertainty surrounded the status of *V. splendidus*-related strains as pathogens of these fish species. Although often recovered from diseased specimens, these bacteria are also found in abundance in marine environments and in the intestines of healthy marine fish larvae. In this regard the possible existence of particular strains pathogenic to cleaner fish, and perhaps distinguishable by use of molecular tools, warranted investigation.

2. Aims of study

Through its diagnostic service provided to the Norwegian aquaculture industry, NVI recovers and cryopreserves large numbers of bacterial isolates from various fish species. This makes the institute strategically well placed when it comes to performing comprehensive studies on bacteria associated with farmed fish in Norway.

The main aims of the present study were to investigate the genetic relationships within two types of bacteria commonly isolated from dead or dying cleaner fish in Norway, and to identify whether both or either populations were dominated by a single or few strains which could represent good candidates for vaccine development. The main focus was on wrasse.

To meet the overall aims, the project was separated into the following objectives:

1. Develop and validate a qPCR protocol for detection of *Aeromonas salmonicida* in fish tissues (Paper I).

2. Establish the bacterial infection status of cleaner fish before and after stocking in salmon cages by culture and *A. salmonicida* qPCR (Paper I).

3. Evaluate *vapA* gene sequencing as a tool for *A. salmonicida* subtyping (Paper II).

4. Characterise *Vibrio splendidus*-related bacteria cultured from diseased cleaner fish by means of MLSA and serotyping (Paper III).

3. Summary of materials and methods

This section will only give a brief overview of the examined materials and most central methods used. Detailed information can be found in Papers I-III, and an introduction e.g. to some of the molecular methods employed has been given previously.

3.1. Materials

In Paper I, bacterial cultures and head kidney tissue samples from wild wrasse (n=412) were collected from 14 locations along the Norwegian coast (Figure 1, Paper I) and examined at NVI Oslo. Head kidneys from farmed cleaner fish (n=64) were kindly donated to us by farmers, but bacterial cultures were not (for logistical reasons) collected from this group. Bacterial cultures from cleaner fish mortalities in salmon farms were investigated as part of a different study (Nilsen et al. 2014), and head kidney tissues (n=131) collected from twelve farms in that project were, again, kindly donated to us for qPCR screening.

Bacterial strains (n=62) used for qPCR development in Paper I are part of the cryopreserved bacterial database archived at NVI Oslo, as are the *A. salmonicida* and *V. splendidus*-related isolates investigated in Paper II and III, respectively. These bacteria primarily consist of historical isolates recovered from fish species held in Norwegian aquaculture facilities. While 333 *A. salmonicida* isolates were reported typed in Paper II, post publication analysis of isolates recovered through 2015 has now (at the time of writing) brought the total number up to 378 (116 from wrasse and 34 from lumpsucker). Of the 112 *V. splendidus*-related isolates sequenced for MLSA in Paper III, 63 derived from wrasse and 12 from lumpsucker.

3.2. Methods

The qPCR assay used in Paper I employed a self-quenching molecular beacon probe (the principles of which are outlined in Figure 11) for specific detection of *A. salmonicida*. The present assay was developed based on a protocol previously described by Keeling et al. (2013), targeting a conserved region of the *vapA* gene which matched all *A. salmonicida* strains sequenced at the time of their publishing. Several modifications were required following introduction of this qPCR protocol to our laboratory, most notably the requirement for a new forward primer to allow inclusion of two novel A-layer types (including the wrasse-associated type V; see later) identified in Paper II. A thorough re-validation of the amended protocol was conducted prior to initiation of the cleaner fish screening. This verified retention of both high specificity and sensitivity for *A. salmonicida*

(in pure culture and fish tissue matrix), and that it detected all described *A. salmonicida* A-layer types with approximately the same sensitivity.

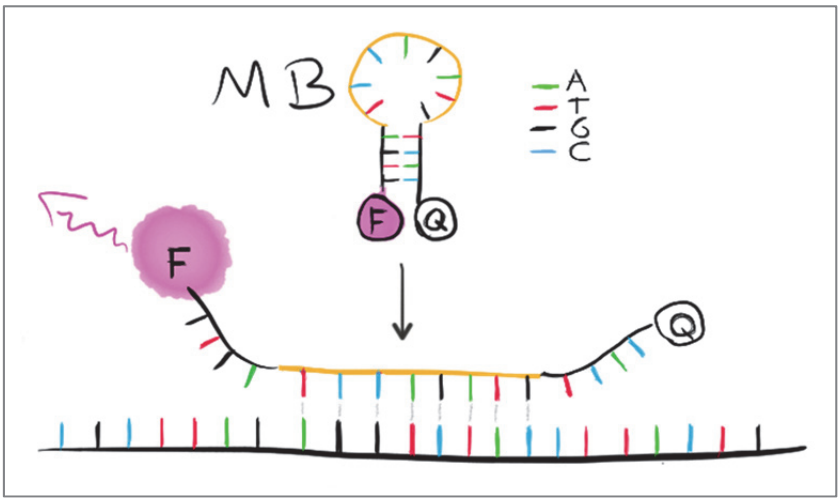

Figure 11: Principles of molecular beacon (MB) based qPCR: The unbound MB has a hairpin shape due to its self-complementary stem (typically 5-7 bp). Emission of fluorescence from the 5' fluorophore (F) is prevented by the proximity of the 3' quencher (Q). Hybridisation in the presence of a single-stranded target locus, matching the MB probe region (yellow; typically 18-30 nucleotides), separates F and Q, and results in emission of fluorescence which can be optically detected. Illustration: Snorre Gulla.

The *vapA* sequencing scheme presented in Paper II employed primers targeting a hypervariable region of this gene. Distinct clusters (comprising three or more isolates and with a 'grouping threshold' of 98% internal sequence identity) identifiable in Maximum Likelihood (ML) dendrograms were assigned A-layer type numbers. If not already publicly available, the near full-length *vapA* sequence (including the region targeted by the qPCR oligonucleotides), in representative strains of all major A-layer types, was also sequenced.

The five housekeeping genes (*rpoD*, *ftsZ*, *pyrH*, *rpoA* and *atpA*) targeted for MLSA in Paper III were selected based on their previously documented ability to resolve phylogenies amongst vibrios (e.g. Thompson, Thompson, et al. 2007, Pascual et al. 2010, Sawabe et al. 2013). Colour-coded ML trees (single gene and concatenated) were visually checked for discrepancies in order to detect putative recombination.

The two polyclonal antisera used in Paper III were produced by immunisation (formaldehyde-inactivated bacterial cells; four injections; one week apart) of rabbits against two *V. splendidus* strains selected based on MLSA results. Slide agglutination testing was performed principally as described by Sørensen & Larsen (1986).

4. Summary of papers

Paper I: *Aeromonas salmonicida* infection levels in pre- and post-stocked cleaner fish assessed by culture and an amended qPCR assay

A previously published molecular beacon qPCR assay for detection of *A. salmonicida* in fish tissue was amended and re-validated. In addition to culture, the modified assay was used to survey bacterial infection levels (with a focus on *A. salmonicida*) in Norwegian cleaner fish species before and after salmon farm stocking. The results revealed that while only 4% of examined wild wrasse and 0% of farmed cleaner fish were infected with *A. salmonicida* prior to salmon farm stocking, 68% of the samples from diseased cleaner fish in salmon farms were positive. Culture showed that few wild wrasse carried systemic bacterial infection in general, while this is one of the most common diagnostic findings in diseased cleaner fish in salmon farms. Overall, this emphasises the relevance of *A. salmonicida* for cleaner fish health, and highlights a need for cleaner fish vaccines against this pathogen.

Paper II: *vapA* (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes

A partial region of the *A. salmonicida vapA* (A-layer) gene was examined in a number of isolates of diverse spatiotemporal origin, and from a wide range of different fish hosts. We found that A-layer typing differentiates *A. salmonicida* subspecies, while also allowing for identification of several previously undescribed subtypes of the bacterium. Moreover, several more or less strong links between A-layer type and host origin (e.g. for wrasse and lumpsucker) could be inferred. In sum, A-layer typing represents an unambiguous, affordable and easily accessible method for high-resolution *A. salmonicida* subtyping. As the *vapA* gene region in question is predicted to encode a surface-exposed and antigenic proportion of the protein, A-layer typing may also be relevant in terms of vaccination strategies.

Paper III: Phylogenetic analysis and serotyping of *Vibrio splendidus*-related bacteria isolated from salmon farm cleaner fish

V. splendidus-related isolates recovered from diseased cleaner fish in Norwegian salmon farms were examined by MLSA (five loci) and compared to isolates of other origins. Slide agglutination testing using two polyclonal rabbit antisera was also conducted. The resulting phylogenetic tree showed one major cluster comprising the *V. splendidus* type strain, and several minor clusters presumably representing distinct Splendidus-clade species. While lumpsucker isolates were distributed throughout the tree, most of the wrasse isolates fell into the major cluster. Extensive genetic and antigenic (micro)diversity existed within the major cluster, and this was also observed amongst isolates recovered from individual mortality episodes. This indicates that fish-to-fish transmission is not the main route by which infection with *V. splendidus*-related bacteria in cleaner fish spreads, and direct opportunistic invasion from environmental sources may be more prevalent.

5. Results and general discussion

Bacterial diseases represent a major obstacle to sustained use of cleaner fish in Norwegian salmon farming, and atypical *A. salmonicida* and *V. splendidus*-related isolates dominate in diagnostic cases. Upon initiation of the present study, the genetic variation amongst these bacteria, and infection levels prior to salmon farm stocking, remained unknown. The project focussed primarily on wrasse.

5.1. Bacterial infection levels in cleaner fish

A survey of bacterial infections in cleaner fish before and after stocking in salmon farms was performed in Paper I. Due to its historical relevance as an important fish pathogen, and thus status as an obvious vaccine target candidate, emphasis was placed upon detection of *A. salmonicida*. While most *V. splendidus*-related strains can (presumably) be readily recovered using culture-based techniques, subclinical *A. salmonicida* infections may go undetected (Austin 1993, Kaku et al. 1999, Reid et al. 2003). In addition to culture, the *A. salmonicida*-specific qPCR protocol modified after Keeling et al. (2013) was thus employed for screening of cleaner fish.

Results from the qPCR survey showed low *A. salmonicida* infection levels in wild wrasse and farmed cleaner fish prior to stocking, compared to the high prevalence seen in diseased cleaner fish in salmon farms ('working' cleaner fish; Figure 12). Furthermore, bacterial loads (inferred from Ct-values) in positive wild wrasse were consistently low, while some very high loads were observed in working cleaner fish. The locations from which positive samples in both groups originated were relatively dispersed.

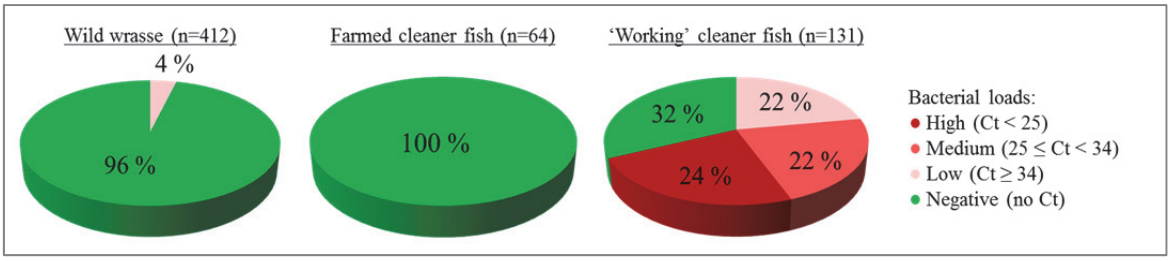

Figure 12: *A. salmonicida* infection levels in cleaner fish inferred from head kidney qPCR Ct-values. Modified after Figure 2, Paper I.

Head kidney cultures from wild wrasse revealed a low bacterial infection prevalence in general (dominated by a few *V. logei*- and *V. splendidus*-related isolates), and no *A. salmonicida* isolates were recovered from this group. Curiously, two (of four) examined

rock cook displayed abundant growth of colonies identified as an *Endozoicomonas* sp. (~97% 16S rDNA similarity). Members of this genus have previously been cultured from various marine invertebrates (Neave et al. 2014), although strains have also been associated with epitheliocystis in cobia, *Rachycentrum canadum* (L.), (Mendoza et al. 2013). The role, if any, of these bacteria in relation to wrasse health remains unknown.

Cultures from diseased cleaner fish in salmon farms were dominated by isolates phenotypically consistent with (in descending order) atypical *A. salmonicida*, *V. splendidus*, unidentified *Vibrio* spp., *Tenacibaculum* spp., *V. logei*, *V. anguillarum*, *Pasteurella* sp., *V. wodanis*, *V. tapetis*, and *V. fischeri* (Nilsen et al. 2014). This correlates relatively well with the historical findings from NVI routine diagnostics on cleaner fish species (Table 1). In no cases involving samples belonging to the 'negative' or 'low' qPCR-categories (Figure 12) had the diagnosis atypical furunculosis been reached from culture and/or histopathology, while all but one case involving 'medium' or 'high' qPCR-category samples had received the diagnosis. This confirms that the subjective definition of the three positive categories was relatively pragmatic.

Taken together, these findings are perhaps unsurprising, but they emphasise the importance of bacterial agents, and *A. salmonicida* particularly, in relation to cleaner fish health. We confirm that these fish are largely free of systemic bacterial infection (in general) prior to stocking, while high infection levels are observed at death in salmon farms. The qPCR-results also indicate the potentially significant role of subclinical *A. salmonicida* carriers, in which covert infections may presumably become activated due to stress in captivity, and subsequently spread throughout cleaner fish stocks. Reduction of disease-related losses, through vaccination against bacterial pathogens prior to stocking, could facilitate a more sustainable and ethically acceptable use of cleaner fish.

5.2. Molecular characterisation of bacteria from cleaner fish

With development of vaccines as a rational goal, one of the primary tasks must be selection of bacterial isolates for vaccine inclusion. To facilitate this we investigated the genetic variation/homogeneity of isolates archived during diagnostic investigations at NVI.

5.2.1. *A. salmonicida* A-layer typing

Methods allowing unambiguous differentiation (typing) of atypical *A. salmonicida* have been lacking. Previously published work identified *vapA* sequence heterogeneity and associated these differences with variations in vaccine protection (Lund, Espelid, et al.

2003, Arnesen et al. 2010). Preliminary work performed at NVI further indicated a possible link between *vapA* sequence type (A-layer type) and host fish species. A comprehensive A-layer typing study was therefore conducted in Paper II, involving isolates of diverse spatiotemporal origin and recovered from a wide range of different fish species.

Sequence analysis of all 378 presently typed isolates (excluding frameshifted mutants; see below) revealed several prominent clusters or A-layer types (Figure 13). All type strains of validly published *A. salmonicida* subspecies (except subsp. *pectinolytica*, which has no *vapA* gene; Lund, Espelid, et al. 2003) fell into distinct positions, and A-layer type I comprised entirely (and exclusively) all examined typical isolates. Several links between A-layer type cluster and host origin(s) could also be deduced.

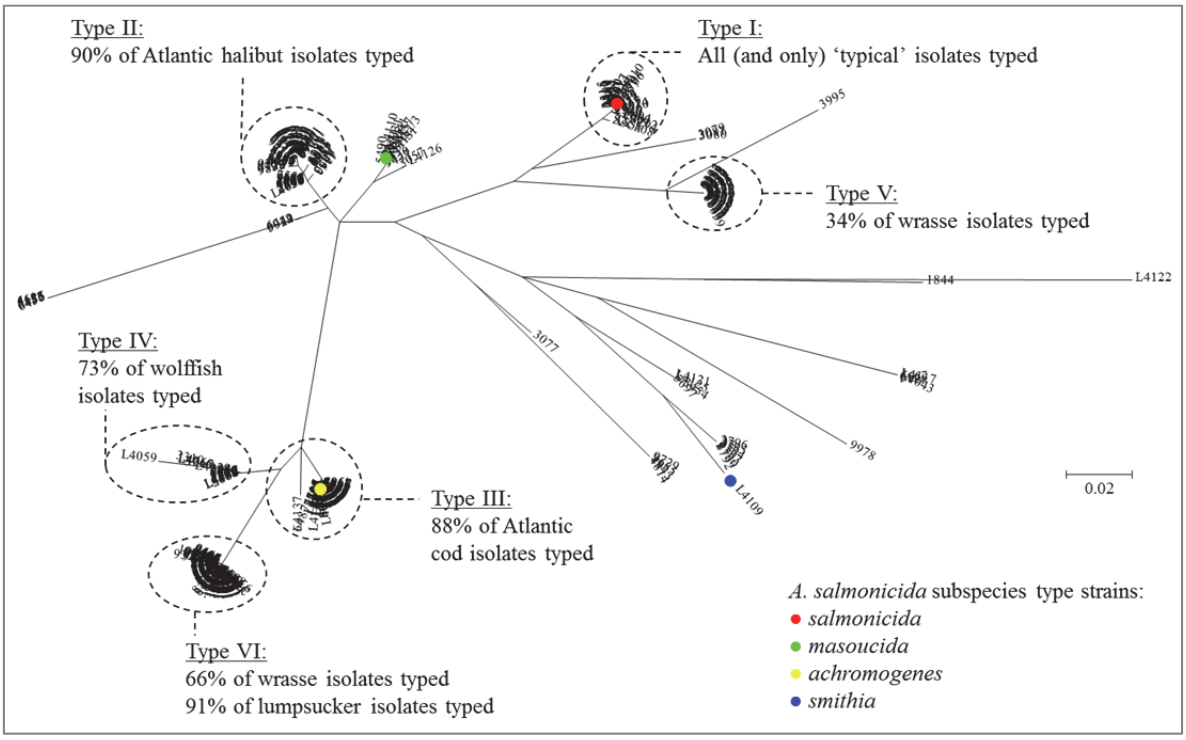

Figure 13: Unrooted ML radiation dendrogram based on *A. salmonicida* partial *vapA* sequences. Subspecies type strains are highlighted with colours, and the six largest A-layer type clusters are indicated and annotated. Modified after Figure 2, Paper II (with addition of isolates from 2015).

The host/A-layer type association was most pronounced for wrasse, from which all 116 examined isolates belonged to either types V or VI (Table 3). The data also suggest that ballan wrasse may be more susceptible to type V, in contrast to goldsinny- and corkwing wrasse which appear more susceptible to type VI. While some variation was identified amongst lumpsucker isolates, type VI clearly dominated. Notably the type V cluster derived, with the exception of one isolate recovered from lumpsucker, entirely from wrasse species.

Table 3: A-layer type and temporal distribution of examined *A. salmonicida* cleaner fish isolates.

Host	A-layer type V		A-layer type VI		Other A-layer types	
	No. of isolates	Temporal distribution	No. of isolates	Temporal distribution	No. of isolates	Temporal distribution
Ballan wrasse	24	2008-2014	14	1997-2014	0	-
Goldsinny wrasse	1	2011	28	2011-2014	0	-
Corkwing wrasse	7	2011-2014	18	1993-2014	0	-
Rock cook	0	-	2	1993-1994	0	-
Cuckoo wrasse	0	-	2	2013-2014	0	-
Unspecified wrasse sp.	7	2011-2014	13	1992-2014	0	-
Lumpsucker	1	2015	31	2011-2015	1 type III; 1 type II	2012; 2014

Although three historical salmon isolates (recovered in 1987, 1994 and 2015) belonged to A-layer type VI, the high prevalence of type V and VI infection amongst cleaner fish (Figure 12 and Table 3), and lack of disease in cohabitating salmon, suggests that salmon are not particularly susceptible to these strains. Alternatively, the absence of infection in salmon could be explained by broad protection awarded by furunculosis vaccines.

The hypervariable *vapA*-region analysed is believed to encode a surface-exposed and antigenic proportion of the A-layer protein (Doig et al. 1993, Lund & Mikkelsen 2004). In this regard, we found that most of the observed nucleotide diversity involved missense- and hydrophobicity-altering mutations, presumably resulting in conformational- and polar variations in the protein. This may conceivably contribute to the A-layer type specific vaccine protection observed in some challenge trials (Lund, Espelid, et al. 2003, Arnesen et al. 2010). It is therefore likely that both A-layer types V and VI should be included in a generic cleaner fish vaccine in order to obtain broad protection against atypical furunculosis in these fish species.

Notably, deleterious *vapA* mutations had rendered 20 of the *A. salmonicida* isolates examined A^- (the majority confirmed by CBB culture), a phenomenon previously associated e.g. with laboratory passage (Ishiguro et al. 1981). Interestingly, analysis of the mutated gene revealed that 17 of these 20 isolates belonged to the type VI cluster. Both old and recent isolates, recovered from both wrasse and lumpsucker, were affected. It remains unknown whether this situation reflects retained infectivity/pathogenicity despite lack of a functional A-layer, or if these strains are particularly prone to acquiring mutational loss of the protein (under the employed culturing conditions). The latter seems more likely, as both

A^+ and A^- type VI colonies were occasionally found on the same agar plate. Moreover, the observed detrimental mutations (frameshifting indels and premature stop codons) were unique in all affected isolates, including some deriving from the same diagnostic case.

5.2.2. MLSA and serotyping of *V. splendidus*

NVI isolates from cleaner fish phenotypically consistent with the species description of *V. splendidus* have repeatedly, through partial 16S rDNA sequencing, been confirmed as Splendidus-clade members. This is a highly diverse and complex taxon however, and an MLSA scheme was therefore developed in Paper III in order to allow for close examination of the phylogeny and population structure of archived cleaner fish isolates. The target loci consisted of partial regions of five housekeeping genes (*rpoD*, *ftsZ*, *pyrH*, *rpoA*, *atpA*). The phylogenetic tree resulting from analysis of concatenated sequences from 112 *V. splendidus*-related isolates essentially consisted of one major- and several minor clusters (Figure 14).

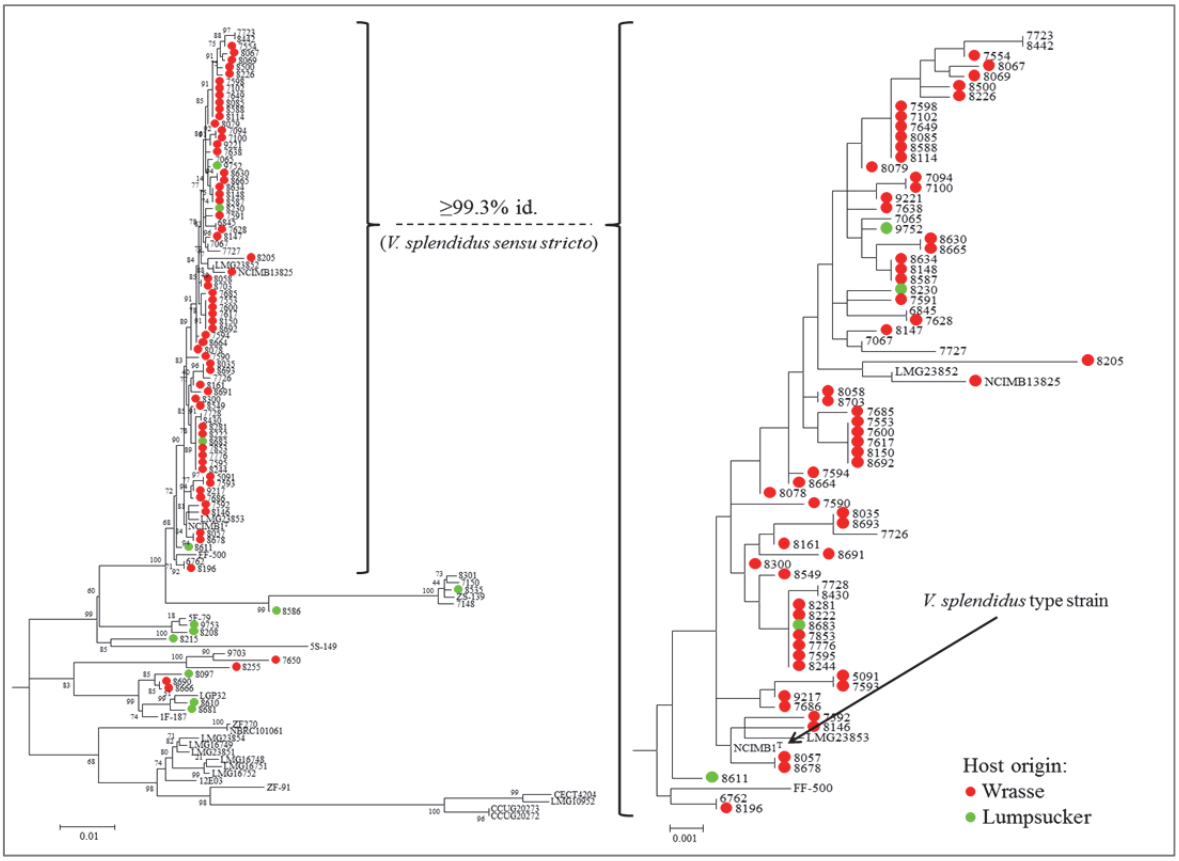

Figure 14: ML tree based on concatenated housekeeping genes (five loci) from *V. splendidus*-related isolates. The compact major cluster (*V. splendidus sensu stricto*), comprising *V. splendidus* NCIMB1^T and most of the wrasse isolates, is magnified on the right. Cleaner fish isolates are colour coded. The tree was rooted against *V. tapetis* NCIMB 13622^T (not shown). Modified after Figure 1 and 2, Paper III.

The 12 examined lumpsucker isolates were relatively dispersed, and displayed no remarkable distribution pattern. In contrast, 59 of the 63 wrasse isolates fell into the major cluster, which comprised also the *V. splendidus* type strain and therefore presumably consists of true members of this species (here termed *V. splendidus sensu stricto*). Despite the compactness of this cluster however (≥99.3% sequence identity), closer examination revealed a high degree of internal microdiversity, with 51 of 78 isolates possessing distinct concatenated sequence types. However, as nearly all the observed heterogeneity consisted of synonymous substitutions, the cluster appeared almost clonal in the corresponding amino acid tree (not shown).

It is possible that this multitude of small, synonymous housekeeping gene dissimilarities amongst *V. splendidus sensu stricto* isolates may be reflected in more extensive, non-synonymous variability in virulence genes encoding antigenic surface proteins. This is due to the fact that while housekeeping genes are under stabilising selection, virulence genes are, as previously mentioned, often under positive selection (Jiggins et al. 2002, Fitzpatrick et al. 2005, Glaeser & Kämpfer 2015). Following slide agglutination of *V. splendidus sensu stricto* isolates, using two polyclonal antisera raised against non-identical members of this cluster, it did indeed become evident that antigenic dissimilarities existed amongst them (Figure 2 in Paper III).

With regard to the relevance of *V. splendidus* as a pathogen of cleaner fish, perhaps the most interesting finding was the genetic variation observed between multiple isolates recovered from individual mortality episodes. In fact, in most instances where two or more isolates from single cleaner fish cases were examined (14/17), genetic (and antigenic) strain variability was observed. This was true for both wrasse- and lumpsucker cases, and clearly shows that clonal expansion of virulent strains is not the primary dynamic during such disease outbreaks. Direct infection from environmental sources (e.g. seawater) may thus occur more frequently than fish-to-fish transmission. Unfortunately, multiple isolates from individual fish were not available for examination. Notably, studies on molluscs have also observed 'intra-outbreak' genetic diversity amongst *V. splendidus*-related strains isolated from diseased specimens, and strain-synergisms have been suggested to play a role in pathogenesis (Gay, Renault, et al. 2004, Gay, Berthe, et al. 2004).

Despite this extent of diversity however, most wrasse isolates, in contrast to lumpsucker isolates, did fall into the *V. splendidus sensu stricto* cluster (Figure 14). This may reflect an

intrinsic predilection for these hosts, although infection trials on wrasse have produced ambiguous results (Bergh & Samuelsen 2006, S. Mortensen, Norwegian Institute of Marine Research, unpublished data). All cleaner fish are undoubtedly subjected to substantial stress during life in captivity however, and disease associated with these bacteria may conceivably reflect opportunistic invasion of a weakened host. A complex interaction between environmental factors, strain composition of bacterioplankton, and fish health, is presumably involved. Vaccination may yet help limit losses during initial post stocking phases, when stress levels are likely to be particularly high. The diverse nature of these bacteria does however complicate selection of representative candidate strains for vaccine development.

5.3. Generalised impact of work

The obvious implications of our results regarding bacterial infection timelines and strain involvement in cleaner fish disease have been discussed above. Some interesting findings which may have a broader significance deserve some additional attention however.

5.3.1. Killing the dogma of 'typical' and 'atypical' *A. salmonicida*

A. salmonicida subsp. *salmonicida* is often perceived as the prevailing member of the *A. salmonicida* species, with atypical strains representing an undefined 'cloud' of variable isolates of largely undescribed significance for disease. It took several decades following its initial description (Emmerich & Weibel 1894), before the existence of strains deviating from the acknowledged phenotype of the species (e.g. non-pigmented) received notable attention (Smith 1963). From this it is easy to assume an almost concentric view on *A. salmoncida* as a species, in which atypical strains would have arisen *from*, rather than *alongside*, typical strains (Figure 15a). The basis for this appearance lies undoubtedly in the strong historical focus on salmonids, wild and farmed, which has entailed an overall sampling bias favouring strains infecting these fish. From Figure 13 it can clearly be inferred however, that *A. salmoncida* comprises a range of specific subtypes (one of which is subsp. *salmonicida*) apparently occupying distinct ecological niches, i.e. as pathogens of different fish lineages. It is likely that these subtypes have evolved simultaneously over time in a traditional 'branching' manner (Figure 15b).

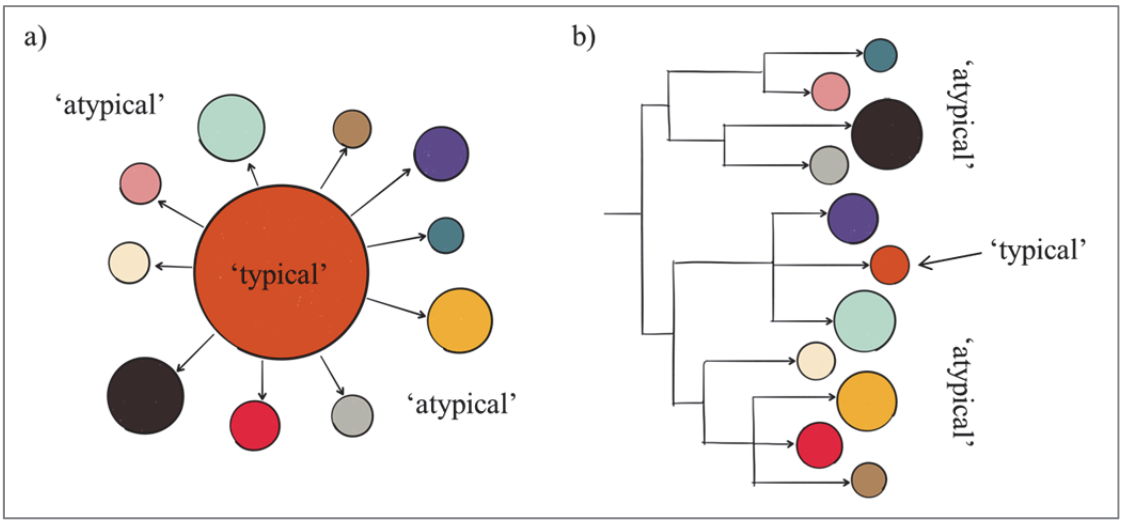

Figure 15: Coloured circles represent theoretical *A. salmonicida* subtypes occupying distinct ecological niches. a): A concentric view placing typical strains, from which atypical ones arise, in the centre as the dominant and founding member of the species. b): Perhaps more likely, all current *A. salmonicida* subtypes have evolved in parallel over time (example does not account for eventual recombination). Illustration: Snorre Gulla.

Phenotypic characterisation of *A. salmonicida* may be ambiguous (McIntosh & Austin 1991, Wiklund & Bylund 1993, Wiklund et al. 1993, Austin 1993, Pedersen et al. 1994, Dalsgaard et al. 1998, Kaku et al. 1999, Koppang et al. 2000), and currently only distinguishes typical from atypical strains. A-layer typing can make the same distinction, which is relevant in terms of the notifiable (in Norway) disease furunculosis in salmonids, but also allows for differentiation of a wide range of atypical strains. Moreover, being based on gene sequence information, the typing-results are unambiguous and easily shared through online databases for inter-laboratory strain comparison. The findings presented in Paper II may (and should) therefore render the uninformative and somewhat misleading distinction typical/atypical *A. salmonicida* obsolete. It could also form the basis for a deeper systematic study of this bacterium, e.g. by WGS.

5.3.2. A-layer type as a putative epidemiological marker

Whether the links between *A. salmonicida* A-layer type and host fish species represent causal- or indicative correlations remains unclear. In the case of a causal relationship, one might expect the distribution of each particular A-layer type to reflect that of the exposed and susceptible host(s). Thus, in the case of non-migratory fish species living in secluded populations, evolution could over time give rise to geographically confined A-layer types,

whereas those associated with migratory fish would show a more widespread geographical distribution.

Wrasse are, as previously mentioned, largely autochthonous (Sundt & Jørstad 1993, 1998, Espeland et al. 2010), and the (almost) entirely wrasse-associated A-layer type V comprises isolates exclusively recovered from 2008 to date (Table 3). This correlates relatively well with the dramatic increase, since 2008/2009, in use of wild-caught wrasse as cleaner fish in Norway (Figure 3). A-layer type V is now apparently ubiquitous along the Norwegian coast (Figure 16).

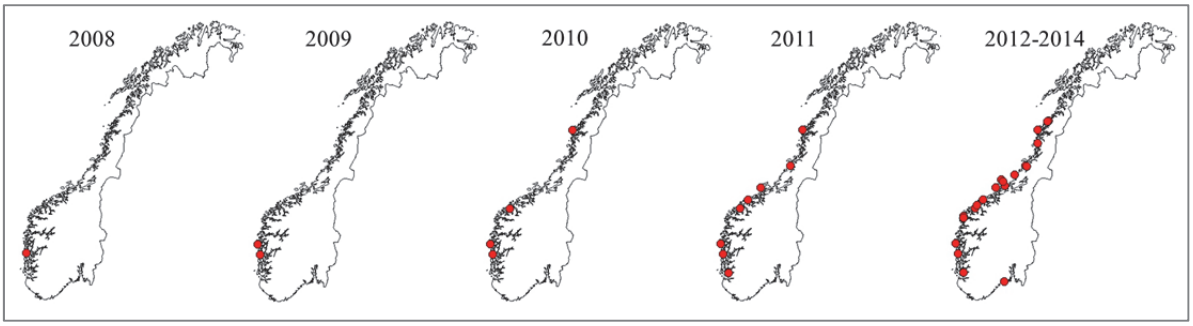

Figure 16: Occurrence along the Norwegian coast of *A. salmonicida* A-layer type V, with year-by-year plots indicating the cumulative collection of locations from which such isolates have been recovered and identified at NVI.

It is tempting to speculate from this observed distribution that A-layer type V may previously have represented a sub-population geographically/physically confined to a secluded population of wild wrasse. Capture, transport and salmon farm stocking of infected fish may eventually have resulted in dissemination of one or more type V strains. True or not, this possibility should act as a reminder of the potential risks associated with uncritical transport of live fish (Treasurer 2012).

On a similar note, A-layer types XI and XIV originate exclusively from two distinct landbased production facilities for Arctic char, *Salvelinus alpinus* (L.), while some A-layer types occasionally recovered from Atlantic salmon have a much broader geographical distribution. Examples of the latter include types I, III and IV from both sides of the Atlantic Ocean, and types I and VII from both sides of the Pacific Ocean (Figure 3a in Paper II). Obviously, the historical worldwide transport of live salmonids may have contributed more or less to the observed spread of these A-layer types. Another route is also possible however, as recent studies have found that wild Norwegian salmon migrate much longer distances than previously assumed, and that they graze in a narrow temperature gradient along the polar front, where biological production is high (Solhaug 2012). Encounters

between European- and North-Eastern American Atlantic salmon, with the possibility for disease transmission, may occur here, and previous studies have indicated that population mixing takes place (Dadswell et al. 2010).

Taken together, the migratory (e.g. Atlantic salmon) or non-migratory (e.g. wrasse and landlocked char) behaviour of susceptible hosts may therefore, conceivably, explain the apparent geographical distribution of some A-layer types. As too few non-Norwegian *A. salmonicida* isolates have been assessed by A-layer typing however, it remains impossible yet to conclude on these matters.

Most of the major *A. salmonicida* A-layer type clusters appear relatively homogeneous (Figure 13) despite largely comprising isolates recovered over more than two decades. This indicates that the *vapA* gene has remained relatively stable over these time spans. The type II cluster constitutes one interesting exception however, as it appears to display some degree of evolutionary change during the sampling period (Figure 5 in Paper II). Members of this cluster originate predominantly from Atlantic halibut, and include a number of isolates collected over a period of fifteen years from a single Norwegian halibut hatchery. Distinct type II sub-clusters can be observed, each of which were recovered during a limited time span from this particular hatchery. A similar pattern can also be observed amongst isolates from another halibut hatchery. It is possible that these observations may represent evolution of 'house strains' during the respective sampling periods.

5.3.3. Heterogeneity within the Splendidus-clade

Our observations in Paper III of genetic and antigenic heterogeneity amongst sympatric *V. splendidus*-related bacteria are in agreement with previous studies (e.g. Gay, Berthe, et al. 2004, Thompson, Pacocha, et al. 2005, Wildschutte et al. 2010). We also found some evidence for recombination between analysed taxa (genes *rpoD*, *atpA* and *ftsZ*), but no attempt was made at inferring the relative contribution of such events (versus point mutation) for diversification in these bacteria.

Notably, several strains acquired from reference collections as *V. splendidus* displayed higher concatenated housekeeping gene sequence identity with distinct (non-*V. splendidus*), and relatively recently published, species in the Splendidus-clade. This illustrates that the nomenclature surrounding 'elderly' strains deposited in culture collections may not necessarily be up to date.

6. Methodological considerations

6.1. Sampling

Cleaner fish, and wrasse especially, seem to undergo particularly fast post mortem autolysis (Nilsen et al. 2014, personal observation), and may rapidly become colonised by saprophytic environmental microbes. This can make interpretation of bacteriological samples from these fish difficult. While *A. salmonicida* is considered an almost obligate fish pathogen (Austin & Austin 2007), it is likely that several recoveries of *V. splendidus*-related strains, abundant in both marine environments (Le Roux & Austin 2006) and in the intestines of wrasse larvae (Birkbeck & Treasurer 2014), may reflect such contamination. The extent of this potential problem is hard to quantify, but it should be borne in mind during diagnostic investigations.

6.2. qPCR screening

One potential drawback of PCR assays targeting DNA molecules lie in their inherent inability to distinguish between live and dead cells. We cannot therefore, with absolute certainty, claim our qPCR detections to represent active infections. Moreover, the possible (and likely) existence of presently undescribed *A. salmonicida* A-layer types, with *vapA* sequence mismatch towards the employed oligonucleotides, represents a potential source for false negative detections. Presumably, this does not constitute a major uncertainty factor for our material however, as 99% of all examined *A. salmonicda* isolates from Norwegian cleaner fish belong to A-layer types V or VI (Table 3), both of which the modified qPCR assay detects.

6.3. A-layer typing

The A-layer typing scheme presented in Paper II differentiates *A. salmonicida* strains with a relatively high resolution. Virulence genes such as *vapA*, which are often under positive selection, may not, however, necessarily reflect true (genome-wide) phylogenetic descent. While A-layer typing is therefore not (alone) suitable for elucidation of phylogenetic relationships, by revealing strong A-layer type/host links (Figure 13) it may be extremely valuable in the applied fields of vaccine development and epidemiological study.

6.4. MLSA loci

Following BLAST searches against the published genome of the *V. splendidus*-related strain LGP 32 it became clear that all five housekeeping genes targeted for MLSA in Paper III lie within chromosome 1 of this strain (Figure 17). This chromosome has indeed been shown to encode most essential cellular processes in this bacterium (Le Roux et al. 2009).

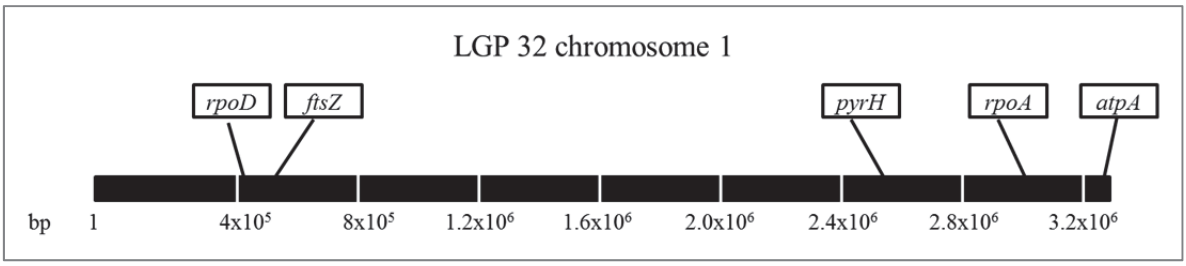

Figure 17: Chromosome 1 of *V. splendidus*-related strain LGP 32 (accession number: FM954972) outlined, with the approximate positioning of housekeeping genes sequenced in Paper III indicated. Illustration: Snorre Gulla.

Due to the risk of linkage, it would perhaps, in retrospect, have been more optimal if the sequenced genes were more evenly dispersed along the chromosome. On the other hand, the gene synteny of strain LGP 32 may not necessarily be shared by all Splendidus-clade members. Unfortunately, no assembled full genome sequences from other strains in the clade are publicly available, but distinct syntenies were observed following BLAST searches against the genomes of more distantly related vibrios (not shown). By using Linkage Analysis (LIAN) software (not shown), we found no indication that linkage represented a problem in our material.

6.5. Antisera used for serotyping

Polyclonal antisera used in Paper III were, as mentioned, raised against isolates within the *V. splendidus sensu stricto* cluster (Figure 14). As distinctive clustering patterns within this major cluster were largely lacking, two isolates with a relatively low sequence identity (99.4%, vs. ≥99.3% for the whole cluster), and recovered from different fish species (ballan wrasse and Atlantic salmon, respectively), were selected. In retrospect, the selection and/or number of isolates used for immunisation may have been suboptimal, as resolute serotyping of all examined *V. splendidus sensu stricto* isolates was not achieved. Alternatively, a more resolving protein typing method, such as Western blotting, could have been employed. The results demonstrated with certainty however, that O-antigen diversity exists within this group of very closely related strains.

7. Main conclusions and future prospectives

Bacterial infections are frequent in Norwegian cleaner fish used for biocontrol of salmon lice, and the most commonly isolated bacteria are atypical *A. salmonicida* and *V. splendidus*-related strains. Infections (in general) occur primarily following transfer to salmon farms, and screening by qPCR shows that *A. salmonicida* (an established cleaner fish pathogen) in particular is almost absent prior to stocking. In contrast, the bacterium was detected (by qPCR) in approximately two thirds of cleaner fish sampled at death in salmon farms. These findings fulfil the first and second objectives of this project and suggest that vaccination, as a preventive measure, could contribute greatly in terms of improving cleaner fish health and welfare. An extended study, involving sampling at various time-points before and after stocking, would facilitate more accurate inference of infection timelines. This could also allow for identification of critical points along the cleaner fish supply chain.

In-depth characterisation of bacterial strains involved in cleaner fish disease has been lacking. We show that A-layer (*vapA*) typing differentiates *A. salmonicida* subspecies, and identifies a range of host-specific subtypes of the bacterium, thus fulfilling the third objective of this project. Examined isolates from Norwegian cleaner fish belong almost exclusively (99%) to two A-layer types (V and VI). The observed diversity in the *vapA* gene may reflect antigenic diversity of importance for immune responses following exposure, and both cleaner fish-associated A-layer types should perhaps be included in an optimal *A. salmonicida* vaccine for these fish. Vaccine trials involving immunisation and challenge using various A-layer types are called for. It would also be interesting to include type VI A^- strains in such an experiment, in order to assess whether these strains may retain pathogenicity in the absence of a functional A-layer.

In contrast to the well-established fish pathogen *A. salmonicida*, the importance of *V. splendidus*-related bacteria in relation to cleaner fish disease is less clear. The executed MLSA and serotyping investigations fulfil the fourth project objective by showing that isolates from these fish are both genetically and antigenically relatively diverse. This is also often the case within individual mortality episodes, indicating that cleaner fish infection with *V. splendidus*-related bacteria does not primarily occur through fish-to-fish transmission. Perhaps more likely, such disease outbreaks reflect secondary invasions of weakened hosts by environmental opportunists. Vaccination against these bacteria may nevertheless improve matters, but vaccine strain selection is difficult due to the variable

nature of involved isolates. A more elaborate investigation of protein diversity amongst *V. splendidus* from cleaner fish, e.g. by use of Western blotting, might help in this regard.

Field trials would eventually be required in order to investigate vaccine protection (*A. salmonicida* and *V. splendidus*) in cleaner fish under the conditions encountered in salmon cages. This would most likely involve farmed cleaner fish, with groups of vaccinated and control fish being continuously monitored by sampling following transfer to salmon farms.

Lastly, it should be pointed out that in addition to atypical *A. salmonicida* and *V. splendidus*-related strains, a range of other bacterial agents have also, during the course of the present study, been frequently isolated from Norwegian cleaner fish at NVI (Table 1). While the role (if any) of *Aliivibrio* spp. with regard to cleaner fish health remains unclear, studies on *V. tapetis* indicate that this species is probably not a primary pathogen of wrasse (S. Gulla, unpublished data). *V. anguillarum*, *V. ordalii, P. anguilliseptica*, and not least the presently unnamed *Pasteurella* sp. (Alarcón et al. 2015), may however represent primary pathogens to wrasse and/or lumpsucker. *Tenacibaculum* spp. and *M. viscosa* may have roles in development of skin lesions. Future studies focussing on the above mentioned bacterial genera/species, in relation to cleaner fish health, are called for.

8. References

Aaen SM, Helgesen KO, Bakke MJ, Kaur K, Horsberg TE (2015) Drug resistance in sea lice: a threat to salmonid aquaculture. Trends Parasitol 31:72–81

Ai-min M, Mao-cang Y, Li-hua H, Qi-lang X, Shao-bo C, Le-zhou S (2010) Study on immune efficacy induced by killed *Vibrio splendidus* vaccine on *Sciaenops ocellatus*. J Hydroecology 3:137–141

Alarcón M, Gulla S, Røsaeg M V, Rønneseth A, Wergeland H, Poppe TT, Nilsen H, Colquhoun DJ (2015) Pasteurellosis in lumpsucker *Cyclopterus lumpus*, farmed in Norway. J Fish Dis:DOI: 10.1111/jfd.12366

Allen-Austin D, Austin B, Colwell RR (1984) Survival of *Aeromonas salmonicida* in river water. FEMS Microbiol Lett 21:143–146

Angulo L, Lopez JE, Vicente JA, Saborido AM (1994) Haemorrhagic areas in the mouth of farmed turbot, *Scophthalmus maximus* (L.). J Fish Dis 17:163–169

Araujo RM, Arribas RM, Pares R (1991) Distribution of *Aeromonas* species in waters with different levels of pollution. J Appl Bacteriol 71:182–186

Arnesen JA, Eggset G (1999) Isolation and characterisation of two extracellular metalloproteases from *Aeromonas salmonicida* ssp. *salmonicida*. J Fish Dis 22:35–43

Arnesen KR, Mikkelsen H, Schrøder MB, Lund V (2010) Impact of reattaching various *Aeromonas salmonicida* A-layer proteins on vaccine efficacy in Atlantic cod (*Gadus morhua*). Vaccine 28:4703–4708

Austin B (1993) Recovery of "atypical" isolates of *Aeromonas salmonicida*, which grow at 37°C, from ulcerated non-salmonids in England. J Fish Dis 16:165–168

Austin B, Austin DA (2007) Bacterial Fish Pathogens - Diseases of Farmed and Wild Fish, 4th edn. Springer Praxis Books, ISBN: 978-1-4020-6069-4

Austin B, Austin DA, Blanch AR, Cerda M, Grimont F, Grimont PAD, Jofre J, Koblavi S, Larsen JL, Pedersen K, Tiainen T, Verdonck L, Swings J (1997) A comparison of methods for the typing of fish-pathogenic *Vibrio* spp. Syst Appl Microbiol 20:89–101

Austin B, Austin DA, Dalsgaard I, Gudmundsdóttir BK, Høie S, Thornton JM, Larsen JL, O'Hici B, Powell R (1998) Characterization of atypical *Aeromonas salmonicida* by different methods. Syst Appl Microbiol 21:50–64

Austin DA, McIntosh D, Austin B (1989) Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* subsp. *smithia* subsp. nov. Syst Appl Microbiol 11:277–290

Balcázar JL, Vendrell D, Blas I de, Ruiz-Zarzuela I, Gironés O, Múzquiz JL (2007) Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using self-quenched, fluorogenic primers. J Med Microbiol 56:323–8

Balebona MC, Zorrilla I, Moriñigo MA, Borrego JJ (1998) Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. Aquaculture 166:19–35

Baumann P, Baumann L, Bang SS, Woolkalis MJ (1980) Reevaluation of the taxonomy of *Vibrio*, *Beneckea*, and *Photobacterium*: Abolition of the genus *Beneckea*. Curr Microbiol 4:127–132

Baumann P, Baumann L, Mandel M (1971) Taxonomy of marine bacteria: the genus *Beneckea*. J Bacteriol 107:268–294

Beall B, Facklam R, Thompson T (1996) Sequencing *emm*-specific PCR products for routine and accurate typing of group A Streptococci. J Clin Microbiol 34:953–8

Beaz-Hidalgo R, Diéguez AL, Cleenwerck I, Balboa S, Doce A, Vos P de, Romalde JL (2010) *Vibrio celticus* sp. nov., a new *Vibrio* species belonging to the Splendidus clade with pathogenic potential for clams. Syst Appl Microbiol 33:311–315

Beaz-Hidalgo R, Doce A, Pascual J, Toranzo AE, Romalde JL (2009) *Vibrio gallaecicus* sp. nov. isolated from cultured clams in north-western Spain. Syst Appl Microbiol 32:111–117

Beaz-Hidalgo R, Magi GE, Balboa S, Barja JL, Romalde JL (2008) Development of a PCR protocol for the detection of *Aeromonas salmonicida* in fish by amplification of the *fstA* (ferric siderophore receptor) gene. Vet Microbiol 128:386–94

Beijerinck MW (1900) Le *Photobacterium luminosum*, bactérie lumineuse de la mer du nord. Arch Néerlandaises des Sci Exactes Nat 23:401–427

Belland RJ, Trust TJ (1985) Synthesis, export, and assembly of *Aeromonas salmonicida* A-layer analyzed by transposon mutagenesis. J Bacteriol 163:877–81

Benagli C, Demarta A, Caminada A, Ziegler D, Petrini O, Tonolla M (2012) A rapid MALDI-TOF MS identification database at genospecies level for clinical and environmental *Aeromonas* strains. PLoS One 7:e48441

Bergh Ø, Hjeltnes B, Skiftesvik AB (1997) Experimental infection of turbot *Scophthalmus maximus* and halibut *Hippoglossus hippoglossus* yolk sac larvae with *Aeromonas salmonicida* subsp. *salmonicida*. Dis Aquat Org 29:13–20

Bergh Ø, Samuelsen OB (2006) Susceptibility of corkwing wrasse *Symphodus melops*, goldsinny wrasse *Ctenolabrus rupestis*, and Atlantic salmon *Salmo salar* smolt, to experimental challenge with *Vibrio tapetis* and *Vibrio splendidus* isolated from corkwing wrasse. Aquac Int 15:11–18

Binesse J, Delsert C, Saulnier D, Champomier-Vergès MC, Zagorec M, Munier-Lehmann H, Mazel D, Roux F Le (2008) Metalloprotease Vsm is the major determinant of toxicity for extracellular products of *Vibrio splendidus*. Appl Environ Microbiol 74:7108–7117

Birkbeck TH, Treasurer JW (2014) *Vibrio splendidus*, *Vibrio ichthyoenteri* and *Vibrio pacinii* isolated from the digestive tract microflora of larval ballan wrasse, *Labrus bergylta* Ascanius, and goldsinny wrasse, *Ctenolabrus rupestris* (L.). J Fish Dis 37:69–74

Biswas G, Sakai M (2014) Loop-mediated isothermal amplification (LAMP) assays for detection and identification of aquaculture pathogens: current state and perspectives. Appl Microbiol Biotechnol 98:2881–2895

Bjordal Å (1988) Cleaning symbiosis between wrasses (Labridae) and lice infested salmon (*Salmo salar*) in mariculture. Int Counc Explor Sea F:17

Bjordal Å (1992) Cleaning symbiosis as an alternative to chemical control of sea lice infestation of Atlantic salmon. World Aquac Work 4:53–60

Björnsdóttir B, Gudmundsdóttir S, Bambir SH, Gudmundsdóttir BK (2005) Experimental infection of turbot, *Scophthalmus maximus* (L.), by *Aeromonas salmonicida* subsp. *achromogenes* and evaluation of cross protection induced by a furunculosis vaccine. J Fish Dis 28:181–8

Bornø G, Lie Linaker M (editors) (2015) Fiskehelserapporten 2014 (Norwegian Farmed Fish Health Report 2014; in Norwegian). Norwegian Veterinary Institute, Harstad, ISSN: 1893-1480

Bowers JM, Mustafa A, Speare DJ, Conboy GA, Brimacombe M, Sims DE, Burka JF (2000) The physiological response of Atlantic salmon, *Salmo salar* L., to a single experimental challenge with sea lice, *Lepeophtheirus salmonis*. J Fish Dis 23:165–172

Butte AJ, Dzau VJ, Glueck SB (2001) Further defining housekeeping, or "maintenance," genes. Focus on "A compendium of gene expression in normal human tissues". Physiol Genomics 7:95–96

Byers HK, Cipriano RC, Gudkovs N, Cranel MSJ (2002) PCR-based assays for the fish pathogen *Aeromonas salmonicida*. II. Further evaluation and validation of three PCR primer sets with infected fish. Dis Aquat Organ 49:139–44

Byers HK, Gudkovs N, Crane MSJ (2002) PCR-based assays for the fish pathogen *Aeromonas salmonicida*. I. Evaluation of three PCR primer sets for detection and identification. Dis Aquat Organ 49:129–38

Chart H, Shaw DH, Ishiguro EE, Trust TJ (1984) Structural and immunochemical homogeneity of *Aeromonas salmonicida* lipopolysaccharide. J Bacteriol 158:16–22

Chu S, Cavaignac S, Feutrier J, Phipps BM, Kostrzynska M, Kay WW, Trust TJ (1991) Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. J Biol Chem 266:15258–65

Cipriano RC, Bertolini J (1988) Selection for virulence in the fish pathogen *Aeromonas salmonicida*, using Coomassie Brilliant Blue agar. J Wildl Dis 24:672–678

Clarridge JE (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 17:840–862

Costello MJ (2009) How sea lice from salmon farms may cause wild salmonid declines in Europe and North America and be a threat to fishes elsewhere. Proc Biol Sci 276:3385–94

Dacanay A, Knickle L, Solanky KS, Boyd JM, Walter JA, Brown LL, Johnson SC, Reith M (2006) Contribution of the type III secretion system (TTSS) to virulence of *Aeromonas salmonicida* subsp. *salmonicida*. Microbiology 152:1847–1856

Dadswell MJ, Spares AD, Reader JM, Stokesbury MJW (2010) The North Atlantic subpolar gyre and the marine migration of Atlantic salmon *Salmo salar*: The "Merry-Go-Round" hypothesis. J Fish Biol 77:435–467

Dalsgaard I, Gudmundsdóttir BK, Helgason S, Høie S, Thoresen OF, Wichardt UP, Wiklund T (1998) Identification of atypical *Aeromonas salmonicida*: inter-laboratory evaluation and harmonization of methods. J Appl Microbiol 84:999–1006

Dalsgaard I, Nielsen B, Larsen JL (1994) Characterization of *Aeromonas salmonicida* subsp. *salmonicida*: a comparative study of strains of different geographic origin. J Appl Bacteriol 77:21–30

Daly JG, Kew AK, Moore AR, Olivier G (1996) The cell surface of *Aeromonas salmonicida* determines *in vitro* survival in cultured brook trout (*Salvelinus fontinalis*) peritoneal macrophages. Microb Pathog 21:447–61

Deady S, Varian SJA, Fives JM (1995) The use of cleaner-fish to control sea lice on two Irish salmon (*Salmo salar*) farms with particular reference to wrasse behaviour in salmon cages. Aquaculture 131:73–90

Denholm I, Devine GJ, Horsberg TE, Sevatdal S, Fallang A, Nolan D V., Powell R (2002) Analysis and management of resistance to chemotherapeutants in salmon lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae). Pest Manag Sci 58:528–536

Diéguez AL, Beaz-Hidalgo R, Cleenwerck I, Balboa S, Vos P de, Romalde JL (2011) *Vibrio atlanticus* sp. nov. and *Vibrio artabrorum* sp. nov., isolated from the clams *Ruditapes philippinarum* and *Ruditapes decussatus*. Int J Syst Evol Microbiol 61:2406–2411

Dipper FA, Bridges CR, Menz A (1977) Age, growth and feeding in the ballan wrasse *Labrus bergylta* Ascanius 1767. J Fish Biol 11:105–120

Doig P, McCubbin WD, Kay CM, Trust TJ (1993) Distribution of surface-exposed and non-accessible amino acid sequences among the two major structural domains of the S-layer protein of *Aeromonas salmonicida*. J Mol Biol 233:753–65

Dror M, Sinyakov MS, Okun E, Dym M, Sredni B, Avtalion RR (2006) Experimental handling stress as infection-facilitating factor for the goldfish ulcerative disease. Vet Immunol Immunopathol 109:279–287

Duperthuy M, Binesse J, Roux F Le, Romestand B, Caro A, Got P, Givaudan A, Mazel D, Bachère E, Destoumieux-Garzón D (2010) The major outer membrane protein OmpU of *Vibrio splendidus* contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*. Environ Microbiol 12:951–963

Duperthuy M, Schmitt P, Garzón E, Caro A, Rosa RD, Roux F Le, Lautrédou-Audouy N, Got P, Romestand B, Lorgeril J de, Kieffer-Jaquinod S, Bachère E, Destoumieux-Garzón D (2011) Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. Proc Natl Acad Sci U S A 108:2993–2998

Effendi I, Austin B (1995) Dormant/unculturable cells of the fish pathogen *Aeromonas salmonicida*. Microb Ecol 30:183–192

Ellis AE, Burrows AS, Stapleton KJ (1988) Lack of relationship between virulence of *Aeromonas salmonicida* and the putative virulence factors: A-layer, extracellular proteases and extracellular haemolysins. J Fish Dis 11:309–323

Emmerich R, Weibel E (1894) Über eine durch Bakterien erzeugte Seuche unter den Forellen. Arch Hyg Bakteriol 21:1–21

Espedal PG, Glover KA, Horsberg TE, Nilsen F (2013) Emamectin benzoate resistance and fitness in laboratory reared salmon lice (*Lepeophtheirus salmonis*). Aquaculture 416-417:111–118

Espeland SH, Nedreaas K, Mortensen S, Skiftesvik AB, Agnalt A-L, Durif C, Harkestad LS, Karlsbakk E, Knutsen H, Thangstad T, Jørstad K, Bjordal Å, Gjøsæter J (2010) Kunnskapsstatus leppefisk - Utfordringer i et økende fiskeri (Current knowledge on wrasse - Challenges in an increasing fishery; in Norwegian). The Norwegian Institute for Marine Research, Fisken og Havet - Project Report 7-2010

Euzéby JP (1997) List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. Int J Syst Bacteriol 47:590–592

Evenberg D, Boxtel R Van, Lugtenberg B, Schurer F, Blommaert J, Bootsma R (1982) Cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*. I. Relationship between autoagglutination and the presence of a major cell envelope protein. Biochim Biophys Acta 684:241–248

Evenberg D, Lugtenberg B (1982) Cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*. II. Purification and characterization of a major cell envelope protein related to autoagglutination, adhesion and virulence. Biochim Biophys Acta 684:249–254

EWOS (2011) EWOS robust. www.ripplesofinnovation.com/robust.html

Faury N, Saulnier D, Thompson FL, Gay M, Swings J, Roux F Le (2004) *Vibrio crassostreae* sp. nov., isolated from the haemolymph of oysters (*Crassostrea gigas*). Int J Syst Evol Microbiol 54:2137–2140

Feil EJ, Cooper JE, Grundmann H, Robinson DA, Enright MC, Berendt T, Peacock SJ, Smith JM, Murphy M, Spratt BG, Moore CE, Day NPJ (2003) How clonal is *Staphylococcus aureus*? J Bacteriol 185:3307–3316

Feil EJ, Maiden MC, Achtman M, Spratt BG (1999) The relative contributions of recombination and mutation to the divergence of clones of *Neisseria meningitidis*. Mol Biol Evol 16:1496–1502

Feil EJ, Spratt BG (2001) Recombination and the population structures of bacterial pathogens. Annu Rev Microbiol 55:561–590

Fijan N (1972) Infectous dropsy in carp - a disease complex. In: Mawdesley-Thomas LE (ed) Diseases of Fish. Symposia of the Zoological Society of London. Academic Press, London, p 39–51

Filliol I, Motiwala AS, Cavatore M, Qi W, Hazbón MH, Bobadilla del Valle M, Fyfe J, García-García L, Rastogi N, Sola C, Zozio T, Guerrero MI, León CI, Crabtree J, Angiuoli S, Eisenach KD, Durmaz R, Joloba ML, Rendón A, Sifuentes-Osornio J, Ponce de León A, Cave MD, Fleischmann R, Whittam TS, Alland D (2006) Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. J Bacteriol 188:759–72

Fitzpatrick DA, Creevey CJ, McInerney JO (2005) Evidence of positive Darwinian selection in putative meningococcal vaccine antigens. J Mol Evol 61:90–98

Fuller DW, Pilcher KS, Fryer JL (1977) A leukocytolytic factor isolated from cultures of *Aeromonas salmonicida*. J Fish Res Board Canada 34:1118–1125

Garduño RA, Moore AR, Olivier G, Lizama AL, Garduño E, Kay WW (2000) Host cell invasion and intracellular residence by *Aeromonas salmonicida*: role of the S-layer. Can J Microbiol 46:660–8

Gay M, Berthe FCJ, Roux F Le (2004) Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. Dis Aquat Organ 59:49–56

Gay M, Renault T, Pons AM, Roux F Le (2004) Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: Taxonomy and host alterations. Dis Aquat Organ 62:65–74

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, Peer Y Van de, Vandamme P, Thompson FL, Swings J (2005) Opinion: Re-evaluating prokaryotic species. Nat Rev Microbiol 3:733–9

Glaeser SP, Kämpfer P (2015) Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst Appl Microbiol 38:237–245

Griffin PJ, Snieszko SF, Friddle SB (1953) A more comprehensive description of *Bacterium salmonicida*. Trans Am Fish Soc 82:129–138

Grimnes A, Jakobsen PJ (1996) The physiological effects of salmon lice infection on post-smolt of Atlantic salmon. J Fish Biol 48:1179–1194

Grøntvedt RN, Jansen PA, Horsberg TA, Helgesen K, Tarpai A (2015) The surveillance programme for resistance to chemotherapeutants in salmon lice (*Lepeophtheirus salmonis*) in Norway 2014. Surveillance programmes for terrestrial and aquatic animals in Norway. Annual report 2014. The Norwegian Veterinary Institute, Oslo. ISSN 1894-5678

Grøntvedt RN, Nerbøvik IKG, Viljugrein H, Lillehaug A, Nilsen H, Gjevre AG (2015) Termisk avlusning av laksefisk - dokumentasjon av fiskevelferd og effekt (Thermal de-lousing of salmonids - Documentation of fish welfare and effect; in Norwegian). Norwegian Veterinary Institute's Report Series 13-2015, ISSN 1890-3290

Gudmundsdottir BK (1996) Comparison of extracellular proteases produced by *Aeromonas salmonicida* strains, isolated from various fish species. J Appl Bacteriol 80:105–113

Gudmundsdóttir BK, Björnsdóttir B (2007) Vaccination against atypical furunculosis and winter ulcer disease of fish. Vaccine 25:5512–5523

Gurtler V, Stanisich VA (1996) New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology 142:3–16

Gustafson CE, Thomas CJ, Trust TJ (1992) Detection of *Aeromonas salmonicida* from fish by using polymerase chain reaction amplification of the virulence surface array protein gene. Appl Environ Microbiol 58:3816–25

Han H-J, Kim D-Y, Kim W-S, Kim C-S, Jung S-J, Oh M-J, Kim D-H (2011) Atypical *Aeromonas salmonicida* infection in the black rockfish, *Sebastes schlegeli* Hilgendorf, in Korea. J Fish Dis 34:47–55

Hedlund BP, Staley JT (2001) *Vibrio cyclotrophicus* sp. nov., a polycyclic aromatic hydrocarbon (PAH)-degrading marine bacterium. Int J Syst Evol Microbiol 51:61–66

Helgesen KO, Romstad H, Aaen SM, Horsberg TE (2015) First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway. Aquac Reports 1:37–42

Hellberg H, Moksness E, Hoie S (1996) Infection with atypical *Aeromonas salmonicida* in farmed common wolffish, *Anarhichas lupus* L. J Fish Dis 19:329–332

Hidaka T, Shibata T, Yanohara Y (1983) On the causative bacteria of head ulcer disease in cultured eels *Anguilla japonica*. Memo Fac Fish Kagoshima Univ 32:147–166

Higuchi R, Dollinger G, Walsh PS, Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. Nat Biotechnol 10:413–417

Hilldén NO (1983) Cleaning behaviour of the goldsinny (Pisces, Labridae) in Swedish waters. Behav Processes 8:87–90

Hiney M, Dawson MT, Heery DM, Smith PR, Gannon F, Powell R (1992) DNA probe for *Aeromonas salmonicida*. Appl Environ Microbiol 58:1039–42

Hirst ID, Ellis AE (1994) Iron-regulated outer membrane proteins of *Aeromonas salmonicida* are important protective antigens in Atlantic salmon against furunculosis. Fish Shellfish Immunol 4:29–45

Hirst ID, Hastings TS, Ellis AE (1994) Utilization of haem compounds by *Aeromonas salmonicida*. J Fish Dis 17:365–373

Hjeltnes B (editor) (2014) Fiskehelserapporten 2013 (Norwegian Farmed Fish Health Report 2013; in Norwegian). Norwegian Veterinary Institute, Oslo, ISSN: 1893-1480

Hunt DE, David LA, Gevers D, Preheim SP, Alm EJ, Polz MF (2008) Resource partitioning and sympatric differentiation among closely related bacterioplankton. Science 320:1081–5

Imsland AK, Reynolds P, Eliassen G, Hangstad TA, Foss A, Vikingstad E, Elvegård TA (2014) The use of lumpfish (*Cyclopterus lumpus* L.) to control sea lice (*Lepeophtheirus salmonis* Krøyer) infestations in intensively farmed Atlantic salmon (*Salmo salar* L.). Aquaculture 424-425:18–23

Ingilæ M, Arnesen JA, Lund V, Eggset G (2000) Vaccination of Atlantic halibut *Hippoglossus hippoglossus* L., and spotted wolffish *Anarhichas minor* L., against atypical *Aeromonas salmonicida*. Aquaculture 183:31–44

Ishiguro EE, Kay WW, Ainsworth T, Chamberlain JB, Austen RA, Buckley JT, Trust TJ (1981) Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. J Bacteriol 148:333–340

Jensen S, Frost P, Torsvik VL (2009) The nonrandom microheterogeneity of 16S rRNA genes in *Vibrio splendidus* may reflect adaptation to versatile lifestyles. FEMS Microbiol Lett 294:207–15

Jensen S, Samuelsen OB, Andersen K, Torkildsen L, Lambert C, Choquet G, Paillard C, Bergh O (2003) Characterization of strains of *Vibrio splendidus* and *V. tapetis* isolated from corkwing wrasse *Symphodus melops* suffering vibriosis. Dis Aquat Organ 53:25–31

Jiggins FM, Hurst GDD, Yang Z (2002) Host-symbiont conflicts: positive selection on an outer membrane protein of parasitic but not mutualistic Rickettsiaceae. Mol Biol Evol 19:1341–1349

Johansen R (editor) (2013) Fiskehelserapporten 2012 (Norwegian Farmed Fish Health Report 2012; in Norwegian). Norwegian Veterinary Institute, Oslo, ISSN: 1893-1480

Johansen L-H, Jensen I, Mikkelsen H, Bjørn P-A, Jansen PA, Bergh Ø (2011) Disease interaction and pathogens exchange between wild and farmed fish populations with special reference to Norway. Aquaculture 315:167–186

Jones CS, Lockyer AE, Verspoor E, Secombes CJ, Noble LR (2002) Towards selective breeding of Atlantic salmon for sea louse resistance: approaches to identify trait markers. Pest Manag Sci 58:559–68

Kaku Y, Yamada Y, Wakabayashi H (1999) Characterization of atypical *Aeromonas salmonicida* isolated from an epizootic ulcerative disease in carp (*Cyprinus carpio*). Fish Pathol 34:155–162

Kay WW, Buckley JT, Ishiguro EE, Phipps BM, Monette JP, Trust TJ (1981) Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. J Bacteriol 147:1077–1084

Kay WW, Phipps BM, Ishiguro EE, Trust TJ (1985) Porphyrin binding by the surface array virulence protein of *Aeromonas salmonicida*. J Bacteriol 164:1332–1336

Kay WW, Trust TJ (1991) Form and functions of the regular surface array (S-layer) of *Aeromonas salmonicida*. Experientia 47:412–4

Keeling SE, Brosnahan CL, Johnston C, Wallis R, Gudkovs N, McDonald WL (2013) Development and validation of a real-time PCR assay for the detection of *Aeromonas salmonicida*. J Fish Dis 36:495–503

Kim DH, Choi SY, Kim CS, Oh MJ, Jeong HD (2013) Low-value fish used as feed in aquaculture were a source of furunculosis caused by atypical *Aeromonas salmonicida*. Aquaculture 408-409:113–117

Kim Y-S, Yoon JW, Han H-J, Suebsing R, Kim J-H (2011) Prevalence and characterization of typical *Aeromonas salmonicida* chum salmon isolates in Korea. Can J Fish Aquat Sci 14:347–354

Kimura T (1969a) A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on “Sakuramasu” (*Oncorhynchus masou*) and Pink salmon (*O. gorbuscha*) rearing for maturity. Fish Pathol 3:45–52

Kimura T (1969b) A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on "Sakuramasu" (*Oncorhynchus masou*) and Pink salmon (*O. gorbuscha*) rearing for maturity. Fish Pathol 3:34–44

Koppang EO, Fjølstad M, Melgård B, Vigerust M, Sørum H (2000) Non-pigment-producing isolates of *Aeromonas salmonicida* subspecies *salmonicida*: isolation, identification, transmission and pathogenicity in Atlantic salmon, *Salmo salar* L. J Fish Dis 23:39–48

Kulkarni A, Caipang CMA, Brinchmann MF, Korsnes K, Kiron V (2009) Loop-mediated isothermal amplification – an assay for the detection of atypical furunculosis caused by *Aeromonas salmonicida* in Atlantic cod, *Gadus morhua*. J Rapid Methods Autom Microbiol 17:476–489

Kvenseth PG (1996) Large-scale use of wrasse to control sea lice and net fouling in salmon farms in Norway. In: Sayer MDJ, Treasurer JW, Costello MJ (eds) Wrasse Biology and Use in Aquaculture, 1st edn. Wiley-Blackwell, ISBN: 978-0-85238-236-3, p 196–203

Küpfer M, Kuhnert P, Korczak BM, Peduzzi R, Demarta A (2006) Genetic relationships of *Aeromonas* strains inferred from 16S rRNA, *gyrB* and *rpoB* gene sequences. Int J Syst Evol Microbiol 56:2743–2751

Laidler LA, Treasurer J, Grant AN, Cox DI (1999) Atypical *Aeromonas salmonicida* infection in wrasse (Labridae) used as cleaner fish of farmed Atlantic salmon, *Salmo salar* L., in Scotland. J Fish Dis 22:209–213

Lasa A, Diéguez AL, Romalde JL (2013) *Vibrio toranzoniae* sp. nov., a new member of the Splendidus clade in the genus *Vibrio*. Syst Appl Microbiol 36:96–100

Le Roux F, Austin B (2006) *Vibrio splendidus*. In: Thompson FL, Austin B, Swings J (eds) The Biology of Vibrios. American Society of Microbiology, ISBN: 978-1555813659, p 285–296

Le Roux F, Binesse J, Saulnier D, Mazel D (2007) Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. Appl Environ Microbiol 73:777–784

Le Roux F, Gay M, Lambert C, Nicolas JL, Gouy M, Berthe F (2004) Phylogenetic study and identification of *Vibrio splendidus*-related strains based on *gyrB* gene sequences. Dis Aquat Organ 58:143–50

Le Roux F, Goubet A, Thompson FL, Faury N, Gay M, Swings J, Saulnier D (2005) *Vibrio gigantis* sp. nov., isolated from the haemolymph of cultured oysters (*Crassostrea gigas*). Int J Syst Evol Microbiol 55:2251–2255

Le Roux F, Zouine M, Chakroun N, Binesse J, Saulnier D, Bouchier C, Zidane N, Ma L, Rusniok C, Lajus A, Buchrieser C, Médigue C, Polz MF, Mazel D (2009) Genome sequence of *Vibrio splendidus*: an abundant planctonic marine species with a large genotypic diversity. Environ Microbiol 11:1959–70

Lehmann KB, Neumann R (1896) Atlas und Grundriss der Bakeriologie und Lehrbuch der speziellen bakteriologischen Diagnositk. (JF Lehmann, Ed.), 1st edn. Munchen

Li W, Raoult D, Fournier P-E (2009) Bacterial strain typing in the genomic era. FEMS Microbiol Rev 33:892–916

Lindstedt B-A (2005) Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. Electrophoresis 26:2567–82

Liu Y, Olaf Olaussen J, Skonhoft A (2011) Wild and farmed salmon in Norway - a review. Mar Policy 35:413–418

Lovdata.no (2008a) Forskrift om drift av akvakulturanlegg - akvakulturdriftsforskriften (Regulation on aquaculture operations): FOR-2008-06-17-822. Norway

Lovdata.no (2008b) Forskrift om omsetning av akvakulturdyr og produkter av akvakulturdyr, forebygging og bekjempelse av smittsomme sykdommer hos akvatiske dyr (Regulation on aquaculture operations): FOR-2008-06-17-819. Norway

Lovdata.no (2012) Forskrift om bekjempelse av lakselus i akvakulturanlegg (Regulation on combatting salmon lice in aquaculture facilities): FOR-2012-12-05-1140. Norway

Lund V, Arnesen JA, Coucheron D, Modalsli K, Syvertsen C (2003) The *Aeromonas salmonicida* A-layer protein is an important protective antigen in oil-adjuvanted vaccines. Fish Shellfish Immunol 15:367–372

Lund V, Arnesen JA, Mikkelsen H, Gravningen K, Brown L, Schrøder MB (2008) Atypical furunculosis vaccines for Atlantic cod (*Gadus morhua*); vaccine efficacy and antibody responses. Vaccine 26:6791–6799

Lund V, Espelid S, Mikkelsen H (2003) Vaccine efficacy in spotted wolffish *Anarhichas minor*: relationship to molecular variation in A-layer protein of atypical *Aeromonas salmonicida*. Dis Aquat Organ 56:31–42

Lund V, Jenssen LM, Wesmajervi MS (2002) Assessment of genetic variability and relatedness among atypical *Aeromonas salmonicida* from marine fishes, using AFLP-fingerprinting. Dis Aquat Organ 50:119–126

Lund V, Mikkelsen H (2004) Genetic diversity among A-proteins of atypical strains of *Aeromonas salmonicida*. Dis Aquat Organ 61:257–262

Lund V, Mikkelsen H, Schrøder MB (2008) Comparison of atypical furunculosis vaccines in spotted wolffish (*Anarhicas minor* O.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). Vaccine 26:2833–40

Lund V, Mikkelsen H, Schrøder MB (2009) Atypical furunculosis vaccines for Atlantic cod *Gadhus morhua*: impact of reattached *Aeromonas salmonicida* A-layer protein on vaccine efficacy. Dis Aquat Organ 85:115–122

Lupiani B, Dopazo CP, Ledo A, Fouz B, Barja JL, Hetrick FM, Toranzo AE (1989) New syndrome of mixed bacterial and viral etiology in cultured turbot *Scophthalmus maximus*. J Aquat Anim Health 1:197–204

Lusedata.no (2014a) Bransjeveileder lakselus - Bruk og hold av leppefisk (Guide on the use and keep of cleaner fish wrasse; in Norwegian). www.lusedata.no/for-naeringen/veiledere-leppefisk

Lusedata.no (2014b) Bransjeveileder lakselus - Bruk og hold av rognkjeks (Guide on the use and keep of cleaner fish lumpsucker; in Norwegian). www.lusedata.no/for-naeringen/veiledere-leppefisk

Lusedata.no (2014c) Bransjeveileder lakselus - Fangst og mellomlagring av leppefisk (Guide on the capture and storage of cleaner fish wrasse; in Norwegian). www.lusedata.no/for-naeringen/veiledere-leppefisk

Lusedata.no (2014d) Bransjeveileder lakselus - Transport av leppefisk (Guide on the transport of cleaner fish wrasse; in Norwegian). www.lusedata.no/for-naeringen/veiledere-leppefisk

Lusedata.no (2014e) Bransjeveileder lakselus - Mottak av leppefisk (Guide to the receipt of cleaner fish wrasse; in Norwegian). www.lusedata.no/for-naeringen/veiledere-leppefisk

Lusedata.no (2014f) Bransjeveileder lakselus - Overvintring av leppefisk (Guide to salmon farm wintering of cleaner fish wrasse; in Norwegian). www.lusedata.no/for-naeringen/veiledere-leppefisk

Lusedata.no (2015) Salmon louse statistics. http://statistikk.lusedata.no

Lutwyche P, Exner MM, Hancock RE, Trust TJ (1995) A conserved *Aeromonas salmonicida* porin provides protective immunity to rainbow trout. Infect Immun 63:3137–42

Lødemel JB, Mayhew TM, Myklebust R, Olsen RE, Espelid S, Ringø E (2001) Effect of three dietary oils on disease susceptibility in Arctic charr (*Salvelinus alpinus* L.) during cohabitant challenge with *Aeromonas salmonicida* ssp. *salmonicida*. Aquac Res 32:935–945

Macián MC, Ludwig W, Aznar R, Grimont PAD, Schleifer KH, Garay E, Pujalte MJ (2001) *Vibrio lentus* sp. nov., isolated from Mediterranean oysters. Int J Syst Evol Microbiol 51:1449–1456

Macpherson HL, Bergh Ø, Birkbeck TH (2012) An aerolysin-like enterotoxin from *Vibrio splendidus* may be involved in intestinal tract damage and mortalities in turbot, *Scophthalmus maximus* (L.), and cod, *Gadus morhua* L., larvae. J Fish Dis 35:153–67

Magnadóttir B, Bambir SH, Gudmundsdóttir BK, Pilström L, Helgason S (2002) Atypical *Aeromonas salmonicida* infection in naturally and experimentally infected cod, *Gadus morhua* L. J Fish Dis 25:583–597

Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG (1998) Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci 95:3140–3145

Markestad A, Grave K (1997) Reduction of antibacterial drug use in Norwegian fish farming due to vaccination. Dev Biol Stand 90:365–369

Martinez-Murcia AJ, Monera A, Saavedra MJ, Oncina R, Lopez-Alvarez M, Lara E, Figueras MJ (2011) Multilocus phylogenetic analysis of the genus *Aeromonas*. Syst Appl Microbiol 34:189–199

Masada CL, LaPatra SE, Morton AW, Strom MS (2002) An *Aeromonas salmonicida* type IV pilin is required for virulence in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 51:13–25

McIntosh D, Austin B (1991) Atypical characteristics of the salmonid pathogen *Aeromonas salmonicida*. J Gen Microbiol 137:1341–1343

Mellmann A, Mosters J, Bartelt E, Roggentin P, Ammon A, Friedrich AW, Karch H, Harmsen D (2004) Sequence-based typing of *flaB* is a more stable screening tool than typing of *flaA* for monitoring of *Campylobacter* populations. J Clin Microbiol 42:4840–4842

Mendoza M, Güiza L, Martinez X, Caraballo X, Rojas J, Aranguren LF, Salazar M (2013) A novel agent (*Endozoicomonas elysicola*) responsible for epitheliocystis in cobia *Rachycentrum canadum* larvae. Dis Aquat Organ 106:31–37

Merino S, Aguilar A, Rubires X, Simon-Pujol D, Congregado F, Tomás JM (1996) The role of the capsular polysaccharide of *Aeromonas salmonicida* in the adherence and invasion of fish cell lines. FEMS Microbiol Lett 142:185–189

Merino S, Aguilar A, Tomás JM, Bonet R, Martinez MJ, Simón-Pujol D, Congregado F (1997) Complement resistance of capsulated strains of *Aeromonas salmonicida*. Microb Pathog 22:315–320

Midtlyng PJ (1996) A field study on intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish Shellfish Immunol 6:553–565

Miyata M, Aoki T, Inglis V, Yoshida T, Endo M (1995) RAPD analysis of *Aeromonas salmonicida* and *Aeromonas hydrophila*. J Appl Bacteriol 79:181–185

Miyata A, Inglis V, Aoki T (1996) Rapid identification of *Aeromonas salmonicida* subspecies *salmonicida* by the polymerase chain reaction. Aquaculture 141:13–24

Munn CB, Ishiguro EE, Kay WW, Trust TJ (1982) Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. Infect Immun 36:1069–1075

Myhr E, Larsen JL, Lillehaug A, Gudding R, Heum M, Hastein T (1991) Characterization of *Vibrio anguillarum* and closely related species isolated from farmed fish in Norway. Appl Environ Microbiol 57:2750–2757

Natrah FMI, Alam MI, Pawar S, Harzevili AS, Nevejan N, Boon N, Sorgeloos P, Bossier P, Defoirdt T (2012) The impact of quorum sensing on the virulence of *Aeromonas hydrophila* and *Aeromonas salmonicida* towards burbot (*Lota lota* L.) larvae. Vet Microbiol 159:77–82

Nealson KH, Wimpee B, Wimpee C (1993) Identification of *Vibrio splendidus* as a member of the planktonic luminous bacteria from the Persian Gulf and Kuwait region with *luxA* probes. Appl Environ Microbiol 59:2684–2689

Neave MJ, Michell CT, Apprill A, Voolstra CR (2014) Whole-genome sequences of three symbiotic *Endozoicomonas* strains. Genome Announc 2:e00802–14

Nicolas JL, Corre S, Gauthier G, Robert R, Ansquer D (1996) Bacterial problems associated with scallop *Pecten maximus* larval culture. Dis Aquat Org 27:67–76

Nilsen A, Erikson U, Aunsmo A, Østvik A, Heuch PA (2010) Mekanisk fjerning av lakselus "FLS avlusersystem" - test av ejektorpumpe fra Flatsetsund Engineering AS (Mechanical louse removal "FLS aavlusersystem" - test of ejector pump from Flatsetsund Engineering AS; in Norwegian). The Norwegian Veterinary Institute's Report Series 11-2010, ISSN: 1890-3290

Nilsen A, Viljugrein H, Røsæg MV, Colquhoun D (2014) Rensefiskhelse - kartlegging av dødelighet og dødelighetsårsaker (Cleaner fish health - a survey of mortalities and causes of death; in Norwegian). The Norwegian Veterinary Institute's Report Series 12-2014, ISSN: 1890-3290

Noonan B, Trust TJ (1997) The synthesis, secretion and role in virulence of the paracrystalline surface protein layers of *Aeromonas salmonicida* and *A. hydrophila*. FEMS Microbiol Lett 154:1–7

NORM/NORM-VET (2015) NORM/NORM-VET Report 2014 - Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Tromsø/Oslo; ISSN:1502-2307 (print) / 1890-9965 (electronic)

Norwegian Directorate of Fisheries (2015) Norwegian Aquaculture Statistics. www.fiskeridir.no/English/Aquaculture/Statistics

Norwegian Institute of Public Health (2015) Pharmaceuticals in Aquaculture (in Norwegian). www.fhi.no/artikler/?id=114175

Norwegian Research Council (2014) New technology can prevent salmon lice. www.forskningsradet.no/prognett-havbruk/Nyheter/New_technology_can_prevent_salmon_lice/1253996529392/p1226994216948

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:e63

O'Brien D, Mooney J, Ryan D, Powell E, Hiney M, Smith PR, Powell R (1994) Detection of *Aeromonas salmonicida*, causal agent of furunculosis in salmonid fish, from the tank effluent of hatchery-reared Atlantic salmon smolts. Appl Environ Microbiol 60:3874–3877

O'hIci B, Olivier G, Powell R (2000) Genetic diversity of the fish pathogen *Aeromonas salmonicida* demonstrated by random amplified polymorphic DNA and pulsed-field gel electrophoresis analyses. Dis Aquat Organ 39:109–119

Olivier G (1990) Virulence of *Aeromonas salmonicida*: Lack of relationship with phenotypic characteristics. J Aquat Anim Health 2:119–127

Optics.org (2014) Laser technique combats sea parasites. www.optics.org/news/5/5/52

Otal I, Martin C, Vincent-Levy-Frebault V, Thierry D, Gicquel B (1991) Restriction fragment length polymorphism analysis using IS6110 as an epidemiological marker in tuberculosis. J Clin Microbiol 29:1252–1254

Parte AC (2014) LPSN - List of prokaryotic names with standing in nomenclature. Nucleic Acids Res 42:D613–6

Pascual J, Macián MC, Arahal DR, Garay E, Pujalte MJ (2010) Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, *recA*, *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* genes. Int J Syst Evol Microbiol 60:154–65

Pavan ME, Abbott SL, Zorzópulos J, Janda JM (2000) *Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. Int J Syst Evol Microbiol 50:1119–1124

Pedersen K, Kofod H, Dalsgaard I, Larsen JL (1994) Isolation of oxidase-negative *Aeromonas salmonicida* from diseased turbot *Scopthhalmus maximus*. Dis Aquat Organ 18:149–154

Phipps BM, Trust TJ, Ishiguro EE, Kay WW (1983) Purification and characterization of the cell surface virulent A protein from *Aeromonas salmonicida*. Biochemistry 22:2934–2939

Potts GW (1973) Cleaning symbiosis among British fish with special reference to *Crenilabrus melops* (Labridae). J Mar Biol Assoc United Kingdom 53:1–10

Press CM, Lillehaug A (1995) Vaccination in European salmonid aquaculture: A review of practices and prospects. Br Vet J 151:45–69

Qadri F, Azim T, Chowdhury A, Hossain J, Sack R, Albert M (1994) Production, characterization, and application of monoclonal antibodies to *Vibrio cholerae* O139 synonym Bengal. Clin Diagn Lab Immunol 1:51–54

Rattanachaikunsopon P, Phumkhachorn P (2012) Detection of *Aeromonas salmonicida* by Reverse Transcription-Multiplex Polymerase Chain Reaction. Biosci Biotechnol Biochem 76:665–670

Reichelt JL, Baumann P, Baumann L (1976) Study of genetic relationships among marine species of the genera *Beneckea* and *Photobacterium* by means of in vitro DNA/DNA hybridization. Arch Microbiol 110:101–20

Reid HI, Duncan HL, Laidler LA, Hunter D, Birkbeck TH (2003) Isolation of *Vibrio tapetis* from cultivated Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 221:65–74

Reith ME, Singh RK, Curtis B, Boyd JM, Bouevitch A, Kimball J, Munholland J, Murphy C, Sarty D, Williams J, Nash JH, Johnson SC, Brown LL (2008) The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. BMC Genomics 9:427

Rockey D, Fryer J, Rohovec J (1988) Separation and *in vivo* analysis of two extracellular proteases and the T-hemolysm from *Aeromonas salmonicida*. Dis Aquat Organ 5:197–204

Roger F, Marchandin H, Jumas-Bilak E, Kodjo A, Lamy B (2012) Multilocus genetics to reconstruct aeromonad evolution. BMC Microbiol 12:62

Rønneseth A, Haugland GT, Colquhoun D, Wergeland HI (2014) Utvikling av smittemodeller på oppdrettet rognkjeks (Development of infection models on farmed lumpsucker; in Norwegian). University of Bergen, FHF Report 1-2014

Sabat AJ, Budimir A, Nashev D, Sá-Leão R, Dijl J m van, Laurent F, Grundmann H, Friedrich AW (2013) Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Eurosurveillance 18:20380

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Santos Y, Pazos F, Toranzo AE (1996) Biochemical and serological analysis of *Vibrio anguillarum* related organisms. Dis Aquat Org 26:67–73

Sawabe T, Kita-Tsukamoto K, Thompson FL (2007) Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. J Bacteriol 189:7932–6

Sawabe T, Koizumi S, Fukui Y, Nakagawa S, Ivanova EP, Kita-Tsukamoto K, Kogure K, Thompson FL (2009) Mutation is the main driving force in the diversification of the *Vibrio splendidus* clade. Microbes Environ 24:281–285

Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AR, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T (2013) Updating the Vibrio clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. Front Microbiol 4:414

Sayer MDJ, Reader JP (1996) Exposure of goldsinny, rock cook and corkwing wrasse to low temperature and low salinity: survival, blood physiology and seasonal variation. J Fish Biol 49:41–63

Schubert RHW (1967) The taxonomy and nomenclature of the genus *Aeromonas* Kluyver and van Niel 1936: Part I. Suggestions on the taxonomy and nomenclature of the aerogenic *Aeromonas* species. Int J Syst Bacteriol 17:23–37

Schwartz DC, Cantor CR (1984) Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell 37:67–75

Seng P, Drancourt M, Gouriet F, Scola B La, Fournier P-E, Rolain JM, Raoult D (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49:543–551

Sevatdal S, Copley L, Wallace C, Jackson D, Horsberg TE (2005) Monitoring of the sensitivity of sea lice (*Lepeophtheirus salmonis*) to pyrethroids in Norway, Ireland and Scotland using bioassays and probit modelling. Aquaculture 244:19–27

Shapiro BJ, Friedman J, Cordero OX, Preheim SP, Timberlake SC, Szabo G, Polz MF, Alm EJ (2012) Population genomics of early events in the ecological differentiation of bacteria. Science 336:48–51

Skiftesvik AB, Blom G, Agnalt A-L, Durif CMF, Browman HI, Bjelland RM, Harkestad LS, Farestveit E, Paulsen OI, Fauske M, Havelin T, Johnsen K, Mortensen S (2014) Wrasse (Labridae) as cleaner fish in salmonid aquaculture – The Hardangerfjord as a case study. Mar Biol Res 10:289–300

Skiftesvik AB, Durif CMF, Bjelland RM, Browman HI (2014) Distribution and habitat preferences of five species of wrasse (Family Labridae) in a Norwegian fjord. ICES J Mar Sci 72:890–899

Smith IW (1963) The Classification of "*Bacterium salmonicida*." J Gen Microbiol 33:263–274

Snitkin ES, Zelazny AM, Thomas PJ, Stock F, Henderson DK, Palmore TN, Segre JA (2012) Tracking a hospital outbreak of carbapenem-resistant *Klebsiella pneumoniae* with whole-genome sequencing. Sci Transl Med 4:148ra116

Sobecky PA, Mincer TJ, Chang MC, Toukdarian A, Helinski DR (1998) Isolation of broad-host-range replicons from marine sediment bacteria. Appl Environ Microbiol 64:2822–2830

Solhaug RM (2012) Tagging wild salmon into the deep north. www.sciencenordic.com/tagging-wild-salmon-deep-north

Sommerset I, Krossøy B, Biering E, Frost P (2005) Vaccines for fish in aquaculture. Expert Rev Vaccines 4:89–101

Spratt BG, Maiden MC (1999) Bacterial population genetics, evolution and epidemiology. Philos Trans R Soc Lond B Biol Sci 354:701–710

Steer AC, Law I, Matatolu L, Beall BW, Carapetis JR (2009) Global *emm* type distribution of group A streptococci: systematic review and implications for vaccine development. Lancet Infect Dis 9:611–616

Stone J, Sutherland I., Sommerville C, Richards R., Varma K. (2000) Field trials to evaluate the efficacy of emamectin benzoate in the control of sea lice, *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* Nordmann, infestations in Atlantic salmon *Salmo salar* L. Aquaculture 186:205–219

Suerbaum S, Smith JM, Bapumia K, Morelli G, Smith NH, Kunstmann E, Dyrek I, Achtman M (1998) Free recombination within *Helicobacter pylori*. Proc Natl Acad Sci U S A 95:12619–12624

Sundt RC, Jørstad KE (1993) Population genetic structure of wrasses used as cleanerfish in Atlantic salmon farming in Norway. Int Counc Explor Sea G:30

Sundt RC, Jørstad KE (1998) Genetic population structure of goldsinny wrasse, *Ctenolabrus rupestris* (L.), in Norway: implications for future management of parasite cleaners in the salmon farming industry. Fish Manag Ecol 5:291–302

Swift S, Karlyshev A V., Fish L, Durant EL, Winson MK, Chhabra SR, Williams P, Macintyre S, Stewart GSAB (1997) Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: Identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules. J Bacteriol 179:5271–5281

Sørensen UBS, Larsen JL (1986) Serotyping of *Vibrio anguillarum*. Appl Environ Microbiol 51:593–597

Sørum H, Holstad G, Lunder T, Håstein T (2000) Grouping by plasmid profiles of atypical *Aeromonas salmonicida* isolated from fish, with special reference to salmonid fish. Dis Aquat Organ 41:159–171

Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB, Swings J (2005) Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. Appl Environ Microbiol 71:5107–5115

Thompson FL, Gomez-Gil B, Vasconcelos ATR, Sawabe T (2007) Multilocus sequence analysis reveals that *Vibrio harveyi* and *V. campbellii* are distinct species. Appl Environ Microbiol 73:4279–85

Thompson FL, Iida T, Swings J (2004) Biodiversity of vibrios. Microbiol Mol Biol Rev 68:403–431

Thompson JR, Pacocha S, Pharino C, Klepac-Ceraj V, Hunt DE, Benoit J, Sarma-Rupavtarm R, Distel DL, Polz MF (2005) Genotypic diversity within a natural coastal bacterioplankton population. Science 307:1311–1313

Thompson FL, Thompson CC, Hoste B, Vandemeulebroecke K, Gullian M, Swings J (2003) *Vibrio fortis* sp. nov. and *Vibrio hepatarius* sp. nov., isolated from aquatic animals and the marine environment. Int J Syst Evol Microbiol 53:1495–1501

Thompson FL, Thompson CC, Li Y, Gomez-Gil B, Vandenberghe J, Hoste B, Swings J (2003) *Vibrio kanaloae* sp. nov., *Vibrio pomeroyi* sp. nov. and *Vibrio chagasii* sp. nov., from sea water and marine animals. Int J Syst Evol Microbiol 53:753–759

Thompson FL, Thompson CC, Swings J (2003) *Vibrio tasmaniensis* sp. nov., isolated from Atlantic salmon (*Salmo salar* L.). Syst Appl Microbiol 26:65–69

Thompson CC, Thompson FL, Vicente ACP, Swings J (2007) Phylogenetic analysis of vibrios and related species by means of *atpA* gene sequences. Int J Syst Evol Microbiol 57:2480–4

Thomson R, Macpherson HL, Riaza A, Birkbeck TH (2005) *Vibrio splendidus* biotype 1 as a cause of mortalities in hatchery-reared larval turbot, *Scophthalmus maximus* (L.). J Appl Microbiol 99:243–50

Thorstad E, Todd C, Uglem I, Bjørn P, Gargan P, Vollset K, Halttunen E, Kålås S, Berg M, Finstad B (2015) Effects of salmon lice *Lepeophtheirus salmonis* on wild sea trout *Salmo trutta* — a literature review. Aquac Environ Interact 7:91–113

Torrissen O, Jones S, Asche F, Guttormsen A, Skilbrei OT, Nilsen F, Horsberg TE, Jackson D (2013) Salmon lice - impact on wild salmonids and salmon aquaculture. J Fsh Dis 36:171–94

Treasurer JW (1996) Wrasse (Labridae) as cleaner-fish of sea lice on farmed Atlantic salmon in West Scotland. In: Sayer MDJ, Treasurer JW, Costello MJ (eds) Wrasse Biology and Use in Aquaculture, 1st edn. Wiley-Blackwell, ISBN: 978-0-85238-236-3, p 185–195

Treasurer JW (2012) Diseases of north European wrasse (Labridae) and possible interactions with cohabited farmed salmon, *Salmo salar* L. J Fish Dis 35:1–8

Trust TJ, Kay WW, Ishiguro EE (1983) Cell surface hydrophobicity and macrophage association of *Aeromonas salmonicida*. Curr Microbiol 9:315–318

Udey JL, Fryer LR (1978) Immunization of fish with bacterins of *Aeromonas salmonicida*. Mar Fish Rev 40:12–17

Umelo E, Trust TJ (1998) Physical map of the chromosome of *Aeromonas salmonicida* and genomic comparisons between *Aeromonas* strains. Microbiology 144:2141–2149

Urakawa H, Kita-Tsukamoto K, Ohwada K (1999) 16S rDNA restriction fragment length polymorphism analysis of psychrotrophic vibrios from Japanese coastal water. Can J Microbiol 45:1001–1007

Urbanczyk H, Ast JC, Higgins MJ, Carson J, Dunlap P V (2007) Reclassification of *Vibrio fischeri*, *V. logei*, *V. salmonicida* and *V. wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *A. logei* comb. nov., *A. salmonicida* comb. nov. and *A. wodanis* comb. nov. Int J Syst Evol Microbiol 57:2823–9

Verner-Jeffreys DW, Shields RJ, Bricknell IR, Birkbeck TH (2003) Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. Aquaculture 219:21–42

Vezzulli L, Pezzati E, Stauder M, Stagnaro L, Venier P, Pruzzo C (2015) Aquatic ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. Environ Microbiol 17:1065–1080

Vos P, Hogers R, Bleeker M, Reijans M, Lee T van de, Hornes M, Friters A, Pot J, Paleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Vågnes Ø, Biering E, Almås K (2014) Development of injection and cohabitation challenge models for atypical *Aeromonas salmonicida* in farmed ballan wrasse (*Labrus bergylta*). The Norwegian Veterinary Institute's Report Series 1-2014, ISSN: 1890-3290

Vågnes Ø, Gulla S, Colquhoun DJ, Biering E (2014) Development of injection and cohabitation challenge models for *Vibrio anguillarum* serotype O1 in farmed ballan wrasse (*Labrus bergylta*). The Norwegian Veterinary Institute's Report Series 10-2014, ISSN: 1890-3290

Waechter M, Roux F Le, Nicolas J-L, Marissal E, Berthe F (2002) Characterization of pathogenic bacteria of the cupped oyster *Crassostrea gigas*. C R Biol 325:231–238

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18:7213–7218

Wiklund T, Bylund G (1993) Skin ulcer disease of flounder *Platichthys flesus* in the northern Baltic Sea. Dis Aquat Organ 17:165–174

Wiklund T, Dalsgaard I (1998) Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid and salmonid fish species: a review. Dis Aquat Organ 32:49–69

Wiklund T, Lönnström L, Niiranen H (1993) *Aeromonas salmonicida* ssp. *salmonicida* lacking pigment production, isolated from farmed salmonids in Finland. Dis Aquat Organ 15:219–223

Wildschutte H, Preheim SP, Hernandez Y, Polz MF (2010) O-antigen diversity and lateral transfer of the *wbe* region among *Vibrio splendidus* isolates. Environ Microbiol 12:2977–2987

9. Errata

- ✓ Page vi: **Diseases of Aquatic Animals (*in press; DOI: 10.3354/dao02938*)** corrected to **Diseases of Aquatic Organisms, 2015, 117(2):121-131**.

- ✓ Page 2: **numbers** corrected to **number**.

- ✓ Page 9: **role as fish pathogens** changed to **role as cleaner fish pathogens**.

- ✓ Page 12, 13, 16, 26 and 44: **phenotypical** changed to **phenotypic**.

- ✓ Page 14 and 22: **taxonomical** changed to **taxonomic**.

- ✓ Page 38: **strains** changed to **colonies**.

- ✓ Page 38: **three categories** changed to **three positive categories**.

- ✓ Page 45: Figure 16 caption changed slightly to avoid possible misconception.

- ✓ Page 50: **circumstances** changed to **conditions**.

- ✓ Page 50: **Gulla** changed to **S. Gulla**.

10. Scientific papers I-III

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Aeromonas salmonicida infection levels in pre- and post-stocked cleaner fish assessed by culture and an amended qPCR assay

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Abstract

Due to increasing resistance to chemical therapeutants, the use of 'cleaner fish' (primarily wrasse, Labridae, species) has become popular in European salmon farming for biocontrol of the salmon louse, *Lepeophtheirus salmonis* (Krøyer). While being efficient de-licers, cleaner fish mortality levels in salmon cages are commonly high, and systemic bacterial infections constitute a major problem. Atypical furunculosis, caused by *Aeromonas salmonicida* A-layer types V and VI, is among the most common diagnoses reached in clinical investigations. A previously described real-time PCR (qPCR), targeting the *A. salmonicida* A-layer gene (*vapA*), was modified and validated for specific and sensitive detection of all presently recognized A-layer types of this bacterium. Before stocking and during episodes of increased mortality in salmon cages, cleaner fish (primarily wild-caught wrasse) were sampled and screened for *A. salmonicida* by qPCR and culture. Culture indicated that systemic bacterial infections are mainly contracted after salmon farm stocking, and qPCR revealed *A. salmonicida* prevalences of approximately 4% and 68% in pre- and post-stocked cleaner fish, respectively. This underpins *A. salmonicida*'s relevance as a contributing factor to cleaner fish mortality and emphasizes the need for implementation of preventive measures (e.g. vaccination) if current levels of cleaner fish use are to be continued or expanded.

Keywords: Aeromonas salmonicida, fish pathogen, qPCR, screening, *vapA*/A-layer, wrasse.

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Introduction

In European salmon farming, the use of so-called cleaner fish has become increasingly popular for biocontrol of the salmon louse, *Lepeophtheirus salmonis* (Krøyer), in recent years. In Norway during 2014, more than fifteen million wild goldsinny, *Ctenolabrus rupestris* (L.), ballan, *Labrus bergylta* (Ascanius), corkwing, *Symphodus melops* (L.), and rock cook, *Centrolabrus exoletus* (L.), wrasse were caught for this purpose (Norwegian Directorate of Fisheries/www.fiskeridir.no 2015). Stocking proportions commonly range between 2 and 10 cleaner fish per 100 salmon (Nilsen *et al.* 2014), depending e.g. on species used, water temperature and geographical region (Lusedata.no 2014a,b). Although farming of lumpsucker, *Cyclopterus lumpus* (L.), and ballan wrasse has been initiated, the current supply of farmed cleaner fish cannot satisfy the large and growing demand.

Cleaner fish mortality is a significant challenge to their sustained use. One recent study registered 33% cumulative mortality within six months of salmon cage stocking, but also pointed out that this was a minimum estimate and predicted the actual proportion to be almost twice as high

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(Nilsen *et al.* 2014). This results in a significant turnover of cleaner fish in salmon farms, involving both highly unfortunate animal welfare aspects and economic consequences for the farmers. The high exploitation of the largely uncharted wild wrasse populations is also questionable (Skiftesvik *et al.* 2014).

Mortality episodes in cleaner fish are often related to bacterial infections, and atypical furunculosis, caused by atypical strains of *Aeromonas salmonicida*, is perhaps the most commonly diagnosed infectious disease in both wrasse and lumpsucker (Johansen 2013; Hjeltnes 2014; Bornø & Lie Linaker 2015). In a recent study, we identified that all isolates of this bacterium cultured from Norwegian wrasse by the Norwegian Veterinary Institute (NVI) in recent years (dating back to 1992) belonged to two distinct A-layer subtypes of *A. salmonicida* (Gulla *et al.* 2015). Whether cleaner fish become infected with this pathogen before or after salmon farm stocking is unclear, but such knowledge will be important to allow development of vaccine strategies against *A. salmonicida* in these fish.

Although disease caused by *A. salmonicida* can usually be readily diagnosed using traditional diagnostic techniques such as bacterial culture and histopathology (Austin & Austin 2007), these methods have a low sensitivity for the detection of covert infections (e.g. subclinical carriers). Moreover, atypical *A. salmonicida* strains are often relatively slow growing (personal observation; Austin & Austin 2007) and may be covered by faster growing opportunists or environmental bacterial contaminants. To obtain a more accurate estimate of the total infection prevalence in different fish groups, a more sensitive screening tool is needed. Here, we screened wild, farmed and stocked (working) cleaner fish from along the Norwegian coast for *A. salmonicida* infection by means of both real-time quantitative PCR (qPCR) and bacterial culture.

Materials and methods

Fish collection and sampling

Between June and September 2013, creel-caught wild wrasse (ballan, goldsinny, corkwing and rock cook) were purchased/donated from commercial fishermen at fourteen locations along the Norwegian coast (approximately 30 apparently healthy specimens per location; 412 in total; Fig. 1). The creels were emptied at least once per day, and the fish were immediately killed with an overdose of benzocaine mixed in sea water. Following visual macroscopic examination (internal and external), the head kidney (HK) was aseptically exposed and a 10 μL inoculation loop used to streak samples onto two blood agar plates (Blood agar base no. 2 [Oxoid AS] 40 g L^{-1}, suppl. w/5% bovine blood) with final NaCl concentrations of 0.5% (BA) and 2% (BA2%), respectively. The HK was then aseptically removed and stored on RNAlater (Invitrogen) at −20 °C prior to further processing. Bacterial cultures were incubated for one week at 22 °C (BA) and 15 °C (BA2%). Eventual dominating colonies observed were then subcultured and identified using established phenotypical diagnostic methods (e.g. Garrity *et al.* 2005; Austin & Austin 2007), in some cases supplemented with partial 16S rRNA gene sequencing (Suau *et al.* 1999), and cryopreserved at −80 °C.

Preserved HK samples on RNAlater from 131 stocked (working) cleaner fish (123 wrasse and 8 lumpsucker) from twelve Norwegian salmon farms (Fig. 1) were also included in the study. These were primarily obtained from dead or moribund specimens sampled during episodes of increased cleaner fish mortality. An additional 64 HK samples from apparently healthy farmed cleaner fish (34 ballan wrasse and 30 lumpsucker, from two distinct farms) prior to salmon farm stocking were also investigated. Most bacterial cultures from working cleaner fish were examined as part of a larger study (see Discussion and Nilsen *et al.* 2014). For logistical reasons, bacterial cultures were not obtained from farmed cleaner fish. The three categories of sampled cleaner fish, that is wild-caught, farmed precage stocking, and mortalities from salmon farms, will be subsequently referred to as 'wild', 'farmed' and 'working', respectively.

Tissue preparation, DNA extraction and qPCR screening

Sterile phosphate-buffered saline (PBS) and HK tissue (~20% w/v) were added to 1.5-mL Eppendorf tubes with one tungsten carbide bead (Qiagen) and homogenized using a TissueLyser II (Qiagen), followed by storage at −20 °C prior to further processing. Using 20 μL homogenate (100 μL for pooled analysis), genomic DNA was

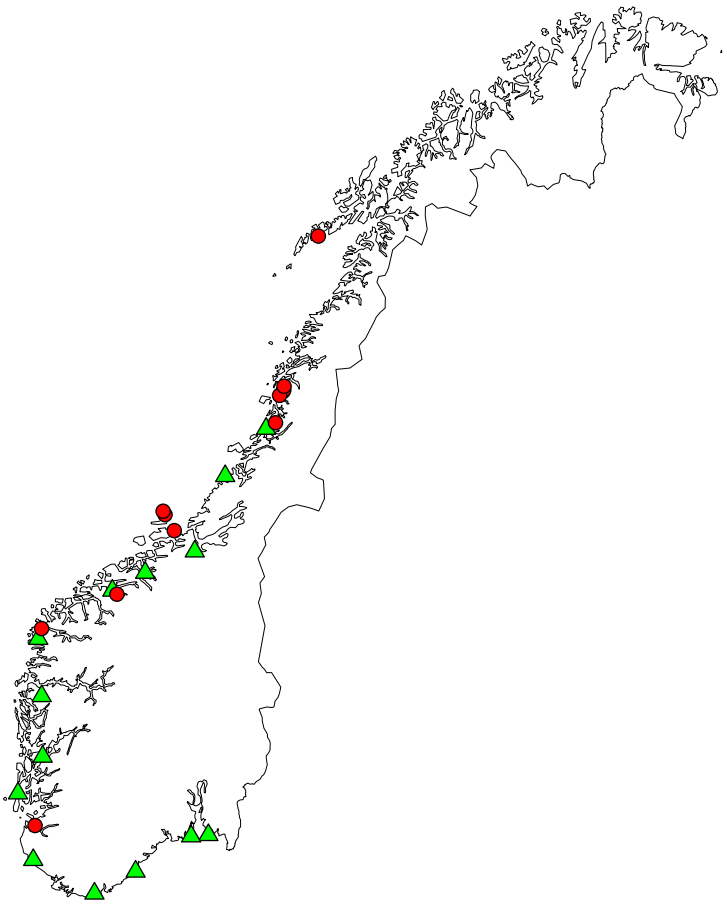

Figure 1 Map indicating the locations along the Norwegian coast from which wild wrasse (fourteen locations; green triangles) and working cleaner fish (twelve salmon farms; red circles) were sampled for screening. Farmed wrasse and lumpsucker were supplied by two distinct cleaner fish farms in western Norway (not shown).

extracted with a Gentra Puregene kit (Qiagen) according to the manufacturer's instructions for tissue samples (including Proteinase K-step), except that the final elution volume was reduced to 25 μL. Extractions were then stored at −20 °C. Homogenates were initially analysed in pools of five, and individually tested if found positive by qPCR.

To control for possible cross-contamination during processing, HK tissue from a single fish, found negative for *A. salmonicida* by culture and qPCR, was included in all homogenization, DNA extraction and qPCR set-ups. Positive (*A. salmonicida* NCIMB 1102^T; DNA at 1 ng per template) and negative (Milli-Q water) template controls were also included in each qPCR run.

Modification of a previously published qPCR protocol

A previously described molecular beacon qPCR assay (Keeling *et al.* 2013), specifically targeting a relatively conserved region of the *vapA* (A-layer) gene of *A. salmonicida*, was modified and employed. Modifications included the design of a new forward primer, while the reverse primer and molecular beacon remained unchanged from the original assay (Table 1).

The modified qPCR set-up was as follows (with changes from the original protocol underlined): each reaction volume consisted of 12.5 μL 2× JumpStart Taq ReadyMix for Quantitative PCR (Sigma-Aldrich), 0.3 μM of each primer, 0.6 μM molecular beacon (Biosearch Technologies), a final concentration of 5.5 mM $MgCl_2$, 0.25 μL Reference Dye for Quantitative PCR (Sigma-Aldrich), 1 μL DNA template, and Milli-Q water amounting to a total reaction volume of 25 μL. An Mx3005P thermal cycler (Stratagene) was used for qPCR, which involved (i) 94 °C for 2 min and (ii) 45 cycles of 94 °C for 15 s, 60 °C for 1 min (w/JOE- and ROX data collection) and 72 °C for 20 s. Data analysis was carried out using MxPro v4.10 software (Stratagene).

Table 1 Oligonucleotides used in this *Aeromonas salmonicida* qPCR assay modified after Keeling *et al.* (2013)

Primer/probe (name)	Sequence (5′–3′)
From Keeling *et al.* (2013)	
F. primer (VapAF1)[a]	TAAAGCACTGTCTGTTACC
Mol. beacon (AS MB)	d CAL Fluor Orange 560-CGCGATC (ACATCAGCAGGCTTCAGAGTC ACTG)GATCGCG-BHQ-1
R. primer (VapAR1)	GCTACTTCACCCTGATTGG
From this study	
F. primer (VapAF3)[a]	ACTGTCTGTTACCCTGCCA

[a]VapAF1 was replaced by VapAF3 in the modified protocol.

Validation of the modified qPCR protocol

Aeromonas salmonicida strains were cultured at 22 °C on either BA for two days (for sensitivity assessment) or in heart infusion broth (HIB) overnight with gentle shaking (for spiking experiments). Other bacterial species used (for specificity assessment) were cultured on suitable media at appropriate temperatures and incubation times. Genomic DNA from bacterial cultures was extracted using a QiaCube (Qiagen) according to the manufacturer's instructions. DNA concentration and purity were assessed with a NanoDrop 1000 (Thermo Scientific), and extracts were stored at −20 °C until needed.

DNA from a diverse panel of 62 bacterial species, subspecies and strains (Table 2) was used for qualitative assessment of qPCR sensitivity and specificity (each at 1 ng DNA per template). In addition to distinct *A. salmonicida vapA* sequence types (A-layer types, including A-layer negative *vapA* mutants; Gulla *et al.* 2015), isolated from various fish species, several closely related aeromonads and various aquatic and terrestrial bacterial species were represented. DNA extracts from HK tissues of four wrasse species (ballan, goldsinny, corkwing and rock cook), lumpsucker and Atlantic salmon, *Salmo salar* (L.), all negative for *A. salmonicida* infection by culture, were also tested.

Cycle threshold (Ct) values resulting from nine consecutive qPCR runs with one single DNA extract from NCIMB 1102^T (at 1 ng DNA per template) were used to estimate the inter-run coefficient of variation (CV). The inter-strain CV was evaluated based on Ct-values from simultaneous qPCR with each of the fifteen *A. salmonicida* A-layer types listed in Table 2.

To evaluate the limit of detection (LOD) for *A. salmonicida* DNA from pure culture, an extraction from NCIMB 1102^T was used to prepare serial dilutions in Milli-Q water (tenfold: 40 ng to 40 fg DNA μL^{-1}; twofold: 40 fg to 5 fg μL^{-1}). All dilutions (ten in total) were then used in triplicate as templates for qPCR. The LOD for *A. salmonicida* in fish HK tissues was evaluated by making tenfold serial dilutions of HIB-cultured NCIMB 1102^T in PBS (undiluted to 10^{-8}). This was carried out on ice to limit bacterial replication. From each dilution, 100 µL was then added to 100 µL aliquots of HK homogenate (*A. salmonicida* negative by culture and qPCR) from ballan wrasse in a triplicate set-up. Additionally, 100 µL PBS was added to one tissue aliquot as a negative control. Prior to DNA extraction as described for tissue homogenates, all suspensions were incubated at room temperature for one hour to allow bacterial cell-adsorption. Resulting DNA extracts were used as templates for qPCR. For retrospective calculation of bacterial concentrations, 100 µL of dilutions 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} was spread (in duplicate) on BA plates for colony-forming unit (CFU) counts.

Results

Screening of cleaner fish – bacterial culture and qPCR

The majority of wild wrasse (ballan, goldsinny, corkwing and rock cook; totally 412) sampled in this study were apparently healthy based on exterior and interior macroscopic examination, and bacterial smears from HK were largely without specific growth (>90%). Bacterial colonies ($n = 22$) subjected to phenotypical characterization, in some cases aided by partial 16S rRNA gene sequencing, were identified as *V. logei*-related (twelve), *V. splendidus*-related (seven), *Endozoicomonas* sp. (two) and *Photobacterium* sp. (one). Additionally, several plates displayed varying amounts of unspecific mixed growth, presumably due to contamination during sampling. No colonies consistent with *A. salmonicida* (i.e. small, 'friable' colonies, with microscopy showing non-motile auto-aggregating coccobacilli) were observed.

Aeromonas salmonicida qPCR (modified protocol) on HK from wild wrasse ($n = 412$), farmed cleaner fish ($n = 64$) and working cleaner fish ($n = 131$) identified infection prevalences of

Table 2 Bacterial isolates/strains used for validation of the modified *Aeromonas salmonicida* qPCR protocol

A. salmonicida A-layer type (Gulla *et al.* 2015) /Bacterial species	Strain designation (evt. w/acc. no for near full-length *vapA*-seq.)	Biological source	qPCR-result
Sensitivity assessment – *A. salm.* A-layer type			
Type I (ssp. *salmonicida*)	NCIMB 1102^T	Atlantic salmon	+
	NVI-4704 (KP184533)	Turbot	+
Type II (atypical)	NVI-4818 (KP184535)	Atlantic halibut	+
	NVI-4953	Atlantic halibut	+
	NVI-8042	Atlantic halibut	+
Type III (ssp. *achromogenes*)	NCIMB 1110^T (AJ749889)	Brown trout	+
	NVI-8143	Atlantic cod	+
Type IV (atypical)	L-4088 (AM937254)	Spotted wolffish	+
	NVI-6641	Wolffish	+
Type V (atypical; wrasse-associated)	NVI-8017 (KP184544)	Ballan wrasse	+
	NVI-8123	Ballan wrasse	+
	NVI-8356[a]	Ballan wrasse	+
Type VI (atypical; cleaner fish-associated)	NVI-8013 (KP184543)	Ballan wrasse	+
	NVI-8198[a]	Lumpsucker	+
	NVI-8232[a] (KP184550)	Goldsinny wrasse	+
Type VII (ssp. *masoucida*)	ATCC 27013^T (AJ749883)	Masu salmon	+
Type VIII (atypical)	NVI-6457	Atlantic salmon	+
Type IX (atypical)	NVI-2950	Trout	+
Type X (atypical)	NVI-5697	Carp	+
Type XI (atypical)	NVI-6449	Arctic char	+
Type XII (atypical)	NVI-2795	Atlantic salmon	+
Type XIII (atypical)	NVI-3071[a]	Atlantic salmon	+
Type XIV (atypical)	NVI-1843 (KP184519)	Arctic char	+
Unnamed type/singleton (ssp. *smithia*)	NCIMB 13210^T (AJ749880)	Common roach	+
Specificity assessment – bacterial species			
A. salm. ssp. *pectinolytica* (*vapA*-deficient)	LMG 19569^T	Polluted river water	–
Aeromonas bestiarum	LMG 13444^T	Diseased fish	–
A. bestiarum	LMG 13448	Faeces	–
A. bestiarum	LMG 13662	Faeces	–
Aeromonas caviae	NCIMB 13016^T	Guinea pig	–
Aeromonas eucrenophila	NCIMB 74^T	Carp	–
Aeromonas hydrophila	LMG 2844^T	Stale canned milk	–
Aeromonas media	NCIMB 2237^T	Fish farm effluent	–
Aeromonas schubertii	NVI-3153	Atlantic salmon	–
Aeromonas sobria	NCIMB 12065^T	Carp	–
Aeromonas veronii	NCIMB 13015^T	Human sputum	–
Arthrobacter globiformis	NCIMB 8907^T	Soil	–
Bacillus cereus	NVI-3588	Unknown	–
Brochothrix thermosphacta	NCFB 1676^T	Pork sausage	–
Carnobacterium piscicola	NCFB 2762^T	Cutthroat trout	–
Escherichia coli	ATCC 25922	Clinical isolate	–
Edwardsiella piscicida	NVI-883	Eel	–
Francisella noatunensis ssp. *noatunensis*	2005/50/F292-6C^T	Atlantic cod	–
Moritella viscosa	NCIMB 2263	Atlantic salmon	–
Nocardia asteroides	NVI-6532	Unknown	–
Pasteurella sp.	NVI-9100	Lumpsucker	–
Pasteurella skyensis	NCIMB 13593^T	Atlantic salmon	–
Photobacterium phosphoreum	NCIMB 1282^T	Unknown	–
Piscirickettsia salmonis	NVI-5692	Atlantic salmon	–
Proteus mirabilis	NCIMB 10823	Unknown	–
Pseudomonas fluorescens	NCIMB 10067	Unknown	–
Renibacterium salmoninarum	NVI-6975	Atlantic salmon	–
Rhodococcus equi	NVI-6122	Unknown	–
Rhodococcus erythropolis	NCIMB 11148^T	Soil	–
Serratia marcescens	NCIMB 10351	Unknown	–
Tenacibaculum sp.	NVI-9148	Wrasse	–
Vibrio anguillarum serotype O1	NVI-8541	Corkwing wrasse	–
Vibrio anguillarum serotype O2a	NVI-8579	Ballan wrasse	–
Vibrio logei	NVI-8547	Goldsinny wrasse	–
Vibrio salmonicida	NCMB 2262^T	Atlantic salmon	–
Vibrio splendidus	NVI-7628	Ballan wrasse	–
Vibrio tapetis	NVI-7627	Ballan wrasse	–
Yersinia ruckeri	NVI-353	Atlantic salmon	–

NVI: cryopreserved at the Norwegian Veterinary Institute, Oslo.

[a]*A. salmonicida* isolates with deleterious mutations in the hypervariable *vapA*-region (A-layer negative; Gulla *et al.* 2015).

3.9%, 0% and 67.9%, respectively. Based on Ct-values (not shown), positive results were further divided into three categories reflecting tentative bacterial loads, i.e. 'low' (Ct ≥ 34) 'intermediary' (34 > Ct ≥ 25) and 'high' (Ct < 25). All positive samples from wild wrasse displayed 'low' bacterial loads, while positive samples from working cleaner fish were distributed relatively evenly among the three categories (Fig. 2).

Original vs. modified qPCR protocol

Introduction of the original *A. salmonicida* qPCR protocol described by Keeling *et al.* (2013) to our laboratory required several modifications to reagent concentrations and thermal cycling parameters (see Materials and methods for details). Further, alignment of the original oligonucleotide sequences against all publicly available *vapA* sequence types (Gulla *et al.* 2015) revealed a single base variation in A-layer types V and XIV (acc. no. in Table 2), resulting in a mismatch with the forward primer (VapAF1; Table 1). qPCR runs with type V isolates using this primer yielded considerably higher Ct-values when compared to other A-layer types (standard curve constant term increased by ~4.5), as exemplified by analysis of A-layer type V strain NVI-8017 and type VI strain NVI-8013 in Fig. 3. Following introduction of the new forward primer (VapAF3), this issue was resolved.

Validation of the modified qPCR protocol

Using the final modified qPCR protocol, all non-*A. salmonicida* bacterial species and control HK tissue samples (six fish species) tested negative, while all *A. salmonicida* isolates (including A-layer negative *vapA* mutants; Gulla *et al.* 2015) tested positive (Table 2). This was with the exception of the *vapA*-deficient ssp. *pectinolytica* (see Discussion). For qPCR Ct-values, the *A. salmonicida* inter-strain CV was 4.9% (fifteen A-layer types), and the inter-run CV was 2.4% (nine runs with NCIMB 1102^T).

The LOD for serial dilutions of NCIMB 1102^T was defined as the lowest DNA concentration which remained in linear relationship with the Ct-values (in $\log_{10}$ scale) for all three replicate runs. This was determined to be 40 fg DNA per

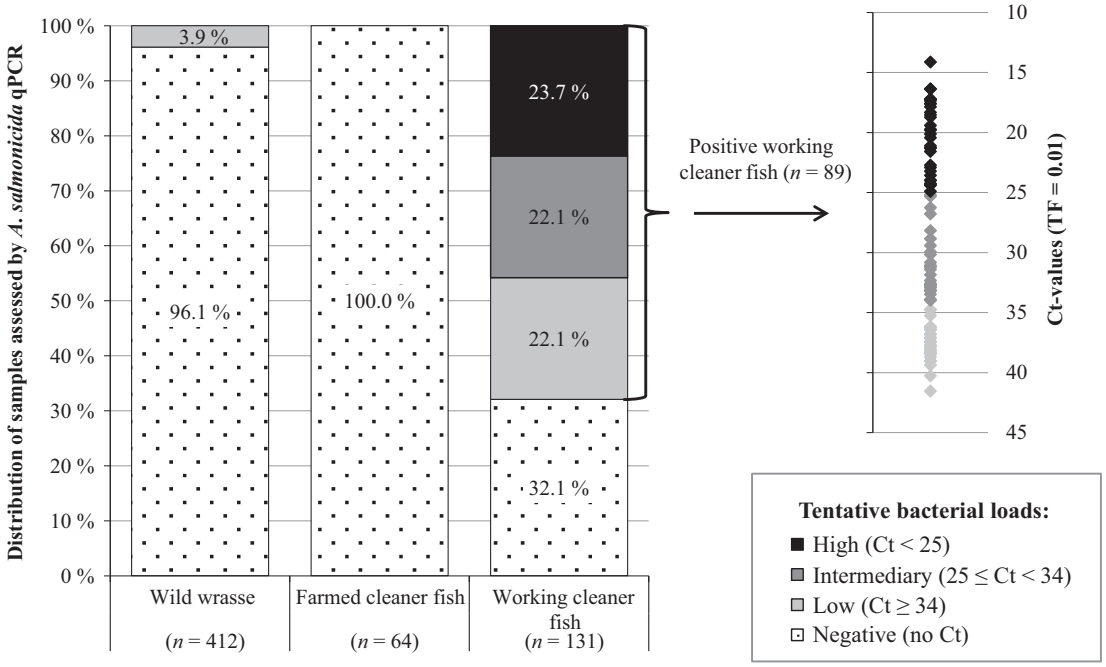

Figure 2 Stacked bar chart (on left) summarizing the results from *Aeromonas salmonicida* qPCR screening of cleaner fish before (wild and farmed) and after (working) salmon farm stocking. A scatter plot (on right) shows the distribution of Ct-values from positive working cleaner fish samples. The results were categorized as negative or positive, with three subordinate categories reflecting tentative bacterial loads (here represented by different bar-/marker fill gradations; see legend).

template, or approximately 7–8 *A. salmonicida* genomes based on the genome size of ssp. *salmonicida* strain A449 (Reith *et al.* 2008), and corresponded to a Ct of ~35 at threshold fluorescence (TF) 0.01 (Fig. 4). At lower DNA concentrations, qPCR was not consistently positive, and eventual Ct-values did not maintain the linear relationship.

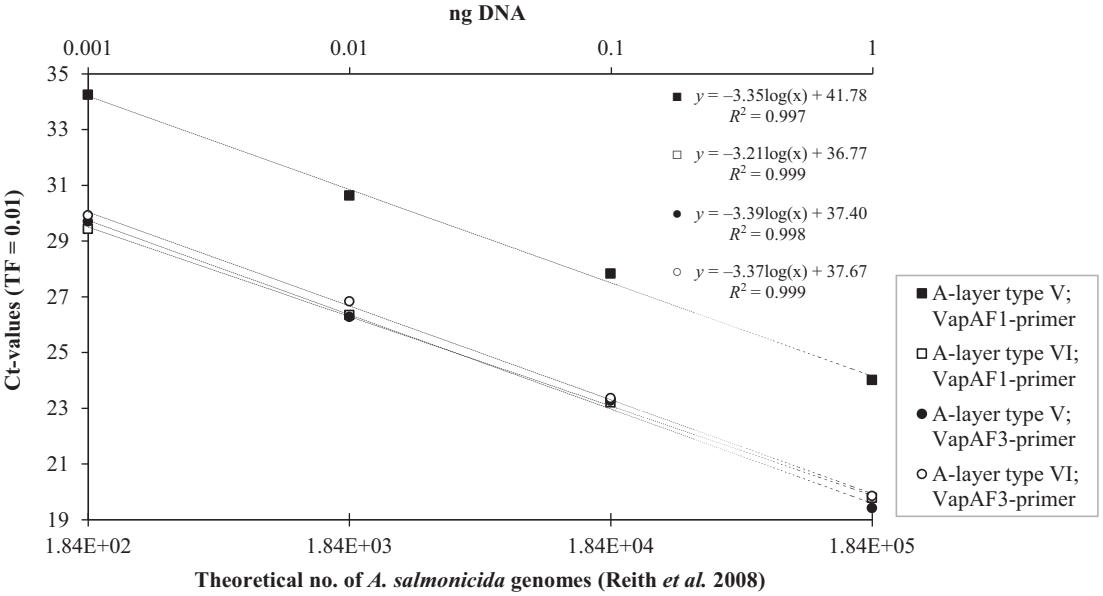

Figure 3 Standard curves based on qPCR of tenfold serial dilutions of DNA from *Aeromonas salmonicida* A-layer types V (strain NVI-8017) and VI (strain NVI-8013), using the two different forward primers VapAF1 (original) and VapAF3 (new) with the modified protocol. Ct-values are plotted against template DNA concentrations in $\log_{10}$ scale (upper *x*-axis – ng DNA; lower *x*-axis – theoretical no. of *A. salmonicida* genomes).

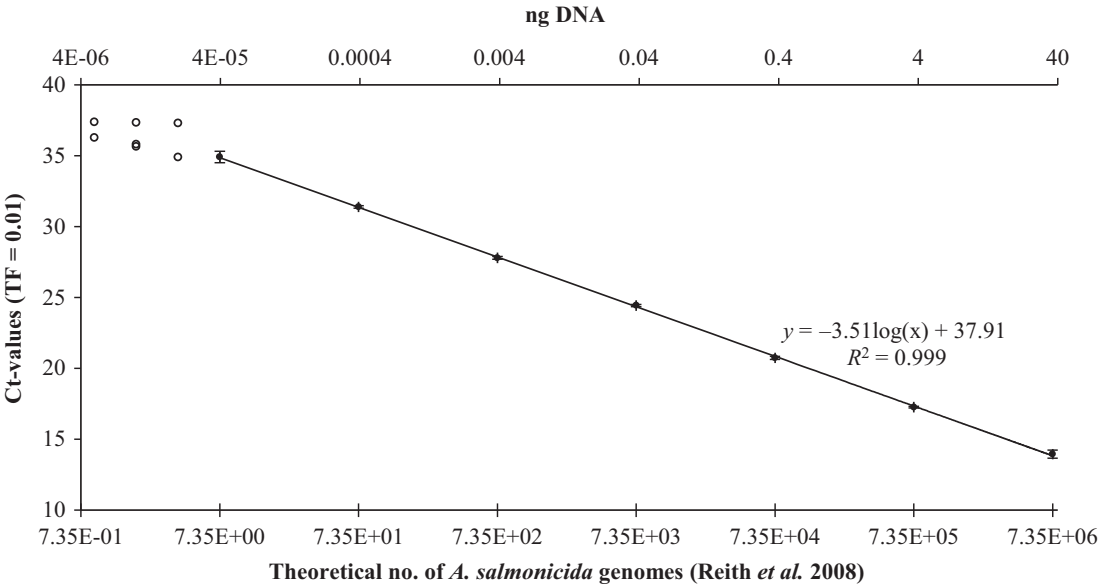

Figure 4 Standard curve based on qPCR of serial dilutions of DNA from NCIMB 1102^T run in triplicate. Ct-values (means [$n = 3$] with standard error bars) are plotted against template DNA concentrations in $\log_{10}$ scale (upper *x*-axis – ng DNA; lower *x*-axis – theoretical number of *Aeromonas salmonicida* genomes). Positive samples with DNA concentrations <40 fg per template (empty circles; not means) were not included in the standard curve data basis.

The LOD for *A. salmonicida* in spiked HK homogenates was evaluated according to the principles described above, except that resulting Ct-values were correlated with the appropriate CFU mL^{-1} in the spiked HK suspensions (log_{10} scale), extrapolated from CFU counts (not shown). The LOD for spiked HK was thus determined to be 1.1×10^3 CFU mL^{-1}, corresponding to a Ct of ~34 at TF 0.01 (Fig. 5), as lower counts were not consistently positive and eventual Ct-values did not display the same linear relationship.

Discussion

In the present study, we investigated *A. salmonicida* infection prevalences in wild Norwegian wrasse, farmed wrasse and lumpsucker, and in working cleaner fish (primarily wild-caught wrasse) sampled during episodes of increased mortality in Norwegian salmon farms. In addition to bacterial culture, a modified version of a previously described molecular beacon qPCR protocol was validated and utilized. Bacterial culture indicated that wild wrasse are largely free of systemic bacterial infections prior to salmon farm stocking, and qPCR showed a considerably higher *A. salmonicida* prevalence in cleaner fish sampled during episodes of increased mortality in salmon farms, compared to wild and farmed specimens. This emphasizes the importance of *A. salmonicida* as a contributing factor to the high cleaner fish losses experienced in Norwegian aquaculture today.

Systemic bacterial infections have previously been found to dominate in working cleaner fish mortalities in Norway. Putative fish pathogens commonly identified include atypical *A. salmonicida*, *Tenacibaculum* sp., *Vibrio anguillarum*, and, from lumpsucker, a recently described *Pasteurella* sp. (Nilsen *et al.* 2014; Alarcón *et al.* 2015; Bornø & Lie Linaker 2015).

Culture-based investigation of wild Norwegian wrasse detected no isolates consistent with the aforementioned bacterial species (including *A. salmonicida*) and indicated a relatively low bacterial infection prevalence. From presumed uncontaminated primary smears, *V. logei*-related and *V. splendidus*-related strains dominated. The role (if any) of these species as pathogens of wrasse is however largely unresolved (Bergh & Samuelsen 2006; Bornø & Lie Linaker 2015; Gulla, unpublished data). Interestingly, two isolates belonging to the genus *Endozoicomonas* (~97% 16S rRNA gene similarity) were cultured in high numbers

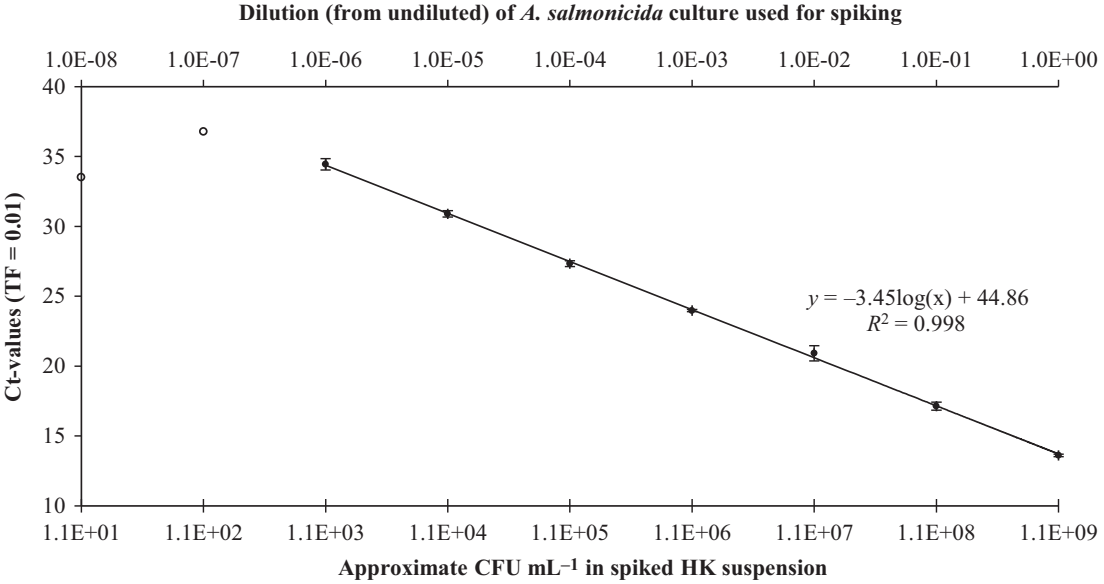

Figure 5 Standard curve based on qPCR of DNA extracts from ballan wrasse HK homogenates spiked (prior to DNA extraction) with serial dilutions of NCIMB 1102^T. Ct-values (means [$n = 3$] with standard error bars) are plotted against the approximate CFU mL^{-1} in the spiked HK suspensions (in log_{10} scale; lower x-axis), with the employed bacterial spiking dilutions also indicated (upper x-axis). Positive samples with CFU mL^{-1} <1100 (empty circles; not means) were not included in the standard curve data basis.

and pure culture from the HKs of two rock cook. However, as only four specimens of this fish species were sampled in this study, it is impossible to draw any conclusions from this observation.

Prior to *A. salmonicida* qPCR screening, several modifications to the original assay (Keeling *et al.* 2013) were required (see Materials and methods and Results for details). This included the need for a new primer (Table 1) due to sequence mismatch against the target gene (*vapA*) of A-layer type XIV and V strains (the latter thus far exclusively cultured from wrasse; Gulla *et al.* 2015), resulting in significantly reduced sensitivity for such isolates (Fig. 3). The modified assay retained 100% analytical specificity and sensitivity (Table 2), and as expected the *vapA*-deficient (Lund, Espelid & Mikkelsen 2003; Gulla *et al.* 2015) *A. salmonicida* ssp. *pectinolytica*, thus far unrelated to disease in fish (Pavan *et al.* 2013), tested negative. We were unable to consistently detect equally low concentrations of *A. salmonicida* pure culture DNA as reported by Keeling *et al.* (2013) (increase in LOD from ~1 to 7–8 genomes per template; Fig. 4). Perhaps more importantly for screening purposes however, the sensitivity for *A. salmonicida* in fish tissues (HK) was increased (reduction in LOD down from 2.2×10^4 CFU mL^{-1} to 1.1×10^3 CFU mL^{-1}; Fig. 5). As the assay was intended for use on field samples stored on RNAlater at −20 °C, we saw no reason to conduct enrichment experiments for improvement of sensitivity.

In accordance with culture-based findings, qPCR screening with the modified assay identified a low proportion of *A. salmonicida*-positive samples from cleaner fish prior to stocking (<4%), compared to ~68% positive samples from working cleaner fish (Fig. 2). Further, all positive samples from wild wrasse represented low bacterial loads, while working cleaner fish samples represented a continuum from low to high bacterial loads. The subjective subdivision of positive samples into categories of 'low', 'intermediary' or 'high' bacterial loads proved to be pragmatic, as it resulted in good corroboration between qPCR and culture-based findings. Particularly, no *A. salmonicida* was cultured from negative or low category samples, whereas the bacterium was cultured from almost all clinical investigations involving intermediary and high category samples. The low-grade positive category is probably underestimated as it comprises only Ct-values ≥34, corresponding to the defined LOD for tissue samples. Eventual underestimation of this category should however be proportionately equal for all investigated fish groups.

Infection trials have verified the pathogenic potential of certain *A. salmonicida* strains towards ballan wrasse (Vågnes, Biering & Almås 2014) and lumpsucker (Rønneseth *et al.* 2014). The variable bacterial loads observed by qPCR among working cleaner fish thus presumably reflect various stages of pathogen invasion, ranging from subclinical infection to severe systemic disease. It is reasonable to assume that capture, storage, transport and salmon farm stocking will entail substantial physical and mental stress for wild-caught wrasse, and although farmed cleaner fish are bred in captivity, transfer to the alien environment of the salmon cages will necessarily involve stress. This will undoubtedly increase the susceptibility of these fish to infectious diseases, and *A. salmonicida* infections may efficiently spread throughout newly introduced, and possibly immunocompromised, cleaner fish batches. This may either be due to an active outbreak in the farm at the time of stocking, or as a result of activated infection in a latent carrier.

Vaccination prior to stocking could presumably help solve this problem, as infection primarily seems to occur following salmon cage transfer. Promising results have previously been reported following atypical *A. salmonicida* vaccination trials performed on several non-salmonid marine fish species under cultivation (Lund, Arnesen & Eggset 2002; Gudmundsdóttir *et al.* 2003; Mikkelsen, Schrøder & Lund 2004), and a recent study on farmed ballan wrasse indicates that protective immunity can also be achieved for this fish species (Colquhoun, unpublished data).

Although the modified qPCR assay used in the present study does not distinguish 'typical' from 'atypical' *A. salmonicida* strains, only atypical isolates have been cultured from Norwegian cleaner fish by the NVI diagnostic service in modern times (historical data going back to 1992). Moreover, all 117 cleaner fish isolates (primarily from Norwegian wrasse) assessed in a recent study (Gulla *et al.* 2015) were verified as atypical *A. salmonicida*, belonging almost exclusively to A-layer types V and VI (98%). It is therefore relatively safe to assume that the majority, if not all, of the samples deemed positive by qPCR reflect infection with atypical *A. salmonicida*.

In summary, the modified qPCR protocol presented here, targeting the *vapA* (A-layer) gene of *A. salmonicida*, enables specific detection of all presently recognized A-layer types of this bacterium. A relatively high sensitivity was achieved for *A. salmonicida* in fish tissues (HK), making the protocol suitable for screening purposes. Supplemented with bacterial culturing, the amended assay was used to screen Norwegian cleaner fish (primarily wild-caught wrasse) for *A. salmonicida* infection. Combined with observations from routine diagnostics at NVI, the results clearly show that systemic bacterial infections, and *A. salmonicida* infection in particular, are largely contracted after salmon cage stocking. High cleaner fish turnover in salmon farms, for example due to *A. salmonicida*-related losses, is intolerable in terms of animal welfare and may also lead to overexploitation of wild wrasse populations. There is therefore an urgent need for the development of preventive measures, such as vaccination, if the current use of cleaner fish in Norwegian salmon farming is to become ethically acceptable.

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References

Alarcón M., Gulla S., Røsaeg M. V, Rønneseth A., Wergeland H., Poppe T.T., Nilsen H. & Colquhoun D.J. (2015) Pasteurellosis in lumpsucker *Cyclopterus lumpus*, farmed in Norway. *Journal of Fish Diseases* [Epub ahead of print]. DOI: 10.1111/jfd.12366.

Austin B. & Austin D.A. (2007) *Bacterial Fish Pathogens - Diseases of Farmed and Wild Fish*, 4th edn. Springer, Netherlands, ISBN: 978-1-4020-6069-4.

Bergh Ø. & Samuelsen O.B. (2006) Susceptibility of corkwing wrasse *Symphodus melops*, goldsinny wrasse *Ctenolabrus rupestis*, and Atlantic salmon *Salmo salar* smolt, to experimental challenge with *Vibrio tapetis* and *Vibrio splendidus* isolated from corkwing wrasse. *Aquaculture International* **15**, 11–18.

Bornø G. & Lie Linaker M. (eds.) (2015) . *Fiskehelserapporten 2014 (Norwegian Farmed Fish Health Report 2014 in Norwegian)*. Norwegian Veterinary Institute, Harstad, ISSN: 1893-1480.

Garrity G., Brenner D.J., Krieg N.R. & Staley J.R. (2005) *Bergey's Manual® of Systematic Bacteriology: Volume 2: The Proteobacteria, Part B: The Gammaproteobacteria*. Springer, US, ISBN: 978-0-387-28022-6.

Gudmundsdóttir S., Lange S., Magnadóttir B. & Gudmundsdóttir B.K. (2003) Protection against atypical furunculosis in Atlantic halibut, *Hippoglossus hippoglossus* (L.); comparison of a commercial furunculosis vaccine and an autogenous vaccine. *Journal of Fish Diseases* **26**, 331–338.

Gulla S., Lund V., Kristoffersen A.B., Sørum H. & Colquhoun D.J. (2015) *vapA* (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes. *Journal of Fish Diseases* [Epub ahead of print]. doi:10.1111/jfd.12367.

Hjeltnes B. (ed.) (2014) *Fiskehelserapporten 2013 (Norwegian Farmed Fish Health Report 2013; in Norwegian)*. Norwegian Veterinary Institute, Oslo, ISSN: 1893-1480.

Johansen R. (ed.) (2013) *Fiskehelserapporten 2012 (Norwegian Farmed Fish Health Report 2012; in Norwegian)*. Norwegian Veterinary Institute, Oslo, ISSN: 1893-1480.

Keeling S.E., Brosnahan C.L., Johnston C., Wallis R., Gudkovs N. & McDonald W.L. (2013) Development and validation of a real-time PCR assay for the detection of *Aeromonas salmonicida*. *Journal of Fish Diseases* **36**, 495–503.

Lund V., Arnesen J.A. & Eggset G. (2002) Vaccine development for atypical furunculosis in spotted wolffish *Anarhichas minor* O.: comparison of efficacy of vaccines containing different strains of atypical *Aeromonas salmonicida*. *Aquaculture* **204**, 33–44.

Lund V., Espelid S. & Mikkelsen H. (2003) Vaccine efficacy in spotted wolffish *Anarhichas minor*: relationship to molecular variation in A-layer protein of atypical *Aeromonas salmonicida*. *Diseases of Aquatic Organisms* **56**, 31–42.

Lusedata.no (2014a) Bransjeveileder lakselus - Bruk og hold av leppefisk (Guide on the use and keep of cleaner fish wrasse; in Norwegian). www.lusedata.no/for-naeringen/veiledere-leppefisk.

Lusedata.no (2014b) Bransjeveileder lakselus - Bruk og hold av rognkjeks (Guide on the use and keep of cleaner fish lumpsucker; in Norwegian). www.lusedata.no/for-naeringen/veiledere-leppefisk.

Mikkelsen H., Schrøder M.B. & Lund V. (2004) Vibriosis and atypical furunculosis vaccines; efficacy, specificity and side effects in Atlantic cod, *Gadus morhua* L. *Aquaculture* **242**, 81–91.

Nilsen A., Viljugrein H., Røsæg M.V. & Colquhoun D. (2014) *Rensefiskhelse – kartlegging av dødelighet og dødelighetsårsaker (Cleaner Fish Health - A Survey of Mortalities and Causes of Death; in Norwegian)*. The Norwegian Veterinary Institute's Report Series 12-2014, Trondheim, ISSN: 1890-3290.

Norwegian Directorate of Fisheries/www.fiskeridir.no (2015) Norwegian Aquaculture Statistics.

Pavan M.E., Pavan E.E., López N.I., Levin L. & Pettinari M.J. (2013) Genome Sequence of the Melanin-Producing Extremophile *Aeromonas salmonicida* subsp. *pectinolytica* Strain 34melT. *Genome Announcements* **1**, e00675–13.

Reith M.E., Singh R.K., Curtis B., Boyd J.M., Bouevitch A., Kimball J., Munholland J., Murphy C., Sarty D., Williams J., Nash J.H., Johnson S.C. & Brown L.L. (2008) The genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* **9**, 427.

Rønneseth A., Haugland G.T., Colquhoun D. & Wergeland H.I. (2014) *Utvikling av smittemodeller på oppdrettet rognkjeks (Development of infection models on farmed lumpsucker; in Norwegian)*. University of Bergen, FHF Report 1-2014.

Skiftesvik A.B., Durif C.M.F., Bjelland R.M. & Browman H.I. (2014) Distribution and habitat preferences of five species of wrasse (Family Labridae) in a Norwegian fjord. *ICES Journal of Marine Science* **72**, 890–899.

Suau A., Bonnet R., Sutren M., Godon J.J., Gibson G.R., Collins M.D. & Doré J. (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and Environmental Microbiology* **65**, 4799–4807.

Vågnes Ø., Biering E. & Almås K. (2014) *Development of Injection and Cohabitation Challenge Models for Atypical Aeromonas Salmonicida in Farmed Ballan Wrasse (Labrus bergylta)*. The Norwegian Veterinary Institute's Report Series 1-2014, Bergen, ISSN: 1890-3290.

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vapA (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes

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Abstract

Sequence variation in a region of the virulence array protein gene (*vapA*; A-layer) was assessed in 333 ('typical' and 'atypical') isolates of the fish pathogenic bacterium *Aeromonas salmonicida*. Resulting similarity dendrograms revealed extensive heterogeneity, with nearly all isolates belonging to either of 14 distinct clusters or A-layer types. All acknowledged *A. salmonicida* subspecies (except ssp. *pectinolytica*, from which no *vapA* sequence could be obtained) were clearly separated, and notably, all isolates phenotypically identified as ssp. *salmonicida* formed a distinct and exclusive A-layer type. Additionally, an array of un-subspeciated atypical strains formed several equally prominent clusters, demonstrating that the concept of typical/atypical *A. salmonicida* is inappropriate for describing the high degree of diversity evidently occurring outside ssp. *salmonicida*. Most representatives assessed in this study were clinical isolates of spatiotemporally diverse origins, and were derived from a variety of hosts. We observed that from several fish species or families, isolates predominantly belonged to certain A-layer types, possibly indicating a need for host-/A-layer type-specific *A. salmonicida* vaccines. All in all, A-layer typing shows promise as an inexpensive and rapid means of unambiguously distinguishing clinically relevant *A. salmonicida* subspecies, as well as presently un-subspeciated atypical strains.

Keywords: Aeromonas salmonicida, A-layer/S-layer, fish, host specificity, molecular typing, *vapA*.

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Introduction

The bacterium *Aeromonas salmonicida* causes severe systemic disease in a wide range of freshwater and saltwater fish species on a worldwide basis (Austin & Austin 2007). Historically, research has focused mainly on the highly homogenous subspecies *salmonicida* (Lehmann & Neumann 1896; Griffin, Snieszko & Friddle 1953) ('typical' *A. salmonicida*), which causes furunculosis in salmonids (Salmonidae). Prior to the development of oil-adjuvanted vaccines in the early 1990s, this disease caused significant economic losses to salmon farming internationally. Recently however, increased attention has fallen on so-called 'atypical' *A. salmonicida* strains, which in Norway may represent a hazard to farmed Atlantic cod, *Gadus morhua* (L.), Atlantic halibut, *Hippoglossus hippoglossus* (L.), wolffish (Anarhichadidae) and the various 'cleaner fish' (i.e. wrasse (Labridae) and lumpsucker, *Cyclopterus lumpus* (L.)) used for biocontrol of the salmon louse, *Lepeophtheirus salmonis* (Krøyer). In contrast to typical, atypical strains are physiologically, biochemically

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and genetically relatively diverse (Austin & Austin 2007). Moreover, although four atypical *A. salmonicida* subspecies have been described, i.e. *achromogenes* (Smith 1963; Schubert 1967), *masoucida* (Kimura 1969a,b), *smithia* (Austin, McIntosh & Austin 1989) and *pectinolytica* (Pavan *et al.* 2000), isolates are frequently identified which cannot be validly assigned to any of these subspecies (Wiklund & Dalsgaard 1998).

As vaccination against furunculosis has been particularly successful in farmed Atlantic salmon, *Salmo salar* (L.), vaccination against atypical *A. salmonicida* would presumably improve the health situation of many cultured non-salmonid fish species. Both commercially available furunculosis vaccines for salmons and experimental atypical vaccines have been utilized in different fish species with varying success (reviewed by Gudmundsdóttir & Björnsdóttir 2007). Undoubtedly, many bacterial factors will be of importance for optimal vaccine protection. Bearing in mind the variable nature of atypical strains, it is therefore not improbable that they may also vary in traits of immunogenic importance. Factors of proposed relevance in this regard include iron-regulated outer membrane proteins (IROMPs) (Hirst & Ellis 1994), LPS (Pyle & Cipriano 1986), a conserved porin (Lutwyche *et al.* 1995) and not least the crystalline, outer membrane protein known as the A-layer (Udey & Fryer 1978; Kay *et al.* 1981). This structure is believed to protect the bacterium from serum complement-mediated killing (Munn *et al.* 1982), protease degradation (Kay & Trust 1991) and to facilitate intracellular macrophage survival (Daly *et al.* 1996). Vaccines based on strains lacking the A-layer have repeatedly proved incapable of inducing strong protective immunity (Lund *et al.* 2003a, 2008a; Lund, Mikkelsen & Schrøder 2008b, 2009; Arnesen *et al.* 2010).

Following sequence analysis of the virulence array protein gene (*vapA*), the gene encoding the A-layer (Chu *et al.* 1991), some strain diversity was identified and several distinct clusters (or A-layer types) appeared in similarity dendrograms (Lund, Espelid & Mikkelsen 2003b; Lund & Mikkelsen 2004; Han *et al.* 2011; Kim *et al.* 2011). A-layer type could further be linked to superior vaccine protection against autogenous strains in some cases (Lund *et al.* 2003b; Arnesen *et al.* 2010), while equally clear correlations could not be deduced in others (Lund *et al.* 2008a; Lund *et al.* 2008b). We therefore decided to examine *vapA* heterogeneity in a large number of *A. salmonicida* isolates of disparate geographical and temporal origin, and from a variety of hosts. From this, we hoped to establish any eventual relationship between A-layer type and the source of isolation. For vaccination purposes, as well as for epizootiological inference, such information could be highly relevant.

Materials and methods

Bacterial isolates

Individual details on all 336 *Aeromonas salmonicida* isolates evaluated in this study are listed in Table S1. Most were clinical isolates obtained through the Norwegian Veterinary Institute's diagnostic services (NVI), in addition to reference and donated strains. Clinical NVI isolates were phenotypically identified as *A. salmonicida* and designated as either typical or atypical in accordance with an in-house-accredited diagnostic method (outlined in Fig. 1). Stock cultures (maintained at −80 °C) were subcultured on blood agar (5% bovine blood) at 22 °C for 48–72 h prior to further processing. Selected isolates cultured on Coomassie Brilliant Blue (CBB) agar (Cipriano & Bertolini 1988) to assess the presence of A-layers were incubated under similar conditions. While in many cases, multiple isolates were sequenced from individual outbreaks, only single representatives from such outbreaks were included in our material unless the sequences showed variation. An additional 27 *vapA* sequences were retrieved from GenBank.

Target gene

Near-full-length *vapA* sequences (approximately 1500 bp) from representatives of all valid *A. salmonicida* subspecies were obtained from GenBank, with the exception of ssp. *pectinolytica* from which no such sequences could be obtained. In addition, near-full-length *vapA* from several atypical isolates was sequenced in our laboratory (as described below). From the majority of isolates, however, only a partial and highly variable *vapA* region (approximately 400 bp) was assessed.

DNA extraction, PCR and sequencing

DNA was extracted by boiling bacterial cells in dH_2O for 7 min, followed by centrifugation and

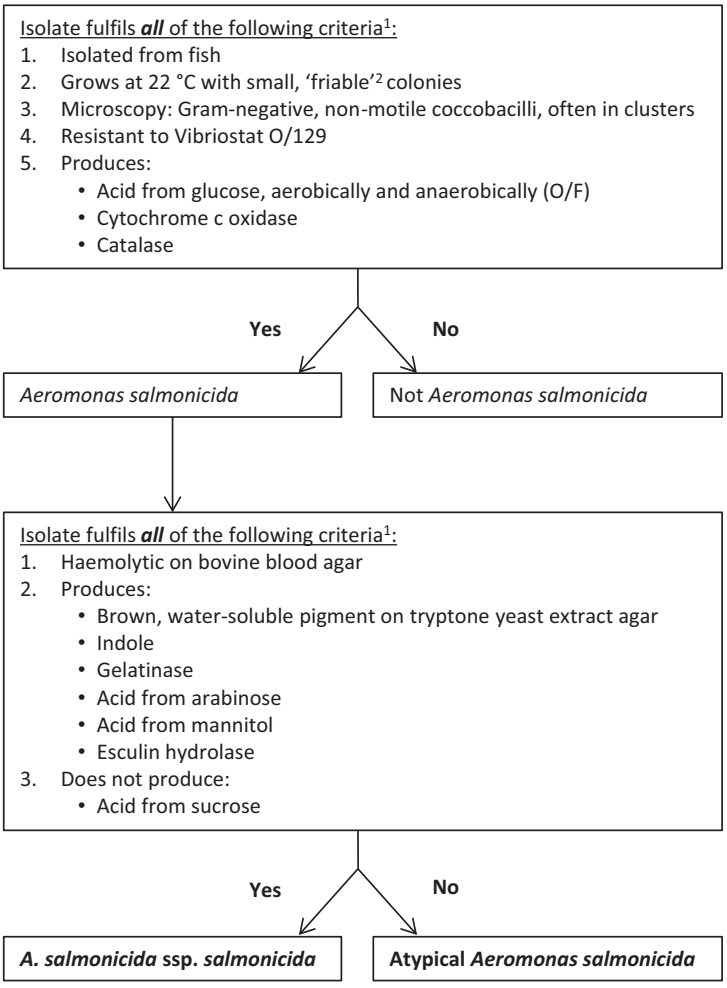

Figure 1 Outline of the phenotypic method (NVI; in-house-accredited) by which the majority of isolates included in the present study were identified as (typical or atypical) *A. salmonicida*.

use of the supernatant as PCR template. This method proved unsuitable for a few isolates, from which DNA was instead extracted using a QiaCube (Qiagen) according to the manufacturer's instructions.

Primers used for PCR amplification and sequencing are listed in Table 1, with base numbering relative to the genome of *A. salmonicida* ssp. *salmonicida* strain A449 (GenBank: CP000644.1). For near-full-length *vapA*, A-layer primers 1 and 6 were used for PCR and A-layer primers 1–6 (in pairs 1 + 3; 2 + 5; 4 + 6) were used for sequencing. When targeting only the hypervariable *vapA* region, primers F2 and R3 (flanking a span of approximately 600 bp) were used for both PCR and sequencing. Each PCR volume consisted of 2.5 μL 10× ThermoPol Reaction Buffer (New England BioLabs), 0.2 mM dNTP (VWR), 0.4 μM of both forward and reverse primers (Invitrogen), one unit *Taq* DNA polymerase (New England BioLabs), 3 μL (if boil-extracted) or 1 μL (if QiaCube-extracted) DNA template, and a final addition of Milli-Q water to reach a total reaction volume of 25 μL. Subsequent PCR thermal cycles involved (i) 3 min at 95 °C; (ii) 30 cycles of 1 min at 95 °C, 1 min at 53 °C and 1 min at 68 °C; and (iii) 4 min at 68 °C followed by cooling to 4 °C indefinitely. This was conducted on a Dyad Dual 96-Well Thermal Cycler (MJ Research).

Upon visual confirmation of PCR products of the expected size by gel electrophoresis (1.5% agarose gel with GelRed staining), purification using

Table 1 Primers used in this study. The protocol in which they were used (near-full-length or partial *vapA* PCR and sequencing) and their nucleotide positioning relative to the genome of *A. salmonicida* ssp. *salmonicida* strain A449 (Accession No.: CP000644.1; *vapA* spanning nucleotides 1497103–1498611) are specified

Primer name	Protocol	Primer orientation	Primer sequence (5′–3′)	Nucleotide positioning	Reference
A-layer 1	Full-length	Forward	ACAGTGCACCGAAGGTTGAT	1497024–1497043	This study
A-layer 2	Full-length	Forward	TCTGCGTCTTGCTCTTGCT	1497540–1497558	This study
A-layer 3	Full-length	Reverse	GTAGCAACCGGATCAGCAGT	1497719–1497700	This study
A-layer 4	Full-length	Forward	TGGCTGCTTTTGGTACACTG	1498061–1498080	This study
A-layer 5	Full-length	Reverse	AGCTTGGCTATCACCTTGGA	1498161–1498142	This study
A-layer 6	Full-length	Reverse	ACGGCAGAGCTTGTCTACCT	1498733–1498714	This study
F2	Partial	Forward	CTGGACTTCTCCACTGCTCA	1497238–1497257	(Lund *et al.* 2003b)
R3	Partial	Reverse	ACGTTGGTAATCGCGAAATC	1497863–1497844	This study

ExoSAP-IT (Amersham Biosciences) was carried out according to the manufacturer's instructions. Sequencing was performed using BigDye Terminator v3.1. according to a protocol previously described (Platt, Woodhall & George 2007) and an Avant 3130*xl* Genetic Analyzer (Applied Biosystems).

Sequence analysis

For contig assembly and manual correction, Geneious v7.1 (Biomatters) was used. Resulting nucleotide and amino acid sequences (excluding frameshifted sequences; see Results) were aligned with ClustalX v2.1 (Larkin *et al.* 2007), and alignments were manually edited in MEGA6 (Tamura *et al.* 2013). A sequence span within the hypervariable *vapA* region, corresponding to nucleotide residues 202–606 (i.e. amino acid residues 68–202) in *vapA* of *A. salmonicida* ssp. *salmonicida* strain A450 (acc. no.: M64655.1), was the target for further analysis and is henceforth referred to as partial *vapA*. Maximum-likelihood (ML) dendrograms were constructed using PhyML v3.0 (Guindon *et al.* 2010) with default settings. Branch support was estimated with the approximate likelihood ratio test (aLRT) (Anisimova & Gascuel 2006). Resulting ML dendrograms were subsequently edited in MEGA6 (Tamura *et al.* 2013). Representatives of all novel *vapA* sequence variants (near full-length if available, and including *vapA* mutants; see Results) have been submitted to GenBank (44 in total; acc. nos. KP184519-KP184562; see Table S1).

A-layer typing

Based on partial *vapA* ML dendrograms, prominent clusters comprising three or more isolates were assigned A-layer type numbers. To aid the process in less obvious cases, a 'grouping threshold' based on pairwise DNA identity was set. Thus, if at any given branching point, the inter-branch pairwise sequence similarity between all sub-branches was consistently <98%, these sub-branches were defined as representing different A-layer types. For the convenience of eventual future research, the A-layer type numbers previously assigned in other studies (Lund *et al.* 2008a; Lund *et al.* 2008b; Arnesen *et al.* 2010) have been maintained here.

Statistical analysis and maps

For some selected fish species or families from which one or two A-layer types seemed remarkably prevalent (see Results), the 95% confidence intervals (CI) for the observed proportions were calculated. This was performed using the Equal or Given Proportions test in R software v 3.1 (R Core Team 2014). Plotted maps based on sampling coordinates were also constructed with this software.

Results

Following alignment (not shown) of near-full-length *vapA* sequences from 32 *A. salmonicida* isolates representing all major A-layer types, it was confirmed that, as previously demonstrated (Lund *et al.* 2003b; Lund & Mikkelsen 2004), most genetic diversity in this gene resides in the deduced hypervariable region.

A total of 336 *A. salmonicida* isolates were included in this study (Table S1), and partial *vapA* sequences could be obtained from 333 of these. Alignment revealed extensive heterogeneity in this area of the *vapA* gene, and most nucleotide

variation involved missense mutations, with the majority of resulting amino acid substitutions involving switches between amino acids of dissimilar hydrophobicity. Pairwise sequence similarities in this gene region were ≥61.7% and ≥80.0%, for amino acids and nucleotides, respectively. ML dendrograms based on protein or DNA appeared roughly similar, except the amino acid tree (not shown) generally featured slightly longer branches. Isolates were separated into several distinct clusters (Fig. 2), most of which were highly conserved. Using the aforementioned grouping threshold, clusters comprising three or more isolates were assigned A-layer type numbers, resulting in the identification of 14 A-layer types (of which 10 were previously unassigned). Additionally, five singleton isolates with unique sequences remain unassigned to any A-layer type. Type numbers I–IV were designated in agreement with those previously proposed (Lund *et al.* 2008a; Lund *et al.* 2008b; Arnesen *et al.* 2010) and should thus accompany equivalent clusters in this study. However, in the case of ATCC 27013 (*A. salmonicida* ssp. *masoucida* type strain; previously A-layer type II), our grouping criteria resulted in reassignment of this strain to the novel type VII. An overview of the A-layer types and their associated isolates (including reference strains) can be found in Table 2.

Most of the major A-layer types (>10 isolates; i.e. type I–VII) originated from widespread locations around the Norwegian coast (Fig. 3). This does not apply to type VII however, which, as far as we know, comprises isolates entirely derived from fish in the Pacific Ocean.

No *vapA* sequence could be obtained from three isolates belonging to ssp. *pectinolytica*, a result consistent with previous studies involving this bacterium (Lund *et al.* 2003b). All type strains of other recognized and validly published (Euzéby 1997; Parte 2014) *A. salmonicida* subspecies, however, were found to represent different A-layer types (Fig. 2 and Table 2). These were type I (ssp. *salmonicida*), type III (ssp. *achromogenes*) and type VII (ssp. *masoucida*), and one unassigned singleton (ssp. *smithia*). Notably, the type I cluster comprised entirely and exclusively the 33 isolates phenotypically identified as ssp. *salmonicida* (Table S1).

When looking at the relative distribution of A-layer types among certain fish species and families, patterns of host specificity seemed evident. This can be seen more clearly in Fig. 4 and Table 3. Figure 4 shows the distribution of

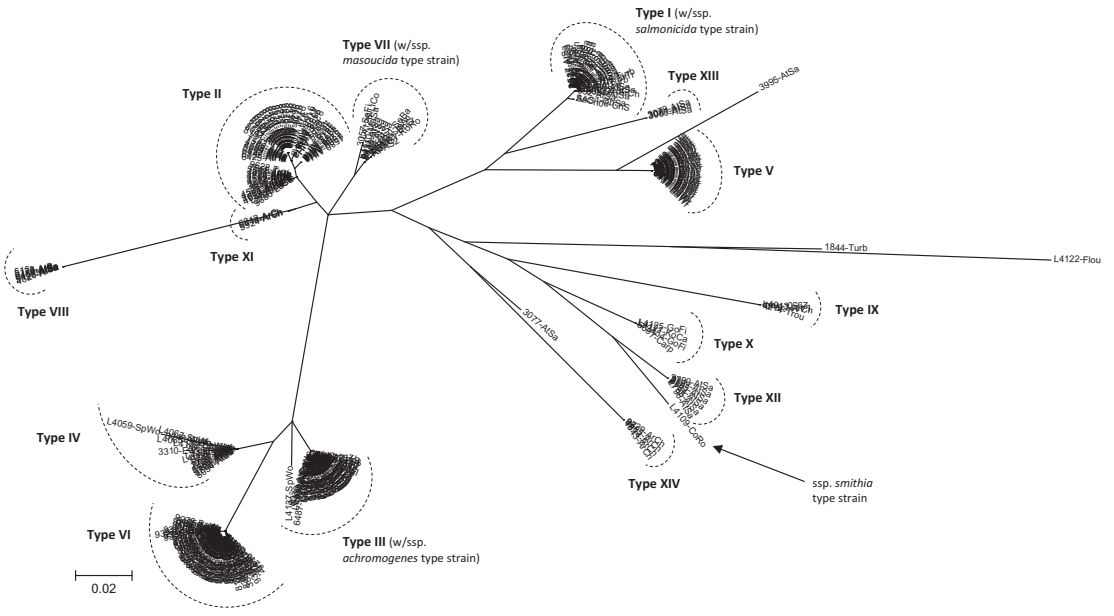

Figure 2 Unrooted radiation ML dendrogram based on partial *vapA* nucleotide sequences (390–423 bp). Frameshifted sequences are excluded (see Results). All assigned A-layer types are designated, and the relative positioning of *A. salmonicida* subspecies type strains (excl. ssp. *pectinolytica*) is indicated.

Table 2 Overview of identified *A. salmonicida* A-layer types and their associated isolates. See Table S1 for individual details on the isolates and accession numbers for submissions

A-layer type	Isolates (evt. reference strains listed last)	Comments
I	NVI-392, NVI-1654, NVI-1665, NVI-2265, NVI-2461, NVI-2500, NVI-2515, NVI-2660, NVI-2761, NVI-2940, *NVI-3094*[a], NVI-3095, NVI-3096, NVI-3151, NVI-3275, *NVI-3294*, NVI-3517, NVI-3706, ***NVI-4704***, *NVI-4757*, NVI-5071, NVI-6416, NVI-7320, NVI-8233, NVI-9776, L4012, L4014, L4017, A449, A450, AsCh08, SAS1; **Reference strains:** L4010 (ssp. *salmonicida* NCIMB1102^T)	• 33 isolates; 84% from salmonids[b] • Seemingly worldwide distribution • Isolated from 1963 to 2014[c]. • Entirely and exclusively comprises isolates phenotypically identified as ssp. salmonicida
II	NVI-2791, NVI-3656, NVI-3842, NVI-3902, NVI-4217, NVI-4363, NVI-4500, NVI-4533, NVI-4580, NVI-4582, NVI-4609, NVI-4670, NVI-4729, NVI-4758, NVI-4786, ***NVI-4818***, NVI-4820, NVI-4871, NVI-4873, *NVI-4953*, NVI-4954, NVI-4977, NVI-5109, NVI-5208, NVI-5582, NVI-5693, NVI-6425, NVI-6437, NVI-6438, NVI-6563, NVI-6725, NVI-6855, NVI-6861, NVI-6959, NVI-6962, NVI-7555, NVI-7566, *NVI-7743*, NVI-7744, NVI-8042, NVI-8141, NVI-8191, NVI-8210, NVI-8291, *NVI-8450*, NVI-8474, NVI-8615, NVI-8771, NVI-8835, NVI-9150, NVI-9254, NVI-9270, *NVI-9338*, NVI-9371, NVI-9416, NVI-9628, NVI-9631, NVI-9686, NVI-9710, NVI-9744, L4050; **No reference strains**	• 61 isolates; 89% from Atlantic halibut • Only Norwegian isolates • Isolated from 1987 to 2014 • Intracluster variation possibly reflecting evolution of a 'house strain' (see Discussion and Fig. 5)
III	NVI-2789, NVI-2797, NVI-2993, NVI-3055, NVI-3067, NVI-3068, NVI-3076, NVI-3093, NVI-3655, NVI-4568, NVI-4881, NVI-5032, NVI-5041, NVI-5170, NVI-5540, NVI-5849, NVI-5915, NVI-5982, NVI-6008, NVI-6069, NVI-6394, *NVI-6402*, NVI-6421, NVI-6424, NVI-6475, NVI-6477, *NVI-6487*, NVI-6492, NVI-6495, NVI-6497, NVI-6522, NVI-6792, NVI-6977, NVI-6993, NVI-7018, NVI-7089, NVI-7321, NVI-7501, NVI-7711, *NVI-8039*, NVI-8125, NVI-8143, NVI-8218, NVI-8317, NVI-9521, L4092, L4099, L4101, L4115, L4124, L4128, L4137; **Reference strains:** L4111 (ssp. *achromogenes* NCIMB1110^T), NVI-1294 (ssp. *achromogenes* NCIMB1109)	• 54 isolates; 55% from Atlantic cod[b] • Only isolates from the Atlantic Ocean[b] • Isolated from 1962 to 2012[c]
IV	NVI-2661, NVI-2799, NVI-2800, NVI-3054, *NVI-3310*, NVI-3746, NVI-4398, NVI-5102, NVI-5986, NVI-6559, NVI-6641, NVI-9780, L4059, L4065, L4067, L4088, L4129; **Reference strains:** L4102 (atypical CECT5200)	• 18 isolates; 61% from wolffish • Only isolates from the Atlantic Ocean • Isolated from 1981 to 2014[c]
V	NVI-6304, NVI-6417, NVI-7256, NVI-7258, NVI-7708, NVI-7746, NVI-7830, NVI-7833, NVI-8011, ***NVI-8017***, NVI-8088, NVI-8095, NVI-8123, NVI-8130, *NVI-8356*[a], NVI-8357, NVI-8552, NVI-8570, NVI-8578, NVI-8640, NVI-9122, NVI-9144, NVI-9250, NVI-9277, NVI-9303, NVI-9346, NVI-9546, NVI-9548, NVI-9599, NVI-9600, NVI-9601, NVI-9673, NVI-9679, NVI-9713, NVI-9775; **No reference strains**	• 35 isolates; all from wrasse • Mainly Norwegian isolates (three Scottish) • Isolated from 2008 to 2014
VI	NVI-2359, NVI-2644, NVI-2652, *NVI-2787*[a], NVI-2884, NVI-2898, NVI-2901, NVI-2919, NVI-2921, NVI-2932, NVI-2948, NVI-2951, NVI-2952, NVI-3038, NVI-3785, NVI-3800, NVI-3815, *NVI-3817*[a], NVI-3906, NVI-4070, NVI-4229, NVI-4676, NVI-4862, NVI-4942, NVI-5177, NVI-6510, NVI-6945, NVI-7834, NVI-7835, ***NVI-8013***, NVI-8014, NVI-8044, NVI-8048, NVI-8126, NVI-8127, NVI-8158, *NVI-8175*[a], NVI-8176, NVI-8195, *NVI-8198*[a], *NVI-8199*[a], NVI-8200, *NVI-8201*[a], NVI-8231, ***NVI-8232***[a], NVI-8289, NVI-8337, NVI-8345, *NVI-8346*[a], NVI-8347, NVI-8419, NVI-8546, NVI-8561, NVI-8575, NVI-8635, *NVI-8838*[a], NVI-9140, NVI-9154, NVI-9155, NVI-9156, NVI-9216, NVI-9276, NVI-9278, NVI-9280, NVI-9304, NVI-9307, *NVI-9321*[a], NVI-9322, *NVI-9324*[a], NVI-9340, *NVI-9355*, *NVI-9358*[a], NVI-9382, NVI-9465, NVI-9496, NVI-9498, NVI-9507, NVI-9508, NVI-9538, NVI-9540, NVI-9543, NVI-9583, *NVI-9687*[a], NVI-9712; **No reference strains**	• 84 isolates; 85% from wrasse • Only Norwegian isolates • Isolated from 1987 to 2014 • Comprises 13 of the 16 isolates with deleterious *vapA* mutations (see Results and Discussion)
VII	*NVI-3057*, NVI-5490, NVI-9430, NVI-9431, *NVI-9432*, *L4126*, TNH3-1, RGM3-7, SHS1, TGM4-1, RFAS2/3; **Reference strains:** L4110 (ssp. *masoucida* ATCC27013^T)	• 12 isolates; all from the Pacific Ocean[b] • Isolated from 1969 to 2014[c]

Table 2 *Continued*

A-layer type	Isolates (evt. reference strains listed last)	Comments
VIII	NVI-4626, NVI-5125, NVI-5153, NVI-5175, NVI-6456, *NVI-6457*; **No reference strains**	• Six isolates; all from Atlantic salmon in one Norwegian county • Isolated from 2002 to 2008
IX	NVI-2950, NVI-4214, L4043, L4117; **No reference strains**	• Four isolates; all Scandinavian • Isolated from 1979 to 2000[c]
X	*NVI-3454*, *NVI-5697*, L4121, L4125; **No reference strains**	• Four isolates; all from Carps • Isolated from 1985 to 2006[c]
XI	NVI-5924, NVI-6012, *NVI-6449*; **No reference strains**	• Three isolates; all from Arctic char in one landlocked location in Norway • Isolated from 2007 to 2008
XII	NVI-2790, NVI-2792, NVI-2793, NVI-2794, NVI-2795, *NVI-2796*; **No reference strains**	• Six isolates; all from Atlantic salmon in two neighbouring Norwegian fjords • Isolated from 1987 to 1988
XIII	*NVI-3071*[a], NVI-3079, *NVI-3080*, NVI-3081; **No reference strains**	• Four isolates; all from At. salmon in east Canada • Isolated from 1986 to 1987[c]
XIV	***NVI-1843***, NVI-4814, NVI-9685, NVI-9729; **No reference strains**	• Four isolates; all from Arctic char in one landlocked location in Norway • Isolated from 1990 to 2014
Singletons	***NVI-1844***, *NVI-3077*, *NVI-3995*, L4122; **Reference strains:** L4109 (ssp. *smithia* NCIMB13210^T)	
No *vapA*	**Reference strains (all ssp. *pectinolytica*):** NVI-5354 (LMG19569^T), NVI-5404 (LMG19570), NVI-5405 (LMG19571)	

NVI-prefix: Stock cultures maintained at −80 °C at the Norwegian Veterinary Institute in Oslo.
Isolates in *italic* or ***bold italic***: *partial* or ***near-full-length*** *vapA* sequence submitted to GenBank (acc. nos. in Table S1).
[a]A-layer negative isolates with deleterious *vapA* mutations.
[b]When disregarding isolates of unknown origin.
[c]Some uncertainties with regard to the time span over which these isolates were collected.

all isolates, with those derived from wrasse, Atlantic halibut, Atlantic cod and wolffish coloured. Table 3 shows the proportion of predominant A-layer types found from particular fish species/families.

For the majority of isolates, partial *vapA* analysis revealed in-frame sequences varying in size between 390 and 423 nucleotides (i.e. 130–141 amino acids). However, nine isolates were identified which possessed unique frameshifting base insertions or deletions (indels), varying in length between insertions of 40 and deletions of 137 nucleotides, respectively. Further, seven isolates displayed singular nonsense mutations resulting in premature stop codons, all of which were localized at different positions within the reading frame. All 16 isolates with either frameshifting or nonsense mutations could be assigned to A-layer types based on the analysis of intact sequence regions, and consequently, 13 of these were identified as representatives of A-layer type VI (Table 2). Furthermore, all 16 isolates were confirmed to be A-layer negative following the appearance of white colonies upon culturing on CBB agar.

Discussion

Following similarity analysis of partial *vapA* sequences from 333 *A. salmonicida* isolates (Table 2, Table S1) of diverse origins, several distinct A-layer types were identified (Fig. 2). In accordance with previous findings (Lund *et al.* 2003b; Lund & Mikkelsen 2004), sequences obtained from type strains of all recognized *A. salmonicida* subspecies (except ssp. *pectinolytica*; thus far unrelated to disease (Pavan *et al.* 2013)) were found to represent different A-layer types. Moreover, all isolates phenotypically identified as ssp. *salmonicida* clustered together as A-layer type I, a type exclusive to such isolates. This is especially relevant in Norway due to legislative differences in management of classical furunculosis and diseases caused by atypical strains. Our results, combined with previous studies (Lund *et al.* 2003b;

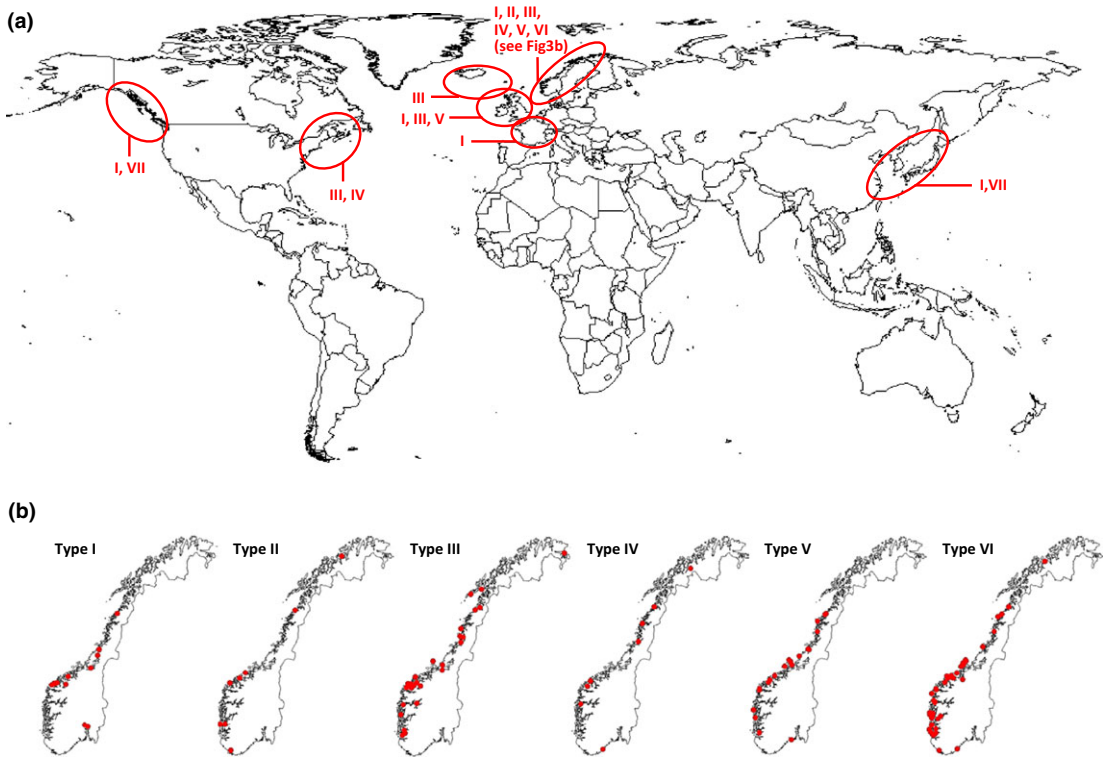

Figure 3 Maps indicating the geographical distribution of assessed *A. salmonicida* isolates by A-layer type. Only A-layer types with >10 isolates (types I–VII) are shown, and isolates of unknown origin are excluded. (a) approximate global distribution and (b) detailed distribution of Norwegian isolates (constituting 86% in total).

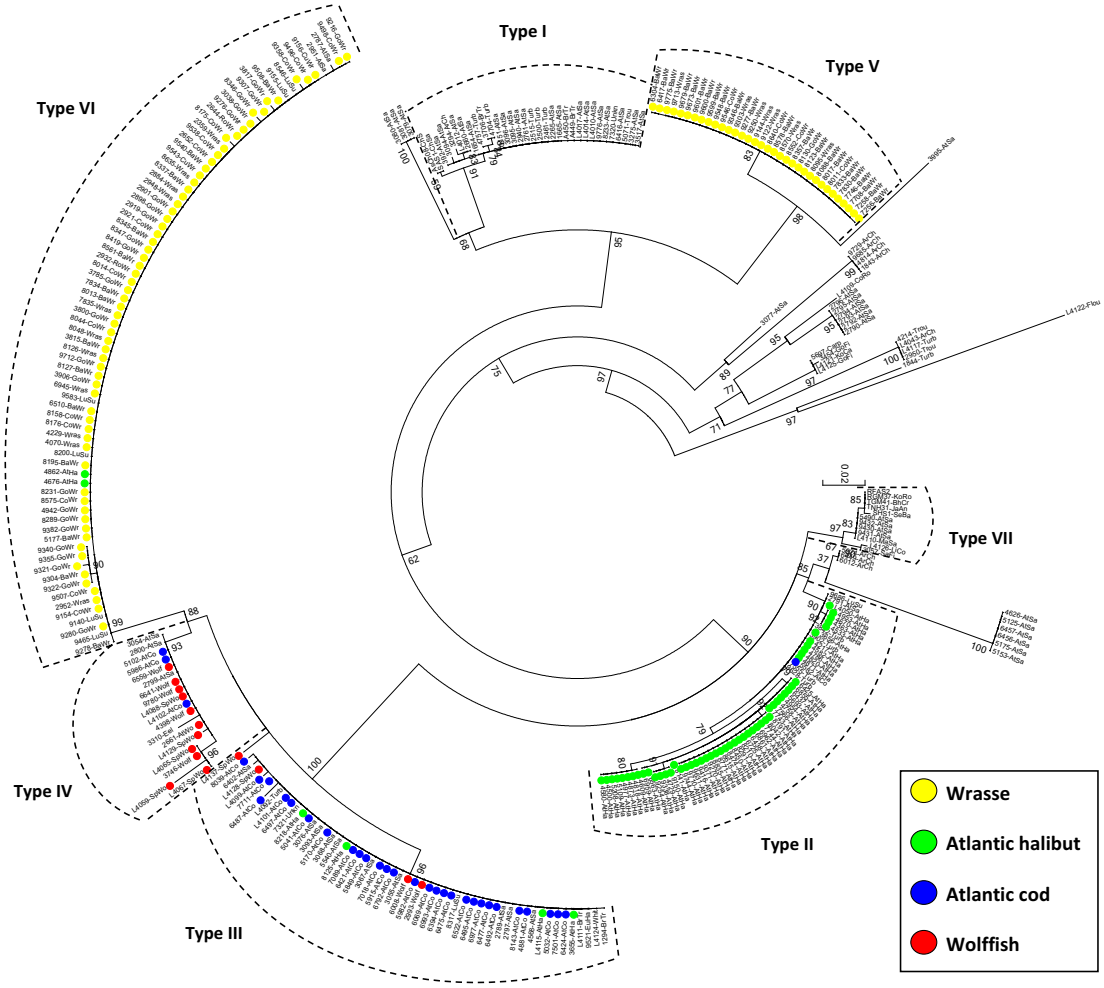

Figure 4 The partial *vapA* ML dendrogram from Fig. 2 in circular presentation (with aLRT branch support values shown) for illustration of host distributions. Major A-layer types (>10 isolates) are indicated by dotted lines, and *A. salmonicida* isolates from some selected fish species/families are coloured according to the legend. For all isolates, designations are followed by four-letter abbreviations indicating the fish species from which they derive. See Table S1 for detailed information on each isolate.

Table 3 Overview of predominant *A. salmonicida* A-layer types in some selected fish species and families

Host fish species/family	No. of isolates and no. of unique sampling sites	No. of isolates in the predominant A-layer type(s)	95% CI for observed proportions
Wrasse (Labridae)	106 (from ≥55 locations)	Types V and VI: all (100%)	95.6–100%
Atlantic halibut	60 (from ≥8 locations)	Type II: 54 (90%)	78.8–95.9%
Atlantic cod	33 (from ≥25 locations)	Type III: 29 (88%)	70.9–96.0%
Wolffish (Anarhichadidae)	15 (from ≥6 locations)	Types III and IV: all (100%)	74.7–100%
Turbot	12 (from ≥3 locations)	Types I and II: 9 (75%)	42.8–93.3%
Lumpsucker	11 (from ≥8 locations)	Type VI: 9 (82%)	47.8–96.8%
Carps (Cyprinidae)	5 (from ≥4 locations)	Type X: 4 (80%)	29.9–98.9%
Atlantic salmon	58 (from ≥33 locations)	N/A (see Discussion)	N/A
Other salmonids (Salmonidae)	20 (from ≥12 locations)	N/A (see Discussion)	N/A

Lund & Mikkelsen 2004; Han *et al.* 2011; Kim *et al.* 2011), thus indicate that A-layer typing may represent an affordable molecular method capable of rapid and definitive separation of all clinically relevant *A. salmonicida* subspecies as well as un-subspeciated atypical strains.

As previously demonstrated (Lund *et al.* 2003b; Lund & Mikkelsen 2004), *A. salmonicida vapA* diversity was found to be mainly restricted to a relatively small portion of the gene. Although extensive heterogeneity in this *vapA* region was evident, we found, following analysis, that most isolates fell into more or less conserved clusters (A-layer types) displaying a low degree of intracluster variation (Figs 2 & 4). Definition of a branch point grouping threshold of 98% was obviously a subjective decision, but one which resulted in a pragmatic separation of assessed isolates. This threshold value should not necessarily be stringently applied in future A-layer studies.

All A-layer types comprising more than ten isolates were collected over a minimum of two decades (except type V; see Table 2) and primarily from widespread locations around the Norwegian coast (except the seemingly 'Pacific' ssp. *masoucida* type VII) (Fig. 3, Table 2). Individual clusters display a relatively low degree of intracluster heterogeneity, indicating that the *vapA* gene has remained quite stable over these time spans. As opposed to the majority of clusters, A-layer type II appears to display some degree of intracluster evolutionary change. This type comprises (among others) isolates collected from a single, relatively intensively sampled, halibut hatchery (Loc. X; Fig. 5), which mostly fall into three distinct subclusters isolated over different (though overlapping) time spans between 2001 and 2014. It is therefore tempting to speculate that this may reflect evolution of a 'house strain' over the thirteen-year collection period. A similar, though less prominent pattern was also observed for type II isolates from another halibut hatchery (Loc. Y).

In contrast to previous studies (Lund *et al.* 2003b), and probably due to the larger number of *A. salmonicida* isolates included in the present work, correlations between certain A-layer types and their respective host fish species could be identified (Fig. 4 and Table 3). Generally, it seems that in all cases where large numbers of isolates were sequenced from individual fish species or families, one or a few A-layer types dominate (exception for salmonids; see below). In particular, the 106 isolates from wrasse species (i.e. Ballan, *Labrus bergylta* (Ascanius), goldsinny, *Ctenolabrus rupestris* (L.), corkwing, *Symphodus melops* (L.), cuckoo, *Labrus mixtus* (L.), and rock cook, *Centrolabrus exoletus* (L.), wrasse) belonged, without exception, to either of two A-layer types (V and VI). Isolates from Atlantic halibut and Atlantic cod also largely represented host-specific types (90% of type II and 88% of type III, respectively). Similar patterns were observed for wolffish, lumpsucker, turbot, *Scophthalmus maximus* (L.), and carps (Cyprinidae) although considerably fewer isolates from these fish groups were examined. The relatively low proportion of salmonid isolates belonging to A-layer type I (35%) reflects the rarity of classical furunculosis in modern salmon farming. The present study also predominantly targeted historical salmonid isolates phenotypically identified as atypical.

Following serial passage, particularly at elevated temperature, *A. salmonicida* strains have been known to lose expression of a functional A-layer (Ishiguro *et al.* 1981), resulting, for example, from mutational *vapA* insertions (Gustafson, Chu & Trust 1994) or deletions (Belland & Trust 1987) (indels). In our material, 16 clinical isolates had acquired either frameshifting indels or nonsense mutations in the *vapA* gene, thus distorting the A-layer. Whether the mutations arose in our laboratories or prior to isolation is unknown, but interestingly, 13 of the 16 isolates belonged to A-layer type VI (Table 2). This might indicate either that isolates belonging to A-layer type VI are particularly prone to acquiring such irreversible *vapA* mutations or that their optimal growth conditions differ from those of other A-layer types. Alternatively, one might hypothesize that type VI isolates may still retain virulence despite lacking a functional A-layer. However, these matters require further investigation.

The hypervariable *vapA* fragment analysed in the present study is assumed to encode a surface exposed and immunogenic portion of the A-layer (Doig *et al.* 1993; Lund & Mikkelsen 2004). In this regard, we found the majority of variable nucleotide sites to involve missense- and hydrophobicity-altering mutations, presumably resulting in A-layers of varying conformational and polar nature. A correlation between A-layer type and the morphology of antigenic epitopes therefore seems likely. As such, this part of the gene has previously been suggested to account for serological strain variations observed upon using A-layer-specific monoclonal antibodies (Lund *et al.* 2003b), and vaccine strains with heterogeneous *vapA* sequences were tested on spotted wolffish,

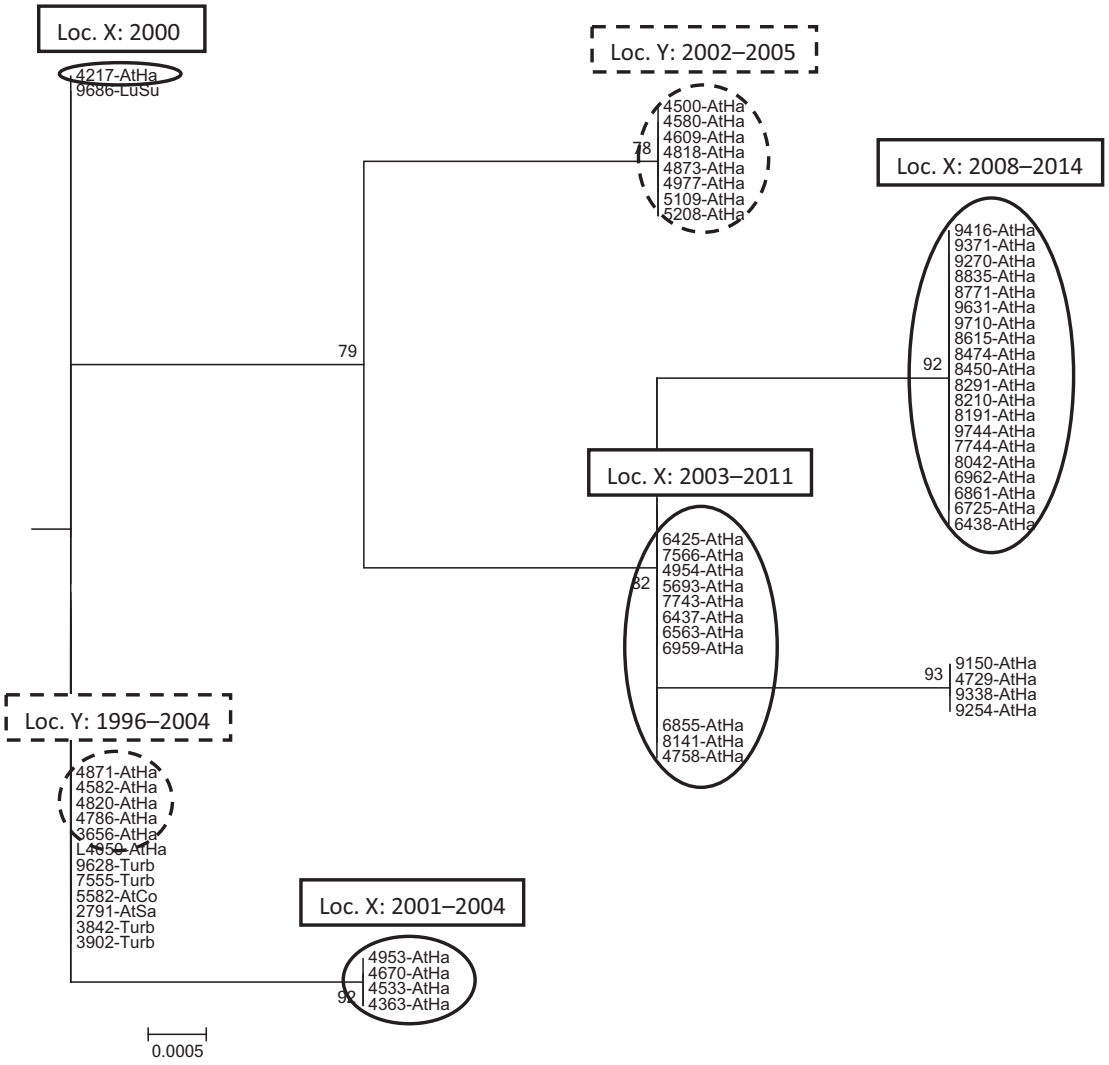

Figure 5 Partial *vapA* ML dendrogram showing only *A. salmonicida* isolates of A-layer type II. Full or dotted circles enclose isolates derived from Loc. X or Y, respectively (two specific Atlantic halibut hatcheries), with text boxes indicating the time spans over which they were collected.

Anarhichas minor (Olafsen), Atlantic halibut and Atlantic cod (Lund *et al.* 2003b, 2008a; Lund *et al.* 2008b; Arnesen *et al.* 2010). These experiments showed a clear correlation between vaccine efficacy and A-layer type only in spotted wolffish and Atlantic cod, and only for certain A-layer types. In Atlantic halibut however, while strain variation was observed, no clear correlations could be made. In the present study, we show that there is in fact a (direct or indirect) link between *A. salmonicida* A-layer type and the frequency with which different types are associated with disease in certain fish species. In our opinion, this warrants further investigation regarding the potential need for fish species- and/or A-layer type-specific vaccines.

In summary, the A-layer typing assay described here, and based on partial *vapA* nucleotide sequences, offers a pragmatic and definite way of differentiating *A. salmonicida* strains. The commonly used concept of typical/atypical *A. salmonicda* is useful only for identifying ssp. *salmonicida* isolates, relevant mostly to salmonids. However, the terminology is uninformative when it comes to describing the array of *A. salmonicida* strains infecting non-salmonids, which

seldom belong to ssp. *salmonicida*. A-layer typing on the other hand, provides clear separation of all recognized subspecies (except ssp. *pectinolytica*), while also distinguishing a range of clusters formed by un-subspeciated strains. Conveniently, isolates from certain fish species or families also show clustering patterns suggesting that the A-layer type may be related to differences in host specificity. Whether this is a causal or just an indicative correlation has not been established however.

As the partial *vapA* primers (Table 1) target only a single gene fragment spanning approximately 600 bp, the sequencing scheme presented here should be relatively affordable and fast, and within the technical range of an increasing number of diagnostic laboratories. Moreover, DNA sequence analysis is undoubtedly less ambiguous than phenotypic characterization, which relies on parameters that may occasionally show inconsistency. For instance, ssp. *salmonicida* isolates lacking pigment production (Wiklund, Lönnström & Niiranen 1993; Koppang *et al.* 2000) and oxidase-negative atypical isolates (Wiklund & Bylund 1993; Pedersen *et al.* 1994) have been reported, both of which contradict established diagnostic criteria (Fig. 1). Through electronic distribution of *vapA* sequence information via online databases, isolates can also be readily compared across laboratories, thus eliminating the need for control strains and facilitating identification of novel A-layer types. As the *vapA* heterogeneity observed might to some extent reflect antigenic variability, A-layer typing also holds potential relevance in the planning of vaccination strategies.

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References

Anisimova M. & Gascuel O. (2006) Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Systematic Biology* **55**, 539–552.

Arnesen K.R., Mikkelsen H., Schrøder M.B. & Lund V. (2010) Impact of reattaching various *Aeromonas salmonicida* A-layer proteins on vaccine efficacy in Atlantic cod (*Gadus morhua*). *Vaccine* **28**, 4703–4708.

Austin B. & Austin D.A. (2007) *Bacterial Fish Pathogens - Diseases of Farmed and Wild Fish*, 4th edn. Springer Praxis Books, Springer Netherlands.

Austin D.A., McIntosh D. & Austin B. (1989) Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* subsp. *smithia* subsp. nov. *Systematic and Applied Microbiology* **11**, 277–290.

Belland R.J. & Trust T.J. (1987) Cloning of the gene for the surface array protein of *Aeromonas salmonicida* and evidence linking loss of expression with genetic deletion. *Journal of Bacteriology* **169**, 4086–4091.

Chu S., Cavaignac S., Feutrier J., Phipps B.M., Kostrzynska M., Kay W.W. & Trust T.J. (1991) Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. *The Journal of Biological Chemistry* **266**, 15258–15265.

Cipriano R.C. & Bertolini J. (1988) Selection for virulence in the fish pathogen *Aeromonas salmonicida*, using Coomassie Brilliant Blue agar. *Journal of Wildlife Diseases* **24**, 672–678.

Daly J.G., Kew A.K., Moore A.R. & Olivier G. (1996) The cell surface of *Aeromonas salmonicida* determines in vitro survival in cultured brook trout (*Salvelinus fontinalis*) peritoneal macrophages. *Microbial Pathogenesis* **21**, 447–461.

Doig P., McCubbin W.D., Kay C.M. & Trust T.J. (1993) Distribution of surface-exposed and non-accessible amino acid sequences among the two major structural domains of the S-layer protein of *Aeromonas salmonicida*. *Journal of Molecular Biology* **233**, 753–765.

Euzéby J.P. (1997) List of bacterial names with standing in nomenclature: a folder available on the internet. *International Journal of Systematic Bacteriology* **47**, 590–592.

Griffin P.J., Snieszko S.F. & Friddle S.B. (1953) A more comprehensive description of *Bacterium salmonicida*. *Transactions of the American Fisheries Society* **82**, 129–138.

Gudmundsdóttir B.K. & Björnsdóttir B. (2007) Vaccination against atypical furunculosis and winter ulcer disease of fish. *Vaccine* **25**, 5512–5523.

Guindon S., Dufayard J.-F., Lefort V., Anisimova M., Hordijk W. & Gascuel O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**, 307–321.

Gustafson C.E., Chu S. & Trust T.J. (1994) Mutagenesis of the paracrystalline surface protein array of *Aeromonas salmonicida* by endogenous insertion elements. *Journal of Molecular Biology* **237**, 452–463.

Han H.-J., Kim D.-Y., Kim W.-S., Kim C.-S., Jung S.-J., Oh M.-J. & Kim D.-H. (2011) Atypical *Aeromonas salmonicida* infection in the black rockfish, *Sebastes schlegeli* Hilgendorf, in Korea. *Journal of Fish Diseases* **34**, 47–55.

Hirst I.D. & Ellis A.E. (1994) Iron-regulated outer membrane proteins of *Aeromonas salmonicida* are important protective antigens in Atlantic salmon against furunculosis. *Fish & Shellfish Immunology* **4**, 29–45.

Ishiguro E.E., Kay W.W., Ainsworth T., Chamberlain J.B., Austen R.A., Buckley J.T. & Trust T.J. (1981) Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. *Journal of Bacteriology* **148**, 333–340.

Kay W.W. & Trust T.J. (1991) Form and functions of the regular surface array (S-layer) of *Aeromonas salmonicida*. *Experientia* **47**, 412–414.

Kay W.W., Buckley J.T., Ishiguro E.E., Phipps B.M., Monette J.P. & Trust T.J. (1981) Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. *Journal of Bacteriology* **147**, 1077–1084.

Kim Y.-S., Yoon J.W., Han H.-J., Suebsing R. & Kim J.-H. (2011) Prevalence and characterization of typical *Aeromonas salmonicida* chum salmon isolates in Korea. *Canadian Journal of Fisheries and Aquatic Sciences* **14**, 347–354.

Kimura T. (1969a) A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on "Sakuramasu" (*Oncorhynchus masou*) and Pink Salmon (*O. gorbuscha*) rearing for maturity. *Fish Pathology* **3**, 34–44.

Kimura T. (1969b) A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on "Sakuramasu" (*Oncorhynchus masou*) and Pink Salmon (*O. gorbuscha*) rearing for maturity. *Fish Pathology* **3**, 45–52.

Koppang E.O., Fjølstad M., Melgård B., Vigerust M. & Sørum H. (2000) Non-pigment-producing isolates of *Aeromonas salmonicida* subspecies *salmonicida*: isolation, identification, transmission and pathogenicity in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* **23**, 39–48.

Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J. & Higgins D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics (Oxford, England)* **23**, 2947–2948.

Lehmann K.B. & Neumann R. (1896) *Atlas und Grundriss der Bakeriologie und Lehrbuch der speziellen bakteriologischen Diagnositk*, 1st Ed., J.F. Lehmann, Munchen.

Lund V. & Mikkelsen H. (2004) Genetic diversity among A-proteins of atypical strains of *Aeromonas salmonicida*. *Diseases of Aquatic Organisms* **61**, 257–262.

Lund V., Arnesen J.A., Coucheron D., Modalsli K. & Syvertsen C. (2003a) The *Aeromonas salmonicida* A-layer protein is an important protective antigen in oil-adjuvanted vaccines. *Fish & Shellfish Immunology* **15**, 367–372.

Lund V., Espelid S. & Mikkelsen H. (2003b) Vaccine efficacy in spotted wolffish *Anarhichas minor*: relationship to molecular variation in A-layer protein of atypical *Aeromonas salmonicida*. *Diseases of Aquatic Organisms* **56**, 31–42.

Lund V., Arnesen J.A., Mikkelsen H., Gravningen K., Brown L. & Schrøder M.B. (2008a) Atypical furunculosis vaccines for Atlantic cod (*Gadus morhua*); vaccine efficacy and antibody responses. *Vaccine* **26**, 6791–6799.

Lund V., Mikkelsen H. & Schrøder M.B. (2008b) Comparison of atypical furunculosis vaccines in spotted wolffish (*Anarhicas minor* O.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). *Vaccine* **26**, 2833–2840.

Lund V., Mikkelsen H. & Schrøder M.B. (2009) Atypical furunculosis vaccines for Atlantic cod *Gadhus morhua*: impact of reattached *Aeromonas salmonicida* A-layer protein on vaccine efficacy. *Diseases of Aquatic Organisms* **85**, 115–122.

Lutwyche P., Exner M.M., Hancock R.E. & Trust T.J. (1995) A conserved *Aeromonas salmonicida* porin provides protective immunity to rainbow trout. *Infection and Immunity* **63**, 3137–3142.

Munn C.B., Ishiguro E.E., Kay W.W. & Trust T.J. (1982) Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity* **36**, 1069–1075.

Parte A.C. (2014) LPSN - List of prokaryotic names with standing in nomenclature. *Nucleic Acids Research* **42**, Database issue D613–D616.

Pavan M.E., Abbott S.L., Zorzópulos J. & Janda J.M. (2000) *Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. *International Journal of Systematic and Evolutionary Microbiology*, **50**, 1119–1124.

Pavan M.E., Pavan E.E., López N.I., Levin L. & Pettinari M.J. (2013) Genome sequence of the melanin-producing extremophile *Aeromonas salmonicida* subsp. *pectinolytica* strain 34melT. *Genome Announcements* **1**, e00675–13.

Pedersen K., Kofod H., Dalsgaard I. & Larsen J.L. (1994) Isolation of oxidase-negative *Aeromonas salmonicida* from diseased turbot *Scopthhalmus maximus*. *Diseases of Aquatic Organisms* **18**, 149–154.

Platt A.R., Woodhall R.W. & George A.L. (2007) Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol. *BioTechniques* **43**, 58, 60, 62.

Pyle S.W. & Cipriano R.C. (1986) Specificity of lipopolysaccharide antigens of *Aeromonas salmonicida*. *Microbios Letters* **31**, 149–155.

R Core Team (2014) R: a language and environment for statistical computing. *R Foundation for Statistical Computing* Open access available at: http://cran.r-project.org

Schubert R.H.W. (1967) The taxonomy and nomenclature of the genus *Aeromonas* Kluyver and van Niel 1936: Part I. Suggestions on the taxonomy and nomenclature of the aerogenic *Aeromonas* species. *International Journal of Systematic Bacteriology* **17**, 23–37.

Smith I.W. (1963) The classification of "*Bacterium salmonicida*". *Journal of General Microbiology* **33**, 263–274.

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.

Udey J.L. & Fryer L.R. (1978) Immunization of fish with bacterins of *Aeromonas salmonicida*. *Marine Fisheries Review* **40**, 12–17.

Wiklund T. & Bylund G. (1993) Skin ulcer disease of flounder *Platichthys flesus* in the northern Baltic Sea. *Diseases of Aquatic Organisms* **17**, 165–174.

Wiklund T. & Dalsgaard I. (1998) Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid and salmonid fish species: a review. *Diseases of Aquatic Organisms* **32**, 49–69.

Wiklund T., Lönnström L. & Niiranen H. (1993) *Aeromonas salmonicida* ssp. *salmonicida* lacking pigment production, isolated from farmed salmonids in Finland. *Diseases of Aquatic Organisms* **15**, 219–223.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Individual details on *A. salmonicida* isolates evaluated in the present study.

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Supporting information

Table S1: Individual details on *A. salmonicida* isolates evaluated in the present study.

Isolate designation	Phenotypic id. + evt. ref.no.	Host	Geographical origin (evt. with county)	Year of first isolation	A-layer type	GenBank *vapA* acc.no.
NVI-392	*salmonicida*	Atlantic salmon	Norway	1989	I	
NVI-1294	*achromogenes* NCIMB1109	Brown trout	UK	1963 (uncertain)	III	
NVI-1654	*salmonicida*	Atlantic salmon	Norway (M&R)	1990	I	
NVI-1665	*salmonicida*	Atlantic salmon	Norway (M&R)	1990	I	
NVI-1843(f.l.)	atypical	Arctic char	Norway	1990	XIV	KP184519
NVI-1844(f.l.)	atypical	Turbot	Norway	1990	singleton	KP184520
NVI-2265	*salmonicida*	Atlantic salmon	Norway (M&R)	1991	I	
NVI-2359	atypical	Wrasse	Norway (HL)	1992	VI	
NVI-2461	*salmonicida*	Turbot	Norway	1992	I	
NVI-2500	*salmonicida*	Turbot	Norway	1992	I	
NVI-2515	*salmonicida*	Turbot	Norway (M&R)	1992	I	
NVI-2644	atypical	Rock cook wrasse	Norway (HL)	1993	VI	
NVI-2652	atypical	Corkwing wrasse	Norway (HL)	1993	VI	
NVI-2660	*salmonicida*	Atlantic salmon	Norway	1993	I	
NVI-2661	atypical	Atlantic wolffish	Norway (AA)	1993	IV	
NVI-2761	*salmonicida*	Atlantic salmon	Norway	prior to 1994	I	
NVI-2787*	atypical	Atlantic salmon	Norway (T)	1987	VI	KP184521
NVI-2789	atypical	Atlantic salmon	Norway	1987	III	
NVI-2790	atypical	Atlantic salmon	Norway (S&F)	1987	XII	
NVI-2791	atypical	Atlantic salmon	Norway (HL)	1987	II	
NVI-2792	atypical	Atlantic salmon	Norway (S&F)	1988	XII	
NVI-2793	atypical	Atlantic salmon	Norway (S&F)	1988	XII	
NVI-2794	atypical	Atlantic salmon	Norway (S&F)	1988	XII	
NVI-2795	atypical	Atlantic salmon	Norway (S&F)	1988	XII	
NVI-2796	atypical	Atlantic salmon	Norway (S&F)	1988	XII	KP184522
NVI-2797	atypical	Atlantic salmon	Norway (NL)	1988	III	
NVI-2799	atypical	Atlantic salmon	Norway (T)	1989	IV	
NVI-2800	atypical	Atlantic salmon	Norway (S&F)	1989	IV	
NVI-2884	atypical	Wrasse	Norway	1994	VI	
NVI-2898	atypical	Goldsinny wrasse	Norway	1994	VI	
NVI-2901	atypical	Goldsinny wrasse	Norway	1994	VI	
NVI-2919	atypical	Goldsinny wrasse	Norway	1994	VI	
NVI-2921	atypical	Corkwing wrasse	Norway	1994	VI	
NVI-2932	atypical	Rock cook wrasse	Norway	1994	VI	
NVI-2940	*salmonicida*	Atlantic salmon	Norway (M&R)	1994	I	
NVI-2948	atypical	Wrasse	Norway (HL)	1994	VI	
NVI-2950	atypical	Trout	Norway (O)	1994	IX	
NVI-2951	atypical	Atlantic salmon	Norway	1994	VI	
NVI-2952	atypical	Wrasse	Norway	1994	VI	
NVI-2993	atypical	Wolffish	Norway	1995	III	
NVI-3038	atypical	Goldsinny wrasse	Norway (AA)	1995 (uncertain)	VI	
NVI-3054	atypical	Atlantic salmon	Norway	prior to 1996	IV	
NVI-3055	atypical	Atlantic salmon	Canada (east)	1987 (uncertain)	III	
NVI-3057	atypical	Sablefish	Canada	prior to 1996	VII	KP184523

NVI-3067	atypical	Atlantic salmon	Unknown	1976 (uncertain)	III	
NVI-3068	atypical	Atlantic salmon	Scotland	prior to 1996	III	
NVI-3071*	atypical	Atlantic salmon	Canada (east)	1986 (uncertain)	XIII	KP184524
NVI-3076	atypical	Atlantic salmon	Scotland	prior to 1996	III	
NVI-3077	atypical	Atlantic salmon	Canada (east)	1988 (uncertain)	singleton	KP184525
NVI-3079	atypical	Atlantic salmon	Canada (east)	1987 (uncertain)	XIII	
NVI-3080	atypical	Atlantic salmon	Canada (east)	1987 (uncertain)	XIII	KP184526
NVI-3081	atypical	Atlantic salmon	Canada (east)	1987 (uncertain)	XIII	
NVI-3093	atypical	Atlantic salmon	Faroe Islands	1986 (uncertain)	III	
NVI-3094*	*salmonicida*	Atlantic salmon	Unknown	prior to 1996	I	KP184527
NVI-3095	*salmonicida*	Atlantic salmon	Unknown	prior to 1996	I	
NVI-3096	*salmonicida*	Atlantic salmon	Norway	1995	I	
NVI-3151	*salmonicida*	Atlantic salmon	Norway (AH)	1995	I	
NVI-3275	*salmonicida*	Atlantic salmon	Norway	1986	I	
NVI-3294	*salmonicida*	Arctic char	Switzerland	1986	I	KP184528
NVI-3310	atypical	Eel	Norway	1986	IV	KP184529
NVI-3454	atypical	Goldfish	Australia	1985 (uncertain)	X	KP184530
NVI-3517	*salmonicida*	Atlantic salmon	Canada (west)	prior to 1990	I	
NVI-3655	atypical	Atlantic halibut	Norway (M&R)	1996	III	
NVI-3656	atypical	Atlantic halibut	Norway (M&R)	1996	II	
NVI-3706	*salmonicida*	Brown trout	Norway (BR)	1996	I	
NVI-3746	atypical	Wolffish	Norway (M&R)	1997	IV	
NVI-3785	atypical	Goldsinny wrasse	Norway (M&R)	1997	VI	
NVI-3800	atypical	Goldsinny wrasse	Norway (M&R)	1997	VI	
NVI-3815	atypical	Ballan wrasse	Norway (HL)	1997	VI	
NVI-3817*	atypical	Goldsinny wrasse	Norway (HL)	1997	VI	KP184531
NVI-3842	atypical	Turbot	Norway (VA)	1998	II	
NVI-3902	atypical	Turbot	Norway (VA)	1998	II	
NVI-3906	atypical	Goldsinny wrasse	Norway (M&R)	1998	VI	
NVI-3995	atypical	Atlantic salmon	Chile	1999 (uncertain)	singleton	KP184532
NVI-4070	atypical	Wrasse	Norway (ST)	1999	VI	
NVI-4214	atypical	Trout	Norway (OL)	2000	IX	
NVI-4217	atypical	Atlantic halibut	Norway (M&R)	2000	II	
NVI-4229	atypical	Wrasse	Norway	2000	VI	
NVI-4363	atypical	Atlantic halibut	Norway (M&R)	2001	II	
NVI-4398	atypical	Wolffish	Norway	2001	IV	
NVI-4500	atypical	Atlantic halibut	Norway (M&R)	2002	II	
NVI-4533	atypical	Atlantic halibut	Norway (M&R)	2002	II	
NVI-4568	atypical	Atlantic salmon	Norway (ST)	2002	III	
NVI-4580	atypical	Atlantic halibut	Norway (M&R)	2002	II	
NVI-4582	atypical	Atlantic halibut	Norway (M&R)	2002	II	
NVI-4609	atypical	Atlantic halibut	Norway (M&R)	2002	II	
NVI-4626	atypical	Atlantic salmon	Norway (NL)	2002	VIII	
NVI-4670	atypical	Atlantic halibut	Norway (M&R)	2003	II	
NVI-4676	atypical	Atlantic halibut	Norway (RL)	2003	VI	
NVI-4704(f.l.)	*salmonicida*	Turbot	Norway (ST)	2003	I	KP184533
NVI-4729	atypical	Atlantic halibut	Norway (HL)	2003	II	
NVI-4757	*salmonicida*	Turbot	Norway (ST)	2003	I	KP184534
NVI-4758	atypical	Atlantic halibut	Norway (M&R)	2003	II	
NVI-4786	atypical	Atlantic halibut	Norway (M&R)	2003	II	
NVI-4814	atypical	Arctic char	Norway (NL)	2003	XIV	
NVI-4818(f.l.)	atypical	Atlantic halibut	Norway (M&R)	2003	II	KP184535
NVI-4820	atypical	Atlantic halibut	Norway (M&R)	2003	II	
NVI-4862	atypical	Atlantic halibut	Norway (RL)	2004	VI	
NVI-4871	atypical	Atlantic halibut	Norway (M&R)	2004	II	

NVI-4873	atypical	Atlantic halibut	Norway (M&R)	2004	II	
NVI-4881	atypical	Atlantic cod	Norway (M&R)	2004	III	
NVI-4942	atypical	Goldsinny wrasse	Norway (HL)	2004	VI	
NVI-4953	atypical	Atlantic halibut	Norway (M&R)	2004	II	KP184536
NVI-4954	atypical	Atlantic halibut	Norway (M&R)	2004	II	
NVI-4977	atypical	Atlantic halibut	Norway (M&R)	2004	II	
NVI-5032	atypical	Atlantic cod	Norway (M&R)	2004	III	
NVI-5041	atypical	Atlantic cod	Norway (M&R)	2004	III	
NVI-5071	*salmonicida*	Trout	Norway (NL)	2004	I	
NVI-5102	atypical	Atlantic cod	Norway (M&R)	2004	IV	
NVI-5109	atypical	Atlantic halibut	Norway (M&R)	2004	II	
NVI-5125	atypical	Atlantic salmon	Norway (NL)	2004	VIII	
NVI-5153	atypical	Atlantic salmon	Norway (NL)	2004	VIII	
NVI-5170	atypical	Atlantic cod	Norway (NL)	2005	III	
NVI-5175	atypical	Atlantic salmon	Norway (NL)	2005	VIII	
NVI-5177	atypical	Ballan wrasse	Norway (HL)	2004	VI	
NVI-5208	atypical	Atlantic halibut	Norway (M&R)	2005	II	
NVI-5354	*pectinolytica* LMG19569 (T)	Water	Argentina	1988 (uncertain)	no *vapA*	
NVI-5404	*pectinolytica* LMG19570	Water	Argentina	1991 (uncertain)	no *vapA*	
NVI-5405	*pectinolytica* LMG19571	Water	Argentina	1991 (uncertain)	no *vapA*	
NVI-5490	atypical	Atlantic salmon	Unknown	prior to 2006	VII	
NVI-5540	atypical	Atlantic salmon	Norway (NL)	2006	III	
NVI-5582	atypical	Atlantic cod	Norway (FM)	2006	II	
NVI-5693	atypical	Atlantic halibut	Norway (M&R)	2006	II	
NVI-5697	atypical	Carp	Norway (HL)	2006	X	KP184537
NVI-5849	atypical	Atlantic cod	Norway (NL)	2007	III	
NVI-5915	*achromogenes*	Atlantic cod	Norway (NL)	2007	III	
NVI-5924	atypical	Arctic char	Norway (T)	2007	XI	
NVI-5982	atypical	Atlantic cod	Norway (NL)	2007	III	
NVI-5986	atypical	Atlantic cod	Norway (NL)	2007	IV	
NVI-6008	atypical	Wolffish	Norway (RL)	2007	III	
NVI-6012	atypical	Arctic char	Norway (T)	2007	XI	
NVI-6069	atypical	Atlantic cod	Norway (NL)	2008	III	
NVI-6304	atypical	Ballan wrasse	Norway (HL)	2008	V	
NVI-6394	atypical	Atlantic cod	Norway (ST)	2008	III	
NVI-6402	atypical	Atlantic salmon	Norway (FM)	2008	III	KP184538
NVI-6416	*salmonicida*	Atlantic salmon	Norway	2008	I	
NVI-6417	atypical	Ballan wrasse	Norway (HL)	2008	V	
NVI-6421	atypical	Atlantic cod	Norway (M&R)	2008	III	
NVI-6424	atypical	Atlantic cod	Norway (NL)	2008	III	
NVI-6425	atypical	Atlantic halibut	Norway (M&R)	2008	II	
NVI-6437	atypical	Atlantic halibut	Norway (M&R)	2008	II	
NVI-6438	atypical	Atlantic halibut	Norway (M&R)	2008	II	
NVI-6449	atypical	Arctic char	Norway (T)	2008	XI	KP184539
NVI-6456	atypical	Atlantic salmon	Norway (NL)	2008	VIII	
NVI-6457	atypical	Atlantic salmon	Norway (NL)	2008	VIII	KP184540
NVI-6475	atypical	Atlantic cod	Norway (NL)	2008	III	
NVI-6477	atypical	Atlantic cod	Norway (RL)	2008	III	
NVI-6487	atypical	Atlantic cod	Norway (M&R)	2008	III	KP184541
NVI-6492	atypical	Atlantic cod	Norway (NL)	2008	III	
NVI-6495	atypical	Atlantic cod	Norway (S&F)	2008	III	
NVI-6497	atypical	Atlantic cod	Norway (RL)	2008	III	
NVI-6510	atypical	Ballan wrasse	Norway (NL)	2008	VI	
NVI-6522	atypical	Atlantic cod	Norway (NL)	2008	III	
NVI-6559	atypical	Wolffish	Norway (NL)	2008	IV	

NVI-6563	atypical	Atlantic halibut	Norway (M&R)	2008	II	
NVI-6641	atypical	Wolffish	Norway (NL)	2008	IV	
NVI-6725	atypical	Atlantic halibut	Norway (M&R)	2009	II	
NVI-6792	atypical	Atlantic cod	Faroe Islands	2009	III	
NVI-6855	atypical	Atlantic halibut	Norway (M&R)	2009	II	
NVI-6861	atypical	Atlantic halibut	Norway (M&R)	2009	II	
NVI-6945	atypical	Wrasse	Norway (VA)	2009	VI	
NVI-6959	atypical	Atlantic halibut	Norway (M&R)	2009	II	
NVI-6962	atypical	Atlantic halibut	Norway (M&R)	2009	II	
NVI-6977	atypical	Atlantic cod	Norway (M&R)	2009	III	
NVI-6993	atypical	Atlantic cod	Norway (M&R)	2009	III	
NVI-7018	atypical	Atlantic cod	Norway (M&R)	2009	III	
NVI-7089	atypical	Atlantic cod	Norway (NL)	2009	III	
NVI-7256	atypical	Ballan wrasse	Norway (HL)	2009	V	
NVI-7258	atypical	Ballan wrasse	Norway (HL)	2010	V	
NVI-7320	*salmonicida*	Unknown	Unknown	prior to 2010	I	
NVI-7321	atypical	Unknown	Unknown	prior to 2010	III	
NVI-7501	atypical	Atlantic cod	Norway (M&R)	2010	III	
NVI-7555	atypical	Turbot	Norway (VA)	2010	II	
NVI-7566	atypical	Atlantic halibut	Norway (M&R)	2010	II	
NVI-7708	atypical	Ballan wrasse	Norway (NL)	2010	V	
NVI-7711	atypical	Atlantic cod	Norway (M&R)	2010	III	
NVI-7743	atypical	Atlantic halibut	Norway (M&R)	2010	II	KP184542
NVI-7744	atypical	Atlantic halibut	Norway (M&R)	2011	II	
NVI-7746	atypical	Ballan wrasse	Norway (M&R)	2010	V	
NVI-7830	atypical	Ballan wrasse	Norway (HL)	2010	V	
NVI-7833	atypical	Ballan wrasse	Norway (HL)	2010	V	
NVI-7834	atypical	Ballan wrasse	Norway (HL)	2010	VI	
NVI-7835	atypical	Wrasse	Norway	2010	VI	
NVI-8011	atypical	Corkwing wrasse	Norway (M&R)	2011	V	
NVI-8013(f.l.)	atypical	Ballan wrasse	Norway (M&R)	2011	VI	KP184543
NVI-8014	atypical	Corkwing wrasse	Norway (M&R)	2011	VI	
NVI-8017(f.l.)	atypical	Ballan wrasse	Norway (M&R)	2011	V	KP184544
NVI-8039	atypical	Atlantic cod	Norway (M&R)	2011	III	KP184545
NVI-8042	atypical	Atlantic halibut	Norway (M&R)	2011	II	
NVI-8044	atypical	Corkwing wrasse	Norway (HL)	2011	VI	
NVI-8048	atypical	Wrasse	Norway (HL)	2011	VI	
NVI-8088	atypical	Ballan wrasse	Norway (M&R)	2011	V	
NVI-8095	atypical	Wrasse	Norway (NT)	2011	V	
NVI-8123	atypical	Ballan wrasse	Norway (RL)	2011	V	
NVI-8125	atypical	Atlantic halibut	Norway (RL)	2011	III	
NVI-8126	atypical	Wrasse	Norway (M&R)	2011	VI	
NVI-8127	atypical	Ballan wrasse	Norway (S&F)	2011	VI	
NVI-8130	atypical	Goldsinny wrasse	Norway (NL)	2011	V	
NVI-8141	atypical	Atlantic halibut	Norway (M&R)	2011	II	
NVI-8143	atypical	Atlantic cod	Norway (ST)	2011	III	
NVI-8158	atypical	Corkwing wrasse	Norway (S&F)	2011	VI	
NVI-8175*	atypical	Corkwing wrasse	Norway (S&F)	2011	VI	KP184546
NVI-8176	atypical	Corkwing wrasse	Norway (HL)	2011	VI	
NVI-8191	atypical	Atlantic halibut	Norway (M&R)	2011	II	
NVI-8195	atypical	Ballan wrasse	Norway (M&R)	2011	VI	
NVI-8198*	atypical	Lumpsucker	Norway (HL)	2011	VI	KP184547
NVI-8199*	atypical	Lumpsucker	Norway (HL)	2011	VI	KP184548
NVI-8200	atypical	Lumpsucker	Norway (HL)	2011	VI	
NVI-8201*	atypical	Lumpsucker	Norway (HL)	2011	VI	KP184549

NVI-8210	atypical	Atlantic halibut	Norway (M&R)	2011	II	
NVI-8218	atypical	Atlantic halibut	Norway (RL)	2011	III	
NVI-8231	atypical	Goldsinny wrasse	Norway (M&R)	2011	VI	
NVI-8232*(f.l.)	atypical	Goldsinny wrasse	Norway (M&R)	2011	VI	KP184550
NVI-8233	*salmonicida*	Atlantic salmon	Norway	1988 (uncertain)	I	
NVI-8289	atypical	Goldsinny wrasse	Norway (M&R)	2012	VI	
NVI-8291	atypical	Atlantic halibut	Norway (M&R)	2012	II	
NVI-8317	atypical	Lumpsucker	Faroe Islands	2012	III	
NVI-8337	atypical	Ballan wrasse	Norway (M&R)	2012	VI	
NVI-8345	unspecified (oxidase -)	Ballan wrasse	Norway (M&R)	2012	VI	
NVI-8346*	atypical	Goldsinny wrasse	Norway (M&R)	2012	VI	KP184551
NVI-8347	atypical	Goldsinny wrasse	Norway (M&R)	2012	VI	
NVI-8356*	atypical	Ballan wrasse	Norway (M&R)	2012	V	KP184552
NVI-8357	atypical	Ballan wrasse	Norway (M&R)	2012	V	
NVI-8419	atypical	Goldsinny wrasse	Norway (M&R)	2012	VI	
NVI-8450	atypical	Atlantic halibut	Norway (M&R)	2012	II	KP184553
NVI-8474	atypical	Atlantic halibut	Norway (M&R)	2012	II	
NVI-8546	atypical	Lumpsucker	Norway (NL)	2012	VI	
NVI-8552	atypical	Corkwing wrasse	Norway (VF)	2012	V	
NVI-8561	atypical	Ballan wrasse	Norway (M&R)	2012	VI	
NVI-8570	atypical	Wrasse	Norway (NL)	2012	V	
NVI-8575	atypical	Corkwing wrasse	Norway (M&R)	2012	VI	
NVI-8578	atypical	Ballan wrasse	Norway (ST)	2012	V	
NVI-8615	atypical	Atlantic halibut	Norway (M&R)	2012	II	
NVI-8635	atypical	Wrasse	Norway (M&R)	2012	VI	
NVI-8640	atypical	Corkwing wrasse	Norway (NL)	2012	V	
NVI-8771	atypical	Atlantic halibut	Norway (M&R)	2012	II	
NVI-8835	atypical	Atlantic halibut	Norway (M&R)	2013	II	
NVI-8838*	atypical	Corkwing wrasse	Norway (M&R)	2013	VI	KP184554
NVI-9122	atypical	Wrasse	Norway (ST)	2013	V	
NVI-9140	atypical	Lumpsucker	Norway (RL)	2013	VI	
NVI-9144	atypical	Wrasse	Norway (ST)	2013	V	
NVI-9150	atypical	Atlantic halibut	Norway (M&R)	2013	II	
NVI-9154	atypical	Corkwing wrasse	Norway (RL)	2013	VI	
NVI-9155	atypical	Lumpsucker	Norway (RL)	2013	VI	
NVI-9156	atypical	Cuckoo wrasse	Norway (RL)	2013	VI	
NVI-9216	atypical	Goldsinny wrasse	Norway (M&R)	2013	VI	
NVI-9250	atypical	Wrasse	Norway (ST)	2013	V	
NVI-9254	atypical	Atlantic halibut	Norway (M&R)	2013	II	
NVI-9270	atypical	Atlantic halibut	Norway (M&R)	2013	II	
NVI-9276	atypical	Goldsinny wrasse	Norway (NT)	2013	VI	
NVI-9277	atypical	Ballan wrasse	Norway (NL)	2013	V	
NVI-9278	atypical	Ballan wrasse	Norway (NL)	2013	VI	
NVI-9280	atypical	Goldsinny wrasse	Norway (ST)	2013	VI	
NVI-9303	atypical	Wrasse	Norway (ST)	2013	V	
NVI-9304	atypical	Ballan wrasse	Norway (ST)	2013	VI	
NVI-9307	atypical	Goldsinny wrasse	Norway (NL)	2013	VI	
NVI-9321*	atypical	Goldsinny wrasse	Norway (ST)	2013	VI	KP184555
NVI-9322	atypical	Goldsinny wrasse	Norway (ST)	2013	VI	
NVI-9324*	atypical	Goldsinny wrasse	Norway (ST)	2013	VI	KP184556
NVI-9338	atypical	Atlantic halibut	Norway (M&R)	2013	II	KP184557
NVI-9340	atypical	Goldsinny wrasse	Norway (ST)	2013	VI	
NVI-9346	atypical	Ballan wrasse	Norway (NL)	2013	V	
NVI-9355	atypical	Goldsinny wrasse	Norway (ST)	2013	VI	KP184558
NVI-9358*	atypical	Corkwing wrasse	Norway (ST)	2013	VI	KP184559

NVI-9371	atypical	Atlantic halibut	Norway (M&R)	2013	II	
NVI-9382	atypical	Goldsinny wrasse	Norway (ST)	2013	VI	
NVI-9416	atypical	Atlantic halibut	Norway (M&R)	2014	II	
NVI-9430(P.X.)	atypical	Atlantic salmon	China	2012 (uncertain)	VII	
NVI-9431(P.X.)	atypical	Atlantic salmon	China	2013 (uncertain)	VII	
NVI-9432(P.X.)	atypical	Atlantic salmon	China	2013 (uncertain)	VII	KP184560
NVI-9465	atypical	Lumpsucker	Norway (RL)	2014	VI	
NVI-9496	atypical	Corkwing wrasse	Norway (S&F)	2014	VI	
NVI-9498	atypical	Corkwing wrasse	Norway (RL)	2014	VI	
NVI-9507	atypical	Corkwing wrasse	Norway (HL)	2014	VI	
NVI-9508	atypical	Ballan wrasse	Norway (S&F)	2014	VI	
NVI-9521	atypical	European Hake	Norway (S&F)	2008	III	
NVI-9538	atypical	Corkwing wrasse	Norway (RL)	2014	VI	
NVI-9540	atypical	Ballan wrasse	Norway (RL)	2014	VI	
NVI-9543	atypical	Cuckoo wrasse	Norway (RL)	2014	VI	
NVI-9546	atypical	Corkwing wrasse	Norway (S&F)	2014	V	
NVI-9548	atypical	Ballan wrasse	Norway (S&F)	2014	V	
NVI-9583	atypical	Lumpsucker	Norway (RL)	2014	VI	
NVI-9599(K.T.)	atypical	Ballan wrasse	Scotland	2013 (uncertain)	V	
NVI-9600(K.T.)	atypical	Ballan wrasse	Scotland	2014 (uncertain)	V	
NVI-9601(K.T.)	atypical	Ballan wrasse	Scotland	2014 (uncertain)	V	
NVI-9628	atypical	Turbot	Norway (VA)	2014	II	
NVI-9631	atypical	Atlantic halibut	Norway (M&R)	2014	II	
NVI-9673	atypical	Ballan wrasse	Norway (ST)	2014	V	
NVI-9679	atypical	Ballan wrasse	Norway (M&R)	2014	V	
NVI-9685	atypical	Arctic char	Norway (NL)	2014	XIV	
NVI-9686	atypical	Lumpsucker	Norway (NL)	2014	II	
NVI-9687*	atypical	Wrasse	Norway (S&F)	2014	VI	KP184561
NVI-9710	atypical	Atlantic halibut	Norway (M&R)	2014	II	
NVI-9712	atypical	Goldsinny wrasse	Norway (M&R)	2014	VI	
NVI-9713	atypical	Wrasse	Norway (S&F)	2014	V	
NVI-9729	atypical	Arctic char	Norway (NL)	2014	XIV	
NVI-9744	atypical	Atlantic halibut	Norway (M&R)	2014	II	
NVI-9775	atypical	Ballan wrasse	Norway (NT)	2014	V	
NVI-9776	*salmonicida*	Atlantic salmon	Norway (NT)	2014	I	
NVI-9780	atypical	Wolffish	Norway (NL)	2014	IV	
L4010	*salmonicida* NCIMB1102 (T)	Atlantic salmon	UK	1963 (uncertain)	I	
L4012	*salmonicida*	Atlantic salmon	Scotland	prior to 1996	I	*AJ749882.1*
L4014	*salmonicida*	Atlantic salmon	Norway	1988 (uncertain)	I	
L4017	*salmonicida*	Atlantic salmon	Norway	1989 (uncertain)	I	*AJ749881.1*
L4043	*achromogenes*	Arctic char	Finland	1992 (uncertain)	IX	*AJ749879.1*
L4050	atypical	Atlantic halibut	Norway (VA)	1995	II	*AJ749892.1*
L4059	atypical	Spotted wolffish	Norway	1997 (uncertain)	IV	*AM937252*
L4065	atypical	Spotted wolffish	Norway	1998 (uncertain)	IV	*AJ749884.1*
L4067	atypical	Spotted wolffish	Norway	1998	IV	*AJ749885.1*
L4088	atypical	Spotted wolffish	Norway	1992 (uncertain)	IV	*AM937254.1*
L4092	atypical	Turbot	Norway	1988 (uncertain)	III	
L4099	atypical	Atlantic cod	Norway (HL)	1993 (uncertain)	III	*AJ749891.1*
L4101	*achromogenes*	Atlantic cod	Iceland	1999	III	*AM937255.1*
L4102	atypical CECT5200	Atlantic cod	Canada (east)	1981 (uncertain)	IV	*AJ749886.1*
L4109	*smithia* NCIMB13210 (T)	Common roach	UK	1988 (uncertain)	singleton	*AJ749880.1*
L4110	*masoucida* ATCC27013 (T)	Masu salmon	Japan	1969 (uncertain)	VII	*AJ749883.1*
L4111	*achromogenes* NCIMB1110 (T)	Brown trout	UK	1962 (uncertain)	III	*AJ749889.1*
L4115	atypical	Atlantic halibut	Norway (M&R)	1992 (uncertain)	III	
L4117	atypical	Turbot	Norway	1979 (uncertain)	IX	

L4121	atypical	Koi carp	Australia	1987 (uncertain)	X	
L4122	atypical	Flounder	Finland	prior to 1996	singleton	*AJ749893.1*
L4124	*achromogenes*	Whiting	Iceland	1992 (uncertain)	III	
L4125	atypical	Goldfish	USA	prior to 1996	X	
L4126	atypical	Lingcod	Canada (west)	1986 (uncertain)	VII	KP184562
L4128	atypical	Spotted wolffish	Iceland	2001 (uncertain)	III	*AJ749890.2*
L4129	atypical	Spotted wolffish	Norway	2001 (uncertain)	IV	*AJ749887.1*
L4137	atypical	Spotted wolffish	Norway	2003	III	*AM937253.1*
A449	*salmonicida*	Brown trout	France	1975 (uncertain)	I	*CP000644.1*
A450	*salmonicida*	Brown trout	France	1978 (uncertain)	I	*M64655.1*
TNH3-1	unspecified	Japanese anchovy	South-Korea	2012	VII	*KC907627.1*
RGM3-7	unspecified	Korean Rockfish	South-Korea	2012	VII	*KC907626.1*
SHS1	unspecified	Seabass	South-Korea	2011	VII	*KC907625.1*
TGM4-1	unspecified	Big-head croaker	South-Korea	2012	VII	*KC907624.1*
AsCh08	*salmonicida*	Chum salmon	South-Korea	2008	I	*GU734698.1*
SAS1	*salmonicida*	Chum salmon	South-Korea	2009	I	*AB521792.1*
RFAS2/3	unspecified	Rockfish	South-Korea	prior to 2009	VII	*AB514574.1*

NVI-prefix: Stock cultures maintained at -80°C at the Norwegian Veterinary Institute in Oslo
*: A-layer negative isolates with deleterious *vapA* mutations
f.l.: Near full-length *vapA* sequence submitted to GenBank (other submissions are of partial *vapA* sequences)
P.X.: Isolate provided by Dr. Peng Xiao, Institute of Oceanology, Chinese Academy of Sciences
K.T.: Isolate provided by Dr. Kim Thompson, Institute of Aquaculture, University of Stirling.
Norwegian county abbreviations used: Finnmark (FM), Troms (T), Nordland (NL), Nord-Trøndelag (NT), Sør-Trøndelag (ST), Møre og Romsdal (M&R), Sogn og Fjordane (S&F), Hordaland (HL), Rogaland (RL), Vest-Agder (VA), Aust-Agder (AA), Vest-Fold (VF), Buskerud (BR), Akershus (AH), Oslo (O) and Oppland (OL).
Accession numbers in *italic*: *vapA* sequences retrieved from GenBank

Phylogenetic analysis and serotyping of *Vibrio splendidus*-related bacteria isolated from salmon farm cleaner fish

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Abstract

Cleaner fish, i.e. various wrasse (Labridae) species and lumpsucker, *Cyclopterus lumpus*, are to an increasing extent used for biocontrol of the salmon louse, *Lepeophtheirus salmonis*, in European salmon farming. Although efficient de-licers, cleaner fish mortality levels in salmon farms are often high. Bacterial infections are common, and *Vibrio splendidus*-related strains are frequently identified during diagnostic investigations. The population structure of 112 *V. splendidus*-related isolates, derived primarily from wrasse spp., was investigated by means of Multilocus Sequence Analysis (MLSA) utilising five housekeeping genes (*rpoD*, *ftsZ*, *pyrH*, *rpoA* and *atpA*). Most isolates were found to be closely related to the *V. splendidus* type strain, yet displayed extensive genetic microdiversity. Slide agglutination testing using polyclonal rabbit antisera further indicated O-antigen variability. Intra-outbreak genetic- and antigenic diversity suggests direct infection from seawater, rather than fish-to-fish transmission, as the main route of infection. The variable nature of isolates involved complicates qualified selection of representative candidate strains, e.g. for infection- and vaccine trials.

Keywords: *Vibrio splendidus*; molecular characterisation; MLSA; serotyping; cleaner fish; salmon louse; biocontrol

Introduction

The use of cleaner fish has in recent years become increasingly popular in European salmon farming for bio-control of the salmon louse, *Lepeophtheirus salmonis*. In Norway, wild-caught goldsinny-, *Ctenolabrus rupestris*, ballan-, *Labrus bergylta*, and corkwing-, *Symphodus melops*, wrasse make up the bulk of cleaner fish used, with over fifteen million captured annually for this purpose (Norwegian Directorate of Fisheries/www.fiskeridir.no 2015). Both lumpsucker, *Cyclopterus lumpus*, and ballan wrasse are now being farmed to meet the growing demand. There are considerable health- and welfare problems related to the use of cleaner fish however, with high mortality levels amongst both wild-caught- and farmed fish following transfer to salmon cages. Over a period of six months one recent study registered 33% cumulative cleaner fish mortality, a figure which was almost certainly an underestimate (Nilsen et al. 2014). This leads to a significant turnover of cleaner fish in salmon farms.

The causes of cleaner fish losses are often unclear, but bacterial infections are amongst the most common diagnostic findings in Norway (Nilsen et al. 2014, Bornø & Lie Linaker 2015). Recognised bacterial fish-pathogens regularly found include atypical *Aeromonas salmonicida*, *Vibrio anguillarum*, *Vibrio ordalii*, *Pseudomonas anguilliseptica* and a recently described *Pasteurella* sp. (Poppe et al. 2012, Bornø & Lie Linaker 2015, Alarcón et al. 2015, Gulla et al. 2015), although the perhaps most frequently isolated bacteria from such investigations are *Vibrio splendidus*-related strains (Johansen 2013, Hjeltnes 2014, Bornø & Lie Linaker 2015).

As is well known, bacterial diseases depend not only on the virulence of the bacterium involved, but also on the environment and the immunological status of the host. In this regard, the pathogenic role (if any) of *V. splendidus* in relation to disease in wrasse and lumpsucker, remains poorly understood. Although some strains have previously been associated with disease in marine animals, including wrasse (Bergh & Samuelsen 2006), the term '*V.*

splendidus' is often (imprecisely) used with reference to a range of phenotypically (Thompson et al. 2004), genetically (Thompson et al. 2005) and antigenically (Wildschutte et al. 2010) diverse bacteria, commonly dominating the marine bacterioplankton (Le Roux & Austin 2006). In recent years, several *V. splendidus*-related taxa have been validly designated as distinct species, and this complex collection of strains/species has collectively, at the sub-genus level, been named the Splendidus-clade (Sawabe et al. 2013).

Sustainable culture of many fish species is dependent on the development and use of effective vaccines against bacterial diseases. It seems certain that comprehensive vaccination programs must also be developed for cleaner fish if the industry is to remain sustainable. In order to shed light on the population structure of *V. splendidus*-related bacteria from fish, and hopefully identify suitable candidate strains for eventual vaccine development, we subjected a representative collection of *V. splendidus*-related isolates from cleaner fish to Multilocus Sequence Analysis (MLSA). The scheme utilises five housekeeping genes previously used in phylogenetic studies of *Vibrio* spp. O-antigen variability was also assessed by slide agglutination testing with polyclonal antisera.

Materials and methods

Bacterial strains and culture

The studied isolates (112) were obtained primarily through the Norwegian Veterinary Institute's (NVI) diagnostic service between 2004 and 2014. A variety of host species were represented, although the majority of isolates were recovered from dead or moribund cleaner fish in Norwegian salmon farms, sampled during periods of increased mortality. Primary cultures were mainly obtained by sowing from aseptically exposed head kidneys onto 5% bovine blood agar with 2% NaCl (BA2%), followed by incubation at 15°C for up to one week. Phenotypic characterisation is often too indiscriminate/versatile in order to differentiate

V. splendidus-related species (Thompson et al. 2004). Unsurprisingly, therefore, investigated cleaner fish isolates differed to some extent from the *V. splendidus* biochemical profile offered by the Bergey's Manual (Garrity et al. 2005), and they were on this basis identified as *V. splendidus*-related (Table 1). The Splendidus-clade affiliation was confirmed for a random selection of isolates through partial 16S rRNA gene sequencing (see below). *V. splendidus* NCIMB1^T and fourteen *V. splendidus* reference strains were also acquired for investigation. Stock cultures (maintained at -80°C) were sub-cultured on BA2% at 15°C for 24-48 h prior to DNA extraction. MLSA sequence data for an additional nine strains was retrieved from GenBank. For details on all isolates/strains, see Table S1.

[Table 1]

DNA extraction, PCR and sequencing

Genomic DNA was obtained by boiling bacterial cells in dH_2O for 7 min, followed by centrifugation and use of the supernatant as PCR template. For some isolates, DNA was isolated using a QiaCube (Qiagen) according to the manufacturer's instructions. Partial sequences of the genes encoding RNA polymerase σ-factor (*rpoD*), cell division protein (*ftsZ*), urydilate kinase (*pyrH*), RNA polymerase α-subunit (*rpoA*) and α-subunit of bacterial ATP synthase (*atpA*) were amplified using primers specified in Table 2. Each PCR reaction volume consisted of 2.5 µl 10x ThermoPol Reaction Buffer (New England BioLabs), 0.2 mM dNTP (VWR), 0.4 µM of both forward- and reverse primers (Invitrogen), 1 unit *Taq* DNA polymerase (New England BioLabs), 3 µl (boil-extracted) or 1 µl (QiaCube-extracted) DNA template, and a final addition of Milli-Q water to reach a total reaction volume of 25 µl. PCR was conducted on a Dyad Dual 96-Well Thermal Cycler (MJ Research) under conditions specified in Table 2. From selected isolates (Table S1) approximately the first third of the 16S rRNA gene (442 bp) was obtained as described by Suau et al. (1999). Following visual confirmation of PCR products of the expected size by gel electrophoresis (1.5% agarose gel

with GelRed staining) and purification (ExoSAP-IT; Amersham Biosciences), products were sequenced using BigDye Terminator v3.1 (protocol: Platt et al. 2007) and an Avant 3130*xl* Genetic Analyzer (Applied Biosystems).

[Table 2]

Sequence analysis

Contigs were assembled and manually corrected (Geneious v7.1; Biomatters), and nucleotide sequences trimmed and (for housekeeping genes) adjusted to reading frame (MEGA6; Tamura et al. 2013) prior to alignment (ClustalX; Larkin et al. 2007). The five partial sequences (trimmed: *rpoD* = 573-576 bp; *ftsZ* = 480 bp; *pyrH* = 456 bp; *rpoA* = 492 bp; *atpA* = 639 bp) were examined individually and as a concatenated sequence (MLSA= 2640-2643 bp) in synteny with chromosome one of *V. tasmaniensis* LGP 32 (Acc. no. FM954972). Upon identification of identical concatenated sequence types in multiple isolates from individual clinical cases (presumably clones), only single representatives from each case were included for further analysis. Eventual selection pressures operating on the housekeeping genes were evaluated with the Codon-based Z-test in MEGA6. The scope was set to 'overall average', and an alternative hypothesis of purifying selection ($d_N < d_S$) was employed with otherwise default settings. Maximum Likelihood (ML) trees were constructed using PhyML v3.0 (Guindon et al. 2010) with default settings, except for the 'proportion of invariable sites' (changed to estimated) and 'number of substitution rate categories' (raised to 8), as previously recommended (Hall 2007). Branch support was evaluated with the approximate Likelihood Ratio Test (aLRT) (Anisimova & Gascuel 2006). Resulting ML-trees were subsequently edited in MEGA6. For each unique concatenated sequence type, the complete set of partial housekeeping gene sequences from a single candidate isolate was submitted to GenBank (Table S1; Acc. nos. KT026600 – KT026964).

Each unique housekeeping gene sequence was assigned an allele type number, and these numbers (one for each of the five separate loci) were used to assign each isolate with a concatenated sequence type number (not shown). These were used as input for eBURST analysis (Feil et al. 2004) with the minimum number of identical loci required for group definition relaxed to three.

In order to detect putative recombination events significantly influencing the phylogenetic signal, colour-coded concatenated- and single housekeeping gene trees were visually checked for conflicting clustering patterns.

Antisera production and serotyping

Two clinical *V. splendidus* isolates (NVI-6762 and NVI-7628 from Atlantic salmon, *Salmo salar*, and ballan wrasse, respectively) were cultured as previously described. Bacterial cells were harvested into 50 ml phosphate-buffered saline (PBS), supplemented with 350 µl 37% formaldehyde and refrigerated overnight. Following two wash cycles with PBS and 400 rpm centrifugation, pelleted cells were re-suspended in PBS and diluted to a McFarland standard of 3-4. Sterility was checked by sowing onto blood agar (2% NaCl) with one week's incubation at 15°C. Adult New Zealand white rabbits were immunised by sub-cutaneous injection (four injections, one week apart) of 0.5 ml antigen solution supplemented with Freund's incomplete adjuvant. Terminal bleeding was conducted two weeks after the final injection.

Slide agglutination testing (Sørensen & Larsen 1986) was conducted in order to assess O-antigen variability amongst selected isolates. Briefly, bacterial cells were suspended in formalin buffer and heat-inactivated by boiling for one hour. Following cooling to room temperature, 15 µl of the suspension was then mixed with 15 µl antiserum on glass slides and observed against a dark background for two minutes with gentle rocking. The agglutination

reaction of each tested isolate against each antiserum was categorised as strong (agglutination within 1 min), weak (agglutination after 1 min) or absent. Auto-agglutination was tested using naïve rabbit antiserum.

Results

Sequence analysis

Genetic variability in the housekeeping genes examined was dominated by synonymous substitutions and purifying selection pressures were identified for all five loci ($p < 0.01$). Average d_S - d_N values were 10.6 (*rpoD*), 6.6 (*ftsZ*), 9.7 (*pyrH*), 5.2 (*rpoA*) and 8.5 (*atpA*). The partial 16S rRNA gene tree showed no particular clustering pattern for the 24 isolates examined. In contrast, individual housekeeping gene trees (112 isolates) displayed more or less consistent topologies, revealing essentially one major- and several minor clusters, as well as one or more singletons (Figure S1).

Analysis of concatenated sequences reinforced the patterns observed in single gene analyses (Figure 1). The major cluster included the *V. splendidus* type strain, almost all of the assessed wrasse-isolates (59/63), and just under half of the lumpsucker-isolates (5/12). Isolates within and outside this 'type strain cluster' are therefore, for the purposes of the present study, referred to as *V. splendidus sensu stricto* and *V. splendidus*-related, respectively. Notably, several *V. splendidus* reference strains and GenBank accessions designated as *V. splendidus* fell outside the main cluster, and many of these showed high sequence identity with type strains of other validly published (Euzéby 1997, Parte 2014) *V. splendidus*-related species (Figure 1 and Table S1). These type strains could not be included in the concatenated analysis presented here however, as full sequence coverage for all five analysed gene regions could not be obtained from public sequence databases.

[Figure 1]

Pairwise sequence identities (PID) for concatenated housekeeping gene sequences for the material as a whole were ≥93.6%, which increased to ≥99.3% when the analysis was restricted to the fish-associated *V. splendidus sensu stricto* cluster (disregarding putative recombinants; see below). Both major- and minor clusters displayed considerable microdiversity, with 51 different concatenated sequence types identified amongst the 78 *V. splendidus sensu stricto* isolates (Figure 2). While only single isolates were examined from individual fish, intra-outbreak sequence variability was identified in the majority of diagnostic cases from which more than one isolate was sequenced (18/21; primarily cleaner fish cases), with as many as four different concatenated sequence types being identified from single investigations. No mutual founding genotype for the *V. splendidus sensu stricto* cluster could be identified using eBURST analysis (not shown), which depicted several minor clonal complexes and singletons.

[Figure 2]

PID-ranges for individual housekeeping genes and concatenated sequences revealed varying taxonomic resolutions both within the *V. splendidus sensu stricto* cluster (intra-cluster), and between this cluster and other *V. splendidus*-related taxa (inter-cluster). When disregarding putative recombinants (see below), intra-cluster PID ranges were ≥98.4% (*rpoD*), ≥98.9% (*ftsZ*), ≥98.5% (*pyrH*), ≥99.4% (*rpoA*), ≥99.2% (*atpA*) and ≥99.3% (MLSA), while inter-cluster PID ranges were 88.5-97.6% (*rpoD*), 96.0-99.4% (*ftsZ*), 91.9-96.3% (*pyrH*), 96.5-99.2% (*rpoA*), 92.5-99.7% (*atpA*) and 93.6-98.3% (MLSA). Only *pyrH*, *rpoD* and *rpoA* (and concatenated sequences) could thus alone distinguish *V. splendidus sensu stricto* from other *V. splendidus*-related taxa (Figure 3; non-overlapping intra- and inter-cluster bars). The discriminatory ability of *rpoD* and the concatenated sequences disappeared, however, following the inclusion of putative recombinants. Due to its multi-copy nature and allele

variability within individual strains, PID-ranges for the partial 16S rRNA gene sequences could not be properly assessed.

[Figure 3]

Comparison of colour coded single gene trees clearly revealed instances of conflicting clustering in the *rpoD*, *atpA* and *ftsZ* trees (Figure 4), presumably due to recombination. Such events in *rpoD* (and to some degree *ftsZ*), alone significantly affected concatenated tree topology. This was in part due to the relatively large contribution of *rpoD* to the combined genetic diversity (Figure 3), and partly to large distances between donor- and recipient clusters of the involved sequences (*ftsZ* and *rpoD*; Figure 4).

[Figure 4]

Slide agglutination

Polyclonal rabbit antisera raised against two genetically distinct *V. splendidus sensu stricto* isolates, yielded positive agglutination (strong or weak) in only around half of the tested candidates in the *V. splendidus sensu stricto* cluster (Figure 2). The majority of isolates displaying positive agglutination reacted with anti-NVI-6762 serum, while few isolates reacted with anti-NVI-7628 serum. No isolates reacted exclusively with anti-NVI-7628 serum. Curiously, anti-NVI-6762 serum yielded only weak/delayed agglutination with the autologous isolate despite several repeated attempts with fresh cultures. No auto-agglutination was observed.

Discussion

In the present study, sequencing of core housekeeping genes (*rpoD*, *ftsZ*, *pyrH*, *rpoA* and *atpA*) was used to investigate the phylogenetic population structure of 112 *V. splendidus*-related isolates, primarily cultured in relation to increased cleaner fish (wrasse and

lumpsucker) mortality in Norwegian salmon farms. The majority of wrasse-isolates proved to be very closely related to *V. splendidus* NCIMB1^T (≥99.3% PID), and presumably represent true examples of this species (i.e. *V. splendidus sensu stricto*). There was however a considerable degree of genetic microdiversity amongst such isolates, often even within individual clinical investigations, and O-antigen variations were also detected. No two lumpsucker-isolates displayed identical concatenated sequence types, and a lesser proportion of these isolates clustered close to the *V. splendidus* type strain.

All five housekeeping genes investigated have previously been extensively used for phylogenetic analysis of vibrios (e.g. Thompson et al. 2007, Pascual et al. 2010, Sawabe et al. 2013). In the present study they were all shown to be under purifying selection ($p < 0.01$), corroborating their suitability for use in phylogenetic studies (Stackebrandt et al. 2002). The taxonomic resolution (PID-ranges) of individual genes varied (Figure 3), and putative recombination events identified in three of the genes (Figure 4) affected PID-values. Nevertheless, the MLSA seemed to efficiently resolve and depict the overall phylogeny of our material (Figure 1). Unsurprisingly, analysis of a variable region of the 16S rRNA gene from selected isolates was unable to sensibly resolve phylogeny amongst these closely related bacteria at any level (Figure S1). This method can therefore only be used to identify isolates as members of the Splendidus-clade, pending further investigation. The applicability of this multi-copy gene for subtyping of *V. splendidus*-related bacteria is further complicated by the fact that intra-cell allele heterogeneity may surpass that observed between distinct strains (Le Roux et al. 2004, Jensen et al. 2009).

Isolates in most minor clusters/singletons in the concatenated five-gene tree showed high sequence identity with type strains of distinct *V. splendidus*-related species (e.g. *V. chagasii*, *V. celticus* and *V. cyclitrophicus*; Figure 1), and most probably belong to these species. Genetic microdiversity was however observed within all clusters, including the major *V.*

splendidus sensu stricto cluster (Figure 2). *V. splendidus*-related taxa are extremely common members of the marine bacterioplankton, and Thompson et al. (2005) estimated >1000 distinct *V. splendidus*-related genotypes to be present within a geographically restricted area. Unsurprisingly, therefore, considering the limited number of isolates examined in the present study, no mutual founding genotype for the major cluster could be identified using eBURST (not shown).

Slide agglutination using polyclonal antisera raised against two *V. splendidus sensu stricto* isolates from ballan wrasse and Atlantic salmon, respectively, and with a relatively low PID (99.4%), yielded ambiguous results (Figure 2). While elucidation of the antigenic relationships was undoubtedly limited by the low number of sera used, the results do show that considerable O-antigen diversity (which apparently cannot be linked to MLSA tree topology) exists within this genetically compact, and presumably conspecific, cluster. This is consistent with the findings of Wildschutte et al. (2010) amongst environmental and animal-associated *V. splendidus*-related isolates with up to 100% MLSA identity.

Interestingly, both genetic- and antigenic (micro)diversity was often (18/21 cases) also observed amongst *V. splendidus sensu stricto* isolates cultured from different fish specimens during individual episodes of increased mortality (Figure 2; dashed curves). Similar findings have previously been reported following investigation of *V. splendidus*-related losses in molluscs (Gay et al. 2004). The apparent lack of clonal expansion within cleaner fish mortality episodes suggests that fish-to-fish transmission is perhaps not the main route of infection during clinical outbreaks, and direct infection from seawater may be more prevalent.

Nevertheless, most wrasse-isolates fell into a single cluster (*V. splendidus sensu stricto*; Figure 1), representing only one sub-taxon within the comprehensive group of environmental *V. splendidus*-related bacteria (Le Roux & Austin 2006). While this may reflect an intrinsic

predilection for these fish, perhaps based on pathogenicity, *V. splendidus* infection trials on wrasse have produced ambiguous results (Bergh & Samuelsen 2006, Vågnes; unpublished data). Moreover, *V. splendidus*-related strains have also been identified as natural members of the intestinal flora in healthy wrasse larvae (Birkbeck & Treasurer 2014).

In addition to virulence, infection and eventual disease progression will, for all infective agents, depend also upon the health- and immune-status of the host. Capture, storage, transport and salmon-cage stocking of wild wrasse undoubtedly entails a range of physical and mental stressors, and although farmed cleaner fish are bred in captivity, transfer to the alien environment of the salmon cages will represent a stressful transition. This presumably increases the susceptibility of these fish to infectious disease. The ability of *V. splendidus*-related bacteria to cause disease in cleaner fish, and the extent of an eventual outbreak, may thus result from a complex interplay between bacterioplankton composition, host predilection and immunocompetence.

As most of the isolates examined in the present study originated from wrasse in Norwegian waters, the occurrence of one dominating cluster (*V. splendidus sensu stricto*) could conceivably also be explained by geographical sampling bias. This seems unlikely however, as Massachusetts seawater-isolates were present in most clusters, and isolates from Norwegian lumpsucker primarily belonged to minor clusters or appeared as singletons (Figure 1).

In summary, the five-gene MLSA used in the present study identified a relatively high degree of microdiversity within distinct *V. splendidus*-related taxa. This was most evident amongst the numerous *V. splendidus sensu stricto* isolates primarily cultured in relation to episodes of increased wrasse (cleaner fish) mortality in Norwegian salmon farms, which also displayed O-antigen dissimilarities. The lack of dominance by one or a few virulent clones amongst

infected specimens, even within individual ‘outbreaks’, indicates that direct infection from seawater may be more prevalent than fish-to-fish transmission. Furthermore, mechanisms underlying *V. splendidus* infection and eventual disease progression in wrasse are probably complex, and may depend both upon the strain in question and not least on the health-status of the fish (i.e. through opportunism). While vaccination against such infection may conceivably help limit *V. splendidus*-related losses, the variable nature of these bacteria complicates selection of representative candidate strains for infection trials and vaccine development.

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References

Alarcón M, Gulla S, Røsaeg M V, Rønneseth A, Wergeland H, Poppe TT, Nilsen H, Colquhoun DJ (2015) Pasteurellosis in lumpsucker *Cyclopterus lumpus*, farmed in Norway. J Fish Dis:DOI: 10.1111/jfd.12366

Anisimova M, Gascuel O (2006) Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst Biol 55:539–552

Bergh Ø, Samuelsen OB (2006) Susceptibility of corkwing wrasse *Symphodus melops*, goldsinny wrasse *Ctenolabrus rupestis*, and Atlantic salmon *Salmo salar* smolt, to experimental challenge with *Vibrio tapetis* and *Vibrio splendidus* isolated from corkwing wrasse. Aquac Int 15:11–18

Birkbeck TH, Treasurer JW (2014) *Vibrio splendidus*, *Vibrio ichthyoenteri* and *Vibrio pacinii* isolated from the digestive tract microflora of larval ballan wrasse, *Labrus bergylta* Ascanius, and goldsinny wrasse, *Ctenolabrus rupestris* (L.). J Fish Dis 37:69–74

Bornø G, Lie Linaker M (editors) (2015) Fiskehelserapporten 2014 (Norwegian Farmed Fish Health Report 2014; in Norwegian). Norwegian Veterinary Institute, Harstad, ISSN: 1893-1480

Euzéby JP (1997) List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. Int J Syst Bacteriol 47:590–592

Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG (2004) eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. J Bacteriol 186:1518–1530

Garrity G, Brenner DJ, Krieg NR, Staley JR (2005) Bergey's Manual® of Systematic Bacteriology: Volume 2: The Proteobacteria, Part B: The Gammaproteobacteria. Springer Science & Business Media, ISBN: 978-0-387-28022-6

Gay M, Berthe FCJ, Roux F Le (2004) Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. Dis Aquat Organ 59:49–56

Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–21

Gulla S, Lund V, Kristoffersen AB, Sørum H, Colquhoun DJ (2015) *vapA* (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes. J Fish Dis:DOI: 10.1111/jfd.12367

Hall BG (2007) Phylogenetic Trees Made Easy: A How-To Manual, 3rd edn. Sinauer Associates, Inc., ISBN: 978-0-87893-310-5

Hjeltnes B (editor) (2014) Fiskehelserapporten 2013 (Norwegian Farmed Fish Health Report 2013; in Norwegian). Norwegian Veterinary Institute, Oslo, ISSN: 1893-1480

Jensen S, Frost P, Torsvik VL (2009) The nonrandom microheterogeneity of 16S rRNA genes in *Vibrio splendidus* may reflect adaptation to versatile lifestyles. FEMS Microbiol Lett 294:207–15

Johansen R (editor) (2013) Fiskehelserapporten 2012 (Norwegian Farmed Fish Health Report 2012; in Norwegian). Norwegian Veterinary Institute, Oslo, ISSN: 1893-1480

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–8

Martens M, Delaere M, Coopman R, Vos P De, Gillis M, Willems A (2007) Multilocus sequence analysis of *Ensifer* and related taxa. Int J Syst Evol Microbiol 57:489–503

Nilsen A, Viljugrein H, Røsæg MV, Colquhoun D (2014) Rensefiskhelse – kartlegging av dødelighet og dødelighetsårsaker (Cleaner fish health - a survey of mortalities and causes of death; in Norwegian). The Norwegian Veterinary Institute's Report Seies 12-2014, ISSN: 1890-3290

Norwegian Directorate of Fisheries/www.fiskeridir.no (2015) Norwegian Aquaculture Statistics.

Parte AC (2014) LPSN - List of prokaryotic names with standing in nomenclature. Nucleic Acids Res 42

Pascual J, Macián MC, Arahal DR, Garay E, Pujalte MJ (2010) Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, *recA*, *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* genes. Int J Syst Evol Microbiol 60:154–65

Platt AR, Woodhall RW, George AL (2007) Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol. Biotechniques 43:58–62

Poppe TT, Taksdal T, Skjelstad H, Svieland C, Vågnes Ø, Colquhoun DJ (2012) Nye arter – nye diagnostiske utfordringer (New species – New diagnostic challenges; in Norwegian). Nor Veterinærtidsskrift 124:19–21

Roux F Le, Austin B (2006) The Biology of Vibrios (Chapter 21: *Vibrio splendidus*) (FL Thompson, B Austin, and J Swings, Eds.). American Society of Microbiology, ISBN: 978-1555813659

Roux F Le, Gay M, Lambert C, Nicolas JL, Gouy M, Berthe F (2004) Phylogenetic study and identification of *Vibrio splendidus*-related strains based on *gyrB* gene sequences. Dis Aquat Organ 58:143–50

Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AR, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T (2013) Updating the Vibrio clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. Front Microbiol 4:414

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kämpfer P, Maiden MCJ, Nesme X, Rosselló-Mora R, Swings J, Trüper HG, Vauterin L, Ward AC, Whitman WB (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52:1043–7

Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 65:4799–4807

Sørensen UBS, Larsen JL (1986) Serotyping of *Vibrio anguillarum*. Appl Environ Microbiol 51:593–597

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–9

Thompson FL, Iida T, Swings J (2004) Biodiversity of vibrios. Microbiol Mol Biol Rev 68:403–431

Thompson JR, Pacocha S, Pharino C, Klepac-Ceraj V, Hunt DE, Benoit J, Sarma-Rupavtarm R, Distel DL, Polz MF (2005) Genotypic diversity within a natural coastal bacterioplankton population. Science 307:1311–1313

Thompson CC, Thompson FL, Vicente ACP, Swings J (2007) Phylogenetic analysis of vibrios and related species by means of *atpA* gene sequences. Int J Syst Evol Microbiol 57:2480–4

Wildschutte H, Preheim SP, Hernandez Y, Polz MF (2010) O-antigen diversity and lateral transfer of the *wbe* region among *Vibrio splendidus* isolates. Environ Microbiol 12:2977–2987

Tables

Table 1: The typical phenotypic profile of *V. splendidus*-related isolates subjected to biochemical assessment in the present study. This profile differs with regards to some parameters from the *V. splendidus* biochemical profile offered by the Bergey's Manual (Garrity et al. 2005).

Culture conditions: Grows on 5% bovine blood agar with 2% NaCl within two days when incubated at 15°C.													
General morphology: 1-4 mm grey/beige, opaque, usually β-haemolytic, colonies, often with production of green diffusible pigment and a strong characteristic smell.													
Microscopy: Motile rods, often curved or pleomorphic. Gram negative following gram staining.													
Biochemical tests with typical reaction, and the proportion of isolates displaying such reaction, indicated													
O/F	ox.	V. O/129	ALO	al.	ge.	in.	ar.	ce.	la.	mt.	ms.	su.	tr.
+/+ 100%	+ 100%	sensitive* 97%	-/-/- 84%	+ 90%	+ 95%	+ 94%	- 100%	+ 97%	- 100%	+ 99%	+ 99%	- 76%	+ 100%

Abbreviations: O/F (aerobic/anaerobic glucose fermentation); ox. (cytochrome c oxidase production); V. O/129 (Vibriostat O/129 sensitivity); ALO (arginine, lysine and/or ornithine metabolism); production of al. (alginase), ge. (gelatinase), in. (indole); production of acid from ar. (arabinose), ce. (cellobiose), la. (lactose), mt. (mannitol), ms. (mannose), su. (sucrose), tr. (trehalose).

*: Several isolates designated Vibriostat O/129 sensitive here displayed small inhibitory zones and/or colonies appearing within the zones.

Table 2: Primers designed and used for PCR and sequencing of partial housekeeping genes, with product sizes and PCR thermal cycle conditions listed.

Gene	**Primer orient.**	**Primer sequence (5'- 3')**	**Prod. size (before/after trim)**	**PCR (initial denat.; #cycles [denat.; anneal.; extent.]; final extent.; end)**
rpoD	forward	TATTGCGAAACGCATTGAAG	744-747/ 573-576 bp.	95°C/3 min; 30x [95°C/1 min; 51°C/1 min; 68°C/1 min]; 68°C/4 min; 4°C/∞
	reverse	CCGATAGACATWCGACGGCT		
ftsZ	forward	CAGYGTRATYCAAATTGGTGG	522/480 bp.	95°C/3 min; 30x [95°C/1 min; 53°C/1 min; 68°C/1 min]; 68°C/4 min; 4°C/∞
	reverse	ATRCSGCTRCCCATCATTGC		
pyrH	forward	GAAGCDCTWCAAGGYGAWGAAG	502/456 bp.	
	reverse	TATCATACARCTCTGCRTCWG		
rpoA	forward	GTAACTCTTGAGCCATTAGAGC	545/492 bp.	
	reverse	TCCATATCGATAACAAGCTTGTC		
atpA	forward	GTTGGTGCTGTTGTAATGGGC	683/639 bp.	
	reverse	AGTAGACGAGAGTGAAGGTAG		

Figures

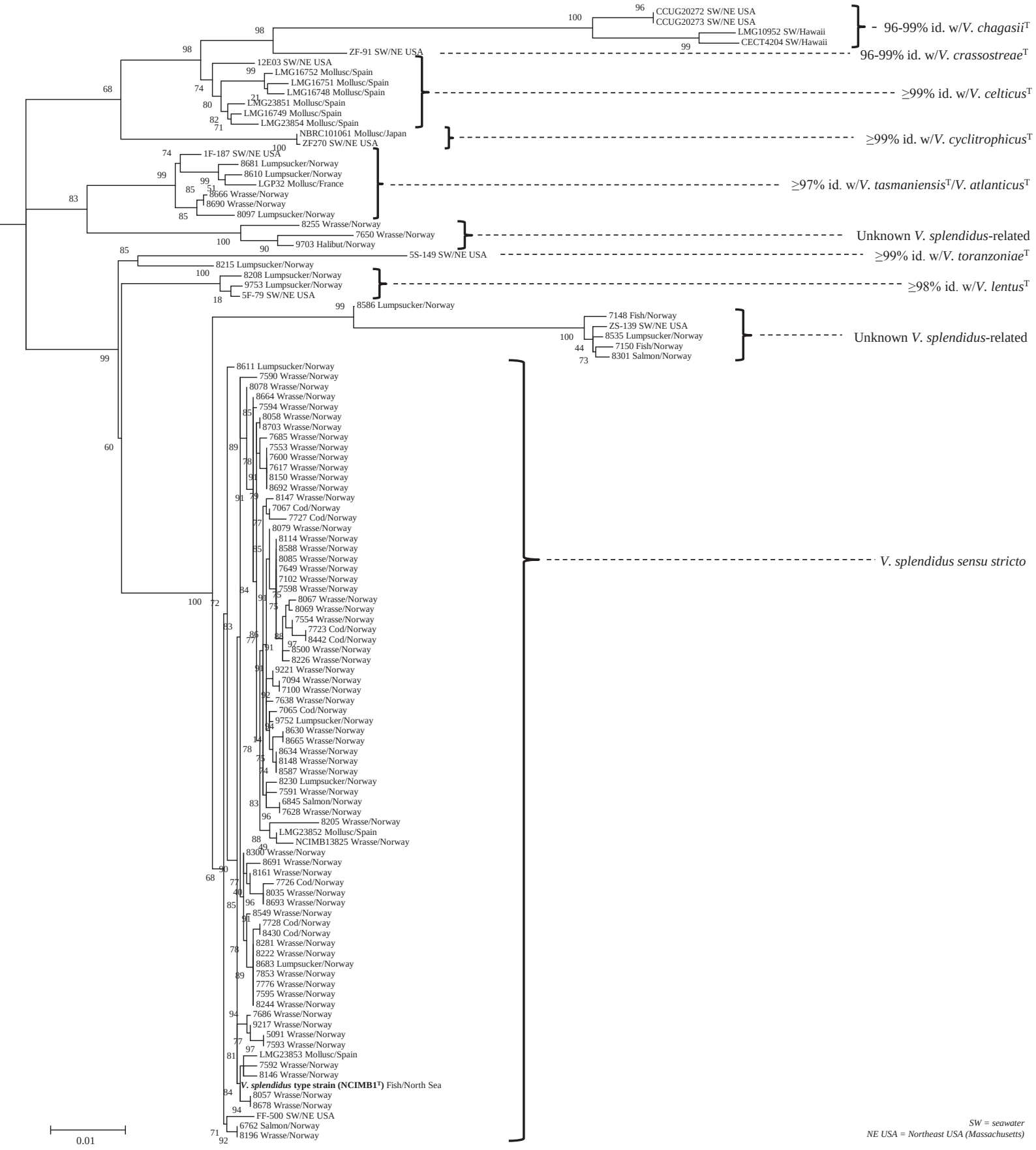

Figure 1: Maximum Likelihood tree based on concatenated housekeeping gene sequences (*rpoD*, *ftsZ*, *pyrH*, *rpoA* and *atpA*) from *V. splendidus*-related isolates, with aLRT branch support values shown. Isolate designations are followed by isolation source (host/geography). The tree was rooted towards the *V. tapetis* type strain (not shown). In addition to the *V. splendidus sensu stricto* cluster, several minor clusters and singletons are labelled according to their putative affiliation with other validly published *V. splendidus*-related species. Accompanying identity percentages represent partial housekeeping gene sequence similarities (following BLAST searches) between the respective clusters and type strains. In no cases could full coverages be obtained for all five genes, which is why the respective type strains were not included for concatenated analysis.

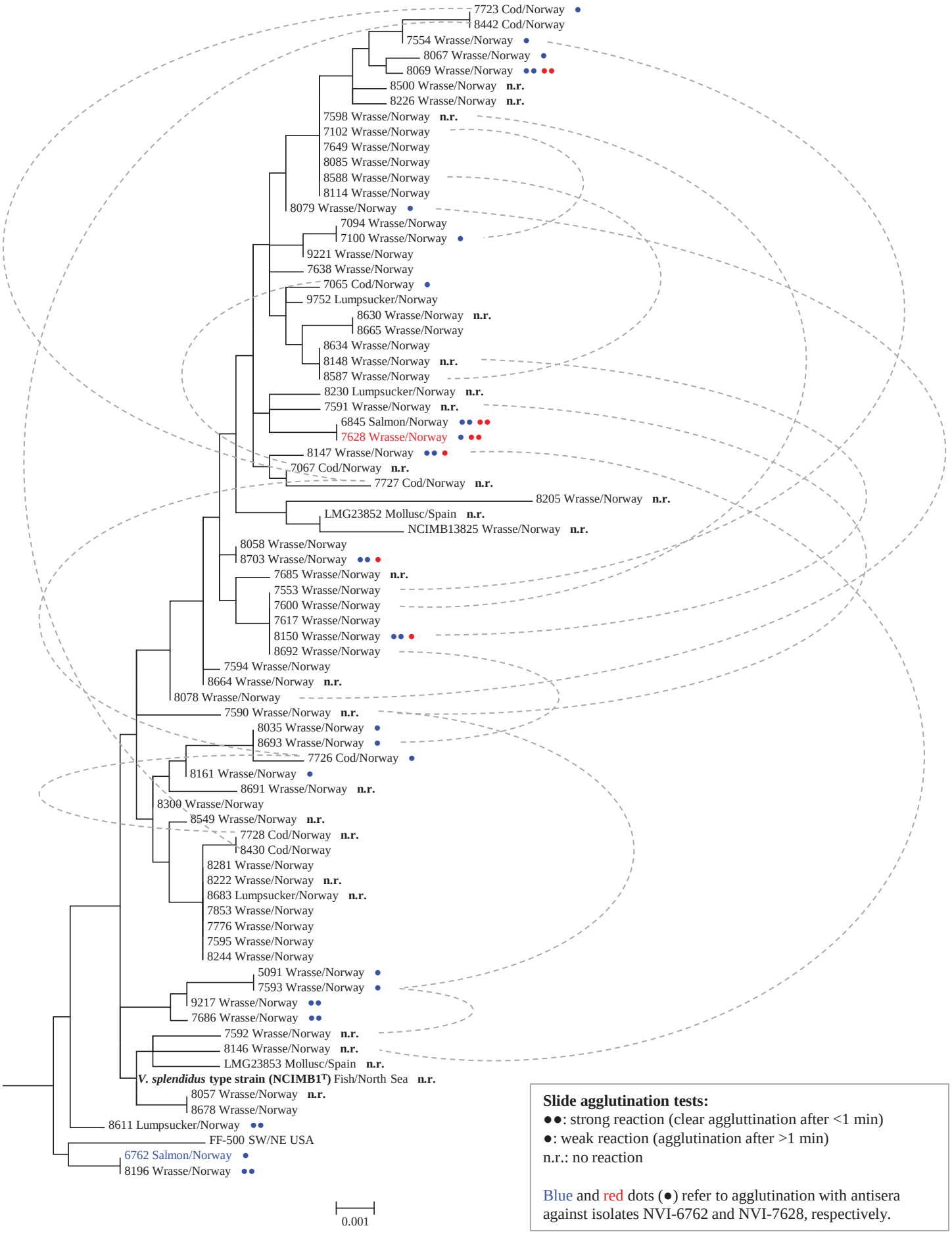

Figure 2: Magnification of the *V. splendidus sensu stricto* cluster from Figure 1, visualising the high degree of genetic microdiversity within this cluster (78 isolates; 51 concatenated sequence types). Selected isolates that were serotyped are followed by an indication of their agglutination reaction (or lack of) towards two polyclonal antisera (see legend). Dashed curves link together isolates cultured from the same clinical case (such relations involving isolates in other clusters/singletons, e.g. from lumpsucker, are not shown).

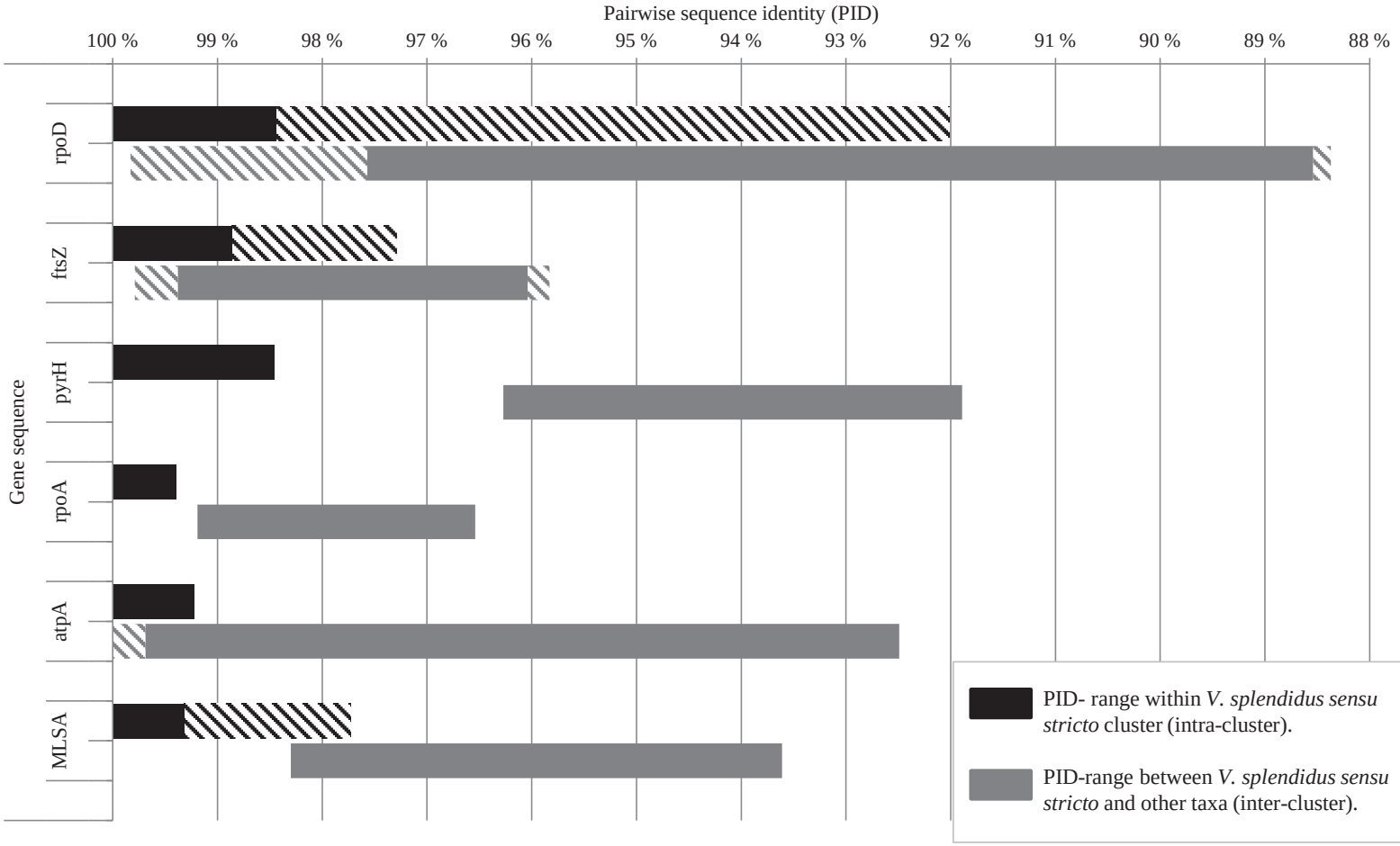

Figure 3: Intra-cluster (amongst *V. splendidus sensu stricto* isolates; black bars) and inter-cluster (*V. splendidus sensu stricto* vs. *V. splendidus*-related; grey bars) taxonomic resolution of individual genes and concatenated sequences, as determined by PID-ranges. Upper and lower bar-values represent, respectively, maximum and minimum PIDs. Partial diagonal striping in lateral bar-segments represent proportions of the observed PID-ranges that can be attributed to putative recombinational events (see text and Figure 4). The presentation form was modified after Martens et al. (2007).

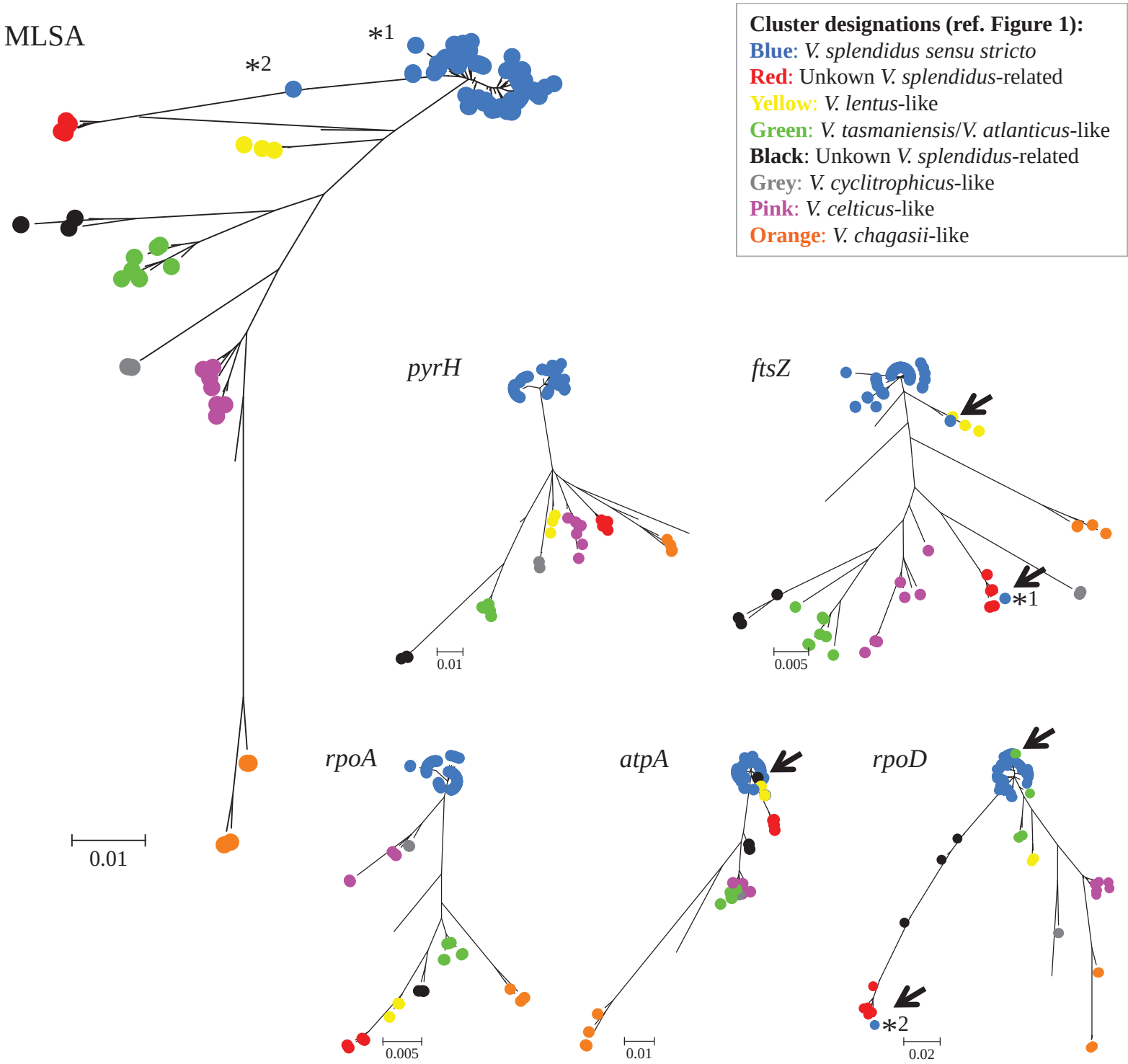

Figure 4: Concatenated- and single housekeeping gene radial trees (unrooted) aimed at highlighting isolates with conflicting clustering across loci due to putative recombination. Non-singleton isolates have been coloured consistently according to clustering in the concatenated tree in Figure 1 (see legend). Although asterisked isolates (*[1]: NVI-8205 and *[2]: NVI-8586) on average displayed relatively low concatenated PID in relation to other isolates in the *V. splendidus sensu stricto* cluster, they have been included as only one of five genes fell outside this cluster. These incidents, and some other clear examples of discrepancies between genes (putative recombinants), are indicated by arrows in the individual housekeeping gene trees.

Supporting information

Table S1: Detailed information on isolates/strains assessed in the present study.

Strain/ isolate id.	Splendidus-clade type strain with the highest housekeeping gene sequence identity	Biological origin	Geographical origin	Evt. accession no. for gene sequence data					
				rpoD	***ftsZ***	***pyrH***	***rpoA***	***atpA***	**16S rRNA**
12E03	*V. celticus*	Seawater	Northeast USA	*AJZD01000366*	*AJZD01000228*	*AJZD01000150*	*AJZD01000310*	*AJZD01000312*	
1F-187	*V. tasmaniensis/V. atlanticus*	Seawater	Northeast USA	*AJZM01000228*	*AJZM01000228*	*AJZM01000071*	*AJZM01000039*	*AJZM01000041*	
5F-79	*V. lentus*	Seawater	Northeast USA	*AJZP01000232*	*AJZP01000232*	*AJZP01000265*	*AJZP01000014*	*AJZP01000303*	
5S-149	*V. toranzoniae*	Seawater	Northeast USA	*AJYX01000052*	*AJYX01000052*	*AJYX01000055*	*AJYX01000006*	*AJYX01000033*	
CCUG20272	*V. chagasii*	Seawater	Northeast USA	KT026600	KT026673	KT026746	KT026819	KT026892	
CCUG20273	*V. chagasii*	Seawater	Northeast USA						
CECT4204	*V. chagasii*	Seawater	Hawaii	*AFWG01000034*	*AFWG01000034*	*AFWG01000013*	*AFWG01000014*	*AFWG01000018*	*AB038030*
NCIMB1(T)	*V. splendidus*	Marine fish	North Sea	*AY751355*	*DQ481635*	*EU118241*	*AJ842725*	*EF601244*	*X74724*
FF-500	*V. splendidus*	Seawater	Northeast USA	*AJZH01000147*	*AJZH01000147*	*AJZH01000120*	*AJZH01000106*	*AJZH01000022*	
LGP32	*V. tasmaniensis/V. atlanticus*	Oyster	France	*FM954972*	*FM954972*	*FM954972*	*FM954972*	*FM954972*	*FM954972*
LMG10952	*V. chagasii*	Seawater	Hawaii	KT026601	KT026674	KT026747	KT026820	KT026893	
LMG16748	*V. celticus*	Oyster	Spain	KT026602	KT026675	KT026748	KT026821	KT026894	
LMG16749	*V. celticus*	Oyster	Spain	KT026603	KT026676	KT026749	KT026822	KT026895	
LMG16751	*V. celticus*	Oyster	Spain	KT026604	KT026677	KT026750	KT026823	KT026896	
LMG16752	*V. celticus*	Oyster	Spain	KT026605	KT026678	KT026751	KT026824	KT026897	
LMG23851	*V. celticus*	Manila clam	Spain	KT026606	KT026679	KT026752	KT026825	KT026898	
LMG23852	*V. splendidus*	Grooved carpet shell	Spain	KT026607	KT026680	KT026753	KT026826	KT026899	
LMG23853	*V. splendidus*	Manila clam	Spain	KT026608	KT026681	KT026754	KT026827	KT026900	
LMG23854	*V. celticus*	Grooved carpet shell	Spain	KT026609	KT026682	KT026755	KT026828	KT026901	
NBRC101061	*V. cyclitrophicus*	Limpet	Japan	KT026610	KT026683	KT026756	KT026829	KT026902	*AB681361*
NCIMB13825	*V. splendidus*	Corkwing wrasse	Norway	KT026611	KT026684	KT026757	KT026830	KT026903	*AY129277*
NVI-5091	*V. splendidus*	Goldsinny wrasse	Norway	KT026612	KT026685	KT026758	KT026831	KT026904	
NVI-6762	*V. splendidus*	Atlantic salmon	Norway	KT026613	KT026686	KT026759	KT026832	KT026905	KT036375
NVI-6845	*V. splendidus*	Atlantic salmon	Norway						
NVI-7065	*V. splendidus*	Atlantic cod	Norway	KT026614	KT026687	KT026760	KT026833	KT026906	KT036376
NVI-7067	*V. splendidus*	Atlantic cod	Norway	KT026615	KT026688	KT026761	KT026834	KT026907	KT036377
NVI-7094	*V. splendidus*	Wrasse	Norway	KT026616	KT026689	KT026762	KT026835	KT026908	
NVI-7100	*V. splendidus*	Corkwing wrasse	Norway						
NVI-7102	*V. splendidus*	Corkwing wrasse	Norway						
NVI-7148	Unknown	Food-fish	Norway	KT026617	KT026690	KT026763	KT026836	KT026909	KT036378
NVI-7150	Unknown	Food-fish	Norway	KT026618	KT026691	KT026764	KT026837	KT026910	KT036379
NVI-7553	*V. splendidus*	Corkwing wrasse	Norway						
NVI-7554	*V. splendidus*	Corkwing wrasse	Norway	KT026619	KT026692	KT026765	KT026838	KT026911	
NVI-7590	*V. splendidus*	Corkwing wrasse	Norway	KT026620	KT026693	KT026766	KT026839	KT026912	
NVI-7591	*V. splendidus*	Corkwing wrasse	Norway	KT026621	KT026694	KT026767	KT026840	KT026913	
NVI-7592	*V. splendidus*	Corkwing wrasse	Norway	KT026622	KT026695	KT026768	KT026841	KT026914	

NVI-7593	*V. splendidus*	Corkwing wrasse	Norway						
NVI-7594	*V. splendidus*	Goldsinny wrasse	Norway	KT026623	KT026696	KT026769	KT026842	KT026915	
NVI-7595	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-7598	*V. splendidus*	Corkwing wrasse	Norway						
NVI-7600	*V. splendidus*	Corkwing wrasse	Norway						
NVI-7617	*V. splendidus*	Ballan Wrasse	Norway						
NVI-7628	*V. splendidus*	Ballan Wrasse	Norway	KT026624	KT026697	KT026770	KT026843	KT026916	
NVI-7638	*V. splendidus*	Ballan Wrasse	Norway	KT026625	KT026698	KT026771	KT026844	KT026917	KT036380
NVI-7649	*V. splendidus*	Goldsinny wrasse	Norway	KT026626	KT026699	KT026772	KT026845	KT026918	KT036381
NVI-7650	Unknown	Goldsinny wrasse	Norway	KT026627	KT026700	KT026773	KT026846	KT026919	KT036382
NVI-7685	*V. splendidus*	Ballan Wrasse	Norway	KT026628	KT026701	KT026774	KT026847	KT026920	
NVI-7686	*V. splendidus*	Corkwing wrasse	Norway	KT026629	KT026702	KT026775	KT026848	KT026921	
NVI-7723	*V. splendidus*	Atlantic cod	Norway						
NVI-7726	*V. splendidus*	Atlantic cod	Norway	KT026630	KT026703	KT026776	KT026849	KT026922	
NVI-7727	*V. splendidus*	Atlantic cod	Norway	KT026631	KT026704	KT026777	KT026850	KT026923	
NVI-7728	*V. splendidus*	Atlantic cod	Norway						
NVI-7776	*V. splendidus*	Goldsinny wrasse	Norway	KT026632	KT026705	KT026778	KT026851	KT026924	KT036383
NVI-7853	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-8035	*V. splendidus*	Corkwing wrasse	Norway	KT026633	KT026706	KT026779	KT026852	KT026925	
NVI-8057	*V. splendidus*	Ballan Wrasse	Norway	KT026634	KT026707	KT026780	KT026853	KT026926	
NVI-8058	*V. splendidus*	Corkwing wrasse	Norway	KT026635	KT026708	KT026781	KT026854	KT026927	KT036384
NVI-8067	*V. splendidus*	Wrasse	Norway	KT026636	KT026709	KT026782	KT026855	KT026928	KT036385
NVI-8069	*V. splendidus*	Wrasse	Norway	KT026637	KT026710	KT026783	KT026856	KT026929	
NVI-8078	*V. splendidus*	Corkwing wrasse	Norway	KT026638	KT026711	KT026784	KT026857	KT026930	
NVI-8079	*V. splendidus*	Corkwing wrasse	Norway	KT026639	KT026712	KT026785	KT026858	KT026931	
NVI-8085	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-8097	*V. tasmaniensis/V. atlanticus*	Lumpsucker	Norway	KT026640	KT026713	KT026786	KT026859	KT026932	KT036386
NVI-8114	*V. splendidus*	Wrasse	Norway						
NVI-8146	*V. splendidus*	Ballan Wrasse	Norway	KT026641	KT026714	KT026787	KT026860	KT026933	
NVI-8147	*V. splendidus*	Ballan Wrasse	Norway	KT026642	KT026715	KT026788	KT026861	KT026934	
NVI-8148	*V. splendidus*	Corkwing wrasse	Norway	KT026643	KT026716	KT026789	KT026862	KT026935	KT036387
NVI-8150	*V. splendidus*	Corkwing wrasse	Norway	KT026644	KT026717	KT026790	KT026863	KT026936	
NVI-8161	*V. splendidus*	Corkwing wrasse	Norway	KT026645	KT026718	KT026791	KT026864	KT026937	KT036388
NVI-8196	*V. splendidus*	Ballan Wrasse	Norway						
NVI-8205	*V. splendidus*	Goldsinny wrasse	Norway	KT026646	KT026719	KT026792	KT026865	KT026938	
NVI-8208	*V. lentus*	Lumpsucker	Norway	KT026647	KT026720	KT026793	KT026866	KT026939	
NVI-8215	Unknown	Lumpsucker	Norway	KT026648	KT026721	KT026794	KT026867	KT026940	KT036389
NVI-8222	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-8226	*V. splendidus*	Ballan Wrasse	Norway	KT026649	KT026722	KT026795	KT026868	KT026941	KT036390
NVI-8230	*V. splendidus*	Lumpsucker	Norway	KT026650	KT026723	KT026796	KT026869	KT026942	
NVI-8244	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-8255	Unknown	Ballan Wrasse	Norway	KT026651	KT026724	KT026797	KT026870	KT026943	
NVI-8281	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-8300	*V. splendidus*	Ballan Wrasse	Norway	KT026652	KT026725	KT026798	KT026871	KT026944	
NVI-8301	Unknown	Atlantic salmon	Norway	KT026653	KT026726	KT026799	KT026872	KT026945	

NVI-8430	*V. splendidus*	Atlantic cod	Norway	KT026654	KT026727	KT026800	KT026873	KT026946	KT036391
NVI-8442	*V. splendidus*	Atlantic cod	Norway	KT026655	KT026728	KT026801	KT026874	KT026947	KT036392
NVI-8500	*V. splendidus*	Wrasse	Norway	KT026656	KT026729	KT026802	KT026875	KT026948	
NVI-8535	Unknown	Lumpsucker	Norway	KT026657	KT026730	KT026803	KT026876	KT026949	
NVI-8549	*V. splendidus*	Goldsinny wrasse	Norway	KT026658	KT026731	KT026804	KT026877	KT026950	
NVI-8586	*V. splendidus*	Lumpsucker	Norway	KT026659	KT026732	KT026805	KT026878	KT026951	
NVI-8587	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-8588	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-8610	*V. tasmaniensis/V. atlanticus*	Lumpsucker	Norway	KT026660	KT026733	KT026806	KT026879	KT026952	
NVI-8611	*V. splendidus*	Lumpsucker	Norway	KT026661	KT026734	KT026807	KT026880	KT026953	
NVI-8630	*V. splendidus*	Goldsinny wrasse	Norway	KT026662	KT026735	KT026808	KT026881	KT026954	
NVI-8634	*V. splendidus*	Wrasse	Norway						
NVI-8664	*V. splendidus*	Ballan Wrasse	Norway	KT026663	KT026736	KT026809	KT026882	KT026955	
NVI-8665	*V. splendidus*	Goldsinny wrasse	Norway						
NVI-8666	*V. tasmaniensis/V. atlanticus*	Goldsinny wrasse	Norway	KT026664	KT026737	KT026810	KT026883	KT026956	
NVI-8678	*V. splendidus*	Ballan Wrasse	Norway						
NVI-8681	*V. tasmaniensis/V. atlanticus*	Lumpsucker	Norway	KT026665	KT026738	KT026811	KT026884	KT026957	
NVI-8683	*V. splendidus*	Lumpsucker	Norway						
NVI-8690	*V. tasmaniensis/V. atlanticus*	Wrasse	Norway	KT026666	KT026739	KT026812	KT026885	KT026958	
NVI-8691	*V. splendidus*	Ballan Wrasse	Norway	KT026667	KT026740	KT026813	KT026886	KT026959	
NVI-8692	*V. splendidus*	Ballan Wrasse	Norway						
NVI-8693	*V. splendidus*	Ballan Wrasse	Norway						
NVI-8703	*V. splendidus*	Wrasse	Norway						
NVI-9217	*V. splendidus*	Wrasse	Norway	KT026668	KT026741	KT026814	KT026887	KT026960	
NVI-9221	*V. splendidus*	Wrasse	Norway	KT026669	KT026742	KT026815	KT026888	KT026961	
NVI-9703	Unknown	Atlantic halibut	Norway	KT026670	KT026743	KT026816	KT026889	KT026962	
NVI-9752	*V. splendidus*	Lumpsucker	Norway	KT026671	KT026744	KT026817	KT026890	KT026963	
NVI-9753	*V. lentus*	Lumpsucker	Norway	KT026672	KT026745	KT026818	KT026891	KT026964	
ZF270	*V. cyclitrophicus*	Seawater	Northeast USA	*AIDR01000142*	*AIDR01000143*	*AIDR01000159*	*AIDR01000078*	*AIDR01000234*	
ZF-91	*V. crassostreae*	Seawater	Northeast USA	*AJZC01000149*	*AJZC01000074*	*AJZC01000276*	*AJZC01000134*	*AJZC01000205*	
ZS-139	Unknown	Seawater	Northeast USA	*AJZE01000014*	*AJZE01000014*	*AJZE01000010*	*AJZE01000117*	*AJZE01000048*	

NVI-prefix: Strains maintained at -80°C at the Norwegian Veterinary Institute in Oslo.

Accession numbers in *italic*: Sequences obtained from GenBank.

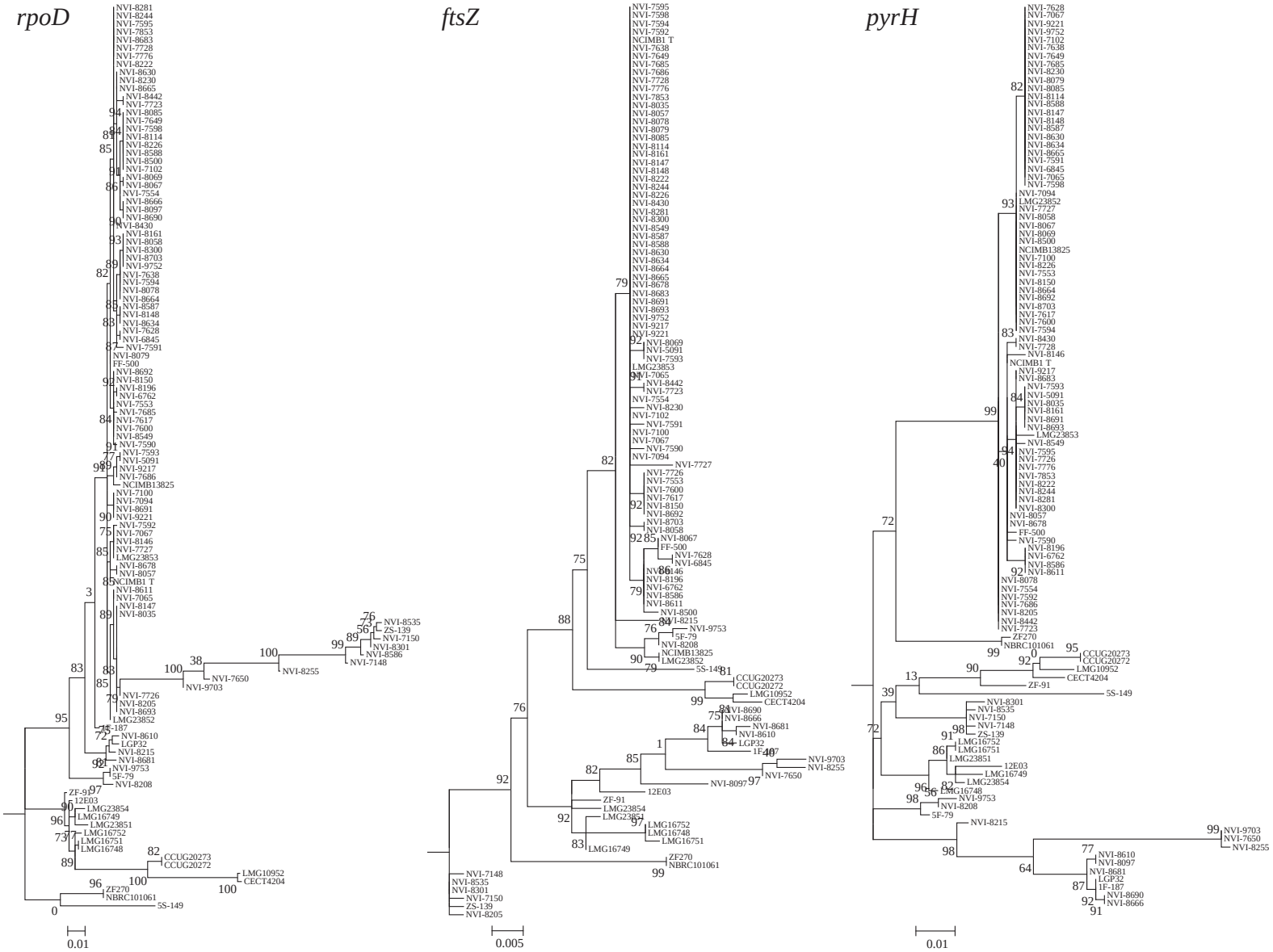

Figure S1: Maximum Likelihood trees based on individual partial gene sequences from *V. splendidus*-related isolates, with aLRT branch support values shown. The trees were rooted towards the *V. tapetis* type strain (not shown).

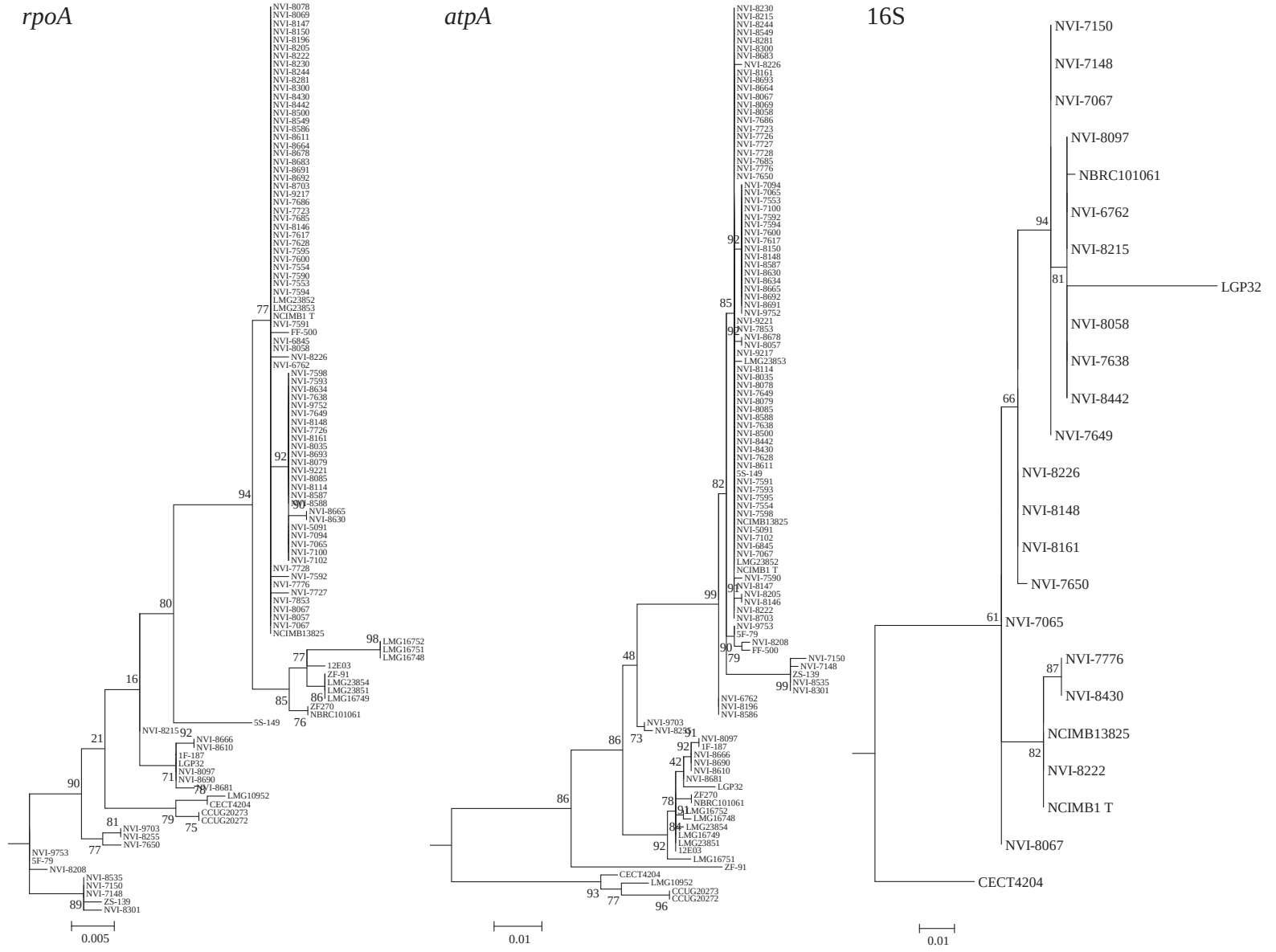

Figure S1 cont.