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EMIDA Call Progress Report

(FINAL)

Project Title: Control Flavobacteriaceae infections in European fish farms.
Acronym: PathoFish
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1. Summary of the project's progress

Consider each of the following items:

- *Main activities and achievements of the consortium*
- *Your opinion on the internal cooperation and added value to the project*

Please state if developments within the projects or outside have caused you to amend any of the project's goals and, if so, in what way

This PathoFish project addressed Topic 4 (Aquaculture) of the EMIDA call. It focused on two fish pathogenic bacteria belonging to the family Flavobacteriaceae: Flavobacterium psychrophilum (Fp) and Tenacibaculum maritimum (Tm). Both are of primary economic significance due to their impacts on fish farming industry.

Meetings:

In order to coordinate the work of the different partners seven meetings were organized during the time-frame of the project.

Website:

A dedicated web site was built up for this project.

http://www6.inra.fr/pathofish

This website encompasses:

- Public pages describing the project and the Partners involved.

- Private pages "For Partners only" are dedicated to the unified protocols used and to the presentation of the meetings and the meeting programs (all items appear as pdf files to be downloaded).

Note: An access to the private pages of this website may be opened upon request to the project coordinator.

Consortium agreement:

A consortium agreement was set up and signed by all partners.

An amendment of the consortium agreement was set up for a one year extension program.

The project goals remained unchanged during all the project. However, the work encompassed two bacterial species (Fp and Tm) and the consortium acknowledged an imbalance in favor of Fp. The obvious reasons (detailed in the following sections) are: the lower than expected availability of Tm strains, the unforseen large diversity of Tenacibaculum species, and the administrative problems of Partner 2 (IZS-Sardinia). Regarding the development of new tools for pathogen control (WP3) and their evaluation (WP4) we chose to only use the qPCR method, targeting Fp gDNA, as it rapidly proved to be very promizing in terms of sensitivity and specificity.

This project gave to all Partners a unique opportunity to exchange technology and knowledge in fish pathology, microbiology, genomics, epidemiology and to develop new diagnostic tools and vaccination strategies.

2. Achievement of planned objectives

Describe the activities that have been performed to meet the objectives set in the proposal.

In order to meet the objectives described in the proposal, we have devised a work plan consisting of five work packages. The completion of the WPs are detailed below.

WP1 - Data acquisition.

Responsible: Partner 1 - Other partners involved: Partners 2, 3, 4, 5 and 8.

Task 1.1 Collecting bacterial isolates from the broadest diversity of countries and fish hosts.

An important aspect of the project was to rationally build and organize a bacterial culture collection. About 200 isolates per country involved in the project (around 1,000 isolates in total) were collected, safely stored, and quality controlled according to the project plan. For instance:

- Partner 2 collected 150 strains, suspected to be Fp, from different rainbow trout organs / tissues (ovarian fluid, spleen, kidney, eye and skin) during clinical outbreaks in Italy occurred mostly in 2011, 2012 and 2013, most of them isolated from the spleen of symptomatic fingerlings. All the strains were analysed by biochemical methods and 16S PCR. Twenty additional strains of suspected Fp, collected in 2002 and 2003 were also included. All of the skin isolates were shown not to belong to Fp, some of them were identified as Chryseobacterium sp.

- A total of 195 isolates from Finland were collected by Partner 3. The isolates were from different fish species (rainbow trout, perch, brown trout, brook trout), from the environment (tank and pond water), sampled during different years (1983-2012). From one fish farm isolates were collected during 7 consecutive years. The collection also contained isolates from Scotland and Estonia.

- Partner 4 provided the majority of Norwegian isolates (about 130 strains) of Fp available in the Norwegian Veterinary Institute collection collected from 1996 to 2012. The collection included isolates from rainbow trout, Atlantic salmon and brown trout . After 2012 strains collected in 2013 and 2014 were provided. In addition, DNA from two strains from rainbow trout collected in South Africa in 2013 was included.

- Partner 5 has selected 210 isolates from Denmark mostly from rainbow trout, but also from brown trout, flounder and stickleback. The isolates were sampled during different years (1988-2012).

- Partner 8 provided all Swiss isolates of F. psychrophilum available from 1993 to 2012 (n = 112).

- Finally, Partner 1 was able to obtain genomic DNA from isolates of F. psychrophilum from Japan (n = 114) and Chile (n = 94) for comparison with isolates obtained in Europe by the other Partners and the ones collected in France and previously published (Nicolas et al. 2008, AEM and Siekoula-Nguedia et al. Vet. Microbiol. 2012).

MLST typing was carried out as planned and the the relationship between isolates was analyzed and published (see WP2).

Note: Because of the reasons detailed below, an extensive sampling was performed for Fp (about 1000 bacterial isolates collected) whereas the sampling of Tenacibaculum strains was more modest (about 200 isolates).

Fp being highly prevalent in European fish farms, many strains were isolated by six Partners (Partners 1, 2, 3, 4, 5 and 8), while Tm strains were supposed to be isolated by three Partners only: Partners 1, 2 (only the ISZ- Sardinia group was involved) and 4. In addition, due to the problems experienced by IZS-Sardinia (see section 4), only 7 Tenacibaculum isolates were provided by Partner 2.

Partner 4 was able to collect about 100 Tenacibaculum isolates from different fish species and isolated since 1996. However, none of the isolates (from Atlantic salmon, rainbow trout, wrasse and cod) genotyped so far (results obtained in WP1, task 1.2) belong to the species T. maritimum. Instead, genotyping revealed that most of them actually belong to other or new (undescribed) Tenacibaculum species (see below). Though interesting, this result made interpretation and exploitation of the data much more complicated.

Therefore, Partner 1 did his best to obtain Tenacibaculum strains outside of the consortium in order to increase the collection: i) the type strains of all 21 Tenacibaculum species, most of them described during the timeframe of the project, were obtained from international culture collections (they were also used as "gold standard" references systematically included in WP1 task 1.2 and WP2); ii) the own retrospective collection of Tm strains of Partner 1 was included (about 60 isolates); and iii) Tenacibaculum strains and genomic DNAs were kindly provided by other colleagues outside of the consortium (about 30 samples) during the first half of the project.

Task 1.2 DNA sequencing.

Two sequence-based approaches were conducted during the project:

- A MultiLocus Sequence Typing (MLST) approach for large-scale analysis (all sampled isolates) was carried out according to the project plan. Isolates (N=36) from Sweden (collected and sequenced outside the consortium) were included in the analysis.

Genotyping of Fp was carried out according to a previously published (and since optimized) MLST scheme. All Fp isolates (about 1000) were genotyped without problem.

Genotyping of Tm strains relied on a MLST scheme that was still under development and unpublished at the beginning of this project. This scheme was initially based on 11 loci obtained from the few complete Tenacibaculum genomes that were available at that time. Some isolates (especially those that did not belong to the clade containing the species T. maritimum) were recalcitrant to amplification at some loci. Since then (see below), as newly sequenced Tenacibaculum genomes became available during the timeframe of this project, an optimized scheme was set up that enabled us to genotype, not only the Tm isolates, but also isolates that belong to other Tenacibaculum species. As of this writing, about 100 Tenacibaculum isolates were successfully genotyped. For the rest of the isolates that Partner 4 has collected (about 80), 7-8 loci out of 11 have been successfully sequenced.

- A whole-genome sequencing approach for in-depth analysis of the genome content of 22 Tenacibaculum and 24 Fp relevant isolates was carried out according to the project plan.

The set of isolates subjected to whole-genome sequencing is composed of: i) 18 Tenacibaculum type strains to serve as "gold standard" references and to optimize the Tenacibaculum MLST scheme as mentioned above; ii) 24 Fp isolates carefully selected out of the 1000 strains according to MLST data [i.e., 2 isolates from France (Partner 1), 2 isolates from Italy (Partner 2), 5 isolates from Finland (Partner 3), 5 isolates from Norway (Partner 4), 5 isolates from Denmark (Partner 5), 2 isolates from Switzerland (Partner 8), 2 isolates from Chile (obtained by Partner 1), and one isolate from Scotland (obtained by Partner 3)] (Chilean and Scottish isolates included for comparative purposes) and iii) more recently and according to the MLST results obtained with Tenacibaculum, four additional Tenacibaculum (undetermined species) isolates from Norway were fully sequenced for comparative purpose.

We used pair-end sequencing-by-synthesis (Solexa, Illumina) for complete genome sequencing. This task was 100% subcontracted and performed by the sequencing platform IMAGIF (https://www.imagif.cnrs.fr/). The very good quality and high number of sequencing reads (>30 M per strain) allowed us to reconstruct almost complete genomes. In this context, we anticipated that optical mapping would be of poor benefit for most of these genomes. According to WP2 results, optical mapping was applied to a limited set of Tenacibaculum genomes.

The planed objectives were perfectly reached for F. psychrophilum but more modestly for Tenacibaculum.

Milestones:

Milestone 1.1: A bacterial strain collection encompassing approximately 1,000 isolates collected all over Europe - Fully accomplished at month 12.

Milestone 1.2: MLST raw data - Fully accomplished at month 16.

Milestone 1.3: Almost complete genome sequences (NGS technology) of 46 isolates - Fully accomplished at month 24.

All deliverables are OK - Fully accomplished at month 24.

WP2 - Data integration

Responsible: Partner 1 - Other partners involved: Partner 7 - Time frame: 12 months (month 7 to month 18)

Computational biologists played a key role in virtually all aspects of this WP using available state-of-the-art methods for genomics, phylogeny, population genetics and statistics.

Task 2.1 Elucidating the relationships between isolates using MLST data.

Using MLST data, we determined the evolutionary relationships between the bacterial isolates. In order to perform such analysis, starting from raw MLST sequence data reads, Partner 1 built a bioinformatic pipeline to: i) check sequence quality; ii) perform assembly of forward and reverse sequencing reads; iii) extract consensus sequences; iv) address allelic profiles and sequence types for all bacterial isolates.

To analyze Fp intra-species diversity, we first used the dedicated eBURST algorithm. This analysis allowed us to: i) compare at a glance all Fp genotypes; ii) determine the genotypes retrieved from European fish farms and to compare them to those of Japanese and Chilean isolates; and iii) identify the main clonal complexes. This analysis confirmed the association between bacterial genotypes and host fish species. Importantly, this analysis allowed us to select isolates for complete genome sequencing (performed in Task 1.2).

A tree obtained by single-linkage hierarchical clustering of the allele type profiles was constructed. It allowed visualizing the alleles shared between all the isolates instead of only between the most closely related as the eBurst diagram does. All the relevant background information available has been superimposed on this tree (including geographical origin, fish host and year). Statistical association between genotype and background information was quantified and tested by AMOVA (Analysis of MOlecular Variance).

Regarding Fp, the results obtained were published as 4 papers in different international journals of high impact factor in the veterinarian field:

- “Molecular epidemiology of Flavobacterium psychrophilum from Swiss fish farms”. Nicole Strepparava, et al. 2013, Dis Aquat Org, 105: 203–210.
- “Population structure of the fish pathogen Flavobacterium psychrophilum at whole-country and model river levels in Japan”. Erina Fujiwara-Nagata et al. 2013, Veterinary Research, 44:34.
- “Introduction, expansion and coexistence of epidemic Flavobacterium psychrophilum lineages in Chilean fish farms”. Ruben Avendano-Herrera, et al. 2014, Veterinary Microbiology, 170: 298–306.
- “Multilocus Sequence Typing Identifies Epidemic Clones of Flavobacterium psychrophilum in Nordic Countries”. Hanne Nilsen et al. 2014, Appl. Environ. Microbiol., 80(9): 2728–2736.

The latter publication encompassed an impressive joined effort of bacterial sampling and analysis by Finland, Norway, Denmark, Sweden (outside the consortium) and France (560 geographically and temporally disparate Fp isolates collected from various sources in the Nordic region between 1983 and 2012).

A publication encompassing the results of the MLST analysis with the isolates obtained by Partner 2 in Italy is under writing:

- “Genetic divergence and population structure of the circulating Flavobacterium psychrophilum strains in Italian fish farms”. Caburlotto G. et al.

To analyze Tenacibaculum intra-species and inter-species diversity, we first used phylogenic tree reconstruction using the concatenated sequences from 11 loci. This analysis allowed us to: i) perform a clustering of bacterial isolates; ii) identify the Tenacibaculum species they belonged to (when possible); iii) identify the isolates that likely belong to yet undescribed Tenacibaculum species (this was the case for most isolates from Norway); and iv) focus on bona fide Tm isolates to analyze intra-species diversity.

This Tenacibaculum MLST scheme was published and now provides a gold standard for future analysis of the diversity within the genus Tenacibaculum. An important outcome was the discovery of pathogenic species in different bacterial lineages.

- "Multilocus Sequence Analysis of the Marine Bacterial Genus Tenacibaculum Suggests Parallel Evolution of Fish Pathogenicity and Endemic Colonization of Aquaculture Systems". Christophe Habib, et al. 2014 Appl. Environ. Microbiol., 80(17):5503.
- A publication based on MLSA/MLST analysis of the Norwegian Tenacibaculum isolates is under writing.

Task 2.2 Comparative genomics using whole-genome sequence data and selection of target genes.

The whole genome sequence data generated in the project were used to build repertoires of the core genome genes and of the variable gene pool. The goal of this task was to identify candidate genes for diagnostic and vaccination purposes.

In order to perform such an analysis starting from raw Solexa sequence data reads, Partner 1 built a bioinformatic pipeline to: i) check sequence quality; ii) perform assembly of all sequencing reads using the Velvet algorithm; iii) extract consensus sequences (contigs and scaffolds); iv) extract coding sequences (CDS) using the Show gene prediction software; and v) perform a semi-automatic annotation of these genomes.

Velvet proved very efficient to assemble all genomes de novo (15 to 109 contigs and 15 to 94 scaffolds). Because of the high quality of de novo assembly, the read mapping initially planed was not used. Instead, it was possible to build a repertoire of core genome genes (i.e. the genes found in all sequenced isolates) from the de novo assembly and to determine the variable gene pool. This work was performed at the species level for Fp and at the genus level for Tenacibaculum.

Regarding Fp:

The whole genomes of 24 Fp isolates were sequenced (in WP1 task 1.2). Together with the published complete genome of Fp JIP02/86 and already available Fp genomes (background of Partner 1) these merged data provided a good picture of the genetic makeup of this species.

Regarding Tm:

The whole genomes of 18 Tenacibaculum type strain species were sequenced (in WP1 task 1.2). These merged data provided a good picture of the genetic makeup of this genus. More recently and according to the MLST results obtained with Tenacibaculum, four additional Tenacibaculum isolates from Norway were fully sequenced for comparative purpose.

The data were subjected to in silico mining to study gene dynamics in terms of presence/absence of genes in the pan-genome and the patterns of sequence evolution in the core genome. In line with the spirit of this project, we paid special attention to regions most likely associated with virulence (toxins, outer-membrane proteins, adhesins, etc.) and to specific genomic traits such as the CRISPR locus and genomic islands. This allowed us i) to identify the genes that were lost or gained in the clade and those for which this occurred repeatedly and ii) to establish the relations of orthology, paralogy and xenology in the set of strains. Gene dynamics was matched against phenotypic characteristics, such as pathogenicity, host-range or presence/absence in particular ecological niches, using phylogenetic reconstruction of ancestral states.

Relevant genes/proteins were selected in silico according to multiple criteria (ubiquity within a bacterial species, exposed at the cell surface or secreted, degree of polymorphism, etc.) for their subsequent use in WP3.2 for the production of vaccine candidates and development of diagnostic/typing tools.

Publications of the whole genome analysis and comparisons are now planned.

Milestones

Milestone 2.1: MLST data analysis: reconstruction of the genealogy of the isolates (including genetic divergence between isolates and population structure) and selection of isolates for whole-genome sequencing. - Fully accomplished at month 24.

Milestone 2.2: Whole-genome sequencing and analysis. - Fully accomplished at month 32.

Deliverables

Deliverable 2.1: MLST sequences (i.e., about 6 Mbp for the genotyping of 1,000 Fp isolates and 114 Tenacibaculum sp. isolates). Release of MLST sequences in the public EMBL database and the establishment of the website for MLST analysis of Tenacibaculum spp (see WP5 for web site address). List of isolates selected for whole-genome sequencing (delivery date initially planed: month 12) - Fully accomplished at month 24.

Deliverable 2.2: 32 automatically annotated genomes and additional detailed manual annotation of specific loci (e.g., virulence-associated genes). All the annotated genome sequences will ultimately be released in public databases (Fully accomplished, publication under construction).

WP3 – Development of new tools for pathogen control.

Responsible: Partner 3 - Other partners involved: Partner 2, 4, 5, 6, 7 & 8 - Time frame: 18 months (month 12 to 30)

Task 3.1 Phenotypic characterization of a subset of bacterial isolates.

Bacterial strains were exchanged between partners (1,2,3,4 and 5).

Altogheter 39 Fp strains were included in the phenotypical analysis. The type strain, NCIMB 1947T was included in all tests.

1) Analysis of bacterial virulence

For Fp (Partner 3, Finland): According to WP2, bacterial isolates (the type strain, NCIMB 1947T was also included for comparison) representative of different MLST groups were selected and studied in vitro and in vivo. Isolates were from different fish species (rainbow trout, Atlantic salmon, coho salmon, brown trout) and from the environment. The isolates from fish were mainly from internal organs. Bacterial virulence in rainbow trout was evaluated by in vivo challenge to discriminate between highly, moderately and non-virulent isolates. Two of the examined isolates were completely non-virulent, five isolates were classified as moderately virulent and five isolates were considered highly virulent.

For Tm (Partner 2 Italy): 300 sea bass fingerlings (4-5 g weight) were introduced in IZSVe wet facilities in June 2012 to be infected with 5 different concentrations of 3 different Tenacibaculum strains: T. maritimum (France), T. mesophilum (Italy) and Tenacibaculum sp. (Norway). Due to an outbreak of parasitical gill disease (Amyloidinum sp.) 70 % of the animal died and the in vivo challenge failed. A smallest virulence trial was carried out with a new batch of healthy sea bass and only with T. maritimum, which was injected IM at three different concentrations (10^6, 10^5, 10^4 cells/fish, 10 fish per group in duplicate). No symptoms or mortality was observed after 1 month of observation: all bacteriological samples from head kidney were negative.

2) Bacterial survival and growth in different in vitro culture conditions (e.g., temperature, salinity, iron-deprived medium, etc.) as well as production of proteolytic enzymes were investigated.

Partner 2 Italy: For Fp and Tm, growth was evaluated at 5, 10, 15, 20, 25 and 30°C. All the strains were tested in duplicate starting from a 10^3 - 10^4 inoculum and incubated for 10 days: O.D. at 525 nm was monitored every 24 hours. All of them, whatever their origin, grew at 5° to 25°C, while no growth was observed at 30°C. The growth was optimal at 15° and 20°C.

Partner 3, Finland: Iron is essential for bacterial growth and bacteria need to have an efficient iron uptake system in order to cause infections in animals. The growth of the strains in "iron limited medium" was examined. Two non-virulent isolates showed a high capacity to grow in iron-limited medium. Three isolates showed a moderate growth and six isolates showed a low growth in the "iron limited medium".

Partner 4, Norway: Hydrolysis of elastin was investigated to discriminate between proteolytic and non-proteolytic isolates (28 strains including NCIMB 1947T). Systemic infection with Fp has been recurrent in rainbow trout reared in brackish water in a particular fjord in Norway. It was of interest to investigate whether some strains have adapted to a more saline environment. Growth at different salinities was measured by absorbance in broth supplemented with different concentrations of NaCl (ranging from 0% to 2.0%). Viability of cells was controlled by culture after 5 days (15 strains; completed month 18; 28 strains completed month 26 including NCIMB 1947T). A few strains grew at 1.25%, no strains showed growth at 1.75%. No strains showed survival at 2.0%.).

Biofilm formation for Fp strains was tested and different factors to encourage biofilm formation were evaluated. An assay using exposure to different salinities was established and the 28 strains of F. psychrophilum were tested. All strains showed some biofilm formation, and for most of the strains this occurred also at increased salinities (0,5 % and 1 %). For some strains the formation of biofilm increased with increased salinity (from 0 to 0,5 %).

3) Serotyping, plasmid profiling and antibiotic resistance.

Partner 5, Denmark: Fp isolates were tested to determine whether they belonged to the three described serotypes, Fd, Th and FpT. All three serotypes were found among the isolates. Plasmids were identified in some Fp isolates used for complete genome sequencing. We first use DNA extraction and gel electrophoresis and identify contigs encompassing these plasmids. The same isolates were tested against 5 different antibiotics with the agar disc diffusion method.

A subset of MLST typed strains were investigated with some of these methods and results are included in a recently published paper:

- "Characteristics of epidemic and sporadic Flavobacterium psychrophilum sequence types" Krister Sundell and Tom Wiklund, Aquaculture, Volume 441, 20 April 2015, Pages 51–56.

4) An intriguing phenotype of DNA degradation was identified in one Fp strain (as well as in F. indicum, a closely related species). This phenotype was linked to the presence of the dnd gene cluster, identified for the first time in this bacterial group. This work was published: "From the Flavobacterium genus to the phylum Bacteroidetes: genomic analysis of dnd gene clusters", by Paul Barbier et al. 2013, FEMS Microbiol Lett 348: 26–35

Task 3.2 Production of new diagnostic tools and innovative vaccine candidates.

Molecular-based diagnostic kit prototypes (real-time PCR and multiplex PCR) were co-developed by Partner 7 according to the combined results of the other Partners in previous tasks (isolate sampling, sequencing of complete genomes and identification of targets using whole genome analysis). Primers and probes were designed by Partner 1 (9 PCR primer pairs and probes),PCR conditions were optimized (mix, temperature) and the best primer pair and probe were retained. The qPCR method is linear in the dynamic range of 10 to 10^5 equivalent genome/µl, detection and quantification limits are 5 and 10 equivalent genome/µl, respectively. The specificty of the method was evaluated against

several bacterial isolates and fish DNA. All isolates of Fp were amplified, no amplification was observed with isolates of other bacterial species or fish DNA.

The method was evaluated in infected rainbow trout (whole fish, kidney, liver and spleen). Ct values ranged from 27 to 31 for whole fish and from 27 to 39 for fish tissues. These very promising results allowed Partner 7 to provide Partners 1, 2, 4, 5 and 8 with diagnostic kit prototypes for use in WP4.2. Due to the promising qPCR technology, most efforts were done with this method and the development of an ELISA kit was abandoned. However 5 recombinant proteins (see after) were used to immunize rabbits and anti-sera directed against these proteins were obtained and evaluated using western blot analysis. We now plan to use them as new tools for immunodectection of the bacteria in fish tissues.

The development of innovative vaccine candidates relied on a reverse-vaccinology approach. This strategy was also used during the timeframe of the project by other groups in the US and in Japan (see for instance Plant KP et al. FEMS Microbiol Lett. 2014 and Kato et al Fish Shellfish Immunol. 2014). In view of the intrinsic difficulties experienced so far in producing an effectetive vaccine this new strategy is very promising (see “Flavobacterium psychrophilum vaccine development: a difficult task”. Gómez E, Méndez J, Cascales D, Guijarro JA. Microb Biotechnol. 2014).

Therefore, 32 relevant proteins were selected in silico according to the results of Task 2.2. The genes (or the gene regions corresponding to a protein domain) encoding the proteins of interest were i) amplified by PCR (86); ii) cloned (86 constructions, 84 transformants obtained) in an ad-hoc plasmid (i.e., pFO4) containing a 6HIS-tag; iii) expressed in a heterologous bacterial host (i.e., E. coli strain T7 express lysY from NEB and subsequently, for those that did not express the protein of interest, in E. coli strain BL21-CodonPlus from Agilent, a strain producing rare tRNA); iii) small scale (2,5ml culture) affinity purification of custom recombinant proteins for all constructs were performed using IMAC columns (27 recombinant proteins were at the same time overexpressed and soluble); and iv) large scale (250ml culture) affinity purification of custom recombinant proteins were performed using IMAC and Superdex 200. These purified recombinant proteins (6 evaluated) were used as vaccine candidates in WP4.

Milestone 3.1: Phenotypic characterization of 6 to 12 bacterial strains (delivery date: month 18). – Fully accomplished at month 32, 2 publications.

Milestone 3.2: To merge phenotypic and clinical information relating to Norwegian isolates with DNA sequences obtained from the MLST typing scheme. – Fully accomplished at month 32.

Note: Clinical information is included in the paper “Multilocus Sequence Typing Identifies Epidemic Clones of Flavobacterium psychrophilum in Nordic Countries”. (see Task 2.1)

Note: Phenotypic data will be included in the publication in preparation under Task 2.2 "Comparative genomics using whole-genome sequences data and selection of target genes".

Milestone 3.3: Cloned proteins, purified proteins and (if possible monoclonal) antibodies. (delivery date: month 24). Fully accomplished at month 36.

Milestone 3.4: ELISA-based diagnostic kit (delivery date: month 30). Abandoned

Note: As we obtained recombinant proteins and rabbit antisera directed against these proteins, they may represent new tools to develop an immunohistochemical method to detect the proteins or bacteria in fish tissues.

Milestone 3.5: PCR- based diagnostic kit (delivery date: month 30). Fully accomplished at month 32.

Deliverables 3.1: 6 to 8 purified soluble recombinant proteins (delivery date initially planned: month 20) – > 8 purified soluble recombinant proteins obtained at month 32.

WP4 – Evaluation of new tools for pathogen control.

Responsible: Partner 6 (during the first 3 years) and Partner 5 (for the one year extension). Other partners involved: Partners 2, 4, 5, 7, 8. Time frame: 9 months (month 28 to month 36). Partner 4 got an extension for a total of 18 months (12 + 6 months). Publication in preparation (partner 4,5,7,8).

The vaccination trials were performed by Partner 2, 3 and 5 both with whole formalin-killed bacteria and recombinant proteins. The vaccine candidates were tested with and without adjuvant (Montanide ISA763AVG, SEPPIC) but none of them provided protection against F. psychrophilum in the tested experimental conditions. An ELISA assay was used to test the rainbow-trout immune response against recombinant proteins and whole cells to find out if the vaccine candidates produced an immune response. Partner 5 has still blood samples from 3 different experiments that need to be tested. The rather disappointing vaccination trials were discussed by all Partners. An efficient, and more importantly reproducible, challenge (i.e., yielding about 70-75% mortality) is still difficult to reach by most Partners. Discrepancies between the results obtained by the different Partners likely arose from: i) the genotype and size of the host fish used and ii) the bacterial strain and the in vitro growth condition used (growth media and temperature, resuspension media, route of infection). However the infection model was significantly improved and a unified experimental infection scheme was formally proposed.

Although the outer-membrane exposed proteins selected were potentially good vaccine candidates, in spite of repeated assays by the different Partners using different types and amounts of recombinant proteins none of them seemed so far to induce protection. Partner 5 has still a trial ongoing with two other recombinant proteins.

The qPCR prototype kit has been tested on field samples (e.g. spleen,ovarian fluid, kidney, gill and brain) and compared with plating. The overall results of qPCR showed 100% specificity but several explanations to the fact that plating is more sensitive than qPCR in some cases (62% sensitivity) were discussed. The Partners agree about the following advantages of qPCR: i) faster results directly from tissues; ii) direct and reliable identification; and iii) results not influenced by mixed infections. However some limitations of qPCR were also discussed in particular the fact that no antibiotic resistance testing could be performed without preliminary culture. Partner 4 observed cases of CWD-like pathology where cells of F. psychrophilum displayed an unusual morphology and could not be isolated; the pathogen, however, was definitely identified using immunohistochemistry and qPCR.

Partner 4, 5, 7 and 8 are planning to produce a common publication.

WP5 - Reporting and dissemination of the results.

Responsible: Partner 1. All other partners involved. Time frame: 36 months

7 meetings have been organized to communicate on the progress of the project.

- 1st, kick-off meeting - April the 4th, 2011. INRA, Paris (France)
- 2nd meeting - November the 14th, 2011. INRA, Paris (France)
- 3nd meeting - June the 8th, 2012. Åbo Akademi University, Turku (Finland)
- 4th, mid-term meeting - December the 4th & 5th, 2012. Paris (France)
- 5th meeting - August the 29th and 30th, 2013. Business Park Dynamo, Tempere (Finland)
- 6th meeting - May the 8th and 9th, 2014. Palazzo Grassi, Chioggia (Italy)
- 7th, closure, meeting - November the 20th and 21st, 2014. INRA, Jouy en Josas and Paris (France)

Some relevant isolates should be rapidly deposited in international culture collections by the different Partners (ongoing).

All DNA sequences (MLST) were deposited in the EMBL Nucleotide Sequence Database (also known as EMBL-Bank that constitutes Europe's primary nucleotide sequence resource) and made freely available to the scientific community. They were also included on dedicated web sites that contain all the tools for analysis and comparisons of MLST data from worldwide origin.

For Fp see: http://pubmlst.org/fpsychrophilum/
Tm see: http://pubmlst.org/tenacibaculum/

Complete genome sequences will be released after publication acceptance.

All details regarding the above-mentioned WP and tasks can be downloaded from the web site: http://www6.inra.fr/pathofish
An access (login and password) to these pages can be opened upon request to the project coordinator.
Seven papers were already published, three are in preparation.
Relevant results were presented in international conferences (e.g.: Flavobacterium 2012, EAFP 2013, FEMS 2013,) and at national level (e.g.: Havbrukskonferansen 2012 and 2014, Frisk Fisk 2013).

3. International collaboration added value

Describe the activities that have been accomplished in collaboration within the consortium. Refer explicitly to joint milestones and deliverables produced.
Describe any sharing of facilities, databases within the consortium.

Eight actors from public institutions and companies have decided to combine their expertise and efforts to generate new scientific knowledge on flavobacteriaceae fish pathogens.
These actors encompasses:
1) Fish pathologists and veterinarians with close connections to the fish farming industry. These Partners were involved in collecting bacterial isolates in fish farms (WP1, task1) and conducted experimental vaccination trials and field-evaluation of new diagnostic tools (WP4, task 1 and 2).
2) Genomicists and bioinformaticians were involved in genome sequencing (WP1, task 2) and analysis (WP2).
3) Bacteriologists and molecular biologists were involved in the phenotypic characterization of a subset of bacterial isolates (WP3, task 1)
4) Companies that were involved in the production of new diagnostic tools and innovative vaccine candidates. (WP3, task 2)

The fact that several European countries have agreed to use the same methods to characterize these bacteria allows now to compare and exchange information.
The project has generated a huge amount of data that we would not have been able to collect without the present international collaboration. The consortium was able to share the sequencing strategy and data integration (e.g. the MLST database has been considerably enriched), the recombinant protein production and dispatching, the diagnostic tools prototypes, bacterial growth media composition and, of primary importance, the biological ressources.

4. Problems and changes in objectives

Describe any difficulties and problems that have hindered the achievement of the planned objectives and any alternative plans or changes with respect to the original proposal.

Italy: Giuseppe Bovo, member of the Istituto Zooprofilattico del Piemonte, Liguria, Valle d'Aosta (IZS PLV) retired in January 2012.

Fulvio Salati, member of the Istituto Zooprofilattico della Sardinia (ISZ-SS) faced administrative difficulties which prevented him from fully participating in the project.

Workpackage leader exchange for WP3 and WP4.

All Partners, except Partner 6, asked and obtained from their funding agencies an extension of the project for one extra year in order to fulfill remaining tasks.

For Flavobacterium psychrophilum no problems were met.

For Tenacibaculum maritimum the number of strains isolated was lower than expected, due to the disengagement of IZS-SS and to the fact that most Norwegian and Italian isolates actually did not belong to the species T. maritimum.

5. Project-derived publications and patents

*Publications **with** the involvement of other partners of the consortium*	- "Molecular epidemiology of Flavobacterium psychrophilum from Swiss fish farms". Nicole Strepparava, Pierre Nicolas, Thomas Wahli, Helmut Segner & Orlando Petrini. 2013, Dis Aquat Org, 105: 203–210. - "Multilocus Sequence Typing Identifies Epidemic Clones of Flavobacterium psychrophilum in Nordic Countries". Hanne Nilsen, Krister Sundell, Eric Duchaud, Pierre Nicolas, Inger Dalsgaard, Lone Madsen, Anna Aspán, Eva Jansson, Duncan J. Colquhoun, Tom Wiklund. 2014, Appl. Environ. Microbiol., 80(9): 2728–2736. The later publication encompassed an impressive joined effort of bacterial sampling and analysis by Finland, Norway, Denmark and France (560 geographically and temporally disparate Fp isolates collected from various sources between 1983 and 2012). - "Multilocus Sequence Analysis of the Marine Bacterial Genus Tenacibaculum Suggests Parallel Evolution of Fish Pathogenicity and Endemic Colonization of Aquaculture Systems". Christophe Habib, Armel Houel, Aurélie Lunazzi, Jean-François Bernardet, Anne Berit Olsen, Hanne Nilsen, Alicia E. Toranzo, Nuria Castro, Pierre Nicolas and Eric Duchaud. 2014 Appl. Environ. Microbiol., 80(17):5503.
*Publications **without** the involvement of other partners of the consortium*	- "Population structure of the fish pathogen Flavobacterium psychrophilum at whole-country and model river levels in Japan". Erina Fujiwara-Nagata, Céline Chantry-Darmon, Jean-François Bernardet, Mitsuru Eguchi, Eric Duchaud and Pierre Nicolas. 2013, Veterinary Research, 44:34. - "Introduction, expansion and coexistence of epidemic Flavobacterium psychrophilum lineages in Chilean fish farms". Ruben Avendano-Herrera, Armel Houel, Rute Irgang, Jean-Francois Bernardet, Marcos Godoy , Pierre Nicolas and Eric Duchaud. 2014, Veterinary Microbiology, 170: 298–306. -"From the Flavobacterium genus to the phylum Bacteroidetes: genomic analysis of dnd gene clusters", by Paul Barbier, Aurelie Lunazzi, Erina Fujiwara-Nagata, Ruben Avendano-Herrera, Jean-Francois Bernardet, Marie Touchon & Eric Duchaud. 2013, FEMS Microbiol Lett 348: 26–35

	-"Characteristics of epidemic and sporadic Flavobacterium psychrophilum sequence types" by Krister Sundell and Tom Wiklund. Aquaculture, Volume 441, 20 April 2015, Pages 51–56.
*Patents **with** the involvement of other partners of the consortium*	
*Patents **without** the involvement of other partners of the consortium*	

6. Brief financial report

	1st year	*2nd year*	*3rd year*	*Total*
Personnel	175301	232510	153967+200540	762318
Equipment	23265	21379	24221+35490	104355
Other costs	106027	126247	133559+106496	472329
Total	304593	380136	311747+342526	1339002

7. Executive summary

The executive summary must not exceed 2 sides in total of A4 and should be understandable to non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Fish provide more than 2.6 billion people with at least 20 percent of their average per capita animal protein intake. Aquaculture accounted for 43 percent of the 106 million tons of food fish in 2004 and its part continues to increase faster than any other animal food-producing sectors. Worldwide, the sector has grown at an average rate of 8.8% per year since 1970. However, and according to the FAO, infectious fish diseases threat the development of this industry and pathogen control has now become a major concern.

Members of two genera in the family Flavobacteriaceae (or flavobacteria), the freshwater Flavobacterium species and the marine Tenacibaculum species, are recognized as major problems for the aquaculture industry in Europe and worldwide.

Three members of the genus Flavobacterium are recognized fish pathogens. Among them, Flavobacterium psychrophilum (Fp) is one of the most prevalent and troublesome infectious agents in salmonid farming worldwide that profoundly impacts the European salmon and rainbow trout production. Fp is the agent of cold water disease (CWD) and rainbow trout fry syndrome (RTFS).

Tenacibaculum maritimum (Tm) is causing a disease mostly characterized by skin and muscle ulcers that is responsible for low but constant mortality in many cultured marine fish worldwide (Avendaño-Herrera et al., 2006). Many European salt-water fish farms suffer Tm infections because of its wide host range that includes many valuable species such as sole, turbot, sea bass, sea bream, meagre, sea trout and salmon.

In order to cope with the diseases, farmers rely on the recurrent use of antibiotics. However, this is incompatible with the development of a sustainable aquaculture. Therefore, disease prevention is likely the most cost-effective control option. Most fish disease management strategies attempt to minimize the risk factors associated with transmission of the pathogens.

At present, accurate diagnosis of the causative agents is difficult and time-consuming and new fast diagnostic tools are urgently needed. They are critical for a fast identification of the pathogen responsible for the outbreaks, for epidemiological investigations (e.g., exploration of the pathogen propagation by global trade of fish and fish eggs, persistence in the environment, etc.) and for quantifying the level of infection in broodstocks.

The diversity and population structure of these pathogens are still poorly understood and the genomic data available are still sparse. The advent of whole-genome sequencing of bacteria and advances in bioinformatics have revolutionized the study of bacterial pathogenesis, enabling the targeting of new diagnostic tools and possible vaccine candidates starting from genomic information.

To improve the control of Fp and Tenacibaculum infections, data at both the epidemiological and molecular levels are urgently needed. This project aimed at filling this scientific gap and immediately

investing the acquired knowledge to provide the fish-farming community with new tools for diagnostic and prophylactic methods. For this reason, eight of the main actors in this field in Europe decided to combine their expertise and efforts to generate new scientific knowledge on Fp and Tm in order to develop rational diagnostic tools and vaccine candidates.

This program allowed to develop and use :

- Unified genotyping schemes based on the multi-locus sequence typing approach for Fp and Tm to characterize at the molecular level the relationships between large numbers of bacterial isolates of the most diverse origins.
Regarding Fp we identified (i) the dominant genotypes encompassed in major clonal complexes ; (ii) a marked association with host fish species ; (iii) an epidemic population structure ; and (iv) the evidence of dissemination with fish trade
Regarding Tenacibaculum, we identified an unexpected species diversity. In the case of T. maritimum, no clonal complexes, no dominant genotypes and no strong association with host fish species were observed, suggesting an endemic population structure with no clues of dissemination with fish trade.

- Whole genome sequence data was used to characterize the gene repertoire of selected strains focusing on virulence mechanisms and host-pathogen interactions. A set of genes/proteins relevant for the development of diagnostic tools and vaccines were selected.

- A sensitive, fast and specific qPCR diagnostic test was therefore developped. This test was used to detect Fp in fish (allowing screening of broodstocks, eggs, ovarian fluid, etc.) and environmental (biofilm and water) samples.

- Expression of Fp recombinant proteins in E. coli and evaluation of their immunogenic properties. This « reverse vaccinology » strategy is a promising way to select and identify proteins that could be used as future vaccine components.

The results of this project have important implications for the aquaculture industry and specifically address the question of the evolutionary process of pathogen emergence at both the strain and species levels. They contribute to the increase in scientific knowledge on the population structure and epidemiology of important fish-pathogenic bacteria allowing risk assessment to prevent the propagation of epidemic clones with aggressive phenotypes.

It is the intention of the EMIDA consortium to publish the executive summary of the project.

Please confirm your agreement to do so.

YES ☒

NO ☐

If no, please explain:

I declare that the information I have given is correct to the best of my knowledge and belief.

Eric DUCHAUD
Name

March the 25th 2015
Date

Coordinator of the PathoFish project
Directeur de Recherche à l'INRA
Head of the team "Infection et Immunité des Poissons"
Position held