Final Report

The Chlafish study has been used to look at the emerging disease epitheliocystis in high value aquaculture species in the Mediterranean. We used cutting edge imaging and genomics techniques to identify, characterise, and sequence the genomes of two novel pathogens.

One aspect of the project focussed on the high value aquaculture fish species gilthead seabream (*Sparus aurata*). These fish suffer increasingly from epitheliocystis, which has been identified in several farms around the Greek coastline. The disease is easily diagnosed in this species due to large white cysts found on the gills. We used gill arch samples from infected, morbid fish which were preserved for microscopy (formalin) and genetic analysis (RNALater and Ethanol). Samples were also taken into sterile sea water and transport medium, from which culture was attempted, but this was not successful. Identification of the bacteria in the cysts used 16S rRNA gene amplification and sequencing indicated that a new, previously undiscovered, type of beta-proteobacterium was present in the samples. Using fluorescent *in situ* hybridisation (FISH) we confirmed that this novel sequence localised to the epitheliocysts and that therefore these pathogens are responsible for the disease.

A similar technique was used to determine the pathogen causing epitheliocystis on the skin and gills of young larvae of sharpsnout seabream (*Diplodus puntazzo*) grown in a mesocosm system. These were preserved and analysed as above. In this case, a novel gamma-proteobacterial species, named *Ca*. Endozoicomonas cretensis, was identified. Using a similar FISH method, this was localised to the cysts in the larvae. The same 16S rRNA gene sequence has been isolated once before, from cobia larvae suffering from epitheliocystis in Colombia [5], indicating that this may be a global issue.

For both fish species, high resolution confocal microscopy and electron microscopy, including three dimensional EM (Focussed Ion Beam-Scanning EM, FIB-SEM), were used to investigate the surroundings of the bacteria within the cysts, especially useful as these pathogens have not been brought into laboratory culture. The two cyst types are easily distinguishable from each other by EM, and also distinguishable from the cysts caused by chlamydial bacteria, which commonly cause epitheliocystis [1-4, 7-10]. *Ca*. Ichthyocystis bacteria have clear double membranes, which bud off into vesicles, and control the host epithelial cell environment to form the interdigitating processes. *Ca*. E. cretensis bacteria are more rod shaped and have filaments projecting from them. Neither have the characteristic lifecycle of Chlamydiae with more dense bodies in the middle of the cyst representing elementary bodies.

The most significant advance from this project is the generation of draft genomes of these novel pathogens from preserved material. These are the first genomes of epitheliocystis agents, and high quality draft sequences have been created in the absence of culture.

Specialised laboratory techniques were used, with many methods trialled, and sequence data was subject to a careful and detailed bioinformatics analysis to obtain the best draft genomes from a mixture of bacterial and host sequencing reads.

The genomic data shows that *Ca*. Ichthyocystis appears to be a stable, obligate intracellular pathogen, as the core genome of this genus is well conserved, yet does not encode pathways for amino acid biosynthesis, implying it is largely dependent on the host cell. There are virulence factors in the form of type 2, 3 and 4 secretion systems, with effectors found within very unusual arrays of duplicated and diversified gene families. Type IV pili are also found within these genomes.

The genome of *Ca*. E. cretensis on the other hand, appears to be undergoing degradation, as many genes have been disrupted by insertion sequence amplification and other methods of psueodgenisation, compared to published genomes of related bacteria within the genus [6]. This generally indicates the beginning of genome reduction, as the pathogen adapts to a new niche.

This research has relevance to high value fish species farmed in the Mediterranean, and therefore to many aquaculture firms and related research groups. In order to raise awareness about this research and its implications, and to encourage future collection of samples to continue the work, this project was presented at the Aquaculture Europe 2014 meeting in San Sebastian, October 2014. In addition, a summary of the work targeted towards those in the industry has been prepared for The Advocate Magazine of the Global Aquaculture Alliance (http://advocate.gaalliance.org/).

As well as these applicable aspects of the project, the discovery of three very diverse bacterial types causing what is diagnosed as the same disease, is a striking finding. And the different ways that these bacteria appear to have arrived at a similar lifestyle is also fascinating. We also recommend our methodology as a thorough way of getting the best data possible on uncultured infectious organisms.

Further information is available here: http://www.fgcz.ch/the-center/people/seth.html

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