Roseobacter-clade bacteria as probiotics in marine larvaculture

Disease caused by fish pathogenic bacteria can cause large scale crashes in marine fish larval rearing units. One of the biggest challenges for aquaculture is the management of these bacterial outbreaks. Vaccines can be admitted to fish but only the juvenile and the adult fish because they need to have a mature immune system. This means that the larvae of the fish, until they are 2-3 weeks old are more prone to bacterial infections. A short term solution is antibiotics but this leaves way for the selection for antibiotic resistance among the pathogenic bacteria, which again can be transferred to human pathogens. Alternatives are therefore needed and one could be the use of probiotic bacteria. Marine bacteria from the Roseobacter clade (Phaeobacter inhibens) have shown great potential as probiotic bacteria, and we have hypothesized that they could be used to antagonize pathogenic fish and crustacean bacteria in the environment of the larvae. The purpose of the present PhD study was to determine if antagonistic Roseobacter clade bacteria occurred in marine aquaculture units. The study would determine their clonal relationship and elucidate the mechanisms by which these potential probiotic bacteria affect the fish pathogens. The efficiency as probionts was studied in cultures of microalgae and live feed organisms in both axenic and none-axenic cultures. Chemical, genetic and bioinformatic tools were used to identify and quantify the production of the antibiotic compound and then compare to reference strains from the Roseobacter clade. Long-term exposure of the fish pathogen to the antimicrobial compound produced by Phaeobacter was done in order to determine risk of resistance and possible genetic and phenotypic effects on the pathogen. The study showed that some of the isolates from the Greek rearing unit could only be identified via 16S rRNA gene sequencing as Phaeobacter sp. following guidelines from a previously published study. Using whole genome sequencing and in silico DNA-DNA hybridization gave an indication of a new Phaeobacter species. Two such strains together with Phaeobacter inhibens DSM 17395 were chosen for further study. The study demonstrated that in axenic live feed cultures, the potentially new species of Phaeobacter were able to antagonize both the fish pathogen Vibrio anguillarum with up to four logarithmic units and the crustacean pathogen Vibrio harveyi with up to two logarithmic units. This corresponded well with results from strains and previous studies. To confirm the in vivo mechanism of action of the antibacterial compound tropodithietic acid (TDA) a defective mutant was included in the study. The mutant was significantly less efficient at antagonizing the pathogen indicating that TDA production is the major contribution to the probiotic action. To further elucidate the probiotic potential of Phaeobacter inhibens, the probiont were added to cultures of Artemia and Dunilla tertiolectra with four added bacterial strains representing aquaculture background microbiota. The Phaeobacter inhibens were able to colonize both cultures and still antagonize the pathogen with up to four logarithmic units. Lastly, the efficiency of the probiont was tested in completely non-axenic cultures of either Artemia or Tetraselmis suecica received from an aquaculture unit. The cultures were inoculated with the pathogen Vibrio anguillarum. The pathogen was reduced with up to three logarithmic units compared to the control. The antibacterial compound tropodithietic acid (TDA), an antiporter that disrupts the proton motive force, is key in the antibacterial activity of several roseobacters. Introducing probiotics at a larger scale requires understanding of any potential side effects of long term exposure of the pathogen to the probionts or any compounds they produce. We here exposed the fish pathogen, Vibrio anguillarum, to TDA for several hundred generations in an adaptive evolution experiment. No tolerance or resistance arose during the 90 days of exposure and whole genome sequencing of TDA-exposed lineages and clones revealed few mutational changes as compared to lineages grown without presence of TDA. Amino acid changing mutations were found in two to six different genes per clone, however, no mutations appeared unique to the TDAexposed lineages or clones. None of the virulence genes of V. anguillarum were affected and infectivity assays using fish cell lines indicated that the TDA- exposed lineages and clones were less invasive than the wild type. Thus, long term TDA exposure does not appear to result in TDA resistance and the physiology of V. anguillarum appears unaffected, supporting the application of TDA-producing roseobacters as probiotics in aquaculture. In summary, this study demonstrates that Phaeobacter inhibens and newly isolated Phaeobacter sp. strains can be used as probiotics against fish and crustacean pathogens both in a short term effect to reduce the concentrations, but more importantly also as in a long term effect as no resistance is seen in the pathogen after continuous exposure of the antibiotic compound TDA. All strains of Phaeobacter sp. were able to colonize cultures of Artemia and microalgae whether it be axenic, defined non-axenic or completely non-axenic.

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